EVALUATION OF DUAL-SEROTYPE ADENOVIRUS-BASED VACCINE-INDUCED CELLULAR IMMUNITY FOLLOWING PREVENTATIVE AND THERAPEUTIC IMMUNIZATION AGAINST SIMIAN IMMUNODEFICIENCY VIRUS

by

Adam Christopher Soloff

B.A., University of Delaware, 2001

Submitted to the Graduate Faculty of

the Department of Infectious Diseases and Microbiology

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2008

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This dissertation was presented

by

Adam Christopher Soloff

It was defended on

August 27th, 2008

and approved by

Kelly Stefano Cole, Ph.D.
Assistant Professor
Director of the Regional Biocontainment Laboratory, Center for Vaccine Research
Departments of Immunology and Molecular Genetics and Biochemistry, School of Medicine
Department of Infectious Diseases and Microbiology, Graduate School of Public Health
University of Pittsburgh

Joanne Flynn, Ph.D.
Professor
Department of Microbiology and Molecular Genetics, School of Medicine
Center for Vaccine Research
University of Pittsburgh

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

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

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

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

EVALUATION OF DUAL-SEROTYPE ADENOVIRUS-BASED VACCINE-INDUCED CELLULAR IMMUNITY FOLLOWING PREVENTATIVE AND THERAPEUTIC IMMUNIZATION AGAINST SIMIAN IMMUNODEFICIENCY VIRUS

Adam Christopher Soloff, Ph.D.

University of Pittsburgh, 2008

ABSTRACT

A vaccine capable of preventing or therapeutically limiting human immunodeficiency virus (HIV) pathogenesis is urgently needed to contain the acquired immunodeficiency syndrome (AIDS) pandemic. Recombinant adenovirus (Ad)-based vectors are being explored as vaccine candidates due to their potent induction of cell-mediated immunity. To circumvent the limitations of vector-specific humoral immunity, novel Ad serotypes impervious to pre-existing immunity against conventional vectors have been developed. Utilizing the nonhuman primate model of HIV infection, we evaluated the immunogenicity of conventional Ad serotype 5- (Ad5) and novel serotype 35- (Ad35) based vaccinations against simian immunodeficiency virus (SIV) infection. In a preventative, proof-of-concept vaccination regimen, immunization against the SIV Gag protein proved highly efficacious, demonstrating robust boosting of Ad5-based vaccine-induced cellular immunity by Ad35-based vectors. Ad5/Ad35-based vaccination induced durable, high-frequency effector T cell responses that were later recalled upon heterologous SIV challenge. Vaccination resulted in modest reductions in SIV viremia, notable given the limited scope of immunization. We then tested the capacity of Ad5/Ad35-based vaccination targeting the SIV Gag, Env, and Nef proteins, with or without IL-15 augmentation, to promote cellular immunity during antiretroviral-treated chronic SIV infection with the goal of limiting rebound viremia following cessation of antiretroviral therapy (ART). Vaccination enhanced both systemic and mucosal antigen-specific cell-mediated immunity, increasing the breadth and strength over innate response to infection. Ad-induced immunity consisted of $CD4^+$ and $CD8^+$ T lymphocyte T_H1 cytokine production of a predominantly monofunctional nature.

Furthermore, vaccination enhanced both central and effector memory $CD4^+$ and $CD8^+$ T cell populations without augmenting naive T cell responses. Although Ad-based immunotherapy transiently restored the systemic central memory $CD4^+$ T cell compartment, vaccination failed to salvage effector memory or mucosal $CD4^+$ T cells. Therapeutic intervention was associated with transient containment of rebound viremia upon ART cessation which vaccination failed to augment. An effective vaccination against HIV represents the most efficient method to end the AIDS pandemic, and is of considerable public health significance. The findings presented herein provide evidence to support the continued evaluation of Ad-based vectors in novel treatment strategies against HIV infection, representing an incremental advancement in the field of HIV vaccine development.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

To begin, I would to thank my research advisor, Dr. Simon Barratt-Boyes, whose encouragement and support has prepared me for whatever challenges may lie ahead. Simon has been a mentor in every sense, and I am fortunate to have had the opportunity to learn from him as a scientist and as an individual.

I would like to thank the members of my graduate committee: Drs. Kelly Cole, Andrea Gambotto, Charles Rinaldo, and Joanne Flynn, for providing the insight and experience necessary to advance my research efforts while assisting in my development as a scientific investigator.

I thank the members of the Barratt-Boyes laboratory members, past and present, for their daily support and assistance.

I would like to thank the staff and faculty members at the Department of Infectious Diseases and Microbiology and Center for Vaccine Research for their tireless efforts.

Finally, I owe an immense debt of gratitude to my family and friends who have inspired and driven me to pursue my life's goals. Without the unconditional love and support from my parents, Dr. Cynthia Scott and Dr. Paul Soloff, I could have never achieved any of the small accomplishments I have made. Finally, I owe a special thanks to Dana Rebich, who has endured me during the stress, suffered with me during the hardships, and rejoiced during the accomplishments of my life.

ABBREVIATIONS

Abbreviations: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; SHIV, SIV/HIV hybrid virus; ART, antiretroviral therapy; STI, structured treatment interruption; Ad5, adenovirus serotype 5; Ad35, adenovirus serotype 35; CTL, cytotoxic T lymphocyte; T_H, T helper lymphocyte; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; IL-, interleukin-; BrdU, 5-bromo-2-deoxyuridine; Ag, antigen; Ab, antibody; NAb, neutralizing antibody; MHC, major histocompatibility complex; HLA, human leukocyte antigen; i.m., intramuscular; i.d., intradermal; Tcm, central memory T cell; Tem, effector memory T cell; PBMC, peripheral blood mononuclear cells; BAL, bronchoalveolar lavage; ELIPSOT, enzyme linked-immunospot assay; ELISA, enzyme linked immuno-sorbent assay; SEB, staphylococcal enterotoxin B; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; MVA, modified vaccinia virus Ankara strain; DMSO, dimethyl sulfoxide; GALT, gut-associated lymphoid tissue; DC, dendritic cell; NK, natural killer cell; APC, antigen presenting cell; TCR, T cell receptor; VLP, virus-like particles; FACS, fluorescence-activated cell sorting.

1.0 INTRODUCTION

Throughout human history, the struggle for survival has been intertwined with the battle against infectious diseases. For the vast majority of human existence, this battle was fought blindly against an unidentified assailant until a technological breakthrough, unheralded initially, lead Antony van Leeuwenhoek to examine the nature of the microbial world. Still unaware of the microscopic enemy, Edward Jenner combined astute observation with cultural traditions to administer the first effective vaccination, inoculating patients with the related cowpox virus to confer protection against lethal smallpox. These simple experiments would prove to be a milestone of modern medicine, providing the first instance of human intervention affecting the course of infectious illness through directly targeting the pathogen responsible. With microbiology still in its infancy, Louis Pasteur excited the world with his miraculous protection of cattle from anthrax, chickens from cholera, and most dramatically, the treatment of a young boy with rabies utilizing attenuated organisms for vaccination. Yet, for as spectacular a showman as Pasteur had become, it was the methodic and dedicated examination of Robert Koch that would finally demonstrate the role of microbes as the causative agents of disease. Through formulating the Koch's postulates, medical science had now identified its enemy.

Well over 300 years since the recognition of microbes, much of modern medicine is still focused on preventing their deleterious effects upon mankind. Whether they are established, historical diseases such as tuberculosis, emerging infectious diseases such as the SARS-coronavirus or zoonotic influenza infections, or re-emerging diseases as is the case with antibiotic resistant bacterial infections, microbial pathogens remain one of the most significant threats to global health. In this context, human immunodeficiency virus type 1 has become known as arguably the most elusive and deadly pathogenic agent in history. In as much, the study of the HIV has pushed the fields of virology and immunology to greater heights, and spawned a new generation of researchers, clinicians, and activists in an attempt to curtail the

massive social and physical destruction caused by disease. With no cure in site, the fight against HIV infection is one of the preeminent battle grounds of modern medical science, whose outcome will influence all aspects of human society on a global scale.

1.1 HIV AND SIV

The human immunodeficiency virus type 1 (herein referred to as HIV) is a member of the *Retroviridae* family of the *Lentivirus* genus. Nucleotide sequence comparisons allow for distinction of three groups of HIV isolates differing between 15-20%, consisting of the M (most) group, further divided into eleven clades (subtypes A-D, F-H, J, and K), group O (outlier) and group N [1]. HIV is an enveloped virus of roughly 110 to 146 nm in length [2]. The HIV genome is composed of two positive single-stranded RNA molecules 9747 base pairs in length containing nine open reading frames coding for structural (Gag, Pol, Env), regulatory (Tat and Rev), and accessory (Vpu, Vpr, Vif, and Nef) proteins. The defining characteristic of HIV is its remarkably error-prone reverse transcriptase, which in conjunction with the influences of immune and drug pressure, as well as host genetics, leads to exceptionally rapid viral evolution.

The HIV lifecycle begins with viral adhesion to its cellular target, with subsequent receptor-mediated entry into the cellular cytoplasm facilitated by recognition of the viral gp120 envelope trimer with the cellular primary and co-receptors, $CD4^+$ and $CCR5^+/CXCR4^+$, respectively [3]. Upon viral entry, the HIV virion is uncoated allowing for the reverse transcription of its RNA genome into linear double-stranded DNA before translocating to the nucleus and becoming permanently integrated into the genome of its host cell, a hallmark of retroviruses. Integrated proviral DNA establishes latent viral reservoirs which may lay dormant for years before the host cell becomes activated, initiating transcription and replicating the viral genes necessary for HIV reproduction [4, 5]. Although viral reproduction occurs primarily in activated $CD4^+$ T cells, resting host cells have been shown to continually produce low levels of progeny virus. Proviral DNA is transcribed, exported to the cytoplasm, and subsequently translated by host cellular mechanisms. Viral proteins congregate at the cellular membrane, where they undergo virion assembly and proteolytic cleavage, allowing for the budding release of infectious progeny.

The simian immunodeficiency virus (SIV), first discovered in 1985 [6], has been shown to infect over 30 species of nonhuman primates [7], and is divided into six major lineages representing genetic variation and host origin [8]. Infection by species-adapted SIV in its natural host is nonpathogenic due to prolonged host-dependant evolution, resulting in sustained moderate SIV viremia with low level immune induction without disease progression [8]. Conversely, host infection by SIV strains of divergent evolution, such as inoculation of Asian macaques with established African SIVmac, closely mimics human HIV infection, resulting in acute and chronic pathogenesis, progression to AIDS, and the development of opportunistic infections [8]. Recently, it has been shown that human HIV infection resulted from three distinct incidences of zoonotic transmission of SIVcpzPtt from the chimpanzee species *Pan troglodytes troglodytes* in western central Africa leading to the emergence of the pandemic M, and non-pandemic O and N clades of HIV [9, 10]. The emergence of SIVcpzPtt itself resulted from cross-species super-infection of chimpanzees with SIVrcm and SIVgsn from red-capped mangabeys and greater spot-nosed monkeys, respectively, allowing for viral recombination and presumably genetic speciation [11, 12]. Speculatively, HIV acquisition by humans and SIVcpzPtt acquisition by chimpanzee occurred through cross-species blood-borne transmission from hunting and preying upon monkeys naturally infected with species-adapted SIV. For these reasons, non-adapted SIV infection of nonhuman primates is currently the most suitable model of HIV infection and intervention [6, 13, 14].

1.2 THE HIV PANDEMIC

1.2.1 History

The HIV pandemic likely originated in western central Africa [15], as it is the only known region where HIV clades M, N, and O, as well as SIVcpzPtt infection of humans and chimpanzees, respectively, are found to coexist [9, 16, 17]. The oldest known HIV isolate, found to be clade M, was derived from a 1959 plasma sample from a man in what is now the Democratic Republic of Congo [18], and evolutionary modeling has estimated the emergence of HIV clade M at around 1930 ± 20 years [19]. In the summer of 1981, a mysterious and

horrifying rash of premature deaths caused by unusually rare diseases was first observed in the United States [20]. In September of 1982, the U.S. Centers for Disease Control and Prevention dubbed this mysterious plague acquired immunodeficiency syndrome (AIDS) due to the progressive loss of immunity seen in effected patients. The following year, independent groups lead by Luc Montagnier, Robert Gallo, and Jay Levy isolated the HIV virus and identified it as the etiologic agent leading to the development AIDS [21-25]. HIV pathogenicity was quickly recognized to include severe depletion of $CD4^+$ T lymphocytes, heightened susceptibility to opportunistic pathogens, and increased incidences of lymphoma and Kaposi's sarcoma [26].

1.2.2 Current Status

As of 2007, there were an estimated 2.5 million new HIV infections and 2.1 million deaths due to HIV, accounting for 33.2 (30.6-36.1) million people living with HIV/AIDS worldwide [27]. No country has been spared by the HIV pandemic, which effects regions through either concentrated epidemics in which pockets of high-risk individuals such as intravenous drug users, sex workers, and men who have sex with men are affected, or as generalized epidemics involving heterosexual transmission which become self-sustaining through high national incidence rates. The highest HIV incident rates are in sub-Saharan Africa, accounting for roughly 68% of people living with HIV/AIDS worldwide, including 90% of child infections and 76% of HIV-associated deaths [27]. HIV transmission is dependent upon the infectiousness of the index case, determined through concentration of cell-associated and cell-free virus transmitted in body fluids, along with the susceptibility of the naïve host [28]. Notably, the susceptibility of each individual to infection varies dramatically and is influenced by genetic factors, such as the presence of specific human leukocyte antigen (HLA) types or mutations within the cellular HIV coreceptor CCR5Δ32 [29, 30], by the presence of sexually transmitted infections which increase skin lesions and HIV target cell numbers in the genital mucosa, and by the presence of physical barriers such as foreskin [28, 31]. HIV infection is predominantly spread through heterosexual intercourse (~80%), with intravenous drug use, unprotected anal intercourse, and mother to child transmission pre- or post-partum remaining significant methods of viral transfer.

1.3 HIV/SIV PATHOGENESIS

1.3.1 Acute Destruction

The preeminent mechanism of HIV pathogenesis is the viral-mediated elimination of $CD4^+$-expressing T helper lymphocytes responsible for providing the cytokine support necessary for the induction and propagation of antiviral cellular and humoral immunity. Within days following the establishment of HIV infection, virus disseminates throughout receptive cellular targets of the body residing predominantly in the genital, intestinal, and respiratory mucosal tracts, as well as the lymph nodes, thymus, and spleen [32-36] primarily via cell-to-cell transmission [37, 38]. Specifically, the majority of viral strains deplete activated, HIV-specific $CD4^+$ effector memory T cells (Tem) bearing the tissue directing CCR5 chemokine receptor [39-42] found in the lamina propria of the mucosal tissues [33, 43, 44]. Yet, this effect is not exclusive, as HIV-mediated depletion of resting $CD4^+$ T lymphocytes has been well documented [34, 45]. The loss of $CD4^+$ Tem cells is highly correlated to immunodeficiency, as they provide antigen (Ag)-specific anamnestic clearance of HIV/SIV, as well as defense against opportunistic infections. Concurrently, HIV-mediated elimination of $CD4^+$ central memory T cells (Tcm) further disrupts T cell homeostasis, as the Tcm population is responsible for $CD4^+$ T cell memory regeneration through clonal expansion and differentiation. In addition, HIV/SIV disease control has been associated with the ability of systemic $CD4^+$ Tcm cells to replenish depleted $CD4^+$ Tem populations systemically and at the mucosa [46]. Traditionally, $CD4^+$ T cell depletion has been monitored in the peripheral blood, yet only 2-5% of total lymphocytes reside there, with the majority of these cells harbored in the gut-associated lymphoid tissue (GALT), estimated at 60%, and subsequent mucosal tissues [47]. Recent studies have demonstrated that the viral-mediated destruction of $CD4^+$ Tem at sites of the mucosa occurs almost immediately, leading to greater than 90% elimination of the mucosal $CD4^+$ Tem population by 14 days post-infection, and is not reflected in the peripheral blood until chronic stages of disease [33, 48-51]. The pathogenesis of acute mucosal $CD4^+$ T cell loss is exemplified by the fact that restoration, either naturally or following ART, of GALT $CD4^+$ Tem cells has been identified in long-term non-progressor macaques [52, 53]. The primary burst of acute HIV viremia is thought to be

sequestered by the virally-reduced frequency of available cellular targets in conjunction with the development of adaptive antiviral immunity.

1.3.2 Viral Latency

Immediately upon HIV infection, proviral integration into host DNA establishes a permanent reservoir in all anatomic locations capable of extended latency. Due to dependence on cellular transcriptional machinery, 99% of viral replication occurs in activated productively infected $CD4^+$ T cells. HIV infected memory T cells which return to a resting state may stably preserve the viral reservoir for the life of the cell, potentially decades [54, 55]. Monocytes, macrophages, and dendritic cells (DC) may contribute to HIV latency through trafficking associated viral particles and nominal productive infection, but these cells contribute minimally to long-term latency [56, 57]. Even as ART is capable of restricting viral replication to undetectable levels, individuals harbor an estimated latent pool of roughly 10^6-10^7 cells, or 0.1-1 HIV infected cells per million T lymphocytes [58-61]. Furthermore, the establishment of the viral reservoir seems unavoidable, as studies have found that ART given before seroconversion fails to prevent latent infection [62]. Mathematical models of viral reservoir decay kinetics have postulated that a current ART regimen would need to be sustained for 60 years to provide potential eradication of the latent pool [63]. Immune activation therapy, aimed at activating latent proviral replication under the protection of ART, would allow for the clearance of latently infected cells through viral cytopathic effects or immune targeting [64]. To this end, studies have employed the cytokines Interleukin-2 (IL-2), IL-6, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), the anti-CD3 mAb OKT3, and the chemical activators cyclophosphamide and valproic acid [65-69]. Immune activation therapy utilizing IL-2 alone or with other agents during ART was found to reduce the number of resting $CD4^+$ T cells containing latent virus and increase proliferation, but failed to lower rebound viremia upon cessation of ART [67-69]. As the presence of the HIV reservoir prohibits viral clearance, novel therapeutic interventions must be combined with ART to inhibit disease progression.

1.3.3 Cellular Pathogenesis: AIDS

1.3.3.1 Regenerative Exhaustion

When compared with nonpathogenic species-adapted SIV infection, the immunopathology of HIV is a consequence of chronic activation [70-74]. Remarkably, measurements of T lymphocyte activation are more precise indicators of disease progression than plasma viral loads [75]. While continual HIV and SIV cytotoxicity eliminates $CD4^+$ T cells and receptive targets, it creates a prolonged inflammatory environment capable of bystander cellular destruction and permanent restructuring of lymphoid and mucosal tissues negatively impacting cellular homeostasis [76-78]. Chronic $CD4^+$ T cell destruction consists of the gradual decline in remaining $CD4^+$ targets characterized by impaired regenerative capacity, draining of the naïve T cell pool, activation-induced cell death, as well as continual viral-mediated killing seen during prolonged infection [36]. Labeling studies utilizing 5-bromo-2-deoxyuridine (BrdU), Ki-67 staining, or ^{2}H-glucose *in vivo* in humans and nonhuman primates have demonstrated disproportionate loss of proliferating $CD4^+$ memory T cells, due to the intrinsic brief life expectancy of activated memory cells in addition to viral cytopathicity and elimination by Ag-specific $CD8^+$ T cells [36, 79-82]. Furthermore, memory $CD4^+$ T cells are inherently more susceptible to apoptosis via activation-induced cell death, Fas-Fas-ligand interactions, and other apoptotic signals present during systemic inflammation [83-85]. Rapid cellular turnover occurs in conjunction with limited repopulation of $CD4^+$ T cells due to age-dependent declines in thymopoiesis [86], suggested to be a key factor in cellular reconstitution during ART or controlled HIV infection [87-92]. Ag-driven expansion of memory $CD4^+$ T cells is limited compared to memory $CD8^+$ T cells [93-95], with fewer activated $CD4^+$ T cells standing-down and returning to long-lived resting states [96-98]. Additionally, acute pathogenesis to GALT immunity and the breach of structural integrity at the intestinal epithelium allow for microbial translocation, further exacerbating chronic immune activation [99, 100]. Generalized activation affects $CD4^+$ T lymphocyte homing and recirculation from the peripheral blood, lymphoid, and extralymphoid effector sites, likely inducing redistribution to areas containing a high concentration of virally infected cells such as the GALT or lung mucosa, creating a positive feedback loop of expansion, recruitment, and local viral-mediated elimination of memory $CD4^+$ T cells [101-103]. Thus, limitations in the generation of $CD4^+$ T cells through age-attenuated

thymic output and Ag-driven peripheral expansion are concurrent with rapid $CD4^+$ T cell loss which progressively exhausts the body's regenerative capacity, removing $CD4^+$ T helper function and promoting the collapse of $CD8^+$ cytotoxic T lymphocyte (CTL) containment of chronic, set-point viremia.

1.3.3.2 Dysfunctional Cellular Immunity

Establishment of chronic, set-point viremia is a consequence of the rapid elimination of available $CD4^+$ T cell substrates for HIV [104] in conjunction with the development of effective T cell-mediated immunity [105-107]. HIV-specific $CD4^+$ and $CD8^+$ T cells become refractory to Ag-specific proliferative induction due to their post-activation state and continual stimulation by chronic viremia [108, 109]. During progressive HIV and SIV infection, the ability of effector T lymphocytes to recognize viral Ag decreases as $CD8^+$ T cells frequently contain T cell receptors (TCR) of low functional avidity and disruptions in signal transduction that together can lead to gaps in the immune repertoire with clonal deletion or exhaustion [110-114]. In addition, dysfunctional HIV-specific CTLs have been shown to lack efficient perforin and granzyme B production, inhibiting cytolytic functions [115, 116]. Notably, CTL-mediated killing and antiviral restriction governed through cytokine production are dramatically diminished or dysfunctional during progressive disease. Rapid dysfunction of $CD4^+$ T lymphocyte production of IL-2, IFN-γ, and TNF-α occurs during SIVmac251 infection in as early as ten days [117]. In addition, skewed maturation of $CD8^+$ T cell populations results in an overabundance of $CD8^+$ Tem cells, often with limited expression of effector molecules such as CD27 and CD28, as well as homing receptors necessary for proper function [118-120]. Furthermore, Ag-specific CTLs over-express the negative regulatory molecule PD-1 during progressive HIV infection, associated with T cell exhaustion, compounding immune dysfunction through both lack of appropriate Ag-specific reaction as well as negative regulatory inhibition of responses [121-123]. The viral-induced disruption of the cellular immune compartment during chronic infection prevents effective clearance of latent cell associated viral reservoirs.

1.4 ANTIRETROVIRAL THERAPY

The utilization of ART has transformed HIV infection into a chronic illness, capable of extending a patient's life expectancy and reducing disease associated morbidity [124]. The restriction of viral replication by ART provides immediate physical benefit, allowing for the replenishment of $CD4^+$ T cell numbers, reduced immune activation, and restoration of Ag-specific cellular immunity and proliferative capacity [125, 126]. Although ART may reduce plasma viral concentrations to undetectable levels, ART is insufficient to provide sterilizing immunity, as latent viral reservoirs are unaffected by current pharmacologic interventions providing continual low-level, cryptic viral replication [127]. In rare instances when ART is initiated early during acute HIV or experimental SIV infection, acute pathogenesis is inhibited, establishing a diminished rate of viral destruction and disease course [128]. Combinations of antiretroviral drugs targeting multiple stages of the HIV lifecycle including entry into host cells, reverse transcription of the RNA genome to DNA, integration of proviral DNA into the host genome, and maturation of formed virions, have enhanced the duration of therapeutic efficacy [129]. Unfortunately, the extraordinarily high rate of HIV replication and genomic error incorporation allow for the development of drug resistant and multi-drug resistant quasispecies to all known inhibitors available [130]. In conjunction, the associated toxicity and financial burden of ART make therapeutic intervention either unavailable or unsustainable for the vast majority of persons living with HIV worldwide.

Early attempts to alleviate drug toxicity and side effects while promoting HIV-specific immunity were performed through structured treatment interruptions (STI) of ART. Therapeutic re-exposure to HIV Ag through STI allowed for contained viremia if ART was initiated during early infection prior to irreparable immunologic damage [131]. Unfortunately, viral rebound and subsequent expansion of the cell-associated viral reservoir during STI nullifies the potential benefit of stimulating naïve and functional cellular memory [132]. Notably, reduced viremia following STI was consistently associated with the functional capacity of $CD4^+$ Tcm cells to produce IL-2 and IFN-γ, of $CD4^+$ and $CD8^+$ Tem cells to produce IFN-γ, and the lymphoproliferative response to recall antigens in all existing therapeutic strategies [40, 133-135]. These findings suggested that therapeutic augmentation of HIV-specific cellular immunity may potentially contain chronic viremia in the absence of life-long ART.

1.5 INTERLEUKIN-15

1.5.1 Biologic Function

Inerleukin-15 (IL-15) is a pleiotropic, immunoregulatory cytokine belonging to the common cytokine receptor γ-chain family with profound influence on innate and adaptive immunity against intracellular pathogens [136]. IL-15 protein production is strictly regulated through translational inhibition via the inclusion of 12 upstream AUG start codons in the 5' UTR of IL-15 mRNA and by secondary RNA structures of the C-terminus, as well as trafficking and translocation restrictions mediated by the presence of distinct long and short signaling peptides encoding for secreted and intracellular cytokine localization, respectively [137-140]. Secreted at times, IL-15 is most frequently membrane-bound in association with the IL-15-specific IL-15Rα, allowing for endosomal recycling and *trans* presentation of the IL-15 complex to neighboring cells following production by DCs, monocytes, and macrophages [141, 142]. The membrane-associated IL-15 presentation from antigen presenting cells (APCs) allows for B and T cell Ag recognition and stimulation in the context of IL-15 co-expressed with co-stimulatory molecules and activation factors, modifying the adaptive T_H1-biased immune response following cell-to-cell activation [141]. As a member of the common cytokine receptor γ-chain family, IL-15 induces some redundant actions with IL-2, IL-21, and others due to shared elements of receptor-mediated signaling through the common family γ_c receptor and IL-2/15Rβ chain subunit leading to Janus kinase (JAK) and signal transducer and activator of transcription systems (STAT) activation [143]. Signaling through its high-affinity heterotrimeric receptor including the IL-15-specific IL-15Rα component activates pathways specialized for stimulating the proliferation of activated $CD4^+$ and $CD8^+$ T cells through FKBP12-mediated activation of p70 S6 kinase, generation of memory $CD8^+$ CTLs, synthesis of immuonoglobulin from B cells, expansion of natural killer (NK) cells, and importantly, the formation and maintenance of cellular memory populations [144-150]. Notably, IL-15 induces the proliferative expansion of $CD4^+$ and $CD8^+$ Tem cells, of virus-specific $CD8^+$ Tcm cells necessary for long-lived immunologic memory, as well as the Ag-independent expansion and differentiation of naive $CD8^+$ T cells [149, 151-153]. Interestingly, IL-15Rα expression is found in a wide variety of cells and tissues, with associated affects ranging from anabolic stimulation of skeletal muscle, growth of mast cells, to microglial

maintenance, illustrating the broad systemic influences of the cytokine [143, 154]. IL-15 promotes survival and inhibits apoptosis of $CD4^+$ and $CD8^+$ T cells induced via cytokine-deprivation, TNF-, and Fas-mediated pathways through the production of the anti-apoptotic proteins Bcl-2, Bcl-X_L, and c-FLIP [155-160]. In addition, IL-15 is a chemoattractant for T lymphocytes, but not B cells, monocytes, or neutrophils [161, 162]. It should be noted that due to its potency as a pro-inflammatory mediator, IL-15 has been associated with autoimmune disorders and T cell-mediated alveolitis in AIDS patients, and should be carefully evaluated in nonhuman primate models [163, 164].

1.5.2 Immunotherapeutic Application

IL-15 is strongly implicated in HIV pathogenesis as IL-15 deficiencies have been identified in patients with progressive infection [165], while IL-15 maintenance is associated with viral containment both during and without ART intervention [166], and high plasma IL-15 levels are associated with viral containment during STI from ART [167]. PBMC from HIV-infected individuals fail to produce adequate amounts of IL-15 upon *ex vivo* stimulation with herpes simplex virus-1 or Staphylococcus aureus compared to healthy donors [165, 168]. Monocyte-derived DCs harvested from progressive AIDS patients have been shown to be severely compromised in their ability to produce IL-15 in response to bacterial lipopolysaccharide (LPS) or Candida albicans [169]. In the context of these findings, IL-15 is uniquely suited as a therapeutic agent for HIV due to its limited mitogenic effects on $CD4^+$ T cells compared to IL-2 treatment, minimizing *de novo* viral synthesis from activated lymphocytes [166, 170, 171]. Furthermore, IL-15 induces Ag-specific expansion and Ag-independent propagation and maintenance of memory $CD8^+$ T cells in the absence of $CD4^+$ T cell help, a valuable characteristic given the profound $CD4^+$ T cell depletion throughout HIV infection [172-174]. Treatment of $CD8^+$ T cells from HIV infected individuals with exogenous IL-15 restored defects in activation, IFN-γ production, and direct cytotoxicity in HIV-specific memory CTLs [171, 175]. Addition of IL-15 to PBMC *ex vivo* modulates the production of the β-chemokines RANTES, MIP-1α, and MIP-1β from $CD4^+$ and $CD8^+$ T cells in HIV infected treatment-naïve, ART responding, and ART failing individuals [176]. Exogenous IL-15 has been shown to salvage fragile lymphocyte populations during HIV infection, enhancing

prolonged survival in culture [175]. Treatment of chronic SIV infected macaques with systemic IL-15 enhanced $CD4^+$ Tem cell function and proliferation, and promoted extralymphoid tissue emigration of T lymphocytes [177]. In conjunction with restoring function, systemic IL-15 treatment during chronic SIV infection bolsters $CD8^+$ Tem and NK cell populations, without affecting $CD4^+$ T cell or macrophage numbers or increasing viral loads [178].

The ability of IL-15 to promote the development of cellular memory and T_H1 immunity makes it an ideal adjuvant for vaccination against intracellular pathogens. Immunization studies in the murine model have demonstrated that co-immunization of plasmid DNA encoding HIV *gag* with an optimized *IL-15* construct enhanced the magnitude of the Ag-specific IFN-γ response over that seen with Ag alone [172]. Additionally, use of IL-15 as an adjuvant promoted superior Ag-specific immunity when compared to IL-2, inducing a more potent and durable Ag-specific $CD4^+$ and $CD8^+$ T cell responses in both murine and nonhuman primate immunization models [179, 180]. Recent studies have demonstrated that delivery of plasmid encoding an optimized *IL-15* in conjunction with a DNA-based multigene SIV vaccine in macaques induced robust antiviral IFN-γ production from $CD4^+$ and $CD8^+$ T cells, enhanced proliferative responses, and provided more rapid control and protection from a SIV/HIV-hybrid virus, SHIV89.6P, challenge over animals receiving SIV vaccination alone [181]. Interestingly, immunization utilizing plasmid SIV *gag* DNA alone in conjunction with plasmid IL-15 co-immunization failed to contain viral SHIV89.6P challenge due to minimal development of cellular and humoral immunity [182]. Finally, recent findings have demonstrated that sequential administration of IL-12 followed by IL-15 plasmid DNA enhanced dual-functional antiviral cytokine responses induced through multigene plasmid DNA immunization during ART-treated, chronic SIVmac251 infection [183].

1.6 ADAPTIVE IMMUNITY TO HIV INFECTION

1.6.1 Humoral Immunity

The humoral immune response to HIV infection is marked by the gradual development of highly functional antibodies (Ab) in conjunction with ongoing mutation and sophisticated viral

escape mechanisms limiting immunologic control. Conventional models have suggested that upon HIV exposure, naïve B cells will take up to four months of maturation and IgG receptor mutation in lymph node germinal centers to develop into plasma cells capable of high avidity Ab production. Recent studies have demonstrated the presence of functional neutralizing antibody (NAb) early during acute HIV infection, dispelling conventional thought that immune control of acute viremia is exclusively based upon cellular immunity [184-186]. Free virion neutralization occurs through Ab-mediated interference with viral fusion and subsequent cellular entry, or by blocking viral uncoating and subsequent replication [187]. HIV-specific NAbs function through binding viral elements necessary for association with the CD4 receptor, the cellular coreceptors CCR5 and CXCR4, or cell-virion fusion domains exposed during conformational shifts necessary for membrane fusion [188]. Within weeks, humoral responses directed against viral structural proteins develop, primarily targeting the gp120 surface unit and gp41 transmembrane region of HIV envelope. Paradoxically, only a small fraction of Ab epitopes in exposed envelope trimers elicit potent neutralization, with conformational and glycosylation-based shielding of functionally optimal epitopes evading immune recognition [189]. Ultimately, the magnitude and breadth of NAb activity increases over time, with robust responses acquired only during chronic infection. Furthermore, immunologic pressure from NAbs drives viral evolution, as NAbs preferentially recognize viral strains preceding their development, but are less effective at targeting contemporary viral isolates [190]. Passive administration of broadly NAbs either systemically or at the vaginal mucosa has been shown to prevent infection of nonhuman primates with a SHIV virus containing the HIV envelope upon an SIV backbone [191-194]. Although viral immune evasion due to selective NAb pressure serves as the most conclusive evidence for the protective role of humoral immunity, studies utilizing passive NAb immunization early, during acute HIV infected humans and nonhuman primates infected with pathogenic SIV strains have demonstrated modest reductions in plasma viremia and rapid viral mutation, further illustrating the utility of focusing NAb responses [192, 195, 196]. By contrast, passive immunization utilizing NAb fails to reduce established, chronic infection, rapidly selecting for viral escape variants [194]. Taken together, these findings illustrate that although the humoral immune response plays a key role in viral suppression during acute and choric HIV/SIV infection, Ab-mediated immunity alone is insufficient to contain viremia during established infection.

1.6.2 Cellular Immunity

Despite massive efforts, the precise correlates of protective immunity against HIV infection remain elusive. While merely one component of the complex and coordinated response that will be necessary to contain HIV infection, the ability of cellular immunity to ameliorate HIV/SIV infection has been well documented. Early findings demonstrated that the reduction in acute HIV viremia is temporally associated with the appearance of antiviral $CD8^+$ T cell-mediated immunity [105, 106]. Studies depleting $CD8^+$ T cells in the rhesus macaque model have correlated the development and maintenance of an adaptive CTL response with control of acute SIV viremia [107, 197, 198] and containment of chronic set point viral loads [199]. Furthermore, containment of rebound SIV viremia following cessation of ART during STI has been associated with robust sustained SIV-specific cellular immunity induced through therapeutic immunization [200, 201]. The protective role of cellular immunity during HIV infection is further supported by the finding that HIV-infected individuals homologous at any of three different loci in human leukocyte antigens (HLA) alleles, limiting T cell repertoire and viral epitope recognition, undergo accelerated disease progression [202]. Interestingly, HLA diversity by itself does not account for virologic control, as qualitative distinctions have been made between long-term non-progressors carrying the HLA-B57, HLA-B27, and HLA-B51 alleles and rapid progressors presenting HLA-B35, illustrating inherent qualitative differences in the ability of epitope-specific T cell responses to contain viremia [203-205].

T cell-dependant restriction of viral infection occurs through the production of soluble mediators, such as inhibitory β-chemokines and cytokines, as well as the direct cell-mediated lysis and induced apoptosis of viral infected cells [206, 207]. Detection of an infected cell through the recognition of major histocompatibility complex (MHC) class I-bound viral peptide by the specialized TCR and co-stimulation signals of an activated CTL initiates the vectorial release of perforin and granzymes A and B to initiate target cell apoptosis [206]. TCR ligation subsequently induces the release of IFN-γ and TNF-α, T_H1 cytokines with broad spectrum antiviral activity including: recruitment of macrophages, NK cells, and T cells, polarization of T cell responses to T_H1 antiviral cytokine profiles, and the upregulation of MHC class I molecules, Ag processing, and Ag transport within locally infected cells [208]. Cellular cytokine-mediated viral clearance is facilitated by the activation of host cellular mechanisms to interrupt viral

replication, such as activation of the RNAse L or PKR pathways to degrade single and double-stranded viral RNA, respectively [208]. Additionally, activated T cells contribute to bystander killing through the production and upregulation of Fas-ligand, allowing for the induction of apoptosis through contact-dependant Fas-FasL-mediated signaling of both infected cells and those in the local vicinity [209]. Generally, MHC class I-restricted CTLs kill predominantly through the perforin/granzyme pathway, whereas $CD4^+$ T cells rely primarily on Fas-FasL-dependant apoptosis due to the limited target cell expression of MHC class II molecules [209]. Additionally, $CD4^+$ T helper function is essential in the propagation and maintenance of both central and effector memory lymphocyte compartments.

HIV/SIV containment has traditionally been associated with the overall magnitude of the Ag-specific $CD8^+$ CTL and $CD4^+$ T helper response, conventionally measured through IFN-γ production and target cell lysis [210-212]. Additionally, increased breadth of $CD8^+$ T cell-restricted epitopes targeted has been associated with viral control at all stages of infection [213]. Recently, our understanding of the composition of T cell-mediated immunity has expanded, as Ag-specific responses from $CD4^+$ Tcm cells were shown to have dramatic influence over HIV/SIV disease progression during natural infection, therapeutic intervention, and vaccine-mediated protection [46, 214]. Concurrently, the ability of memory $CD4^+$ and $CD8^+$ T cells to proliferate upon HIV/SIV stimulation, allowing for repopulation of effector T cells and regeneration of $CD4^+$ T helper support, is associated with reduced viremia [46, 215]. These factors are intrinsically linked to the ability of $CD4^+$ Tcm cells to differentiate, expand, and produce IL-2, regenerating the $CD4^+$ Tem and sustaining memory $CD8^+$ T cell levels [46, 215, 216]. Yet, these factors alone are insufficient for viral containment, as individuals with progressive AIDS have been identified with potent and broad T cell-mediated immune responses. Detailed evaluation of cellular immunity has revealed that not all responses are created equal. Studies involving treatment-naïve HIV-positive individuals have associated Gag-specific $CD8^+$ T cell-mediated IFN-γ production with superior control of plasma viral load, and Env-specific responses with lack of virologic containment, suggesting an immunologic advantage in targeting evolutionarily constrained viral epitopes [217]. Similarly, CTL responses mediated by the rare HLA-B*1503 allele were found to control HIV clade B infection when subdominant epitopes were presented, but failed to control clade C infection in the absence of subdominant epitope presentation [218]. Furthermore, the diversity of the antiviral effector

cytokine profile has been implicated in HIV elite controllers, individuals who innately control HIV viremia, and ART responders, with heterogeneic polyfunctional profiles including IL-2, IFN-γ, TNF-α, CD107, MIP-1α, and MIP-1β associated with superior viral restriction [219-222]. Taken together, these findings suggest that the qualitative natural of the cellular immune responses against HIV is equal to, if not more important than its magnitude.

1.7 HIV IMMUNE EVASION

HIV replication results in roughly 10^9 new virions produced each day, with an estimated viral mutation rate of approximately one nucleotide alteration in every 10^5 bases due to the lack of proofreading activity by the reverse transcriptase enzyme [223]. Containing a viral genome of 10^4 bases, a mutation may be expected to occur at least once at every possible location in the HIV genome each day. With such extreme evolutionary flexibility, HIV rapidly evolves to escape selective immunologic pressure. Viral escape from CTL-mediated elimination occurs through mutations in epitope sequences, ablating peptide presentation, antigenic processing, and subsequent recognition by $CD8^+$ T cells [224]. CTL escape has been shown to occur rapidly during acute infection, as well as continually throughout progressive disease in both chronic HIV and SIV infection [225-228]. Following SIV infection of five sibling macaques, animals capable of specific Env and Nef CTL epitope recognition had potent immunologic responses to infection with concomitant CTL escape mutations, but prolonged survival, whereas related animals lacking effective CTL recognition profiles during primary infection progressed rapidly, illustrating the associated cost in viral fitness following CTL escape [226]. Similarly, rapid viral escape was observed *in vivo* following adoptive transfer of clonally expanded Nef-specific CTLs into an HIV-infected patient, demonstrating the clinical relevance of the escape phenomenon [229].

In contrast to the altered processing and presentation mechanisms seen in CTL escape, viral escape from Ab-mediated humoral immunity is predominantly facilitated by physical occlusion and changes in structural confirmation, ablating Ab epitope recognition [190]. Ab-mediated selective pressure was demonstrated *in vitro* following growth of HIV in the presence of the gp120 targeting NAb G3-4, leading to rapid viral variants expressing single amino acid

substitutions ablating Ab epitope recognition [230]. Not surprisingly given the precise conformational dependence of Ab epitope recognition, single amino acid changes in gp41 were shown to alter the exposure of multiple NAb binding domains in gp120, and amino acid substitutions in envelope have been shown to cooperate in reducing the overall sensitivity to neutralization in the presence of neutralizing antiserum [231-233]. Escape of the molecular clone SIVmac239 was demonstrated *in vivo* following the development of strong Ab-mediated selective pressure, leading to a heightened rate of nonsynonomous substitutions within the variable regions of the envelope protein [234]. Finally, numerous reports of HIV escape following selective humoral immune pressure have been noted clinically. Dramatic evidence of successful viral evasion was demonstrated by the reduced ability of antiserum from the latter stages of infection to neutralize contemporary viral isolates, suggesting ineffective humoral immunity [184, 186, 235-237]. Concurrent with immune evasion, escape mutations incur a cost on the collective evolutionary fitness of the viral isolate, reducing replicative capacity and quickly reverting in the absence of immunologic pressure [238-240]. Although epitope escape undermines immunologic control of viral replication, the associated reduction in viral fitness may balance the overall pathologic detriment to the individual.

1.8 HIV VACCINES

1.8.1 Traditional Vaccine Limitations

The goal of a conventional vaccine is to present innate, unaltered Ag from the pathogen of interest in a manner that promotes local immunity and subsequently establishes durable humoral and cellular memory responses capable of preventing disease pathogenesis. The most effective vaccines to date have been comprised of live, attenuated pathogens as used in the oral polio, BCG tuberculosis, and yellow fever vaccines, along with chemically inactivated organisms utilized in vaccines against influenza, cholera, and hepatitis A. Immunization utilizing protein subunits of target Ag, such as the hepatitis B surface Ag, have also proven effective, albeit with reduced immunogenicity, necessitating multiple rounds of vaccine boosting. Regrettably, these approaches have largely failed against –immunodeficiency virus infections. Over fifteen years

ago, researchers demonstrated that nonhuman primates immunized with a live attenuated form of pathogenic SIVmac239 containing a largely deleted *nef* gene were capable of resisting infection from homologous wild-type SIVmac239 as well as heterologous SIVmac251 [241]. Although initial findings suggested that live attenuated SIV vaccines were well tolerated, further vaccination studies in infant and adult nonhuman primates demonstrated that attenuated viruses retained virulence, with progressive disease associated with continuous low-level viremia [242-244]. These findings were corroborated clinically after a small cohort of individuals in Australia developed progressive disease following transfusion-borne infection with a *nef*-deficient HIV strain [245, 246]. Similarly, immunization utilizing the virulence-attenuated SIVmac-$_{1A11}$ strain was shown to prevent early disease but not infection from a pathogenic SIV strain [247]. Formalin-inactivated whole virus immunogens have provided effective immunity in the SIV model of nonhuman primate infection, but protection was restricted to near homologous challenge strains [248]. Nearly a decade later, the first phase III HIV vaccine trial advanced the bivalent recombinant gp120 subunit protein immunization into clinical evaluation [249]. Although initially touted as a success after conferring a level of protection upon chimpanzees infected with HIV-IIIB [250], VaxGen's subunit protein immunization provided no significant protection from HIV infection in clinical trials [251, 252]. Failure to induce protective immunity was due to the inability of subunit protein gp120-based immunization to induce high-titer Ab, relevant NAb responses, or effective antiviral cellular immunity. The inherent flaws of conventional vaccine modalities necessitate the creation and testing of novel immunization strategies against HIV infection.

1.8.2 Novel Vaccine Strategies

1.8.2.1 DNA Vaccines

DNA vaccines consist of a foreign pathogen-associated gene of interest, often optimized for expression in eukaryotic cells, which is cloned into a bacterial plasmid [253]. Advancements in DNA vaccine delivery, including liposome-mediated, electroporation, and gene guns systems, in conjunction with affordable production and stable room temperature storage make DNA vaccines attractive candidates for the treatment of HIV. Furthermore, DNA vaccination is capable of inducing both humoral and cellular Ag-specific immunity, largely due to potent

transfection of APCs and subsequent stimulation of both $CD4^+$ and $CD8^+$ T cells. Unfortunately, naked DNA has limited immunogenicity. The innate unmethylated cytidine-phosphate-guanosine (CpG) dinucleotide motifs of the plasmid vector may be augmented through inclusion of additional toll-like receptor agonists, cytokines, co-stimulatory molecules, and classical vaccine adjuvants to increase DNA vaccine immunogenicity [254]. As DNA vaccines fail to elicit vector-specific immunity, multiple booster immunizations may be delivered to further enhance immunity. DNA immunization in nonhuman primates elicited robust immunity. IL-2 cytokine augmented DNA-based vaccination expressing SIV *gag* and HIV *env* was able to protect monkeys against a minimally pathogenic, less stringent SHIV89.6 challenge, associated with the induction of Ag-specific CTL expansion and proliferation along with NAb responses [210]. In contrast, DNA vaccination has shown limited efficacy against pathogenic SIVmac251, SIVsmE660, or SIVmac239 infection, in light of substantial immune induction [255, 256]. Clinical trials utilizing multiclade DNA immunization targeting the HIV *gag, pol, nef,* and *env* genes have demonstrated the induction of mediocre antiviral CTL responses (~50%), but strong lymphoproliferative responses against HIV Gag protein (~93) [257].

1.8.2.2 Poxvirus-Based Vectors

Recombinant poxvirus-based vectors confer the ability to efficiently infect a wide variety of host cells, possess ample room for transgenic inserts, and elicit natural innate and adaptive immune response against an intracellular viral pathogen. To avoid safety concerns associated with replicating vaccinia vectors in potentially immunocompromised populations, poxvirus vectors such as MVA and the avian canarypox (ALVAC) and fowlopx have been engineered to undergo an abortive replication cycle in human cells. Problematic in all recombinant viral systems, host cell infection by poxvirus-based vectors produces both viral and transgenic proteins, leading to the development of vector-specific immunity capable of inhibiting immunogenicity upon homologous boosting. Initial immunization studies in chimpanzees and macaques demonstrated the ability of poxvirus-based vectors, particularly MVA, to induce potent $CD4^+$ and $CD8^+$ T cell-mediated immunity, as well as NAb responses to SIV proteins [258-260]. Yet, similar to other vaccine modalities, poxvirus-based vaccination has proven effective in protecting against the virulence attenuated SHIV89.6 strain only [261], while they failed to provide prolonged containment of pathogenic SIV species such as SIVsmE660, even

when administered to Mamu-A*01-expressing animals prone to viral control [262-264]. Notably, recent immunization studies have suggested the MVA and NYVAC vectors may be superior in the induction of qualitatively advantageous, polyfunctional cellular immune responses consisting of perforin and granzyme expression, production of IL-2, IFN-γ, TNF-α, and MIP-1β, and the marker for degranulation, CD107a [265]. Currently, poxvirus-based vaccines are being evaluated in clinical efficacy trials against HIV, but have demonstrated lower immunogenicity than expected with ALVAC-induced Gag-, Pol-, and Env-specific CTL responses seen in roughly 50% of vaccinees [266, 267].

1.8.2.3 Virus-Like Particle Vaccines

As HIV/SIV-specific cellular immunity can ameliorate but not prevent infection, a putative AIDS vaccine will optimally promote strong humoral immunity in conjunction with T cell-mediated responses. Virus-like particles (VLPs) are uniquely suited for the induction of humoral immunity, as they present functional, conformationally relevant virion-associated viral envelope and structurally proteins in a safe context devoid of the viral RNA genome [268]. HIV and SIV VLPs self-assemble following expression of *gag* and *env* genes in permissive cell lines, creating mature particles expressing high levels of viral Env and capable of budding at the cell surface [269, 270]. In addition, VPLs bind cellular receptors facilitating virion fusion and entry into host cells expressing $CD4^+$ and the appropriately matched CXCR4 or CCR5 coreceptor for the particular VLP Env [271]. Intranasal immunization of mice with SIV VLPs was shown to induce mucosal IgA and systemic IgG responses, as well as T cell-mediated release of IFN-γ and IL-4 in cervical lymphocytes and splenocytes, which were enhanced through the addition of a cholera toxin adjuvant [272]. Incorporation of HIV Env proteins with reduced glycosylation and selective deletions in the variable loops has lead to enhanced immunogenicity of these vectors [273]. Immunization with DNA plasmids sufficient for VLP formation *in vivo* induced strong NAb responses and CTL-mediated killing of target cells in *Macaca fascicularis*, leading to partial control of SIVmne challenge as seen by reduced cell free and cell-associated virus detection and inhibition of $CD4^+$ T cell loss [274]. Given their likeness to the highly effective live-attenuated or inactivated particle vaccines for SIV, VLPs contain great potential as HIV immunogens [275].

1.8.2.4 Heterologous Prime-Boost Immunizations

Plasmid DNA, VLPs, and recombinant viral vectors are each associated with unique profiles of immune induction, as well as limitations for clinical use. Combining heterologous vaccination systems allows for induction of tailored and often superior immune responses while circumventing restrictions such as vector-specific immunity and lack of immunogenicity. Currently, DNA priming in conjunction with viral vector boosting has shown promise. The DNA priming component of this regimen is capable of establishing more robust humoral immune responses, both NAb and quantitative Ab titer, then viral vector priming, and provides more even expansion of $CD4^+$ and $CD8^+$ T cell responses to Ag. The limited immunogenicity of DNA vaccines can be combated with multiple boosts, adjuvants, and cytokine but will provide only modest cellular immunity. In contrast, viral vector priming, most commonly with Ad or MVA, establishes broad and potent T cell-mediated immunity, but rarely induces strong antiviral humoral immunity due to predominant intracellular delivery and production of vector-associated transgenes. Although numerous vaccination regimens have protected against inadequately pathogenic SHIV89.6P challenge, a finding skewed by overrepresentation of Mamu-A*01-expressing animals in immunization groups, a study comparing vaccination utilizing DNA, MVA, or Ad5 either alone or in combinations, demonstrated a roughly 5 fold enhancement of Ag-specific tetramer positive $CD8^+$ T cells in DNA primed/Ad5 boosted groups as compared to other regimens [211]. Follow up investigations correcting for macaque genetic variability found that homologous Ad5 immunization was superior at increasing the magnitude of Ag-specific tetramer positive and IFN-γ production, but did not control pathogenic intrarectal SIVmac239 challenge to the extent of DNA/Ad5 regimen, suggesting a qualitative immunologic advantage of the heterologous prime-boost regimen [276]. Similar studies demonstrated that DNA/Ad5 prime-boost regimens could mitigate acute and chronic infection upon low-dose SIVmac239 challenge through Ag-specific cellular immunity and retention of T cell memory and $CD4^+$ T cell populations alone in Mamu-A*01-expressing macaques [277]. Furthermore, DNA/Ad5 prime-boost regimens have been shown to preserve memory $CD4^+$ Tcm populations in macaques following pathogenic SIVmac251 infection, correlated with prolonged survival [278]. Similarly, studies evaluating DNA/poxvirus prime-boost strategies have demonstrated increased peak frequencies of Ag-specific tetramer positive and IFN-γ producing T cells in animals receiving DNA priming followed by MVA, fowlpox, or recombinant vaccinia boosting, but control of the

virulence attenuated SHIV89.6P was achieved following either homologous DNA alone or in conjunction with viral vector boosting [279].

1.8.3 Therapeutic Vaccination

As a preventive vaccine is currently unavailable, diverse therapeutic strategies aimed at prolonging asymptomatic HIV infection and optimally inducing sterilizing host immunity are being pursued. The most successful therapeutic vaccination studies to date have demonstrated a one thousand fold decreases in chronic SIV plasma RNA and reductions in cell associated DNA through repeated immunization of macaques with aldrithiol-2 inactivated, SIV-pulsed autologous DCs in the absence of ART [280]. Preliminary human immunization trials utilizing DCs loaded with aldrithiol-2 inactivated autologous virus achieved an 80% median reduction among all recipients for roughly four months, with 8 of 18 subjects containing plasma viremia for greater than one year post-vaccination without ART [281]. This study suggests that the nature of antigenic presentation to the immune system may dramatically influence the host's ability to respond to HIV/SIV infection. Practical application of a therapeutic HIV vaccination will occur during ART suppressed viremia, commonly after acute infection has impaired host immunity. During ART intervention, studies have demonstrated that poxvirus-based therapeutic vaccination of macaques during chronic SIVmac251 infection restores broad Ag-specific CTL and lymphoproliferative responses, absent during ART alone [201, 282, 283]. Furthermore, transient SIVmac251 suppression was achieved in 75% of macaques upon STI following poxvirus-based therapeutic vaccination during a six month course of ART, associated with enhanced Ag-specific lymphoproliferation and CTL target lysis [200].

1.9 ADENOVIRAL VECTORS

1.9.1 Adenovirus-Based Vaccination

Adenoviruses are medium sized (90-100nm), non-enveloped, icosohedral viruses containing a linear, non-segmented, double-stranded DNA genome of roughly 35 kilobase pairs. There are 51 serologically distinct types of human-associated adenovirus, falling into six subgenera (A-F), with several serotypes capable of causing respiratory illness, gastroenteritis, or conjunctivitis. Adenoviruses infect a broad range of both resting and dividing cell types, including potent APCs such as myeloid and plasmacytoid DCs [284]. Deletion of the adenoviral E1 and E3 genes eliminates replication capacity and MHC downregulation, respectively, and provides room to insert a foreign transgene of interest, such as a vaccine Ag. Codon optimization of transgenic inserts and inclusion of heterologous regulatory elements such as the high-output CMV immediate/early promoter increase transgenic protein production [285, 286]. Recombinant infectious adenovirus is then created by co-transfecting a packaging cell line engineered to express Cre recombinase with the adenoviral backbone (Adψ) DNA and a plasmid containing the nonviral transgene of interest (pAdlox) [287]. Cre recombinase can catalyze recombination between the Adψ containing E1 flanking *loxP* sites and pAdlox containing transgene flanking *loxP* sites in a two step process, removing the packaging site from Adψ then transferring the recombinant genes from pAdlox, creating E1-substituded Ad vectors [287]. Additionally, Cre-*lox* recombination supplies negative selection pressure on non-recombinant viruses, allowing for rapid outgrown of desirable recombinants [287]. Recombinant adenovirus is easily grown and purified from cell culture, allowing for rapid and affordable mass production.

1.9.2 Immunogenicity of Ad-Based Vaccines

The utility of Ad-based vectors as HIV vaccines lies largely in their ability to induce T_H1 cellular immune responses, paramount in protection from intracellular pathogens. Immunogenicity studies in nonhuman primates have demonstrated the efficiency of recombinant Ad-based vectors to induce robust INF-γ ELISPOT responses and target cell lysis, as well as modest NAb titers in an escalating dose-dependent manner [288]. Comparative analysis of

DNA, MVA, and Ad-based immunizations against the SIV Gag protein found that Ad-based vectors promoted the highest quantitative IFN-γ production from Ag-specific T cells amongst the regimens tested [288]. Ad-based vaccination against the SIVmac239 Gag protein induced high-frequency $CD4^+$ and $CD8^+$ T cell-mediated IFN-γ responses against both dominant and subdominant epitopes that were later recalled upon heterologous challenge with pathogenic SIV/DeltaB670 [289]. Although arguably superior in promoting the overall magnitude of T_H1 cellular immunity, Ad5-based vaccination has been shown to preferentially elicit $CD8^+$ T cell-mediated production of IFN-γ and TNF-α, either individually or combined, potentially due to high-dose vector-driven terminal differentiation of both Ag-specific $CD4^+$ and $CD8^+$ T cells [290-293]. In contrast, recent studies providing detailed characterization of the cytokine profiles elicited by the rare serotype Ad26- and Ad48-based vectors in rhesus macaques demonstrated a significant enhancement of polyfunctional IFN-γ^+TNF-α^+IL-2^+ and overall IL-2 production from both $CD4^+$ and $CD8^+$ memory T cell populations as compared to immunization utilizing Ad5-based vectors [294]. Given that DCs have been shown to express CD46, the receptor facilitating entry of subgroup B and D Ad, but not the coxsackievirus and adenovirus receptor (CAR) utilized by Ad5, suggests distinct biologic pathways of Ad infection which may potentially influence the dynamics and character of immune induction [295-297]. As recent findings have implicated polyfunctional cellular immune responses in vaccine efficacy and HIV disease control, methods to improve on the potency of Ad-based immunization must be explored [292].

1.9.3 Limitations of Adenoviral Vectors

Inherent in the use of viral vectors as immunogens are the natural humoral and cellular antiviral immune responses directed against the vector virions and derived proteins. Ad-specific immune responses are manifested by an initial Ab-mediated clearance targeting the capsid hexon, fiber, and penton base proteins, followed by T cell-mediated elimination of cells expressing Ad proteins prior to and following novel Ad protein synthesis [298-301]. As expected, NAb responses are generally serotype specific, whereas $CD4^+$ and $CD8^+$ T cell-mediated immunity targeting homologous sequences in structural proteins can be cross-reactive, with subgroup C primed $CD4^+$ T cell-clones proliferating in responses to subgroup A, B, and C exposure [302]. In as much, the presence of pre-existing Ad-specific immunity dramatically

reduces the immunogenicity of the vaccine vector, attenuating its efficacy [288, 303]. Therefore, the prevalence of pre-existing vector-specific immunity in various populations will determine the potential utility for serotype-specific Ad-based vaccination regionally [304]. Pre-existing NAb responses against the common Ad5, subgroup C, were found in over 80% and 35% of adults in Africa and the United States, respectively, whereas NAb titers against rare Ad35, subgroup B, were present in roughly 2.5% and 6% of these same populations, respectively, with similar seroprevalence among HIV infected and uninfected individuals [298, 305, 306]. Ad-specific NAb responses were shown to develop rapidly, with infants in Sub-Saharan Africa developing an 80% seroprevalence of Ad5-specific Abs by 18 months of age [307]. Given these limitations, novel approaches at circumventing pre-existing anti-Ad immunity, such as vaccination with Ad transduced DCs, are being evaluated [308].

1.9.4 Heterologous Adenoviral Immunization

To circumvent the limitations of pre-existing vector-specific immunity, Ad vectors of rare prevalence in human populations with unrelated serology are being utilized in heterologous prime-boost regimens. Development of novel, alternative vectors based on rare human Ad serotypes from subgroup B (Ad11, Ad34, Ad35, Ad50), subgroup C (Ad6), and subgroup D (Ad24, Ad26, Ad48, Ad49) and nonhuman ovine, bovine, porcine, and chimpanzee serotypes (AdC1, AdC6, AdC7, AdC32, AdC33, AdC68) have been established to compliment traditional Ad5-based vectors (subgroup C) [304, 309-317]. Early immunogenicity studies in mice demonstrated that rare serotyped vectors, specifically Ad35, AdC6, and AdC68 are capable of boosting transgene-specific immunity either in conjunction with Ad5 or as independent prime-boost regimens in the presence of pre-existing Ad5-specific NAb titers [311, 318, 319]. It was later shown that even the rare serotyped Ad11- and Ad35-based vectors, both subgroup B, were susceptible to low-level cross-reactive vector-specific NAb targeting regions of homology in the hexon hypervariable regions [318]. Consistent with these findings, vaccination of mice with a subgroup B/D combination Ad35- and Ad49-based prime-boost regimen proved more immunogenic than utilizing multiple Ad vectors within the same subgroup, demonstrating that even rare vectors must be sufficiently distinct to avoid cross-reactive immunity and optimal immunogenicity [320]. Immunization studies against HCV in macaques utilizing heterologous

Ad6/AdC32 regimens provided durable $CD4^+$ and $CD8^+$ T cell-mediated immunity susceptible to alternative serotype boosting 2 years after primary vaccination [321]. However, recent immunogenicity studies in the nonhuman primate have demonstrated that Ad vectors based on serotype 5, 26, and 48 individually promote unique, vector-specific cellular immune induction profiles which influence the subsequent ability of heterologous Ad-based boosting [294]. Notably, this report suggests that Ad5-based vectors promote restricted transgene-specific cellular immunity consisting of $CD8^+$ T cell dominated responses devoid of IL-2 production which limit the capacity of heterologous Ad-based boosting to alter the functionality of Ag-specific cellular immunity [294]. Combined, these findings illustrate the potential utility of heterologous Ad-based vaccination to optimally induce HIV-specific immunity, and are among the leading vaccine candidates for the induction of T cell-mediated immunity.

1.10 PUBLIC HEALTH SIGNIFICANCE

In the absence of a preventive vaccine for HIV infection, alternative methods for the treatment of HIV should be explored. The prohibitive cost, development of drug-resistant virus, and inability to induce sterilizing immunity to HIV infection are limiting factors to continual ART. Transient immune reconstitution and therapeutic vaccination during ART suppressed viremia may provide the necessary stimulation to eradicate the persistent viral reservoirs responsible for eventual treatment failure. Our immunotherapy proposes a temporary treatment of –immunodeficiency virus infection, eliminating the cost and duration of current treatment models with the potential for persistent disease containment. Therapeutic vaccine-mediated reductions in viremia would inhibit HIV pathogenesis, preventing disease progression while decreasing the potential for further viral transmission [322, 323].

2.0 HYPOTHESIS AND SPECIFIC AIMS

The cellular immune response to HIV infection is implicated in controlling acute viremia, containing set-point chronic viral loads, and establishing durable suppression of disease associated pathogenesis allowing for long-term non-progressor status and extended survival without pharmacologic intervention. Therefore, a prophylactic vaccine aimed at preventing HIV infection or a therapeutic vaccine used to inhibit disease progression and transmission should promote high frequency, qualitatively superior T cell-mediated antiviral immunity. Recombinant Ad-based vectors are among the preeminent vehicles for the induction of transgene-specific cellular immunity, but have been limited by the development of vector-specific immunity. Utilizing novel Ad35-based vectors in conjunction with conventional Ad5-based vectors, we have examined the capacity of heterologous Ad-based immunization to induce efficacious antiviral cellular immunity in the nonhuman primate model of HIV infection. We hypothesize that dual-serotype Ad-based vaccination will elicit superior cellular immune responses over those attained by homologous Ad-based vaccination, and that in a model of therapeutic intervention, heterologous vaccine induce immunity will be capable of controlling chronic viremia. The specific aims of this project are:

Aim 1: To measure the potential for novel Ad35-based vectors to enhance transgene-specific cellular immunity previously induced through conventional Ad5-based vaccination. Hypothesis: Serologically distinct Ad35-based vectors will be capable of enhancing cellular immune responses induced through Ad5-based vaccination due to lack of vector-specific Ab-mediated cross-neutralization. In a proof-of-concept trial, rhesus macaques received four priming immunizations utilizing Ad5-based vectors encoding the SIVmac239 *gag* transgene, followed by boosting with either continual Ad5-based vaccination or Ad35-based vectors. Vaccinated and naïve control animals were then challenged with pathogenic SIV/DeltaB670. To

evaluate the magnitude of vaccine-induced cellular immunity between groups, longitudinal IFN-γ ELISPOT analysis was performed. IFN-γ ELISPOT matrix analysis and fine epitope mapping were utilized to provide detailed characterization of the Ag-specific breadth elicited by vaccination and in responses to infection. MHC restriction of identified epitopes was performed through IFN-γ ELISPOT following magnetic bead depletion of $CD4^+$ and $CD8^+$ T cells from peripheral blood mononuclear cells (PBMC). Subsequently, SIV immune evasion from vaccine-induced T cell-mediated responses was evaluated by comparison of sequence analysis from plasma viral isolates with known previously identified epitope targets.

Aim 2: To evaluate the efficacy of therapeutic dual-serotype Ad-based vaccination during ART-treated chronic SIV infection to limit rebound viremia following STI through the induction of antiviral cellular immunity. Hypothesis: Immunotherapeutic vaccination utilizing Ad5- then Ad35-based vectors during established SIV infection will augment antiviral T cell-mediated immunity capable of restricting rebound viremia following ART cessation. Building upon our previous trial, we next examined the potential of Ad5/Ad35-based delivery of the codon optimized SIVmac239 *gag, env,* and *nef* transgenes, with or without *IL-15* augmentation, to boost cellular immunity during ART-treated SIVmac251 infection. Ag-specific cellular immune responses to Gag, Env, and Nef proteins in PMBC were longitudinally measured by IFN-γ ELISPOT. Subsequently, multiparametric flow cytometry was utilized to measure production of the T_H1 cytokines IFN-γ, TNF-α, and IL-2 in memory populations of $CD4^+$ and $CD8^+$ T lymphocytes from PBMC and bronchoalveolar lavage (BAL) samples at timepoints of peak response. Fluorescence-activated cell sorting (FACS) analysis and cellular quantitation was performed to evaluate absolute memory $CD4^+$ and $CD8^+$ T cells in peripheral blood, and total percentages of $CD4^+$ T cells in BAL throughout infection. Plasma viral RNA levels were measured, and immune correlates of viral control were identified.

Aim 3: To assess the ability of immunotherapeutic vaccination with or without IL-15 augmentation to effect the dynamics of T cell phenotype and proliferation systemically and at the lung mucosa. Hypothesis: Administration of Ad-based immunotherapeutic vaccination will promote peripheral expansion and proliferation of $CD4^+$ and $CD8^+$ T cell memory populations that will be further enhanced upon IL-15 delivery. Throughout therapeutic

intervention, we utilized multiparametric FACS analysis to longitudinally monitor alterations in $CD4^+$ and $CD8^+$ T cell memory phenotype and proliferation following SIVmac251 infection, ART treatment, and dual-serotype Ad-based immunization. Cellular proliferation was determined by Ki-67 detection in PBMC and BAL samples, comparing immunized animals with mock vaccinated or non-infected controls, respectively. Absolute cell enumeration was performed in PBMC and population percentages examined in BAL samples.

3.0 CHAPTER ONE: HETEROLOGOUS ADENOVIRUS-BASED VACCINATION INDUCES ROBUST ANTIGEN-SPECIFIC CELLULAR IMMUNITY WHICH IS RECALLED UPON SIV INFECTION

Adam C. Soloff[1,3], Wentao Gao[5], Xiangdong Liu[1,3], Kevin N. Brown[1,3], Edward Nwanegbo[5], Premeela A. Rajakumar[6], Paul D. Robbins[6], Michael Murphey-Corb[1,6], Richard D. Day[1,2], Andrea Gambotto[3,5,6,7], and Simon M. Barratt-Boyes[1,3,4]

Departments of Infectious Diseases and Microbiology[1], and Biostatistics[2], Graduate School of Public Health, and Center for Vaccine Research[3], Departments of Immunology[4], Surgery[5], Molecular Genetics and Biochemistry[6], and Medicine[7], School of Medicine, University of Pittsburgh, Pittsburgh, PA. 15261 USA.

This chapter is adapted from a publication of the *Journal of General Virology*, 2006, Volume 87, pages 139-149 to reflect my contributions to the completed study.

3.1 PREFACE

The following chapter is in fulfillment of specific aim 1. The described study was a large scale collaborative effort within the University of Pittsburgh involving the Barratt-Boyes lab for immunologic analysis, the Viral Vector Core for creation of adenoviral vectors, and the Primate Facility for Infectious Disease Research for animal procedures and maintenance. Specifically, Adam C. Soloff performed all ELISPOT analysis subsequent to wk 22 post-vaccination, roughly 100 wks, and assisted in the study design, analysis, and manuscript preparation. This work included longitudinal determination of Ag-specific responses, evaluation of $CD4^+$ and $CD8^+$ T cell-mediated fractional response through T cell-specific Ab depletion, identification of epitope targets, and additional fine mapping of precise epitope sequences. Kevin Brown performed partial Ag-specific ELISPOT analysis from study initiation to wk 22 post-vaccination. Xiangdong Liu carried out RT-PCR assays for viral sequencing. Premeela A. Rajakumar and Michael Murphey-Corb provided viral load measurements. Edward Nwanegbo preformed Ad-specific NAb assays. Richard D. Day ran statistical analysis on viral load measurements. Wentao Gao, Paul D. Robbins, and Andrea Gambotto manufactured all Ad vectors utilized in the study. The published manuscript was authored primarily by Simon M. Barratt-Boyes, who conceived and designed the study.

3.2 ABSTRACT

The ability of Ad-based vaccines to induce potent T cell-mediated immunity to transgenes is limited by the immunogenicity of the vector, which induces serotype specific NAb responses. We have examined the potential for a novel Ad35-based vaccine to boost cellular immunity to SIV Gag protein previously delivered by a conventional Ad5-based vaccine in rhesus macaques. Eight animals received four immunizations each of Ad5 expressing the codon optimized SIVmac239 *gag* gene in two parts (Ad5-Gag), followed by two boosts utilizing either continued Ad5-Gag or alternative Ad35-Gag. Subsequently, animals were challenged intrarectally with the heterologous primary isolate SIV/DeltaB670. Initial immunizations with Ad5-Gag induced broad and potent T cell responses to SIV Gag protein measured by IFN-γ

ELISPOT. Repeated Ad5-Gag immunization was ineffective in boosting T cell responses, associated with an elevated Ad5 NAb response. In contrast, boosting with Ad35-Gag substantially increased the strength of T cell responses to Ag. Vaccination induced $CD4^+$ and $CD8^+$ T cell responses to several previously unreported epitopes. These responses were recalled upon heterologous viral challenge suggesting viral epitopes targeted by vaccination were conserved between distantly related SIV strains. Notably, immunization provided reductions in post-challenge viral load demonstrating acute and chronic viral inhibition. The capacity of dual-serotype Ad-based vaccination to induce broad T cell-mediated immunity to multiple conserved epitopes illustrates the potential power of this vaccine regimen for HIV-1.

3.3 INTRODUCTION

Developing an effective HIV vaccine has been hindered by safety concerns involved with classical attenuated and inactivated virus, and the limited immunogenicity of subunit protein and plasmid DNA-based immunogens [324]. Live, recombinant viral vectors have received increased attention due to their innate ability to stimulate cellular immune responses. Viral vector immunogens including MVA, poxvirus, vesicular stomatitis virus, Ad5 and others have shown promise in prophylactic vaccination models against SIV infection models in nonhuman primates [211, 212, 261, 325-327]. Furthermore, studies comparing plasmid DNA, MVA, and Ad5-based immunizations have demonstrated the superior ability of Ad vectors to induce Ag-specific cellular immunity [211, 288], and are currently being advanced in clinical trials [328]. Ad5-based vaccinations against such emerging pathogens as Ebola virus, the SARS-coronavirus, and H5N1 influenza are currently being explored [329-331]. Regrettably, repeated administration of Ad-based immunizations are limited by the induction of vector-specific cellular and humoral immunity, reducing the capacity for continual immune induction from homologous vectors [332-335]. In conjunction, a high prevalence of serum Ab titres to Ad5 have been demonstrated in the global human population due to previous naturally occurring adenoviral infections [297, 305, 306].

To circumvent pre-existing vector specific immunity, development of alternative vectors based on rare human Ad serotypes (Ad35, Ad11, Ad24, Ad26, Ad4, Ad34) and chimpanzee

serotypes (AdC6, AdC7, AdC68) have been pursued [304, 310-313, 317]. Serial immunization regimens utilizing heterologous Ad vectors have demonstrated the ability of discordant Ad-based vaccination to boost transgene specific cellular immune responses in the presence of pre-existing Ad-specific immunity [312]. As natural, pre-existing immunity against Ad35 is found to a dramatically reduced extent compared to responses against Ad5, vectors based on an Ad35 backbone would not experience the restrictions of vector-specific humoral immunity associated with high Ad5 seroprevalence [297, 305, 306, 336]. Additionally, previous studies have demonstrated that Ad5-specific Ab responses fail to cross-neutralize Ad35-based vectors, allowing for the combination of these vectors in prime-boost immunization regimens [297, 319]. Findings in the murine model have supported this platform, demonstrated the potential utility of Ad35 immunization to boost SIV Gag-specific immunity in the presence of pre-existing Ad5-specific humoral responses [319].

In the presented study, we have evaluated the ability of Ad35-based vectors to enhance Gag-specific cellular immunity previously induced through Ad5-based vaccination in a sequential, heterologous immunization strategy against SIV in adult Indian rhesus macaques. As macaques naturally lack pre-existing immunity to either Ad5 or Ad35, the nonhuman primate model of HIV infection provides on optimal situation to examine the immunogenicity of this vaccination platform. To determine the extent of immune induction, we performed detailed characterization of the breadth and strength of transgene-specific cellular immunity following vaccination and upon subsequent viral challenge with the pathogenic biologic isolate SIV/Delta B670. Intrarectal viral challenge with SIV/DeltaB670 provided a stringent and relevant model of mucosal HIV infection, consisting of a CCR5 tropic virus with a median time to death of 11 months [337-339]. At its initiation, this study represented the first examination of the heterologous Ad5/Ad35-based immunization regimen in the nonhuman primate model of HIV infection.

3.4 MATERIALS AND METHODS

3.4.1 Generation and expression of recombinant Ad vectors

Replication-defective Ad5-based vectors excluding the E1/E3 genes were produced by Cre-*lox* recombination as described previously [287, 308]. Briefly, a *Sal*I-*Not*I fragment containing the transgenic segment of interest was inserted into the pAdlox shuttle plasmid and subsequently cotransfected with the E1/E3 deleted Adψ5 helper virus into the Ad packaging cell line CRE8. Recombinant Ad was purified by cesium chloride density gradient centrifugation, dialyzed, and stored at -80^{0}C. Vectors were constructed to include two codon-optimized fragments of the SIVman239 *gag* gene expressing the full-length protein as Ad5-p17 and Ad5-p45 (collectively, Ad5-Gag). Segmented expression of the Gag protein allowed for potential presentation of subdominant epitopes that may otherwise be restricted by competition from regions containing immunodominant epitopes [340]. Construction of replication-competent Ad35-based vectors excluding the E3 gene alone was performed utilizing the loxP recombination method previously described to incorporate identical Gag transgenes as described above, creating Ad35-p17 and Ad35-p45 (Ad35-Gag) [310]. Briefly, SalI–NotI fragments of codon-optimized gag p17 or gag p45 were cloned into the Ad35 shuttle plasmid pAd35E3. Plasmids were linearized with EcoRV and cotransfected with NotI-digested Ad35 helper virus Ad35E3/EYFP DNA into CRE8 cells. The resultant Ad35-based vectors were produced in HEK293 cells. Western blot analysis utilizing the SIV p17- and p27-specific monoclonal Abs, KK59 and 2F12 was performed to confirm segmented Gag protein expression from lysates of HEK239 and 239T cells transfected with Ad5- and Ad35-based vectors [308] (Gao, W. unpublished data).

3.4.2 Animals

Eleven colony-bred, adult Indian rhesus macaques (*Macaca mulatta)* where housed at the University of Pittsburgh Primate Facility for Infectious Disease Research and maintained in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. MHC determination was carried out via contract with the Wisconsin National Primate Research Center.

Molecular typing of the MHC class I Mamu-A*01, A*02, A*08, A*11, B*03, B*04, and B*17 alleles was performed.

3.4.3 Immunization and SIV challenge

All Ad manipulation was performed at 4°C with minimization of vector disturbance. Ad-based immunizations were rapidly thawed and resuspended in cold sterile saline at a concentration of 10^{11} viral particles per 150 μl. Immunization was administered in less than 1 h from viral thawing. Ad5- and Ad35-based vectors containing p17 or p45 were delivered at separate sites by intramuscular (i.m.) injection in the lateral thigh or by intradermal (i.d.) injection in the inguinal region, respectively. All animals were challenged through atraumatic inoculation of an undiluted stock of pathogenic SIV/DeltaB670 into the rectum as previously described [341].

3.4.4 IFN-γ ELISPOT assays

Effector T cell-mediated responses to SIV Gag were analyzed in previously frozen PBMC by IFN-γ ELISPOT assay as described [308]. Briefly, 96-well high protein binding membrane-coated plates (Millipore, Bedford, MA) were incubated overnight with 10 μg/ml monoclonal IFN-γ capture Ab (MD-1) (U-Cytech, Utrecht, The Netherlands) in 0.1 M carbonate buffer at 4°C. Subsequently, cells were plated at $1x10^5$ to $3x10^5$ per well in the presence of peptide Ag, dimethyl sulfoxide (DMSO) negative control, or staphylococcal enterotoxin B (SEB) positive controls and incubated for 24 h, 37°C, at 5% CO_2. Following stimulation, cells were disposed of, and plates underwent successive overnight incubations at 4°C using biotinylated IFN-γ detection Ab at 10 μg/ml (U-Cytech) and streptavidin-alkaline phosphatase conjugate (Bio-Rad, Hercules, CA). Spots were developed using chromogenic alkaline phosphatase substrate (Bio-Rad) and enumerated using an AID ELISPOT reader (Cell Technology, Columbia, MD). Individual 15-mer peptides at >80% purity representing the Gag, Pol, Env and Nef sequences of SIVmac239 and overlapping by 11 amino acids (NIH AIDS Research & Reference Reagent Program, Bethesda, MD) were dissolved in DMSO and used as Ag. Gag peptides were used in pools of eight peptides, 30-32 peptides (final concentration 3.1 – 3.9 μg/ml) or individually (5 μg/ml), as described (7). Env and Nef peptides were used as single pools of 212 peptides (0.6 μg/ml) and

64 peptides (1.6 μg/ml), respectively. Pol peptides were split into two pools of 131 and 132 peptides (1 μg/ml). For detailed epitope analysis of MHC class I and II responses respectively, 9-mer and 15-mer peptides were synthesized and HPLC purified to >93% (Sigma Genosys, The Woodlands, TX). Significant responses were three times that of background with a minimum number of 50 spots per 10^6 cells.

3.4.5 NAb responses to Ad vectors

Serum NAb responses to Ad5 and Ad35 vectors were detected through inhibition of infection by serotype matched reported viruses as previously demonstrated [305]. The human lung carcinoma cell line A549 (American Type Culture Collection, Manassas, VA) was cultured to confluence in 150- by 25-mm tissue culture dishes, harvested through trypsin disruption, washed, and counted utilizing trypan blue for apoptotic cell exclusion. Cells were then seeded at 10^5 per well in a 96-well flat-bottom plate. Four-fold serum dilutions from individual macaques (1:8, 1:32, 1:128, and 1:512) were pre-incubated with 10^8 viral particles of either E1/E3-deleteed Ad5-EGFP or E3-deleted Ad35-EYFP, expressing the fluorescent extra green or yellow fluorescent protein (EGFP/EYFP), respectively, for 1 h at 37^0C prior to combination with previously distributed A549 cells. Plates were incubated for 24 h at 37^0C. Cells were then harvested, washed, and EGFP or EYFP expression was analyzed using a FACScan flow cytometer and Cell Quest software (Becton Dickinson, Mountain View, CA). A minimum of 1,000 events were collected. The end-point titre was calculated as the highest serum dilution that inhibited >50% of infection. Significance determined by Student's *t* test. A549 cell line was chosen for providing equivalent infection qualities and kinetics between Ad5 and Ad35 viral serotypes.

3.4.6 Virus quantitation

Quantitation of virion-associated RNA in plasma was determined through real-time PCR as described previously [341]. From 1 ml plasma, virions were pelleted, total RNA was extracted utilizing Trizol reagent (Life Technologies, Rockville, MD), and 20 μl per sample was analyzed in a 96-well plate. cDNA synthesis was accomplished through the addition of 50 mM MgCl, 1xPCR buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 0.75 mM dGTP, 0.75 mM dATP, 0.75

mM dCTP, 0.75 mM dTTP, 1 U of RNase inhibitor, 1.2 U of murine leukemia virus reverse transcriptase (RT), 2.5 μM random hexamers, and 10% total viral RNA. Following mixing and a 10 min incubation at room temperature, PCR was initiated by the addition of RT and 30 μl master mix containing 1xPCR buffer A, 5.5 mM $MgCl_2$, 2.5 U of Amplitaq Gold, 200 mM deoxyribonucleoside triphosphates (dNTPs), 450 nM each primer, and 200 nM probe. The primers used were 5'AGGCTGGCAGATTGAGCCCTGGGAGGTTTC3' and 5'CCAGGCGGCGACTAGGAGAGATGGGAACAC3', and the probe used was 5'TTCCCTGCTAGACTCTCACCAGCACTTGG3'. The probe was labeled in the 5' position with the fluorescent reporter dye 6-carboxyfluorescein and in the 3' position with the quencher dye 6-carboxymethylrhodamine. Successive incubations as room temperature for 10 min, 42°C for 12 min, 99°C for 5 min, and 4°C for 5 min completed the reaction. Amplification was performed on a Prism 7700 sequence detection system undergoing the following conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s. Standard curves were generated through serial dilutions of in vitro transcribed LTR-containing control plasmid ranging from 10^8 to 10^0 and run in triplicate.

3.4.7 Viral sequencing

To perform viral sequence analysis, cell-free plasma was obtained and viral RNA was isolated utilizing the viral RNA mini kit (Qiagen, Valencia, CA) as per company instructions. To amplify the *gag* gene, first-strand cDNA synthesis was primed with random hexamers or the *gag*-specific primer BGAGR: 5'-GCGCTGCAGTGGGAGTTGCCCTGGTGTCAGT-3' and reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). PCR-amplified fragments containing 90% of the *gag* gene were generated by using the primers BGAGF: 5'-GGCGAATTCATGGGCGTGAGAAACTCCGTCTTG-3' and BGAGR with the Expanded High Fidelity PCR system (Roche Applied Science, Indianapolis, IN), as per manufacturer's instructions, using an annealing temperature of 53°C. For analysis of individual cloned viral cDNA sequences, amplicon DNA was purified from agarose gels and cloned into the pGEM-TA vector (Promega, Madison, WI) prior to transformation into bacteria. Plasmid DNA was sequenced with a 3770 DNA analyzer (Applied Biosystems, Foster City, CA).

Sequence data were aligned with SIVmac239 (GenBank accession no. M33262) using CLUSTAL W.

3.4.8 Statistical analyses

Statistical comparisons of animal-specific cellular immunity were achieved through paired sample *t* tests while NAb determinations were measured using Student's *t* Test. Longitudinal evaluations of cellular immunity or viral load between immunized and control groups were attained by calculating the cumulative average response from the summed means of individual values per animals over a predetermined interval. Mean values per immunized and control treatment groups were then compared over sequential time points using the non-parametric binomial (sign) test [342], examining the consistency of binary differences (±) between the two groups across time [343].

3.5 RESULTS

3.5.1 Study characteristics

In a proof-of-concept study, eight adult, Indian rhesus macaques received four immunizations each of an Ad5-based vaccine encoding the SIVmac239 *gag* transgene in as p17 and p45. Segmentation of the Gag protein was performed to allow for the optimal expression and potential recognition of subdominant epitopes. Vaccination was delivered at roughly wks 0, 4, 16, and 26. Subsequently, two animals received continued boosting with conventional Ad5-based vectors, while the six remaining monkeys received heterologous boosting utilizing the novel Ad35-based vectors encoding identical *gag* transgenes (Table 1). Eight immunized and three naïve control animals were then challenged through atraumatic inoculation with SIV/DeltaB670 at roughly three months following the final immunization. Animals were monitored for disease progression, and followed to a study endpoint defined as greater than 20%

weight loss or the development of non-treatable opportunistic infections. Study animals were males of equivalent age, with one Mamu-A*01 expressing individual per treatment group.

Table 1: Animal characteristics and immunization schedule.

Table I

Animal characteristics and immunization schedule – Prophylactic Vaccine Trial

Animal	Age (Years), Gender	Mamu MHC Class I	Vaccine Route	Ad5 (wk)	Ad35 (wk)	SIV/DeltaB670 Challenge (wk)
M1601	6, M	NA	I.M.	0, 4, 16, 26, 56, 65	-	77
M7801	3, M	A*08	I.M.	0, 5, 16, 26	56, 65	84
M1701	5, M	NA	I.M.	0, 4, 16, 26	64, 73	84
M2201	6, M	A*01	I.M.	0, 5, 16, 26	63, 72	84
M1501	5, M	A*08	I.D.	0, 4, 16, 26, 51, 60	-	72
M2301	5, M	A*08	I.D.	0, 4, 16, 26	51, 60	72
M9700	3, M	A*01, B*01	I.D.	0, 4, 16, 26	43, 52	64
M10201	3, M	A*08	I.D.	0, 4, 16, 26	43, 52	64
M3398	8, M	A*02, B*17	-	-	-	0
M15001	5, M	A*01	-	-	-	0
M14301	3, M	A*01, A*02	-	-	-	0

I.M. – Intramuscular

I.D. – Intradermal

3.5.2 Ad35-based booster vaccination enhances Ad5-based vaccine primed immunity to SIV Gag

Upon immunization with Ad5-Gag, all animals demonstrated a rapid and potent Gag-specific cellular immune response measured by IFN-γ ELISPOT assay (Fig. 1, left). Peak responses following Ad5-Gag immunizations were identified following two to four immunizations, with frequencies of Gag-specific effector T cells in uncloned PBMC ranging from 1:1000 to 1:500 for most study animals. We found no apparent differences in the development of cellular immunity following either i.m. or i.d. vaccination. In agreement with previous studies, we observed the immediate induction of Ad5-specific NAb that were incapable of cross-neutralizing Ad35-based vectors (Fig. 1, right). As expected, repeated Ad5-Gag immunization displayed reduced efficacy in the presence of high titre Ad5-specific NAb. Upon continued boosting immunization with Ad5-Gag, animals M1501 and M1601 displayed dramatically inhibited expansion of Ag-specific immunity, representing roughly 30% and 50% of previously attained peak values. Notably, the persistence of Ad5-specific NAb in serum decreased in the absence of Ad5 exposure following the fourth Ad5-Gag immunization, showing a 40 fold reduction in Ab titre in the 27.3±2.8 weeks between priming and boosting immunizations. This decline in Ab titre potentially allowed for the measure of immune induction identified after continual Ad5-Gag booster vaccination. By contrast, boosting using Ad35-Gag provided substantial immune enhancement, attaining higher frequency Gag-specific T cell responses in all vaccinees over those found following repeated Ad5-Gag alone. Interestingly, the development of Ad35-specific NAb titres were delayed and muted, with one third of the animals failing to mount a response until the second Ad35-Gag immunization. Additionally, Ad35 exposure produced weaker, transient NAb titres, with responses falling to undetectable levels between the first and second Ad35-Gag immunizations in 83% of animals.

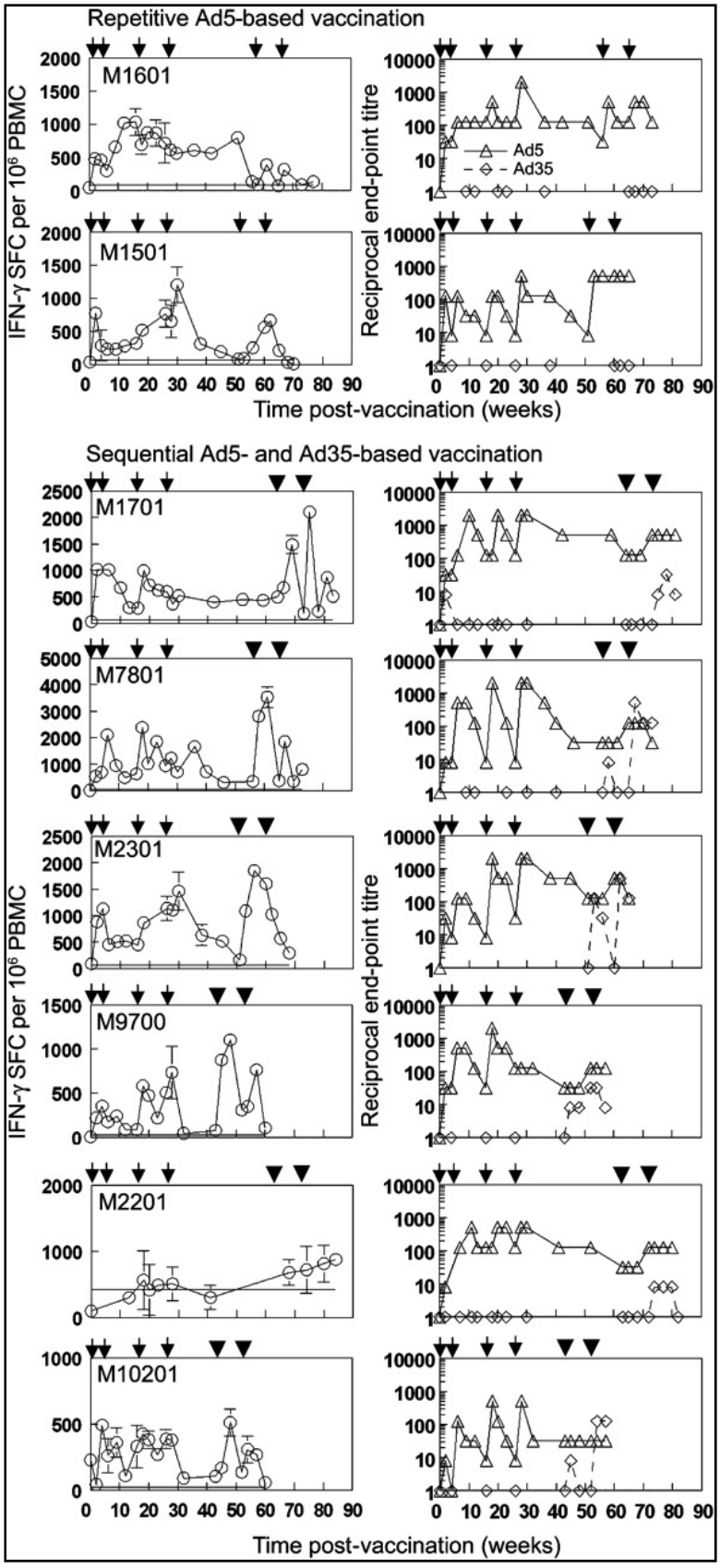
Repetitive Ad5-based vaccination
M1601
M1501
IFN-γ SFC per 10⁶ PBMC
Reciprocal end-point titre
Ad5
Ad35
Time post-vaccination (weeks)
Sequential Ad5- and Ad35-based vaccination
M1701
M7801
M2301
M9700
M2201
M10201
IFN-γ SFC per 10⁶ PBMC
Reciprocal end-point titre
Time post-vaccination (weeks)

Figure 1: Ad35 boosts SIV Gag-specific immunity induced by Ad5.
Monkeys were immunized and boosted with replication-defective Ad5-p17 and Ad5-p45 alone (arrows, top panels) or prior to boosting with replication-competent Ad35-p17 and Ad35-p45 (arrowheads, bottom panels). Left, PBMC were incubated with four pools of Gag peptides and IFN-γ-producing cells were quantified 24 h later. Shown are mean ± SEM of triplicate determinations for all pools combined after subtraction of background. Thresholds for significance are shown by horizontal lines denoting mean background responses over all time points for each animal. Right, Ad5- and Ad35-specific neutralizing Ab titres in serum.

3.5.3 Dual-serotype Ad-based vaccination induced broad T cell responses to Gag

We next sought to characterize the development of Gag-specific epitope recognition upon Ad5-Gag priming and subsequent Ad35-Gag boosting vaccinations. This was achieved through IFN-γ ELISPOT analysis of overlapping peptide matrices. Peptide matrix design provides that a specific peptide is contained in two discrete pools of eight peptides each, allowing for the discrimination of candidate epitopes when responses to both unique pools are positive. Candidate peptides are then testing individually to confirm reactivity [308]. Animal M1701 displayed a strong response to two regions in Gag represented as p35/p36 and p68/p69, likely representing two specific epitopes found in sets of 15mer peptides containing common 11aa sequences (Fig. 2a). Ad35-Gag vaccination did not alter the specificity of the response. Similarly, M7801 displayed reactivity against the p14/p15 and p35/p36 regions of Gag, demonstrating potent enhancement of identical responses following Ad5-Gag prime and Ad35-Gag boost. Animal M2301 responded to eight different peptides over the course of vaccination, representing six regions of Gag protein. As expected, animals M2201 and M9700 expressing the Mamu-A*01 allele developed dominant responses to the p45 and p46 peptides, containing the MHC class I-restricted, immunodominant $CD8^+$ T cell epitope CM9 ($Gag_{181-189}$) (Fig. 2a, data not shown) [344].

We next sought to define whether the identified peptides were MHC class I- or class II-restricted epitopes, representing responses mediated by $CD8^+$ or $CD4^+$ T cells, respectively. To this end, we used magnetic bead Ab depletion to remove either $CD4^+$ or $CD8^+$ T cells from PBMC prior to testing peptide epitopes by IFN-γ ELISPOT analysis. Analysis revealed that IFN-γ responses to p68 and p69 in M1701 and M2301, respectively, were completely abrogated upon removal of $CD8^+$ but not $CD4^+$ T cells, illustrating that these epitopes are MHC class I-restricted (Fig. 2b). Similarly, IFN-γ release from p35 stimulation of M7801 was lost in the

absence of $CD8^+$ T cells. Additionally, CM9 responses in M2201 and M9700 were found to be MHC class I-restricted, $CD8^+$ T cell-dependant as previously reported (data not shown). In contrast, the p14/p15 Gag region recognized by M7801 was found to be MHC class II-restricted, as IFN-γ production was eliminated upon the removal of $CD4^+$ T cells. Taken together, these findings demonstrate that Ad-based vaccination is capable of enhancing the breadth of both $CD4^+$ and $CD8^+$ T cell responses.

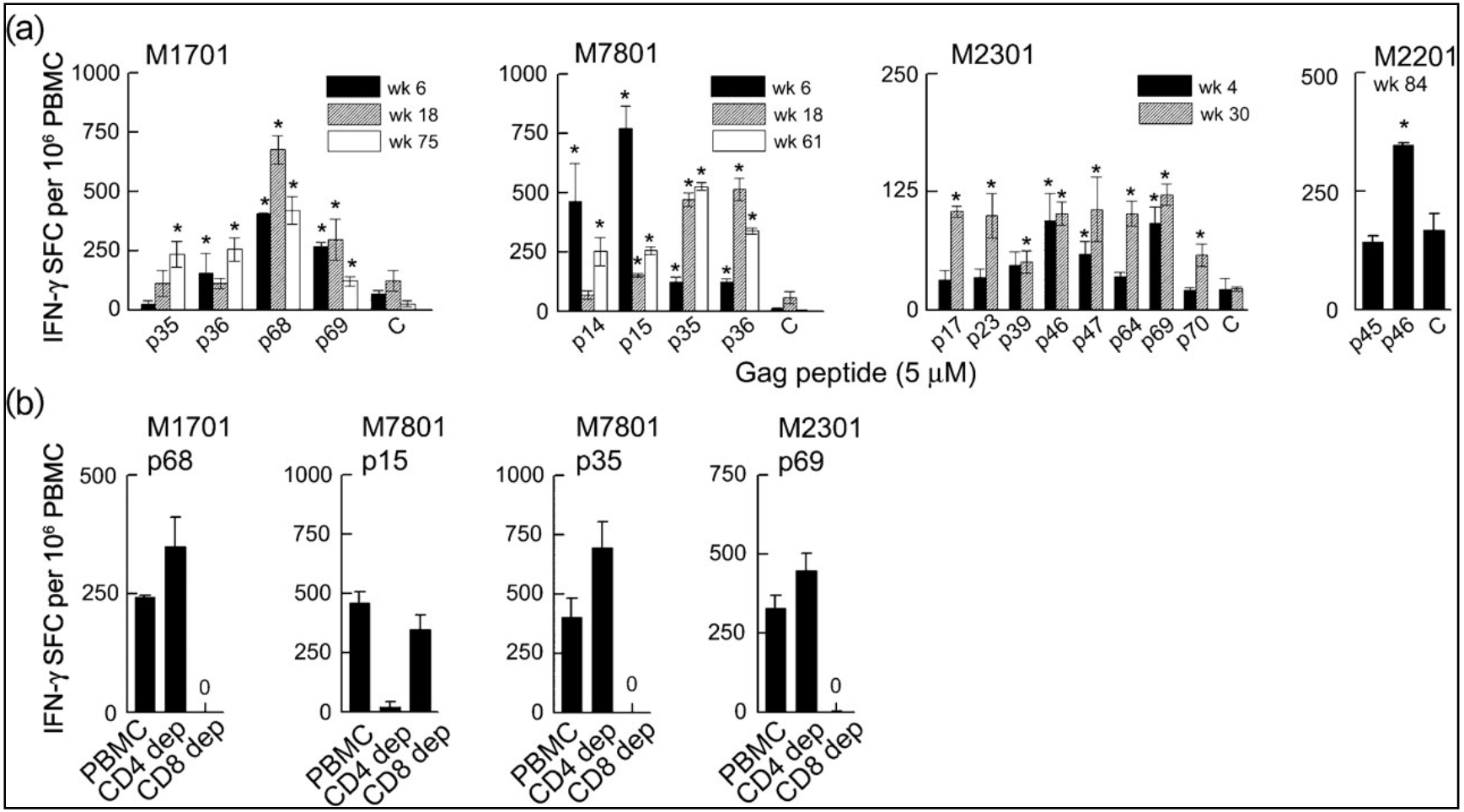

Figure 2: Vaccination induces broad Gag-specific cellular immunity.
(A) PBMC were incubated with diluents or individual Gag peptides at various times after immunization as indicated, and IFN-γ-producing cells were quantified 24 h later. Positive responses as defined in Materials and Methods are indicated by asterisks. Shown are mean ± SEM of triplicate determinations. (B) PBMC from animals M1701, M7801, and M2301 before and after Ab-mediated depletion of CD4+ or CD8+ T cells were incubated with individual 15mer peptides as indicated and IFN-γ-producing cells were quantified 24 h later. Shown are mean ± SEM of triplicate determinations based on absolute number of cells in the assay with or without depletion. C, diluent control; dep, depleted.

3.5.4 Vaccine induced cellular immunity is recalled upon viral challenge

Given the stark diversity of HIV, a non-autologous vaccination must direct immunity against viral regions of high structural conformity and thus evolutionary constraint. To this end, eight immunized and three treatment naïve control animals were mucosally challenged by intrarectal inoculation of the pathogenic biologic isolate SIV/DeltaB670 11-12 wks after final boost. Vaccine targeted SIVmac239 and challenge SIV/DeltaB670 are distantly related viral strains, sharing an 8% Gag sequence dissimilarity (data not shown), providing a stringent and realistic exposure modeling natural HIV infection [337, 339]. The two animals receiving repetitive Ad5-Gag immunizations produced recall responses following SIV challenge, although of varying dynamics. M1601 developed a strong and transient Gag-specific recall response, with Ag-specific T cell frequencies reaching 1:500 per uncloned PBMC (Fig. 3a) before disappearing for the remainder of the study. M1501 displayed a low frequency response to SIV challenge that was sustained. Unfortunately, M1501 died due to anesthetic related complications unrelated to SIV infection at 15 wks post-challenge, precluding further observations. Animals receiving the Ad5/Ad35 prime-boost immunization regimen generally developed robust cellular immunity following SIV challenge, although considerable variation between animals was seen (Fig. 3a). Notably, post-challenge Gag-specific recall responses were strongly associated with the magnitude of peak vaccine induced immunity during vaccinations. In contrast, the IFN-γ response to infection among non-vaccinated control animals was minimal, with only two control animals developing moderate responses (Fig. 3b). When the mean Gag-specific IFN-γ response was compared between Ad5/Ad35 immunized and control animals, Gag-specific immunity was significantly greater in vaccinees for at least 25 wks post-infection (Fig. 3c). To demonstrate that augmentation in post-challenge immunity was due to Gag-specific vaccination, we examined T cell responses to the SIV Env, Nef and Pol proteins before and after SIV challenge. We found no differences in Ag-specific cellular immunity targeting peptide pools comprising the Env, Nef or Pol Ags when Ad5/Ad35 immunized animals were compared with control infected animals at during establishment of set-point viremia (wks 5-10) and during chronic infection (wks 23-25) (Fig. 3d).

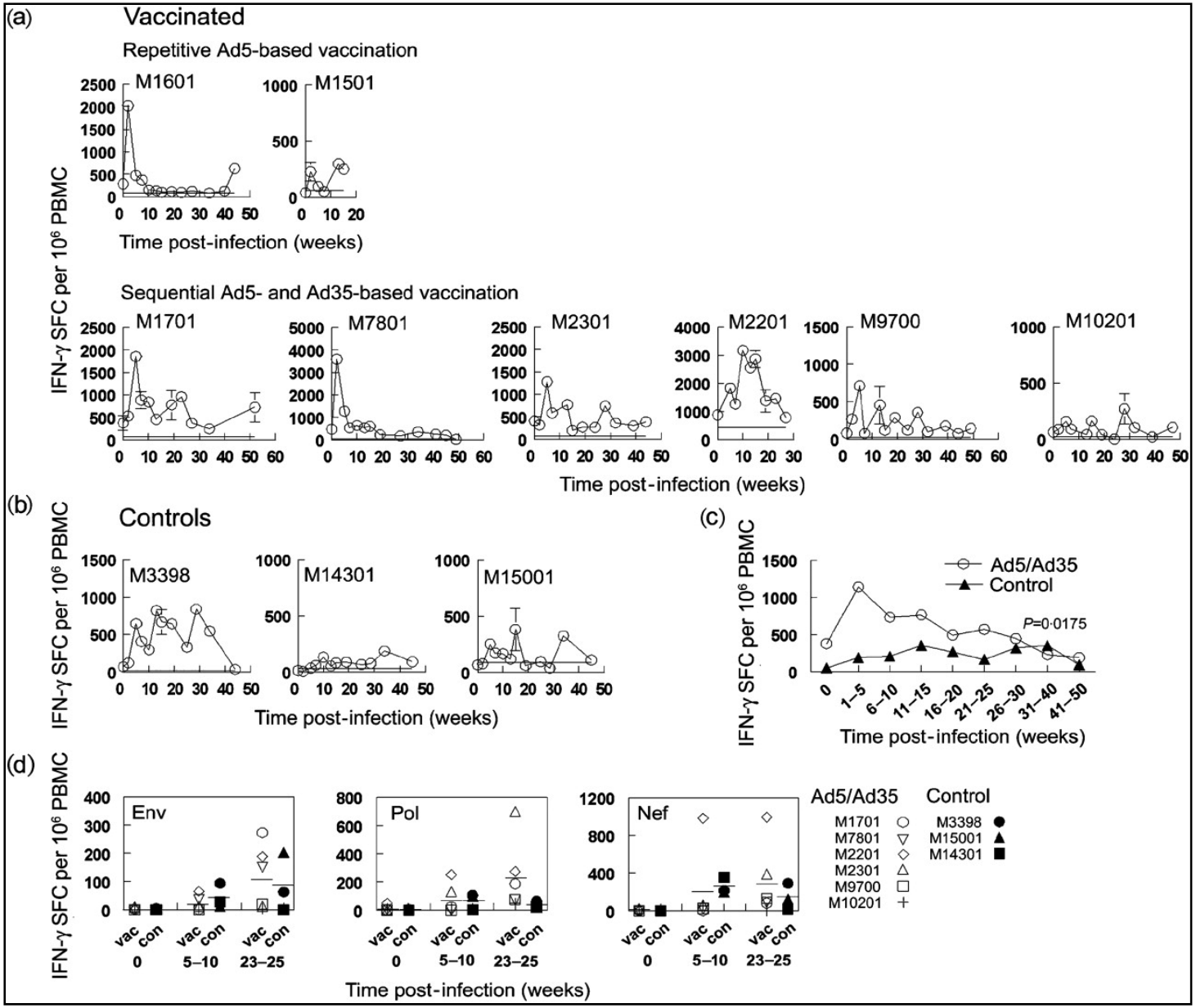
(a)
Vaccinated
Repetitive Ad5-based vaccination
M1601
M1501
IFN-γ SFC per 10⁶ PBMC
Time post-infection (weeks)
Sequential Ad5- and Ad35-based vaccination
M1701
M7801
M2301
M2201
M9700
M10201
Time post-infection (weeks)
(b)
Controls
M3398
M14301
M15001
IFN-γ SFC per 10⁶ PBMC
Time post-infection (weeks)
(c)
Ad5/Ad35
Control
P=0·0175
IFN-γ SFC per 10⁶ PBMC
0
1–5
6–10
11–15
16–20
21–25
26–30
31–40
41–50
Time post-infection (weeks)
(d)
IFN-γ SFC per 10⁶ PBMC
Env
Pol
Nef
vac
con
0
5–10
23–25
Time post-infection (weeks)
Ad5/Ad35
M1701
M7801
M2201
M2301
M9700
M10201
Control
M3398
M15001
M14301

Figure 3: Vaccination induces durable recall responses following SIV infection.
Vaccinated (A) or control (B) monkeys were challenged with SIV/DeltaB670 by atraumatic intrarectal inoculation. PBMC were incubated with diluents or Gag peptide pools at various times after challenge and IFN-γ-producing cells were quantified 24 h later. Shown are mean ± SEM of triplicate determinations for all Gag peptides after subtraction of background. Thresholds for significance are shown by horizontal lines denoting mean background responses over all time points for each animal. (C) Mean Gag-specific IFN-γ responses of Ad5/Ad35-vaccinated and control groups at intervals after virus challenge. Responses over time were compared using a binomial test. (D) Responses of Ad5/Ad35-vaccinated and control animals to Env, Pol, and Nef peptide pools at intervals after virus challenge. Shown are mean responses of triplicate determinations after subtraction of background for each animal, with the mean for the group represented by a horizontal line. If more than one sample was analyzed during the interval indicated, the stronger response is shown. Vac, vaccinated; con, control.

3.5.5 Novel and recalled Gag-specific epitopes identified following SIV infection

Given the degree of genetic variation between the SIVmac239 immunization strain and SIV/DeltaB670 challenge virus, we examined whether epitope specific responses developed upon vaccination were conserved upon heterologous viral challenge. To this end, we employed peptide matrices and IFN-γ ELISPOT analysis. M1701 responded vigorously to infection, developing recall responses to the identical p35/p36 and p68/p69 regions induced through vaccination (Fig. 4). Notably, M1701 developed rapid and potent recall responses to SIV challenge, demonstrating superior IFN-γ production to the immunodominant p68 epitope at 5 wks post-infection to that seen previously following vaccination. Upon infection, M2301 responded to six of eight vaccine induced epitopes, failing to develop recall IFN-γ production to p23 or p47 stimulation (Fig. 4). Peptide mapping revealed M7801 developed recall responses to all previously identified epitopes, but interestingly, responded to two novel epitopes, p1 and p67-p69, following SIV challenge (Fig. 4). Weak IFN-γ production was transiently identified to the $CD4^+$ T cell restricted p14/p15 epitope following viral infection, suggesting rapid elimination of virally targeted, SIV-specific $CD4^+$ Tem cells. As expected, Ag-specific recall responses from the Mamu-A*01-expressing M2201 were directed against the immunodominant and conserved CM9 coding sequence as identified previously, approaching a frequency of 0.2% in unseparated PBMC. This is in contrast to the failure to develop *de novo* CM9-specific immunity from the Mamu-A*01-expressing control animals M14301 and M15001 (Fig. 3, data not shown), illustrating the utility of preventative vaccine-induced CTL memory expansion. Finally, primary epitope-specific responses to SIV infection from M3398, the only control animals for which a

robust post-challenge Gag-specific response was detected, were directed against the p68/p69 region (Fig. 4). Taken together, these data indicate that epitope-specific vaccine-mediated responses were capable of rapid and potent recall upon heterologous infection with SIV/DeltaB670.

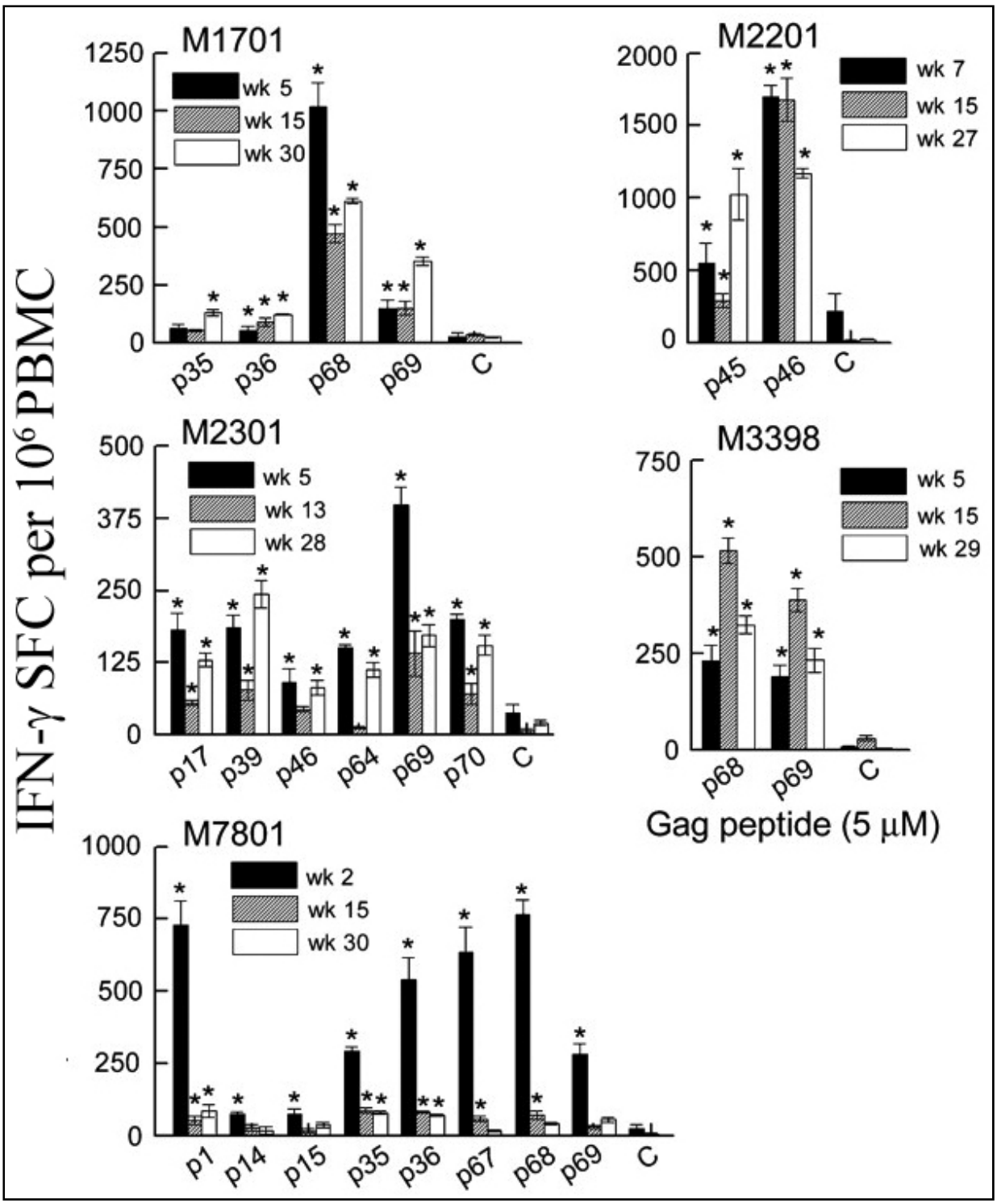

Figure 4: Epitope response following SIV/DeltaB670 challenge.
PBMC were incubated with diluents or individual Gag peptides at various times after SIV infection as indicated, and IFN-γ-producing cells were quantified 24 h later. Positive responses as defined in Materials and Methods are indicated by asterisks. Shown are mean ± SEM of triplicate determinations. C, diluents control.

3.5.6 Limited viral control following heterologous SIV challenge

We then utilized a sensitive RT-PCR assay to measure alterations in post-challenge SIV/DeltaB670 viremia, and the potential impact of Gag-specific Ad-based vaccination. Regrettably, analysis of the two Ad5/Ad5 immunized animals was prevented by the anesthesia related death of M1501. Interestingly, M1501 was capable of restricting set-point viremia to roughly $2x10^3$ RNA copies per ml in the presence of only moderate Gag-specific IFN-γ production, suggesting a qualitative advantage that went undetected in this study (Fig. 5a). Upon SIV challenge, Ad5/Ad35 immunized animals displayed a trend towards lowered viral load when compared to controls (Fig. 5a). Although a great deal of variation was seen among Ad5/Ad35 immunized animals, two of six, M17010 and M2301 attained undetectable viral loads for multiple timepoints until wk 44 post-infection (Fig. 5a). When mean viral loads over pre-determined intervals were compared between the Ad5/Ad35 immunized and control groups, we found a statistically significant temporal reduction in viremia with Ad5/Ad35 immunized group presenting lower viral loads at 11 of 12 timepoints ($p=0.003$) (Fig. 5b). Comparison of survival between control and Ad5/Ad35-vaccinated groups showed a trend towards longer survival periods among immunized animals ($P=0.094$), with groups achieving median times to death from AIDS at 41.0 and 49.3 wks respectively (Fig. 5c). Animal M2301 remained alive at 70 wks post-infection, and the duration of the study. These data demonstrate the measurable effect of vaccination, even of limited antigenic scope, upon viral control.

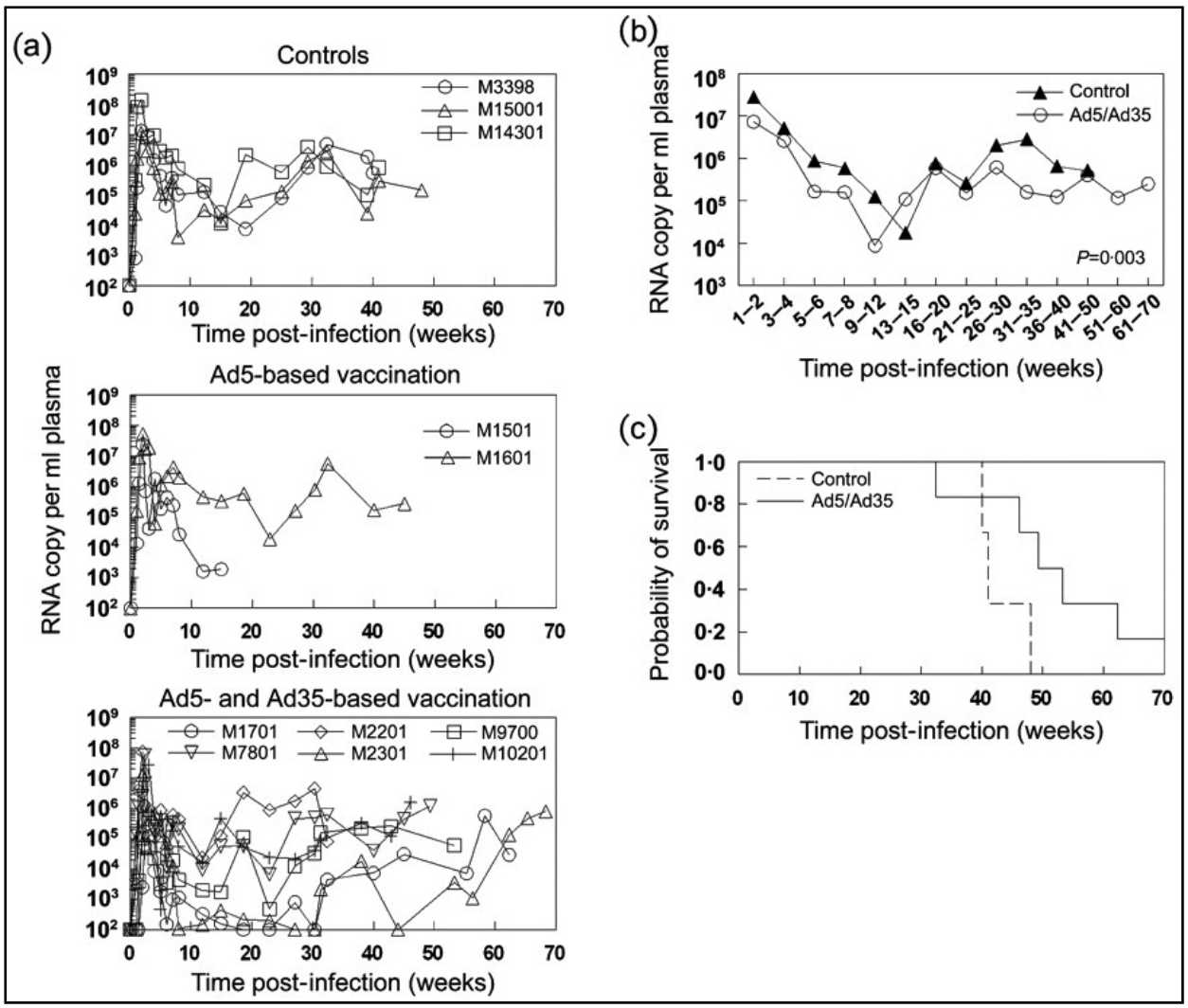

Figure 5: Post-challenge SIV/DeltaB670 viral load and survival.
(A) Plasma virus load of control animals and animals vaccinated with Ad5-based or Ad5- and Ad35-based vectors. Final measurements are at the time of sacrifice due to AIDS except for animal M1501 which died of unrelated causes at wk 15, and animal M2301 which remains alive and free from disease at wk 70 post-infection. (B) Mean virus loads of Ad5/Ad35-vaccinated and control groups at predetermined intervals after challenge. (C) Kaplan-Meier survival curves for Ad5/Ad35-vaccinated and control groups.

3.5.7 Fine mapping of novel $CD8^+$ and $CD4^+$ T cell-mediated epitopes

To define precise viral targets, we utilized walking 9mer or 15mer peptides overlapping by a single amino acid to span previously targeted regions containing MHC class I- or class II-restricted epitopes, respectively. Four animals expressing the MHC Mamu-A*01-expressing allele, M2201, M9700, M14301, and M15001 all had immunodominant responses to the CM9 epitope ($Gag_{181-189}$) as previously acknowledged (data not shown). Fine mapping of the p35/p36 region within the capsid protein revealed that the responses of M1701 and M7801 were directed towards peptides GL9 ($Gag_{141-149}$) and GS9 ($Gag_{142-150}$), suggesting the optimal epitope is an 8 amino acid sequence common to both peptides (Fig. 6). IFN-γ release mediated by the p68/p69 region was found to be directed against two distinct 9mer sequences, GY9 ($Gag_{270-278)}$ and QP9 ($Gag_{272-280)}$ within capsid in PBMC from M7801 following SIV challenge (Fig. 6). By contrast, the epitopes within the same region, p68/p69, recognized by M1701, M2301, and M3398 could not be identified using 9mer peptides, suggesting a larger peptide sequence may be targeted. Using walking 15mers, we identified the $CD4^+$ T cell-mediated p14/p15 matrix protein response of M7801 to be directed primarily against GT15 (Gag_{56-70}) (Fig. 6).

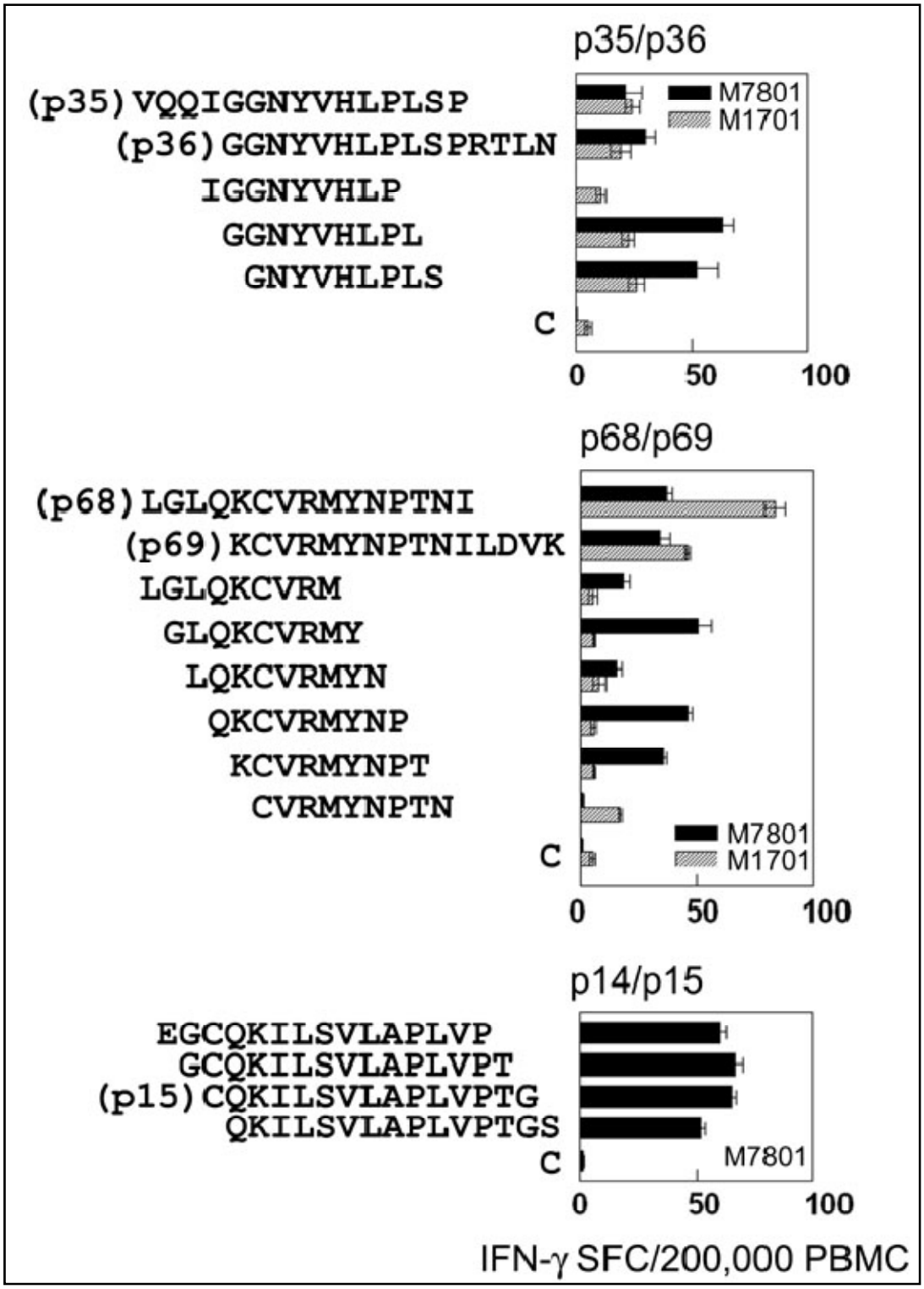

Figure 6: Fine mapping of vaccine induced epitopes.
PBMC from animals M1701 and M7801 were incubated with walking 9mer (p35/p36 and p68/p69) and 15mer (p14/p15) Gag peptides overlapping by one amino acid or diluents control at times of optimal immunity following SIV infection, and IFN-γ-producing cells were quantified 24 h later. Shown are mean ± SEM of triplicate determinations. C, diluents control.

3.5.8 $CD8^+$ T cell-mediated epitope escape mutations and SIV/DeltaB670 variability

To examine the effect of $CD8^+$ T cell-mediated immune pressure on viral mutation, we longitudinally compared viral sequences in the known immunodominant CM9 epitope and regions containing the previously unidentified epitopes listed in this study. Although isolates from the SIV/DeltaB670 challenge virus were heterogeneous at flanking regions compared to SIVmac239, known to effect CM9 recognition during SIVmac251 infection, all sequences expressed the intact CM9 epitope. In the presence of potent Gag-specific $CD8^+$ T cell-mediated immunity composed exclusively of CM9-specific responses, animal M2201 had a rapid increase in plasma viremia at wk 15 post-infection leading to death at wk 32, suggesting cellular immunity towards this epitope was no longer effective. Sequence analysis revealed characteristic 2^{nd} anchor residue CM9-specific $CD8^+$ T cell-mediated escape mutations were present in the circulating virus of M2201 by wk 15 and wk 23, consisting of 63 and 100% of isolates (Fig. 7a) [345]. Of the four animals expressing the Mamu-A*01 allele (Table 1), all had measurable CM9-specific IFN-γ responses, and three subsequently developed CM9-specfic $CD8^+$ T cell-mediated escape mutations coincident with increases in viral load (Fig. 7a). M15001 had 100% viral escape at within CM9 by wk 19 post-infection. Similarly, M9700 displayed viral escape in 85% of isolates by wk 39 post-infection. Notably, the only Mamu-A*01-expressing animal to not show evidence of CM9 viral escape, M14301, mounted inferior Gag-specific immunity following viral infection (Fig. 3b), illustrating the association between effective immune pressure and viral escape. Interestingly, mutations within the CM9 sequence but outside positions known to affect peptide binding, were also identified in viruses isolated from animals M1701 and M7801, which did not express the Mamu-A*01 allele (Fig. 7b). Altered CM9 sequences failed to induce *de novo* $CD8^+$ T cell-mediated IFN-γ responses (Fig. 7b), suggesting that SIV/DeltaB670 is capable of limited viral mutation within traditionally constricted regions in the absence of immune pressure. No consistent extraepitopic mutations associated with any CM9 mutations could be identified throughout this investigation.

We next examined if T cell-mediated immunity could direct viral escape mutations in the novel GL9, GY9, QP9 and GT15 epitopes. Sequence analysis of the SIV/DeltaB670 viral inoculum showed minimal variation in the MHC class II-mediated GT15 epitope region, which

was later found to consistently include the unaltered epitope in plasma viral isolates collected from M7801 at wks 2 and 15 post-infection (Fig. 7c). As IFN-γ response to GT15 stimulation was lost by wk 15 post-infection, this suggests elimination of virally targeted SIV-specific $CD4^+$ T cells instead of epitope escape. The SIV/DeltaB670 inoculum was found to contain a position 5 valine to threonine (V145T) alteration in the MHC class-I restricted GL9 epitope compared to the consensus SIVmac239 vaccination strain that was preserved throughout the course of infection in M7801 (Fig. 7c). Peptides corresponding to GL9 with either the valine or threonine at Gag_{145} were found to induce equivalent IFN-γ responses upon stimulation of uncultured PBMC from M7801 (Fig. 7c). Additionally, the immunogenic region containing the GY9 and QP9 MHC class I-restricted peptides targeted by M1701 was shown to be identical in both vaccine and challenge strains, and was conserved without mutation at wk15 post-infection in this animal (Fig. 7d).

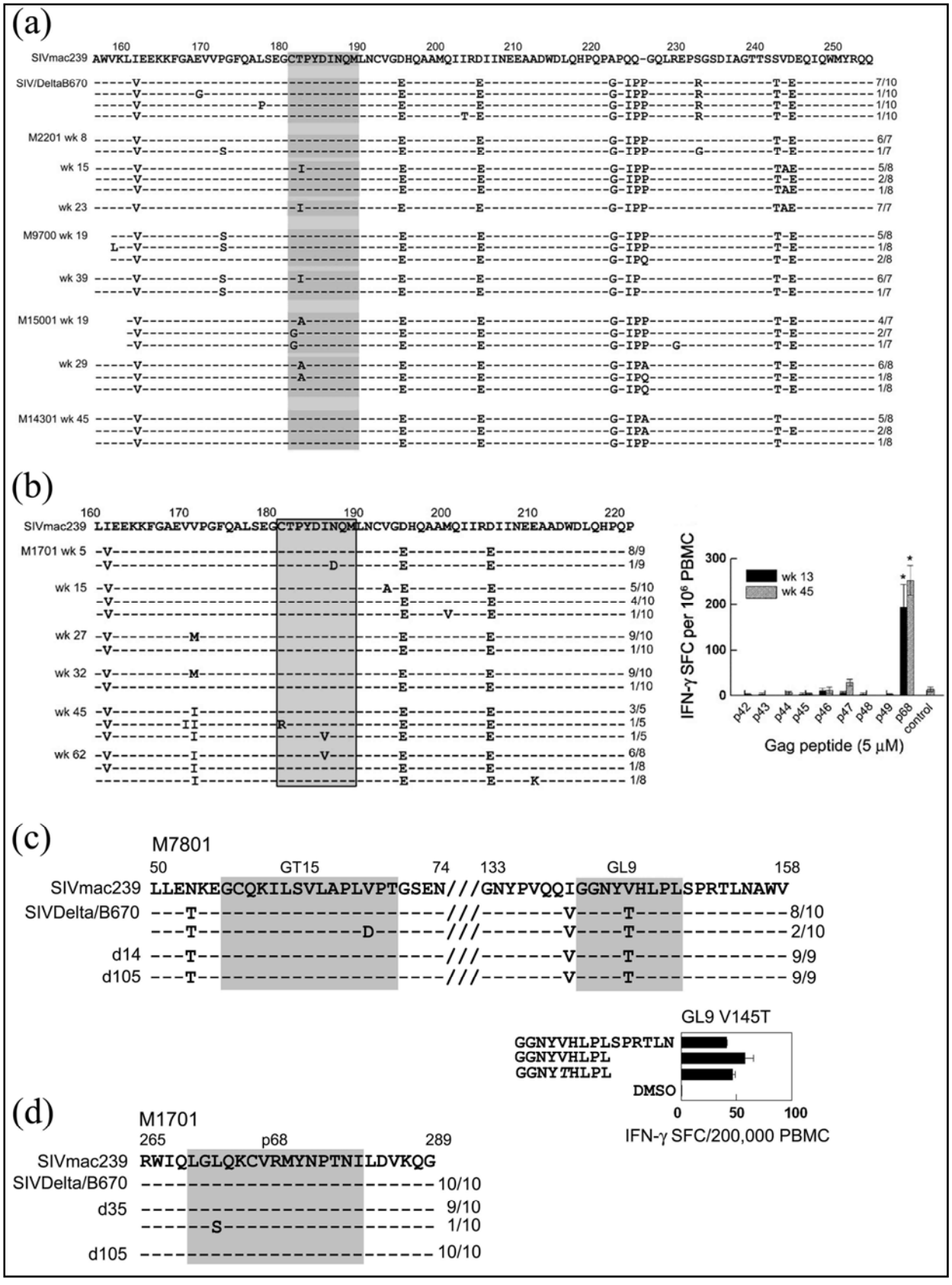
(a)
SIVmac239 AWVKLIEEKKFGAEVVPGFQALSEGCTPYDINQMLNCVGDHQAAMQIIRDIINEEAADWDLQHPQPAPQQ-GQLREPSGSDIAGTTSSVDEQIQWMYRQQ
SIV/DeltaB670 7/10
1/10
1/10
1/10
M2201 wk 8 6/7
1/7
wk 15 5/8
2/8
1/8
wk 23 7/7
M9700 wk 19 5/8
1/8
2/8
wk 39 6/7
1/7
M15001 wk 19 4/7
2/7
1/7
wk 29 6/8
1/8
1/8
M14301 wk 45 5/8
2/8
1/8
(b)
SIVmac239 LIEEKKFGAEVVPGFQALSEGCTPYDINQMLNCVGDHQAAMQIIRDIINEEAADWDLQHPQP
M1701 wk 5 8/9
1/9
wk 15 5/10
4/10
1/10
wk 27 9/10
1/10
wk 32 9/10
1/10
wk 45 3/5
1/5
1/5
wk 62 6/8
1/8
1/8
IFN-γ SFC per 10^6 PBMC
wk 13
wk 45
p42 p43 p44 p45 p46 p47 p48 p49 p68 control
Gag peptide (5 μM)
(c)
M7801
SIVmac239 LLENKEGCQKILSVLAPLVPTGSEN///GNYPVQQIGGNYVHLPLSPRTLNAWV
SIVDelta/B670 8/10
2/10
d14 9/9
d105 9/9
GL9 V145T
GGNYVHLPLSPRTLN
GGNYVHLPL
GGNYTHLPL
DMSO
IFN-γ SFC/200,000 PBMC
(d)
M1701
SIVmac239 RWIQLGLQKCVRMYNPTNILDVKQG
SIVDelta/B670 10/10
d35 9/10
1/10
d105 10/10

Figure 7: $CD8^+$ T cell-mediated epitope escape mutations and SIV/DeltaB670 variability.
Sequence comparison between SIVmac239 vaccine strain, SIV/DeltaB670 inoculum, and viruses isolated from plasma from animals expressing (A) Mamu-A*01, M2201, M9700, M15001, and M14301, (B) non-Mamu-A*01-expressing animal M1701, (C) animal M7801, and (D) animal M1701 at times post-challenge. (A, B) The Mamu-A*01 restricted CM9 epitope is depicted by the shaded region. (B) IFN-γ responses to the CM9-flanking sequences in SIV/DeltaB607 in PBMC by ELISPOT shown at times post-infection as indicated in M1701. (C) SIV/DeltaB670 mutations in immunodominant T cell epitopes, depicted as shaded boxes, and concurrent IFN-γ responses to the GL9 mutation in PBMC by ELISPOT in animal M7801 at times post-infection. (D) SIV/DeltaB670 mutations in immunodominant T cell epitopes, depicted as shaded boxes, in the p68/p69 region from times post-infection of M1701.

3.6 DISCUSSION

At the inception of this investigation, second generation adenoviral vectors based on non-Ad5 serotypes were being extensively pursued as immunogens for vaccination against HIV and other infectious diseases [304, 346-348]. Ad35 has risen to the forefront of heterologous Ad development based on a relatively low global seroprevalence compared to Ad5, and its ability, as a group B virus, to evade cross-neutralization by Ad5-specific humoral immunity [297, 305, 306, 310, 336]. Previous studies in the murine model have demonstrated the immunogenicity of Ad35 vectors to boost transgene-specific cellular immunity in the presence of pre-existing Ad5-specific NAb responses [319]. Our current study extends these findings, providing the first evidence that Ad5/Ad35-based vaccination is immunogenic in the non-human primate model. Furthermore, these results support those of similar sequential vaccination regimens utilizing recombinant heterologous simian Ad species which have proven to induce robust cellular immunity [311]. We found Ad35-based immunization boosted existing Gag-specific T cell-mediated responses induced through Ad5-based priming without expanding their repertoire, suggesting that transgene delivery and processing is consistent between vectors. In contrast, SIV/DeltaB670 infection produced both anamnestic as well as novel Gag-specific epitope responses, potentially due to sequence variation or differences in Ag processing and presentation. Although the data presented were attained using replication competent Ad35-based vectors, we have subsequently developed E1/E3-deleted, replication-defective Ad35-based vectors further enhancing the safety of immunization [310].

This study demonstrates that vaccination targeting conserved epitopes can induce preserved cellular immunity capable of targeting a heterologous challenge strain, providing acute

and chronic viral inhibition. Published sequence analysis confirmed that the vaccine targeted molecular clone SIVmac239 and SIV/DeltaB670 quasispecies are truly heterologous, based on 14% dissimilarity in the predicted amino acid sequence of the viral envelop proteins. Despite significant overall variability, we immunized animals utilizing vectors expressing the relatively conserved Gag protein which retains 94% sequence homology between SIVmac239 and SIV/DeltaB670 (data not shown). We found remarkable consistency between the recognized epitope repertoires induced through vaccination and upon infection, suggesting anamnestic responses capable of viral detection and containment. Previous findings by Vogel et. al., have demonstrated differences in Ag-specific epitope recognition upon DNA/MVA immunization against nearly all viral proteins from SIV17E-Fred and SIVmacJ5 and subsequent challenge with the closely related pathogenic SIVmac239, characterizing significant addition and loss of targeted epitopes during acute and chronic infection [349]. Our current findings utilizing SIV/DeltaB670 challenge are similar in nature to the DNA/MVA model, with modest inhibition of acute viremia without pronounced containment of disease progression [350]. Notably, viral inhibition in our model was achieved following Gag-based immunization alone, as recent reports have implicated the breadth of cellular immunity targeting multiple viral proteins, both structural and regulatory proteins, in the control of SHIV and SIVmac251 infection following immunization [351, 352].

Detailed epitope characterization identified an immunogenic region of the capsid protein ($Gag_{269\text{-}291}$) that was conserved and targeted within both SIVmac239 and SIV/DeltaB670. $CD8^+$ T cell-mediated responses directed against this region were identified in four unrelated animals. The novel 9mer epitopes GY9 ($Gag_{270\text{-}278}$) and QP9 ($Gag_{272\text{-}280}$) were identified in one vaccinee, yet specific epitope responses from the remaining three animals could not be defined. As the animals responding to this region lack a consensus MHC genotype, it is possible that $Gag_{269\text{-}291}$-directed immunity is conferred by a single undefined allele common among the study responders. Regardless of epitope definition and MHC restriction, IFN-γ response to this region was associated with heightened control of post-challenge viremia, as two of three $Gag_{269\text{-}291}$-responsive animals maintained undetectable viral loads for several months. Interestingly, we did not detect stable $CD8^+$ T cell-mediated epitope escape mutations within the $Gag_{269\text{-}291}$ region, M1701 who had previously contained viremia died of AIDS at wk 62 post-infection with the wild-type $Gag_{269\text{-}291}$ sequence preserved in all viral clones isolated at the time of death,

suggesting that although immunogenic, the $Gag_{269-291}$ region may be of lesser importance in virologic control. It is evident that broad vaccine induced cellular immunity targeting multiple structurally conserved Ags and evolutionarily restricted viral epitopes will be necessary to establish durable control of heterologous viral infection as supported by the current and similar investigations [326, 341, 351, 353].

$CD8^+$ T cell-mediated epitope escape occurs continually throughout SIV/HIV infection, with immunologic T cell-mediated pressure driving viral mutations inhibiting Ag processing, presentation or recognition, often accompanied by reduced viral fitness [224, 240, 354]. The capsid-embedded, Mamu-A*01-restricted CM9 epitope is widely conserved among SIV stains and other lentiviruses, including SIV/DeltaB670 as shown here, and is highly immunogenic among SIV species [355]. CM9 is generally resistant to escape mutation due to accompanying defects in virion maturation and Gag protein production [239, 356-358], and CM9 escape is associated with loss of virologic control and the development of AIDS [345, 357, 359]. Two valine-for-isoleucine compensatory mutations flanking the CM9 sequence at positions 161 and 206 of Gag (I161V and I206V) have been shown to restore proper virion production and in vitro fitness during SIVmac239 and SHIV-89.6P infection [360, 361], and can be stably transmitted to susceptible hosts in the absence $CD8^+$ T cell-mediated targeting [362]. Here we show that SIV/DeltaB670 is capable of undergoing classic position two anchor residue escape mutation in the absence of temporally associated compensatory flanking mutations in the presence of potent CM9-specific $CD8^+$ T cell-mediated responses. CM9 escape occurred relatively early in this model, suggesting mutation had only minor effects on viral fitness. Furthermore, in non-Mamu-A*01-expressing animals the CM9 epitope voluntarily sustained mutations in the absence of measurable epitope-specific T cell response. Interestingly, the protein sequence of SIV/DeltaB670 flanking CM9 is hypervariable when compared to SIVmac239, including a stable I161V substitution, with a dissimilarity of 12% compared to just 6% for the entire Gag protein. Our findings suggest that structural flexibility and stable mutations in the SIV/DeltaB670 capsid impart the ability to undergo early CM9 escape without dramatic fitness loss. In support, Mamu-A*01-expressing macaques lack a survival advantage over animals expressing other genotypes during SIV/DeltaB670 infection (data not shown), in contrast to SIVmac239 or SIVmac251 infection [363, 364].

HIV/SIV targeting by $CD4^+$ T cells is a crucial factor in controlling viral replication and maintaining $CD8^+$ T cell-mediated responses [133, 212], and is primary goal of effective vaccination. We currently show that dual-serotype Ad-based vaccination induced predominantly $CD8^+$ T cell-mediated responses, consisted with earlier reports [288]. Yet, we have identified a novel $CD4^+$ T cell-dependant epitope, GT15 ($Gag_{156-170}$), in the matrix protein induced by Ad5-priming and sustained through Ad35-based boosting. Although the GT15 epitope sequence is conserved between SIVmac239 and SIV/DeltaB670, detectable IFN-γ responses against this region were of low frequency and transient duration following viral challenge. These findings are consistent with previous reports by Vogel, et. al., showing lack of vaccine-induced $CD4^+$ T cell-mediated recall responses upon challenge with a closely related viral strain [349]. Potentially, $CD4^+$ T cell-dependant immunity may be lost due to rapid elimination of SIV-specific $CD4^+$ T cells following viral challenge, as findings have indicated that HIV/SIV preferentially replicate in activated, virus-specific $CD4^+$ T cells [39]. Together these findings illustrate the difficulty in inducing sustained Ag-specific $CD4^+$ T helper immunity following viral infection, and suggest intervention to salvage $CD4^+$ T cells post-infection may restore vaccine primed immunity.

3.7 CONCLUSIONS

The presented findings demonstrate the ability of Ad35-based vaccination to be utilized in conjunction with Ad5-based immunization, due to the lack of cross-neutralizing vector-specific Ab responses. Furthermore, ability of dual-serotype Ad-based vaccination targeting the Gag protein alone to limit viremia upon pathogenic SIV challenge highlights the superior immunogenicity of Ad-based vectors. Together, these findings strongly support the further examination and optimization of Ad-based vaccination regimens for the treatment of HIV infection.

4.0 CHAPTER TWO: IMMUNOTHERAPEUTIC ADENOVIRUS-BASED VACCINATION INDUCES ROBUST $CD8^+$ T CELL-BIASED IMMUNITY BUT FAILS TO CONTROL VIREMIA IN CHRONICALLY $SIV_{MAC}251$ INFECTED MACAQUES

4.1 PREFACE

The following chapter is in fulfillment of specific aim 2 and has been submitted for peer-reviewed publication. The described therapeutic vaccination study was a collaborative effort within the University of Pittsburgh involving the Barratt-Boyes lab for immunologic analysis, the Viral Vector Core for creation of Ad vectors, and the Department of Laboratory Animal Research at the Primate Facility for Infectious Disease Research for animal procedures and maintenance. Specifically, Adam C. Soloff performed all experiments excluding RT-PCR viral load determinations which were run by Xiangdong Liu. A. Soloff completed all data analysis and statistical evaluation, and was the primary author of the submitted manuscript with assistance from Dr. Barratt-Boyes. A. Soloff assisted in study design. Wentao Gao and Andrea Gambotto manufactured all Ad vectors utilized in the study. Richard D. Day was consulted on statistical analysis.

4.2 ABSTRACT

Effective approaches to immunotherapy for HIV infection are being sought with the goal of increasing immune control of virus in infected individuals. We tested a prime-boost immunotherapy strategy using recombinant adenovirus serotype 5 (Ad5) and Ad35-based vaccines given during two cycles of antiretroviral therapy in monkeys with chronic SIVmac251 infection. Vectors independently expressed SIV *gag*, *env*, and *nef* genes as well as bioactive

rhesus *IL-15*, used here as a potential vaccine adjuvant. Vaccination increased the frequency of circulating T cells specific for all three targeted viral Ag although this effect was lost with IL-15 delivery. Ag-specific $CD8^+$ T cells produced combinations of IFN-γ, TNF-α and/or IL-2, whereas Ag-specific $CD4^+$ T cells exclusively produced IFN-γ, with responses represented within both central and effector memory $CD4^+$ and $CD8^+$ T cell populations. Notably, vaccination did not change the proportion of $CD8^+$ or $CD4^+$ T cells producing one or more cytokines relative to control animals. Immunization transiently and incompletely restored peripheral blood $CD4^+$ Tcm numbers but failed to reverse $CD4^+$ Tem cell decline. Immunotherapy did not reverse profound mucosal $CD4^+$ T cell loss but induced Ag-specific $CD8^+$ T cell responses of a primarily monofunctional nature at the lung mucosa. Nevertheless, Ad-based vaccination had no impact on virus load. When analyzed independent of treatment group, virus control was correlated with polyfunctional blood $CD4^+$ and $CD8^+$ T cell responses and maintenance of blood $CD4^+$ Tcm cells and mucosal $CD4^+$ T cells. These data demonstrate that Ad-based immunotherapy given during chronic SIV infection, after loss of blood and mucosal $CD4^+$ T cells, induces robust but largely monofunctional $CD8^+$ T cell-biased responses that are incapable of controlling virus load.

4.3 INTRODUCTION

Advancements in the therapy of HIV infection have lead to a dramatic reduction in disease-associated morbidity and mortality, but have not been successful in preventing disease progression due to the emergence of viral drug resistance [365]. Although ART serves to restrict viral replication, providing a window of immunologic salvation and opportunity for restoration of $CD4^+$ T lymphocyte populations, it fails to eliminate latent viral reservoirs capable of regenerating infection upon treatment cessation [60, 366, 367]. Paradoxically, effective ART reduces the antigenic exposure necessary to drive virus-specific host immunity, leading to a loss of *de novo* immunologic response and a reduction in effector capabilities [368, 369]. Therefore, novel therapeutic strategies aimed at bolstering antiviral immunity and restoring immunologic balance during ART-treated HIV infection are urgently needed [370].

Therapeutic vaccination should aim to augment antiviral T cell-mediated immune responses and maintain $CD4^+$ Tcm populations, both strongly associated with control of infection. Virus-specific cellular immunity has been implicated as a dominant factor in the initial control of primary viremia [105, 106], maintenance of lowered chronic set-point viral loads [197, 199], and control of rebound viremia during STI [200, 201]. CTLs that target a breadth of viral epitopes, particularly in the Gag region, have been associated with reduced viremia in untreated HIV infection [214, 371]. Maintenance of cellular immune responses in mucosal tissues is also likely to be important in control of infection [372]. Additionally, recent studies have demonstrated that durable containment of HIV is predominantly a function of the qualitative polyfunctional characteristics of the cellular immune response rather than simply the magnitude of response [219, 221, 292]. Working in conjunction with the antiviral effector response, $CD4^+$ Tcm lymphocytes aid in the preservation and regeneration of T lymphocyte Tem populations during the course of HIV infection of humans and SIV infection of non-human primates [36]. In addition, augmentation of $CD4^+$ Tcm cells has been associated with vaccine-mediated protection against SIV infection [214].

There has been significant interest in the use of cytokines to augment vaccine-induced immune responses to infection, particularly focused on IL-15. Loss of IL-15 has been observed in progressive HIV infection [166, 373], potentially leading to homeostatic disruption of T lymphocyte memory populations. Conversely, elevated plasma IL-15 concentrations are associated with control of viremia during STI within chronic HIV infection [167]. Cytokine chemotherapy utilizing IL-15 has been shown to augment $CD4^+$ and $CD8^+$ T cell memory populations in viral infection [177, 374] and enhance antiviral cellular immunity [172, 179-181]. In addition, IL-15 treatment has been found to rescue Ag-specific T cell effector and proliferative responses from dysfunctional HIV-infected PBMC *in vitro* [168, 175, 375]. However, recent reports have observed increases in acute SIV viremia and ablation of therapeutic vaccine-mediated reductions in SIV viral loads following systemic administration of high-dose IL-15 [376, 377]. As a result of these contradictory reports, the therapeutic benefits of IL-15 in promoting antiviral immunity remain unclear.

Therapeutic vaccine trials have employed inactivated virus, poxvirus vectors, DNA, and Ag-pulsed dendritic cells among many other approaches with varying degrees of success in controlling HIV and SIV infection [200, 280, 378, 379]. Due to their potent ability to induce

transgene-specific cellular immunity, adenoviral (Ad)-based vectors have emerged as one of the most promising candidates for HIV vaccine development [288, 304], although enthusiasm has been tempered recently following disappointing results from a large vaccine trial [380]. Ad-based approaches to immunotherapy for HIV infection have not been reported to date. To circumvent vector-specific immunity, Ad vectors based on rare human and nonhuman primate serotypes have been developed, providing vectors impervious to cross-neutralization and appropriate for prime-boost immunization regimens [310-312, 320, 381]. In the current study, we employed a heterologous immunization regimen utilizing Ad serotype 5 (Ad5) - and Ad35-based vectors [289] to enhance antiviral cellular immunity against the Gag, Env, and Nef proteins during infection of rhesus macaques with pathogenic SIV. To address the potential for therapeutic efficacy of IL-15, a subset of animals additionally received Ad-based vaccines expressing the rhesus *IL-15* gene. To provide a realistic model of clinical HIV infection, we delayed intervention until establishment of chronic infection and administered vaccines during two periods of ART interspersed by a STI.

4.4 MATERIALS AND METHODS

4.4.1 Animals and SIV infection

Twenty-one colony-bred Indian rhesus macaques (*Macaca mulatta)* were housed at the University of Pittsburgh Primate Facility for Infectious Disease Research and maintained in accordance with institutional regulations. Molecular MHC class I typing for the rhesus macaque alleles Mamu-A*01, A*02, A*08, A*11, B*01, B*03, B*04 and B*17 was carried out through a contract with the Wisconsin National Primate Research Center. Animals were i.v. infected with 1,000 $TCID_{50}$ of uncloned, pathogenic SIVmac251 provided by Chris Miller (University of California, Davis). The principal criterion for inclusion in the study, for which fifteen animals qualified, was a median set-point SIV plasma viral load $\geq 10^5$ RNA copies/ml and $\leq 10^7$ RNA copies/ml from wk 6 to wk 12 when ART was initiated. These animals were stratified based on gender and expression of Mamu-A*01 (R479, R480, R487), and subsequently randomized into three groups receiving Adψ vectors lacking expression of transgene (control, n=4), Ad-based

vectors expressing SIV *gag, env,* and *nef* genes (Ad-SIV, n=5), or Ad-based vectors expressing SIV *gag, env,* and *nef* and rhesus *IL-15* (Ad-SIV/IL-15, n=6).

4.4.2 ART and therapeutic vaccination

All animals received ART consisting of 9-[2-(phosphonylmethoxy)propyl]adenine (PMPA; 20mg/kg/day, s.c. injection) and 2'-deoxy-5-fluoro-3'-thiacytidine (FTC; 30mg/kg/day, s.c. injection), from wk 12 to wk 24 post-infection (PI, ART cycle-1) and again from wk 32 to wk 44 PI (ART cycle-2). PMPA and FTC were provided by Michael Miller (Gilead Sciences, Inc). Ad5-based vectors were given by i.m. injection during ART cycle-1 at wk 16 ($5x10^{10}$ viral particles per vector) and at wk 22 ($1x10^{11}$ viral particles per vector), and Ad35-based vectors were given by the same route and dose during ART cycle-2 at wk 36 and wk 42 PI. Control-treated animals received injections of similar amounts of the Adψ5 and Ad ψ35 vectors, respectively.

4.4.3 Recombinant Ad vectors

Ad5- and Ad35-based vectors expressing *egfp* or codon-optimized SIVmac239 *gag, env,* or *nef* or lacking transgene were developed as previously described [289, 310]. *IL-15* was RT-PCR amplified from total RNA extracted from healthy macaque PBMC following 24 h stimulation with IL-2 and SEB using the sense (5'CGTCGACGGATCCGCCACCATGAGAATTTCGAAACCACATTTGAG3') and anti-sense (5'GCGAATTCTCAAGAAGTGTTGATGAACATTTGGACAATATGTAC3') primers and subcloned into the pAdlox shuttle plasmid. Incorporation of the IL-2 signaling peptide sequence (SP2) and C-terminal stop codon and FLAG epitope into the *IL-15* expression plasmid was performed through successive rounds of PCR utilizing the listed primer sequences based on previous reports [140]. The stop codon was inserted upstream of the FLAG sequence to prevent expression of the FLAG epitope and remove the potential for production of anti-FLAG Ab *in vivo*. Ad5- and Ad35-based vectors containing the optimized SP2.IL-15.FLAG sequence were created using established methods [289, 310]. All Ad vectors were E1/E3-deleted with the exception of Ad35 containing the *env* transgene, which was E3-deleted due to technical

difficulties in generating E1/E3-deleted Ad35-*env*. Protein expression by Ad5- and A35-based vectors expressing SIV genes was confirmed by Western blot analysis of infected HEK293 cells as described [289] (data not shown).

4.4.4 IL-15 production and bioactivity

Cytokine production was measure by human IL-15-specific ELISA kit using the rhesus cross-reactive capture (G243-935) and detection (G243-886) Abs (BD PharMingen, San Jose, CA). ELISAs were performed on supernatants harvested 48 h after transfection of HEK293T cells with pAdlox plasmids or infection with Ad5- or Ad35-based vectors at an MOI of 100. MTT cell proliferation assays were performed to measure the capacity of Ad-produced IL-15 to propagate the IL-2/IL-15 dependant CTLL-2 cell line [172]. Briefly, CTLL-2 cells were incubated for 24 h with either exogenous IL-2 or culture supernatants and then incubated for 6 h in the presence of 10µl MTT reagent prior to lysis and detection of absorbance at 570 nm wavelength.

4.4.5 Measurement of SIV RNA in plasma

Viral RNA was isolated from cell-free plasma of SIV-infected monkeys and SIV RNA levels quantified by real-time PCR as described previously [289]. Plasma SIV RNA levels were detected to a sensitivity of 10^3 copies/ml with lesser values reported at a baseline of 10^4 copies/ml.

4.4.6 IFN-γ ELISPOT

Effector T cell responses to SIV Ag were analyzed in previously cryopreserved PBMC by IFN-γ ELISPOT assay using pools of overlapping peptides representing the Gag, Env, and Nef proteins of SIVmac239 as previously described [289]. Responses that were two times that of DMSO background with a minimum number of 50 spots per 10^6 PBMC were scored as positive.

4.4.7 T cell phenotype and intracellular cytokine staining

Phenotypic characterization and detection of Ag-specific cytokine production from T cells within cryopreserved PBMCs were performed simultaneously as previously described with minor modifications [382]. All Abs listed in this study were procured from BD Biosciences (BD Biosciences, San Jose, CA) unless otherwise specified. Briefly, cells were stimulated at $1x10^6$ to $5x10^6$ cells per test with final concentrations of 1.0μg/ml anti-CD49d (9F10) and Gag, Env, or Nef peptide pools, or 50ng/ml PMA and 1.0μg/ml ionomycin (Sigma, St. Louis, MO) for 6 h with brefeldin A (10μg/ml, Sigma) present for the last 5 h. Fresh bronchoalveolar lavage (BAL) samples were treated similarly using the dominant Ag pool identified through IFN-γ ELISPOT. Samples were labeled to exclude dead cells by incubation with amine reactive UV Live/Dead dye (Molecular Probes, Carlsbad, CA) or 0.5μg/ml ethidium monoazide (Sigma) followed by fluorescent light fixation for 15 min, and then stained for surface Ag using mAbs specific for CD3 conjugated to Alexa488 (CD3-Alexa488; SP34-2), CD4-AmCyan (L200, NIH AIDS Research and Reference Reagent Program), CD8-APC-Cy7 (RPA-T8), CD28-PE-Cy5 (28.2), and CD95-Alexa647 (DX2). Cells were then fixed and permeabilized by treatment with BD Cytofix/Cytoperm solution followed by labeling with mAbs specific for IFN-γ-PE-Cy7 (4S.B3), TNF-α-Pacific Blue (Mab11, eBioscience, San Diego, CA), and IL-2-PE (MQ1-17H12) for 30 min at 4^oC in the presence of permeabilizing staining buffer. Cells were collected immediately on a BD LSRII flow cytometer (Beckman Dickenson) using FACSDiva software for analysis. A minimum of 300,000 events were collected with values greater than three times DMSO background scored as positive.

4.4.8 T lymphocyte quantitation

Absolute quantitation of peripheral blood T lymphocytes was performed using TruCount beads (BD Biosciences) based on manufacturer's suggestions. Briefly, 50μl of peripheral whole blood collected in EDTA was added to TruCount tubes containing CD3-PE, CD4-FITC, and CD8-PE-Cy5 specific mAbs, and incubated at room temperature for 20 min prior to the addition of BDfix/lyse solution (Beckman Dickenson). Cells were collected the same day on a BD LSRII with 2,500-10,000 $CD3^+$ T cell events analyzed using FACSDiva software. Absolute numbers of

memory T cells were calculated from the known quantity of $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells per µl whole blood. Baseline values were determined by averaging 2-3 pre-infection timepoints.

4.4.9 Statistical analysis

Given the small sample sizes used in this study, nonparametric analysis was performed throughout this investigation. Comparisons between treatment groups were carried out using the Mann-Whitney U test, while assessments of variation within each group were made using the Wilcoxon ranked-sum test. SPSS 14.0 software (SPSS Inc., Chicago, IL) was utilized for these calculations. Correlation coefficients were calculated using the Spearman rank-order test and performed using Graphpad PRISM 5 (Graphpad Software, Inc., La Jolla, CA). All *P* values are two-sided with significance considered to be $P < 0.05$.

4.5 RESULTS

4.5.1 Ad-mediated delivery of bioactive rhesus *IL-15*

To incorporate IL-15 administration into our immunotherapy regimen, we first developed and tested Ad-based vectors expressing rhesus macaque IL-15. Transfection by pAdlox encoding rhesus *IL-15* resulted in notable cytokine production compared to control transfection with pAdlox-*egfp* (Fig. 8a). Removal of C-terminal translational inhibition through inclusion of a FLAG epitope provided a 4-fold increase in IL-15 production to 208±7 pg/ml, consistent with previous reports [140]. Insertion of the IL-2 signal sequence, facilitating cytokine secretion, on its own into the pAdlox-IL-15 plasmid had minimal effect, whereas addition of the IL-2 signal sequence together with the FLAG epitope resulted in a 5-fold increase in IL-15 production over the unaltered IL-15-expressing plasmid to 260±36 pg/ml (Fig. 8a). Ad5- and Ad35-based vectors expressing SP2.IL-15.FLAG (Ad5-IL-15 and Ad35-IL-15, respectively) were subsequently tested for the capacity to induce IL-15 expression following 48 h infection of HEK293T cells. Ad5-IL-15 and Ad35-IL-15 transduction resulted in high-level expression of

IL-15, with levels of 1,988±11 pg/ml and 1,008±6 pg/ml, respectively (Fig. 8b). Control transduction with Ad5-EGFP and Ad35- EGFP or mock transduction of cells produced undetectable levels of IL-15 (Fig. 8b). The reduced levels of cytokine production by Ad35- as compared to Ad5-based vectors are due to differences in post-infection replicative capacity and subsequent protein production within HEK293T cells, and are not observed in cells of hematopoietic origin (data not shown). Finally, we examined the bioactivity of Ad-derived IL-15 using the IL-2/IL-15 dependant CTLL-2 cell line. Viable and proliferating CTLL-2 cells cultured for 24 h in the presence of exogenous IL-2 were standardized to 100% (Fig. 8c). When CTLL-2 cells were cultured in the presence of supernatants from HEK293T cells transduced with Ad5-IL-15 or Ad35-IL-15, we observed five and three fold enhancement of cellular proliferation, respectively, over that induced by IL-2, demonstrating functional bioactivity. Non-transduced supernatants lacking IL-2 or IL-15 failed to sustain CTLL-2 cells, leading to rapid cell death and undetectable MTT measurement (Fig. 8c). These data indicate that Ad-based vectors encoding an optimized rhesus macaque *IL-15* expression construct are highly effective at generating bioactive IL-15 in infected cells.

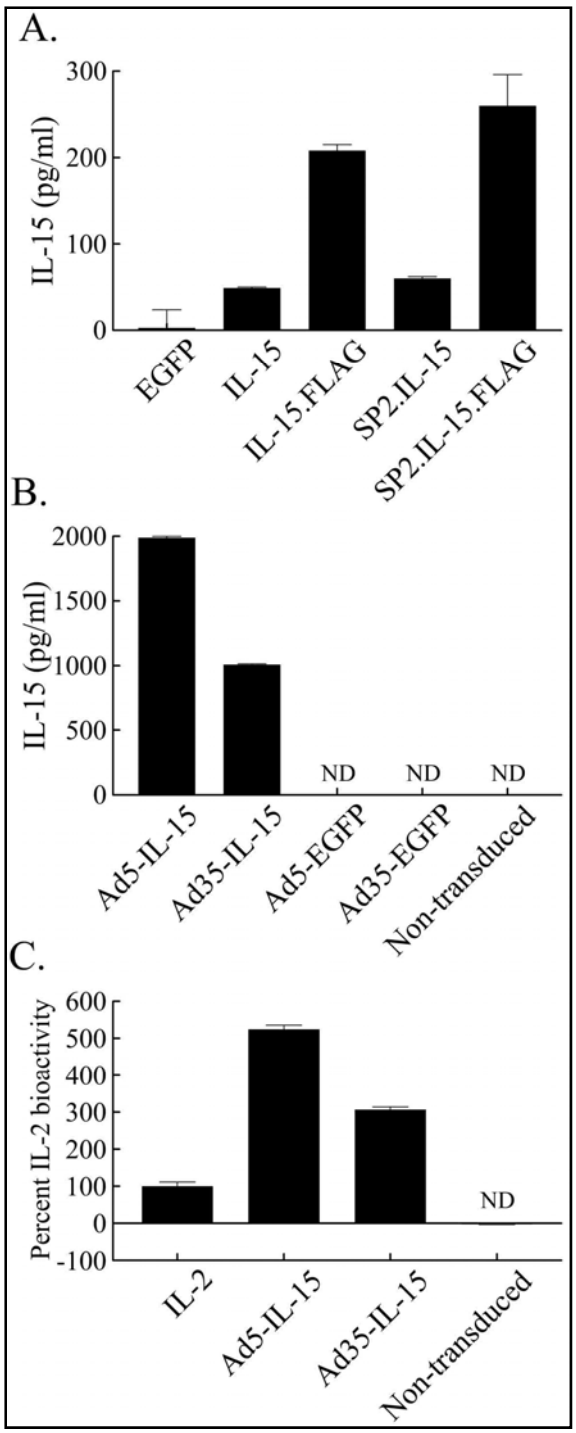

Figure 8: Expression and bioactivity of rhesus IL-15 following plasmid transfection and recombinant Ad infection.

(A) Presence of rhesus IL-15 in HEK293T cell supernatants following transfection with pAdlox expressing *egfp, IL-15*, or *IL-15* including the IL-2 signaling peptide sequence and/or c-terminal FLAG epitope as determined by ELISA. (B) Presence of rhesus IL-15 in HEK293 T cell supernatants following infection with Ad5- or Ad35-IL-15, Ad5- or Ad35-EGFP, or mock transduction as determined by ELISA. (C) Bioactivity of rhesus IL-15 in HEK293 T cell supernatants following Ad5- or Ad35-IL-15 infection or mock transduction as determined by CTLL-2 cell propagation using the MTT assay. Propagation was standardized against CTLL-2 cell activity in response to IL-2. Shown are means of triplicate determinations with SEM. ND = not detected.

4.5.2 Therapeutic vaccination enhances the strength and breadth of Ag-specific immunity

To determine the capacity of Ad-based immunotherapy, with and without IL-15, to enhance Ag-specific immune responses in nonhuman primates with established SIV infection, infected macaques received Ad5- and Ad35-based immunizations during two cycles of ART as described in Materials and Methods (Table 2). IFN-γ ELISPOT analysis was performed bi-weekly to measure the magnitude of SIV-specific cellular immune responses over time. Animals that received Ad5-*gag*, Ad5-*env,* and Ad5-*nef* (collectively called Ad5-SIV) alone at wk 16 and wk 22 during ART cycle-1 had significantly increased cumulative IFN-γ responses to all three viral proteins at wk 22 and wk 24 as compared to control animals, with an average frequency of around 1 Ag-specific cell per 1,000 PBMC (Fig. 9a, c). In contrast, animals receiving Ad5-SIV with Ad5-IL-15 had limited responses to vaccination as compared to controls (Fig. 9a, c), although when all vaccinated animals were evaluated irrespective of Ad-IL-15 delivery statistically significant differences were maintained (Fig. 9b). IFN-γ ELISPOT responses returned to baseline levels in vaccinated animals during the first STI, but boosting with Ad35-SIV at wk 36 and wk 42 during ART cycle-2 rapidly expanded the frequency of virus-specific IFN-γ producing cells as compared to controls, with significant differences at wk 38 and wk 42 reaching an average frequency of around 1 Ag-specific cell per 300 PBMC (Fig. 9a, c). As in the first treatment cycle, animals that received the combination of Ad35-SIV/IL-15 had modest increases that were not significantly different than control animals (Fig. 9a, c), whereas all vaccinated animals analyzed collectively, irrespective of IL-15 delivery, maintained significant increases in IFN-γ ELISPOT responses over controls (Fig. 9b). During the second STI, IFN-γ ELISPOT responses in both SIV and SIV/IL-15-vaccinated groups were greater than those of mock-treated animals, but did not attain significance (Fig. 9a, b). Notably, intermittent cycles of ART followed by STI in the absence of immunotherapy failed to enhanced T cell responses to virus, as animals in the control group maintained relatively stable frequencies of Ag-specific T cells over the entire course of the study (Fig. 9a-c).

To examine whether vaccination influenced the breadth of T cell responses against targeted viral Ags, we evaluated the IFN-γ ELISPOT responses against Gag, Env, and Nef proteins individually. Immunization with both Ad5-SIV and Ad35-SIV, irrespective of IL-15

immunotherapy, significantly increased peak IFN-γ responses against each viral protein from those identified at the initiation of ART cycle-1 (wk 12) and ART cycle-2 (wk32), respectively (Fig. 9d). In contrast, mock immunized animals failed to develop significant enhancement of peak Gag-, Env-, or Nef-specific immunity during either ART cycle (Fig. 9d). These data indicate that vaccination with Ad-SIV vectors was effective at enhancing the magnitude and antigenic breadth of the IFN-γ response to SIV, but that addition of IL-15 in the vaccine regimen provided no detectable benefit.

Table 2: Animal characteristics and immunotherapy schedule.

Table 2. Animal characteristics and immunization schedule

ID	Age, (years) Gender	Mamu MHC Class I	ART Cycle 1*			ART Cycle 2*			Endpoint* (AIDS)
			Ad-ψ5	Ad5-SIV (*gag, env, nef*)	Ad5-IL-15	Ad-ψ35	Ad35-SIV (*gag, env, nef*)	Ad35-IL-15	
R478	4, M	A*02, A*08, B*01	16, 22	-	-	36, 42	-	-	NA†
R480	4, M	A*01	16, 22	-	-	36, 42	-	-	NA
R484	4, M	A*11	16, 22	-	-	36, 42	-	-	NA
M5306	4, F	ND‡	16, 22	-	-	36, 42	-	-	NA
R180	7, F	B*01	-	16, 22	-	-	36, 42	-	45
R479	4, M	A*01	-	16, 22	-	-	36, 42	-	NA
R483	5, M	ND	-	16, 22	-	-	36, 42	-	42
R486	6, M	A*08, B*17	-	16, 22	-	-	36, 42	-	NA
R489	4, M	A*08	-	16, 22	-	-	36, 42	-	NA
R183	8, F	B*17	-	16, 22	16, 22	-	36, 42	36, 42	44
R481	4, M	ND	-	16, 22	16, 22	-	36, 42	36, 42	NA
R485	7, M	ND	-	16, 22	16, 22	-	36, 42	36, 42	34
R487	5, M	A*01, B*01	-	16, 22	16, 22	-	36, 42	36, 42	NA
M5506	4, F	A*08	-	16, 22	16, 22	-	36, 42	36, 42	NA
M5606	4, F	A*08	-	16, 22	16, 22	-	36, 42	36, 42	NA

* Weeks post-SIVmac251 infection

† Not attained by study completion

‡ None detected

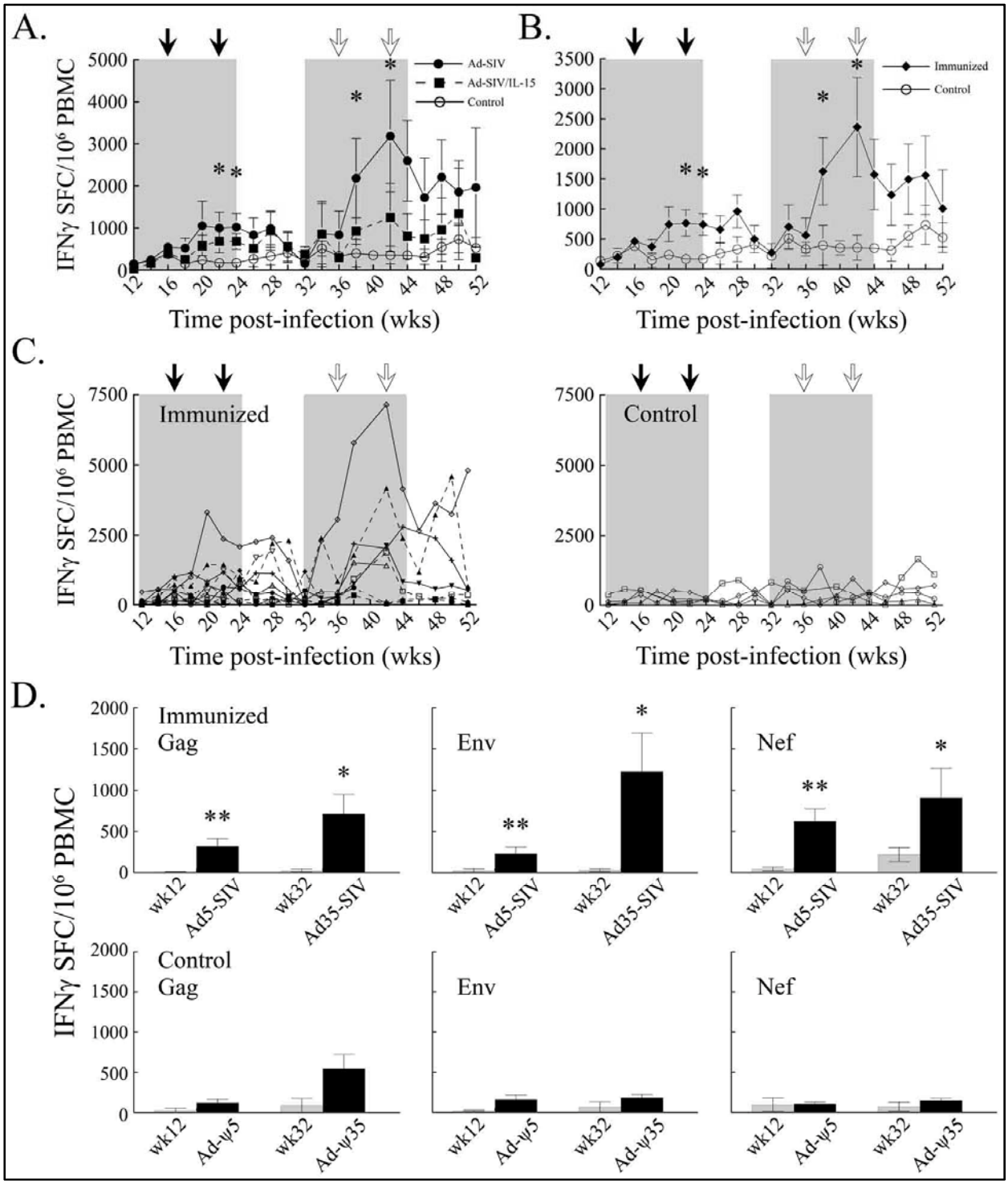
A.
IFNγ SFC/10⁶ PBMC
Ad-SIV
Ad-SIV/IL-15
Control
Time post-infection (wks)
B.
Immunized
Control
Time post-infection (wks)
C.
Immunized
Control
IFNγ SFC/10⁶ PBMC
Time post-infection (wks)
D.
Immunized
Gag
Env
Nef
Control
Gag
Env
Nef
IFNγ SFC/10⁶ PBMC
wk12
Ad5-SIV
wk32
Ad35-SIV
Ad-ψ5
Ad-ψ35

Figure 9: Robust enhancement of cellular immunity following Ad5- and Ad35- based immunotherapy in SIV infected monkeys.
PBMC were harvested from SIV-infected monkeys at intervals after initiation of ART at wk 12 and tested for Ag-specific IFN-γ production by ELISPOT assay. (A-C) All animals received two cycles of ART as depicted by shaded regions and therapeutic or mock-vaccination with Ad5- and Ad35-based vaccines at times indicated by closed and open arrows, respectively. (A) Cumulative IFN-γ responses against Gag, Env, and Nef peptide pools for animals receiving Ad-ψ5 control vectors (open circles), Ad-SIV (closed circles), or Ad-SIV together with Ad-IL-15 (closed squares). Shown are mean ± SEM. (B) Cumulative IFN-γ responses against Gag, Env, and Nef peptide pools for animals receiving Ad-ψ5 control vectors (open circles) or Ad-SIV vaccination irrespective of Ad-IL-15 delivery (closed diamonds). Shown are mean ± SEM. (C) Individual cumulative IFN-γ responses against Gag, Env, and Nef peptide pools for immunized animals (left) receiving Ad-SIV (solid lines) or Ad-SIV plus Ad-IL-15 (dotted lines) or control animals receiving Ad5ψ and Ad35ψ (right). (D) IFN-γ responses to Gag, Env, and Nef peptide pools in PBMC from vaccinated (top) and control (bottom) animals, comparing responses at the initiation of ART cycle-1 (wk 12) and ART cycle-2 (wk 32) with peak responses following Ad5- and Ad35-based immunotherapy, respectively. Animals receiving Ad-SIV alone or in combination with Ad-IL-15 (top graphs) are depicted as solid and dotted lines, respectively. *=P<.05, **=P<.01.

4.5.3 Ad-based immunotherapy induces polyfunctional $CD8^+$ but monofunctional $CD4^+$ T cell responses to SIV during established infection

We next analyzed the functional character of vaccine-induced T cells, focusing on production of the Th1 cytokines IFN-γ, IL-2, and TNF-α, as coexpression of these cytokines by $CD4^+$ and $CD8^+$ T cells is associated with virologic control in HIV-1-infected individuals [219, 222, 383]. Utilizing multiparameter flow cytometry, we compared systemic T cell responses at the initiation of ART cycles-1 and 2 with peak responses following Ad5- and Ad35-based immunotherapy, respectively, as previously determined by IFN-γ ELISPOT (Fig. 10a). For simplicity, vaccinated animals were assessed collectively, irrespective of Ad-IL-15 delivery, for responses induced by individual Gag, Env, and Nef peptide pools combined. Ad5-SIV immunotherapy increased the frequency of monofunctional Ag-specific $CD8^+$ T cells producing either IFN-γ or TNF-α but not IL-2 (Fig. 10b). In addition, the magnitude of polyfunctional $CD8^+$ T cells producing combinations of IL-2 and IFN-γ, IFN-γ and TNF-α or all three cytokines was increased in response to Ad5-based immunization (Fig. 10b). However, the proportion of total Ag-specific $CD8^+$ T cells producing two or three cytokines in response to Ag stimulation was not increased in vaccinated animals relative to controls and remained minor, with a mean of 19% and 1%, respectively, for all vaccinated animals (Fig. 10d). Boosting with Ad35-SIV further expanded the frequency of virus-specific $CD8^+$ T cells producing IFN-γ alone but had no impact on polyfunctional T cell responses (Fig. 10b). In the case of Ag-specific $CD4^+$ T cells,

Ad5-based vaccination increased only the frequency of cells producing IFN-γ alone and had no impact on $CD4^+$ T cells producing more than one cytokine (Fig. 10c, d). This effect was lost upon Ad35 boosting, as the peak frequency of Ag-specific $CD4^+$ T cells producing any combination of cytokines was not significantly different from the baseline frequency at wk 32 (Fig. 10c). Moreover, the proportion of $CD8^+$ and $CD4^+$ T cells producing one, two, or three cytokines as a result of Ad5 and Ad35 vaccinations was essentially the same as control animals receiving mock Ad-based vaccination (Fig. 10d). As expected, no increase in the frequency of Ag-specific $CD8^+$ or $CD4^+$ T cells was observed in control animals during either ART cycle for any cytokine (Fig. 10b, c). These data indicate that Ad-based immunization bolstered the strength of antiviral cell-mediated immunity, but vaccination had limited impact on the qualitative nature of this response, particularly with respect to the $CD4^+$ T cell response.

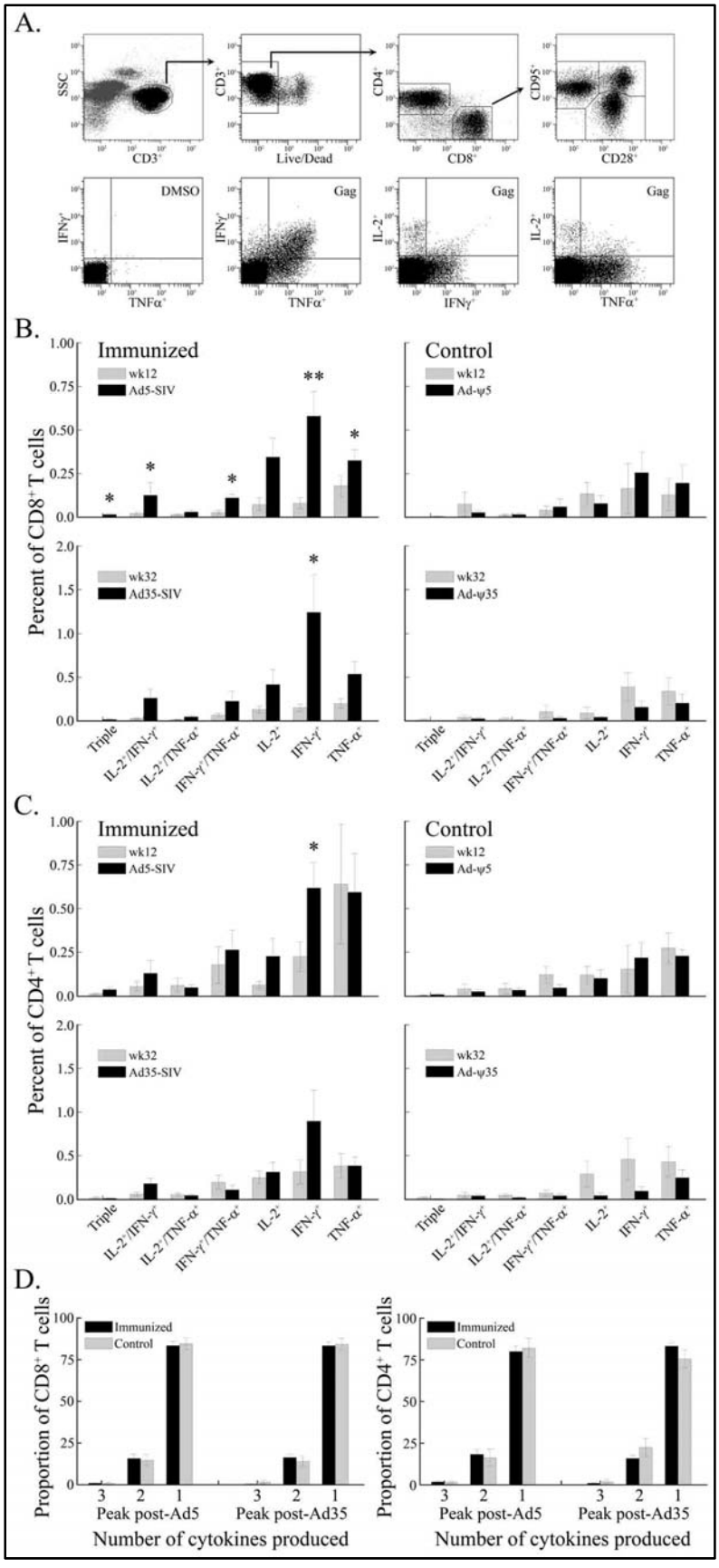
A.
SSC
CD3+
CD3+
Live/Dead
CD4+
CD8+
CD95+
CD28+
DMSO
Gag
Gag
Gag
IFNγ+
TNFα+
IFNγ+
TNFα+
IL-2+
IFNγ+
IL-2+
TNFα+
B.
Immunized
wk12
Ad5-SIV
Control
wk12
Ad-ψ5
wk32
Ad35-SIV
wk32
Ad-ψ35
Percent of CD8+ T cells
1.00
0.75
0.50
0.25
0.0
2.0
1.5
1.0
0.5
0.0
Triple
IL-2+/IFN-γ+
IL-2+/TNF-α+
IFN-γ+/TNF-α+
IL-2+
IFN-γ+
TNF-α+
C.
Immunized
wk12
Ad5-SIV
Control
wk12
Ad-ψ5
wk32
Ad35-SIV
wk32
Ad-ψ35
Percent of CD4+ T cells
1.00
0.75
0.50
0.25
0.0
2.0
1.5
1.0
0.5
0.0
Triple
IL-2+/IFN-γ+
IL-2+/TNF-α+
IFN-γ+/TNF-α+
IL-2+
IFN-γ+
TNF-α+
D.
Proportion of CD8+ T cells
Immunized
Control
100
75
50
25
0
3
2
1
3
2
1
Peak post-Ad5
Peak post-Ad35
Number of cytokines produced
Proportion of CD4+ T cells
Immunized
Control
100
75
50
25
0
3
2
1
3
2
1
Peak post-Ad5
Peak post-Ad35
Number of cytokines produced

Figure 10: Polyfunctional $CD8^+$ and monofunctional $CD4^+$ T cell responses induced by Ad-based immunization of SIV-infection monkeys.
(A) Representative flow cytometry dot plots depicting the gating strategy for the identification of live $CD4^+$ and $CD8^+$ T cell subsets (top) and $CD8^+$ T cells expressing combinations of IFN-γ, TNF-α, and IL-2 following stimulation with Gag peptides or mock-stimulation (DMSO, bottom). (B, C) Frequency of $CD8^+$ (B) and $CD4^+$ (C) T cells in PBMC expressing IFN-γ, IL-2, or TNF-α either alone or in combination from animals receiving Ad-SIV immunization, irrespective of Ad-IL-15 administration, or mock immunized controls (Adψ). Shown are mean values ± cumulative responses to Gag, Env, and Nef peptide pools at the initiation of ART cycle-1 (wk 12) and ART cycle-2 (wk 32) compared to peak responses following Ad5- and Ad35-based immunization, respectively. (D) Proportion of Ag-specific $CD8^+$ (Left) and $CD4^+$ (Right) T cells producing 1, 2, or 3 cytokines at the peak response following immunization with Ad5-SIV and Ad35-SIV (Immunized) or Ad-ψ5 and Ad-ψ35 (Control). *=P<.05, **=P<.01.

4.5.4 Differential effects of Ad-based immunotherapy on $CD4^+$ and $CD8^+$ T cell memory subsets in the peripheral blood

We next determined whether $CD8^+$ and $CD4^+$ T cell responses induced by vaccination in chronic infection were mediated by central memory or effector memory subsets or both. $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells were identified as Tcm ($CD28^+CD95^+$) or Tem ($CD28^-CD95^+$) [384] and SIV-specific cytokine production was measured in these cells at the time of peak response following Ad-based immunization, irrespective of IL-15 administration, using multiparametric flow cytometry (Fig. 10a). Both $CD8^+$ Tcm and $CD8^+$ Tem cells were rapidly and significantly increased in number in the peripheral blood by wk 12 of infection relative to pre-infection levels, and vaccination beginning at wk 16 did not impact this response (Fig. 11a, c). However, Ad5-SIV immunization significantly increased the peak frequency of Ag-specific $CD8^+$ Tcm cells producing one cytokine relative to control animals, and this effect was extended to $CD8^+$ Tcm cells producing two cytokines following the Ad35 boost (Fig. 11b, d). Similar enhancement of peak Ag-specific responses following Ad5- and Ad35-based vaccination from $CD8^+$ Tem cells producing one or more cytokine combinations was observed, but failed to attain statistically significant variation relative to mock immunized control animals (Fig. 11b, d). These findings indicate that immunization with Ad5 and Ad35 induces durable $CD8^+$ T cell responses from both central and effector memory compartments during established infection.

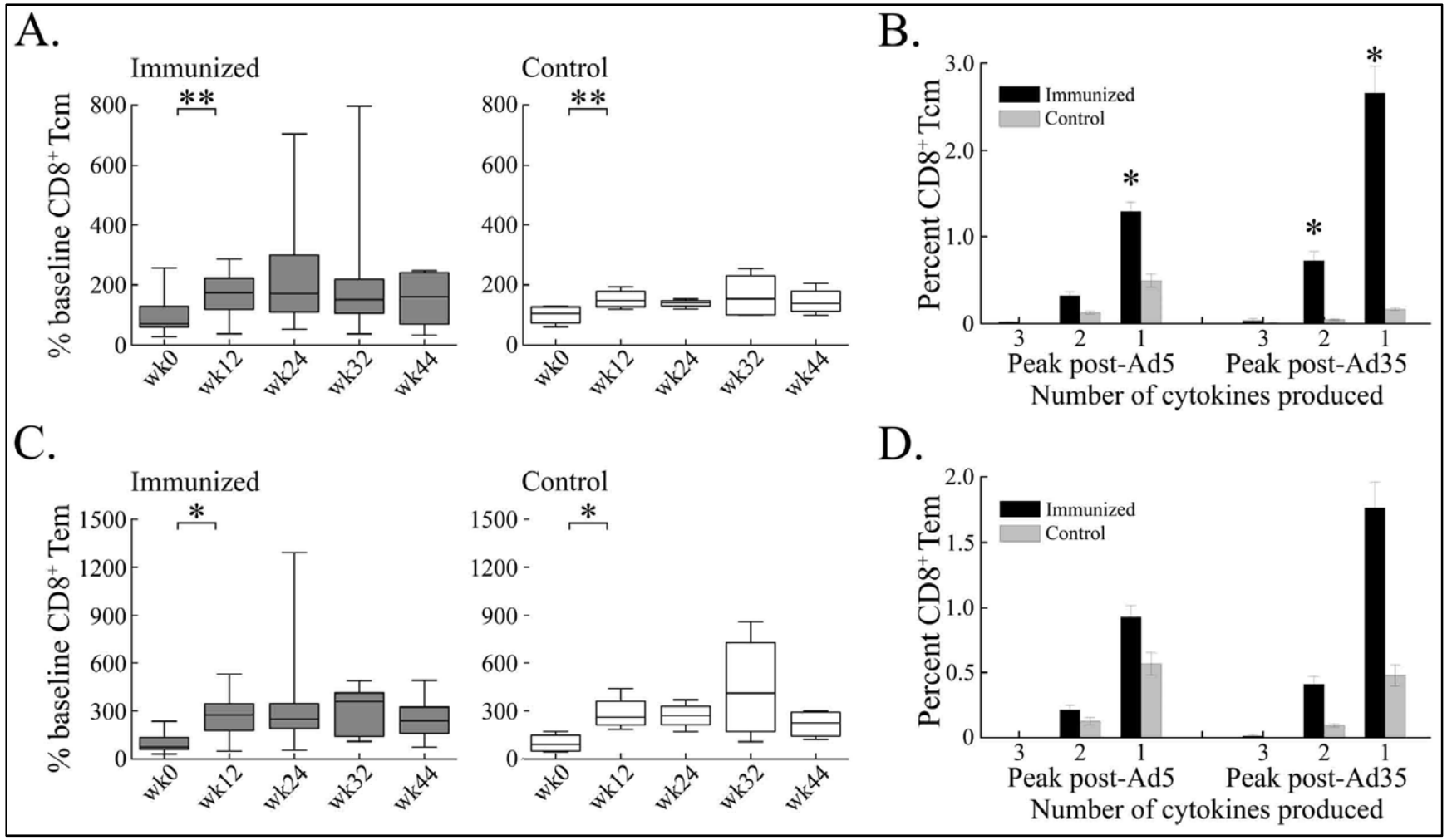

Figure 11: Ad-based immunization induces primarily Ag-specific CD8$^+$ Tcm in the peripheral blood of SIV-infected monkeys.
(A, C) Changes in the number of circulating CD8$^+$ Tcm cells (A) and CD8$^+$ Tem cells (C) in animals at various intervals after immunization with Ad-SIV with and without Ad-IL-15 (left, closed) or control vectors (right, open) depicted as percent change from baseline. Shown are box-whisker plots depicting median and interquartile ranges with SEM. (B, D) Frequency of cumulative SIV-specific CD8$^+$ Tcm (B) and CD8$^+$ Tem (D) responses consisting of 1, 2 or 3 cytokine profiles following Ad5- (left) and Ad35- (right) administration for Ad-SIV immunized animals, irrespective of IL-15 administration, and mock immunized controls. Shown are mean ± SEM. (*=P<.05, **=P<.01).

In contrast to $CD8^+$ T cell subsets, there was a rapid loss of circulating $CD4^+$ T cells in all study animals prior to ART at wk 12 that corresponded to a precipitous decline in Tcm cells (Fig. 12a), consistent with previous reports [36]. Notably, Ad5 prime and Ad35 boost vaccinations resulted in transient increases in the number of $CD4^+$ Tcm cells between wk 12 and 24 and wk 32 and 44, respectively, which translated into a partial normalization of total $CD4^+$ T cells to 80% of pre-infection levels. No recovery of $CD4^+$ Tcm cells was observed in control animals over this period, indicating that this effect was due to vaccination and not antiretroviral therapy alone (Fig. 12a). There was a trend towards an increase in the peak frequency of Ag-specific $CD4^+$ Tcm cells producing 1 or 2 cytokines in response to Ad5 and Ad35 vaccination although this did not reach significance (Fig. 12b). $CD4^+$ Tem populations increased between the time of infection and initiation of antiretroviral therapy at wk 12 but were gradually eliminated from the peripheral blood of all study animals over time (Fig. 12c). Ad5-based immunization resulted in high peak frequencies of Ag-specific $CD4^+$ Tem cells relative to controls, with 12% and 4% of the population producing 1 and 2 cytokines, respectively; however boosting with Ad35 was incapable of sustaining this response (Fig. 12d). Notably, peak Ag-specific $CD4^+$ Tem responses following Ad35-based vaccination were reduced by 5 fold compared to those attained during Ad5 administration within both immunized and control groups (Fig. 12d). These results indicate that Ad-based immunotherapy initiated during chronic SIV infection was effective at transiently correcting $CD4^+$ Tcm loss and boosting the frequency of Ag-specific $CD4^+$ Tcm cells. However, $CD4^+$ Tem cell numbers were progressively lost over time, and increases in Ag-specific cells in this subset were limited to Ad5 priming and were lost upon boosting with Ad35.

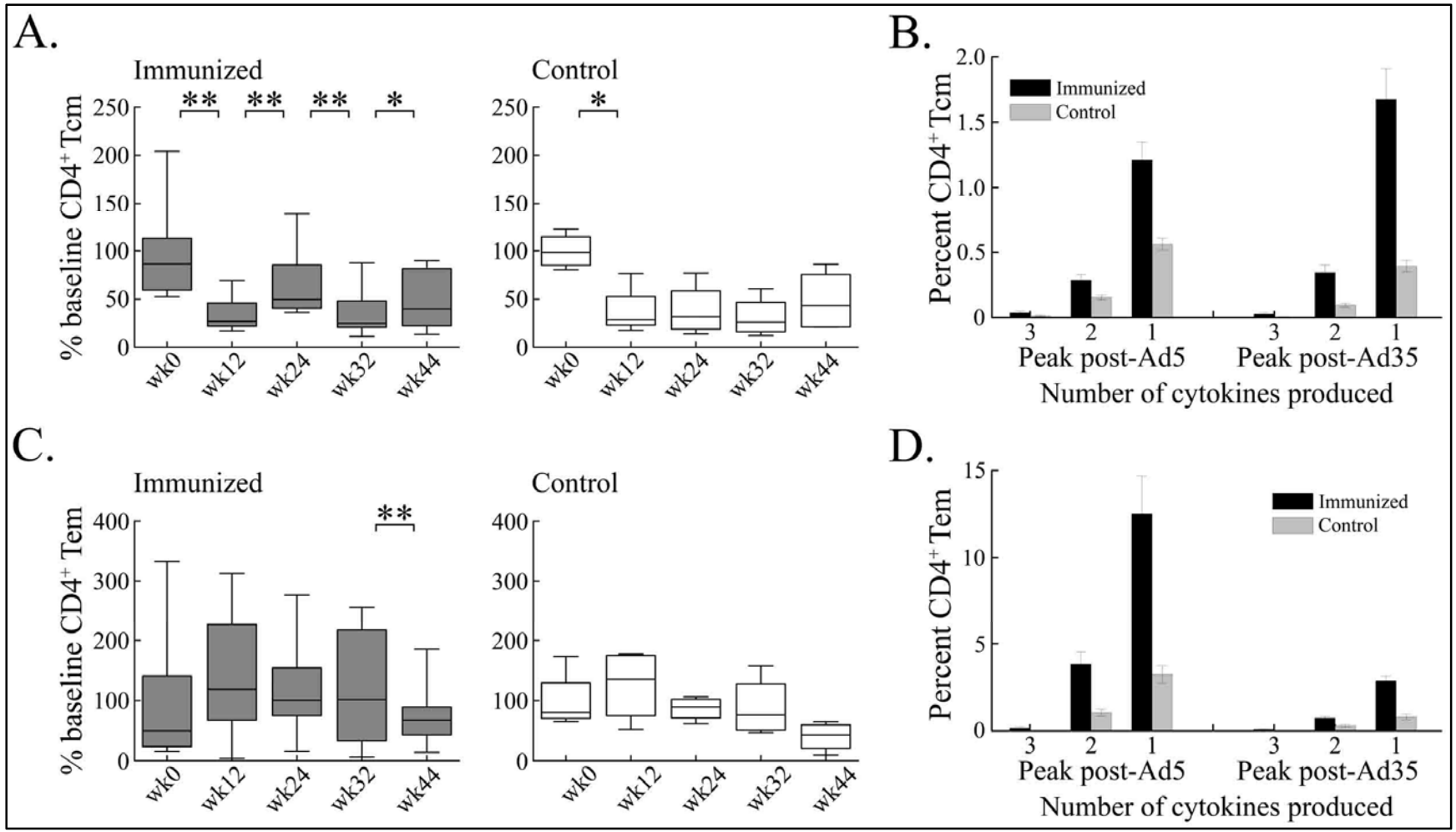

Figure 12: Ad-based immunization transiently restores CD4$^+$ Tcm but not CD4$^+$ Tem cells in SIV-infected monkeys.
(A, C) Changes in the number of circulating CD4$^+$ Tcm cells (A) and CD4$^+$ Tem cells (C) in animals at various intervals after immunization with Ad-SIV with and without Ad-IL-15 (left, closed) or control vectors (right, open) depicted as percent change from baseline. Shown are box-whisker plots depicting median and interquartile ranges with SEM. (B/D) Frequency of cumulative SIV-specific CD4$^+$ Tcm (B) and CD4$^+$ Tem (D) responses consisting of one, two, or three cytokine profiles following Ad5- (left) and Ad35- (right) administration for Ad-SIV immunized animals, irrespective of IL-15 administration, and mock immunized controls. Shown are mean ± SEM. (*=P<.05, **=P<.01).

4.5.5 Ad-based immunotherapy enhances primarily monofunctional Ag-specific $CD8^+$ T cell responses in mucosa

Using BAL samples as a readily available source of mucosal T cells, we examined the effects of therapeutic immunization within mucosal tissues. We detected a near complete elimination of $CD4^+$ T lymphocytes in all animals prior to ART which was maintained for the duration of the study (Fig. 13a, b), consistent with previous reports [215]. We next examined whether Ad-based immunotherapy, irrespective of Ad-IL-15 administration, enhanced Ag-specific mucosal T cell responses. We focused on the production of IFN-γ and IL-2 by $CD8^+$ T cells directed against the dominant Ag identified by IFN-γ ELISPOT PBMC analysis. Intracellular cytokine analysis was performed prior to and 2 wks following Ad5- and Ad35-based immunizations. Animals immunized with Ad5-SIV had increased peak frequencies of Ag-specific mucosal $CD8^+$ T cells producing IL-2 but not IFN-γ or both cytokines compared to responses measured at ART cycle-1 initiation (Fig. 13c). Interestingly, Ad35-SIV immunization expanded peak frequency of polyfunctional IL-2^+ IFN-γ^+ $CD8^+$ T cells responding to viral Ag without further enhancement of monofunctional IL-2 or IFN-γ production, although the proportion of dual cytokine-producing cells relative to cells producing either cytokine alone was minor (Fig. 13c). These data indicate that systemic Ad-based immunotherapy has the potential to enhance Ag-specific $CD8^+$ T cell immunity at mucosal tissues during chronic SIV infection despite the massive loss of $CD4^+$ T cells from that compartment.

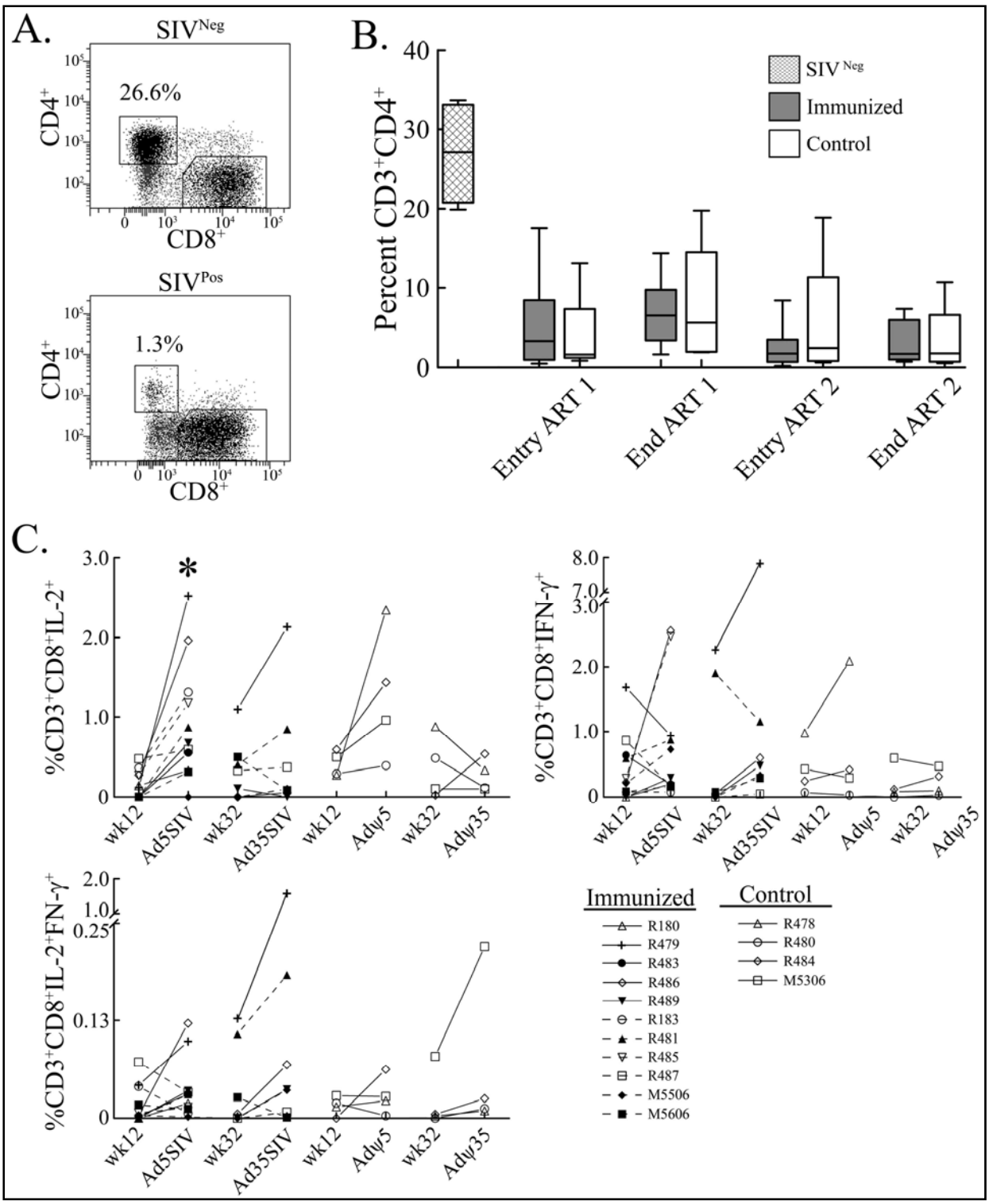
A.
SIV^Neg
26.6%
CD4+
CD8+
SIV^Pos
1.3%
B.
Percent CD3+CD4+
SIV Neg
Immunized
Control
Entry ART 1
End ART 1
Entry ART 2
End ART 2
C.
%CD3+CD8+IL-2+
%CD3+CD8+IFN-γ+
%CD3+CD8+IL-2+FN-γ+
wk12
Ad5SIV
wk32
Ad35SIV
Adψ5
Adψ35
Immunized
R180
R479
R483
R486
R489
R183
R481
R485
R487
M5506
M5606
Control
R478
R480
R484
M5306

Figure 13: Ad-based immunotherapy enhances cellular immunity at the lung mucosa.
(A) Representative flow cytometry dot plots showing T cell subsets in BAL from a SIV-naïve monkey and a SIV-infected monkey (wk 11 PI) highlighting profound $CD4^+$ T cell loss following infection. Numbers represent the percentage of $CD4^+$ T cells in each sample. (B) The percentage of $CD4^+$ T cells in BAL isolated from SIV-naïve animals (n=6) and SIV-infected animals receiving Ad-SIV immunotherapy with and without Ad-IL-15 (closed) or mock immunotherapy (open) at the initiation and termination of ART cycle-1 (wk 12 and wk 24, respectively) and the initiation and termination of ART cycle-2 (wk 32 and wk 44, respectively). Shown are box-whisker plots depicting median and interquartile ranges with SEM. (C) Proportion of $CD8^+$ T cells in BAL expressing IFN-γ, IL-2, or both from animals receiving immunotherapy with Ad-SIV with and without Ad-IL-15 (dotted and solid lines, respectively) or mock immunotherapy (Adψ). Shown are responses to Gag, Env, or Nef peptide pools at the initiation of ART cycle-1 (wk 12) and ART cycle-2 (wk 32) compared to peak responses measured 2 wks after immunization with Ad5- and Ad35-based vectors, respectively. *=$P<.05$.

4.5.6 Ad-based immunotherapy fails to contain virus load during chronic SIV infection

To determine whether the enhanced systemic and mucosal cellular immune responses induced through Ad-based immunotherapy had an effect on viral containment in SIV-infected monkeys, we quantified cell-free virus in plasma at intervals after infection. Upon the initiation of ART cycle-1, all animals had around a 2-log reduction in virus load indicating responsiveness to ART, although the extent of virologic control varied between animals over the course of therapy (Fig. 14). Interestingly, animals receiving Ad5-SIV immunotherapy either alone or with Ad5-IL-15 had no evidence of virus control relative to animals not receiving immunotherapy (Fig. 14a). Following ART cycle-1 cessation, the majority of animals experienced a transient reduction in virus load that was rapidly reversed regardless of whether animals had received Ad-based immunotherapy during the preceding period (Fig. 14). Initiation of ART cycle-2 resulted in an average reduction in virus load of 1 to 1.5 logs for each group, and there was no detectable influence of Ad35-based immunotherapy on plasma viremia during this period (Fig. 14). Following cessation of ART at wk 44, the average plasma virus load remained relatively low in control animals and animals receiving Ad5- and Ad35-based SIV immunotherapy alone, being roughly 2-logs lower than at the initiation of ART at wk 12 (Fig. 14). In contrast, animals treated with Ad5- and Ad35-SIV together with the respective Ad-based IL-15 delivery tended to have increased virus loads subsequent to the second STI (Fig. 14). These findings indicate that Ad-based immunotherapy administered in the context of ART was not effective at controlling SIV virus loads in chronically-infected animals.

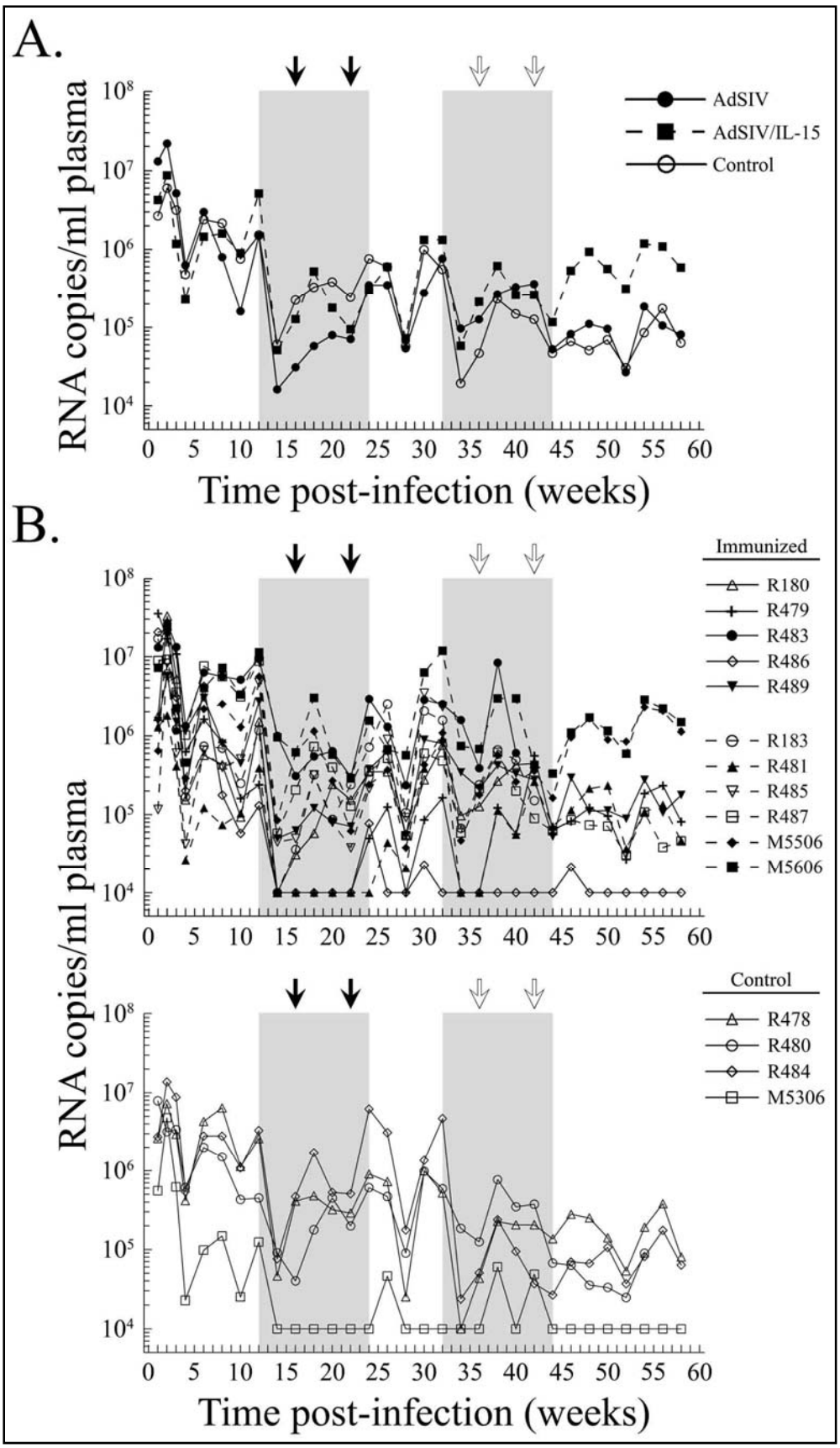
A.
RNA copies/ml plasma
Time post-infection (weeks)
AdSIV
AdSIV/IL-15
Control
B.
RNA copies/ml plasma
Time post-infection (weeks)
Immunized
R180
R479
R483
R486
R489
R183
R481
R485
R487
M5506
M5606
Control
R478
R480
R484
M5306

Figure 14: Ad-based immunotherapy fails to control viremia in chronically infected animals on ART.
(A) Median plasma virus loads at intervals after SIV infection for animals receiving Ad-SIV (closed circles), Ad-SIV with Ad-IL-15 (closed square) or mock (open circle) immunotherapy. (B) Individual virus loads at intervals after SIV infection for animals receiving Ad-SIV with and without Ad-IL-15 (dotted and solid lines, respectively, top) or mock immunotherapy (bottom). The periods of ART are depicted by shaded regions and the times of immunotherapy with Ad5- and Ad35-based vaccines are indicated by closed and open arrows, respectively.

4.5.7 Strength of T cell response, T cell function, and $CD4^+$ Tcm cells correlate with virus control

To better understand why the robust cellular immune responses to SIV induced through immunotherapy failed to control viremia, we evaluated data from all animals irrespective of treatment for potential factors that correlated with virus control. The strength of peak Gag- and Env-specific IFN-γ ELISPOT responses each correlated inversely with virus load during ART, and the magnitude of the cumulative IFN-γ ELISPOT response at 12 wks PI prior to therapeutic intervention was inversely correlated with sustained virus control measured at 52 wks PI, indicating the importance of the strength and breadth of the cellular immune response (Fig. 15a). However, the quality of cellular immunity was also important in controlling virus in infected animals. The peak frequency of $CD8^+$ T cells producing both IFN-γ and TNF-α during the first ART cycle was strongly correlated with reduced viremia at that time and at 56 wks PI (Fig. 15b). Similarly, the proportion of polyfunctional Ag-specific $CD4^+$ T cells expressing two or three cytokines was inversely correlated with virus load during the first ART cycle (Fig. 15b). The maintenance of $CD4^+$ Tcm cells both early (wk 12) and late (wk 24 or 44) in infection was negatively correlated with virus load and positively correlated with Ag-specific dual cytokine-producing $CD8^+$ Tcm cells in blood, supporting the role of $CD4^+$ T cell help in the preservation of effective memory $CD8^+$ T cell responses (Fig. 15c) [385]. Finally, the number of mucosal $CD4^+$ T cells during chronic infection was inversely correlated with virus load (Fig. 15d). Interestingly, this association was not observed when comparing $CD4^+$ T cell levels in the peripheral blood with viral loads (data not shown), illustrating the sensitivity of BAL $CD4^+$ T cell measurements as a predictive indicator of disease outcome [215]. Expression of the Mamu-A*01 allele (in animals R478, R480 and R487) did not correlate with virus control, and no other detected MHC allele was observed to influence viral load in this model (data not shown). These correlative data indicate that both quantitative and qualitative $CD8^+$ and $CD4^+$ T cell responses

along with maintenance of peripheral and mucosal $CD4^+$ T cells are associated with control of viremia in chronic SIV infection.

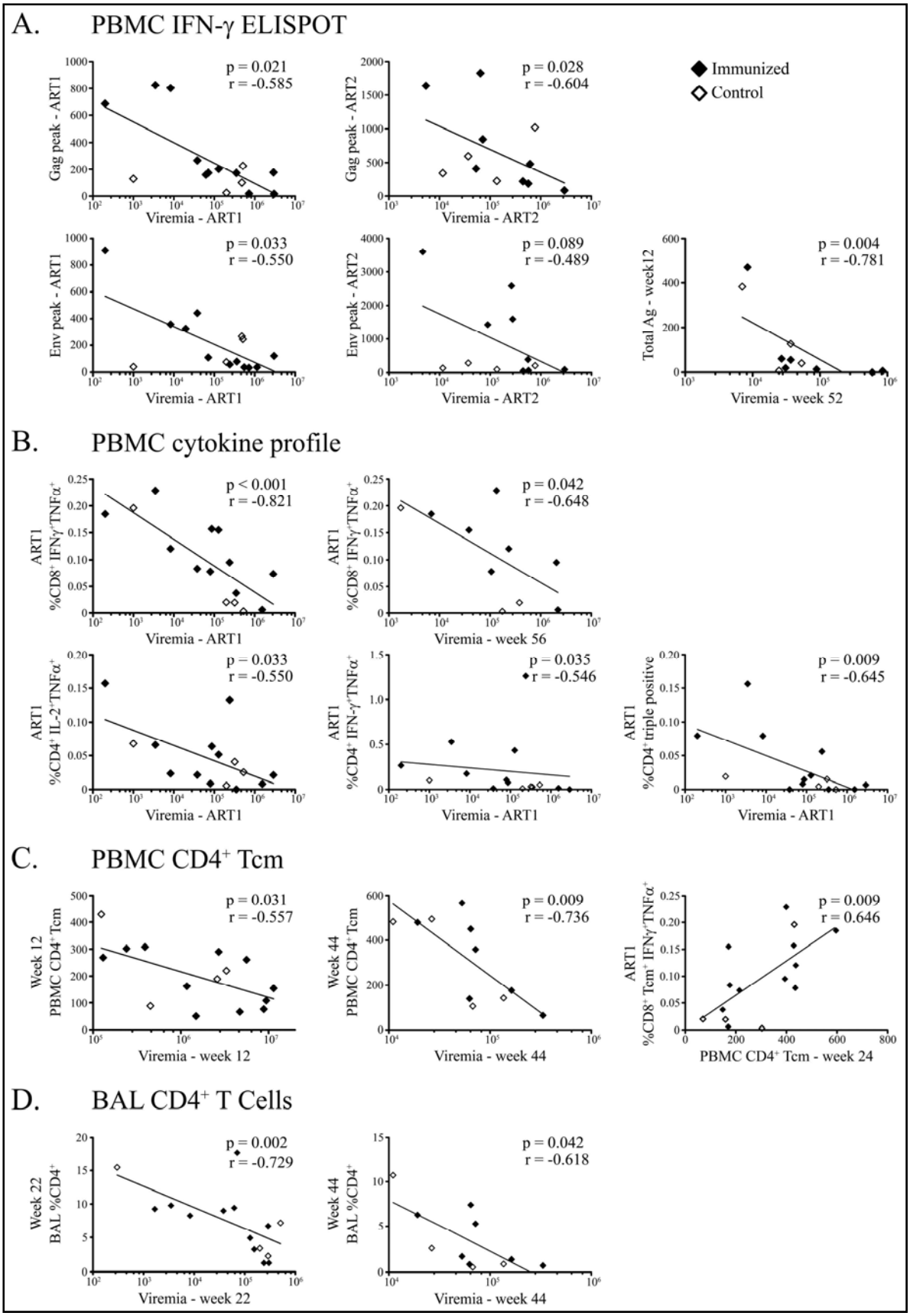
A.
PBMC IFN-γ ELISPOT
Immunized
Control
Gag peak - ART1
p = 0.021
r = -0.585
Viremia - ART1
Gag peak - ART2
p = 0.028
r = -0.604
Viremia - ART2
Env peak - ART1
p = 0.033
r = -0.550
Viremia - ART1
Env peak - ART2
p = 0.089
r = -0.489
Viremia - ART2
Total Ag - week12
p = 0.004
r = -0.781
Viremia - week 52
B.
PBMC cytokine profile
ART1 %CD8+ IFNγ+TNFα+
p < 0.001
r = -0.821
Viremia - ART1
ART1 %CD8+ IFNγ+TNFα+
p = 0.042
r = -0.648
Viremia - week 56
ART1 %CD4+ IL-2+TNFα+
p = 0.033
r = -0.550
Viremia - ART1
ART1 %CD4+ IFN-γ+TNFα+
p = 0.035
r = -0.546
Viremia - ART1
ART1 %CD4+ triple positive
p = 0.009
r = -0.645
Viremia - ART1
C.
PBMC CD4+ Tcm
Week 12 PBMC CD4+ Tcm
p = 0.031
r = -0.557
Viremia - week 12
Week 44 PBMC CD4+ Tcm
p = 0.009
r = -0.736
Viremia - week 44
ART1 %CD8+ Tcm+ IFNγ+TNFα+
p = 0.009
r = 0.646
PBMC CD4+ Tcm - week 24
D.
BAL CD4+ T Cells
Week 22 BAL %CD4+
p = 0.002
r = -0.729
Viremia - week 22
Week 44 BAL %CD4+
p = 0.042
r = -0.618
Viremia - week 44

Figure 15: Control of chronic SIV infection is correlated with the magnitude and quality of cellular immunity and maintenance of peripheral $CD4^+$ Tcm and mucosal $CD4^+$ T cells.
(A) The peak IFN-γ ELISPOT response in PBMC to Gag or Env during ART cycle-1 and 2 inversely correlated with virus load at the same time PI, and the cumulative IFN-γ ELISPOT response to Gag, Env, and Nef combined at wk 12 PI inversely correlated with virus load at wk 52 PI. (B) The peak frequency of $CD8^+$ T cells in PBMC expressing IFN-γ and TNF-α in response to Gag, Env, and Nef stimulation during ART cycle-1 correlated inversely with virus load at the same time PI and at wk 56 PI, and the peak frequency of $CD4^+$ T cells in PBMC expressing either IL-2 and TNF-α, IFN-γ and TNF-α, or IFN-γ, IL-2, and TNF-α in response to Gag, Env, and Nef stimulation during ART cycle-1 correlated inversely with virus load at the same time PI. (C) The number of $CD4^+$ Tcm cells in blood at wk 12 and wk 44 PI, respectively, correlated inversely with virus load at the same time PI, and the number of $CD4^+$ Tcm in blood at wk 24 PI correlated positively with the peak frequency of blood $CD8^+$ Tcm cells producing IFN-γ and TNF-α in response to Gag, Env, and Nef stimulation during ART cycle-1. (D) The proportion of $CD4^+$ T cells in BAL at wk 22 and wk 44 PI, respectively, correlated inversely with virus load measured at the same time PI. Each symbol represents an individual animal either receiving Ad-SIV with and without Ad-IL-15 (closed symbols) or mock (open symbols) immunotherapy. P-values and Spearman's rank-order correlation coefficient (r) values are presented.

4.6 DISCUSSION

Our findings demonstrate the potent ability of Ad-based vectors to elicit virus-specific cellular immune responses during ART-treated, chronic SIV infection in rhesus macaques, extending the use of Ad-based vectors for the first time beyond prophylactic vaccination in this model [211, 289, 294, 304, 381, 386]. Therapeutic vaccination readily enhanced the magnitude of IFN-γ-mediated cellular immunity against all targeted viral Ags, promoted CTL responses both in the circulation and mucosa, and increased an array of T_H1 cytokines produced by Ag-specific $CD8^+$ T cells, all factors that have been shown to aid in the containment of disease progression [372, 387, 388]. Yet, Ad-based immunotherapy was not effective at controlling virus load during ART or the period of rebound viremia following STI. While these findings were limited by the relatively small number of animals used, the results are in agreement with previous studies showing that therapeutic immunization utilizing poxvirus-based vectors during ART-treated HIV infection had only minor influences on rebound viremia following ART cessation [389, 390]. Furthermore, the data are also consistent with recent findings that vaccination with replication-defective Ad5-based vectors failed to prevent HIV-1 infection in a large cohort of human volunteers [380].

Recent studies have associated the maintenance of polyfunctional $CD4^+$ and $CD8^+$ T cells producing multiple cytokine and chemokine combinations with long-term non-progression, viral containment during STI, and effective cytotoxic control of viral replication during HIV infection

[219-222, 383]. Similarly, we observed a strong relationship between polyfunctional $CD4^+$ and $CD8^+$ T cell-mediated immunity and control of chronic SIV viremia. However, therapeutic vaccination with Ad-based vectors mainly induced $CD8^+$ T cell responses in blood and mucosa and these were characterized primarily as monofunctional, with less than 20% of cells producing two cytokines and less than 1% producing three cytokines. In addition, while the proportion of blood $CD4^+$ T cells producing IFN-γ increased in response to Ad5-based immunotherapy the total number of $CD4^+$ T cells steadily declined over time, resulting in a decrease in the number of Ag-specific $CD4^+$ T cells during ART cycle-2. Moreover, the fraction of each $CD4^+$ or $CD8^+$ T cell subset producing 1, 2 or 3 cytokines subsequent to vaccination did not differ from those observed in control animals, suggesting that immunization served to merely enhance the magnitude of the innate response already established against SIV infection without altering its polyfunctional character. Consistent with our data is the finding that Ad5-mediated immunity invokes skewed $CD8^+$ to $CD4^+$ T cell responses and restricted polyfunctional cytokine expression as compared to alternative vectors based on Ad26 or Ad48 serotypes in a prophylactic vaccine model [294]. Therapeutic Ad5-based priming vaccination in our model may have established qualitatively deficient antiviral immunity, ameliorating the potential of Ad35-based boosting as seen in mice [294]. These findings are corroborated by a recent report showing that high-dose Ad5 exposure induced qualitatively inferior cellular immunity and protection against *Leishmania major* infection of mice [291].

$CD4^+$ Tem cells provide crucial support against opportunistic infections in HIV and SIV infected individuals and are an essential mediator in reducing disease pathogenesis [215, 391]. A recent study indicated that the ability to repopulate the $CD4^+$ Tem compartment during progressive SIV infection of monkeys is associated with the regenerative capacity of $CD4^+$ Tcm cells to expand and differentiate [46]. Our data showed that maintenance of $CD4^+$ Tcm cells both early and late in infection was directly correlated with the frequency of polyfunctional $CD8^+$ T cells and inversely correlated with virus load. Encouragingly, we found a significant expansion of $CD4^+$ Tcm cells present in peripheral blood following Ad-based immunotherapy during ART. However, this recovery was relatively short-lived and was not associated with any detectable recovery of $CD4^+$ Tem cells. It is possible that the relatively brief duration of ART administered in our study, together with incomplete control of virus load while on therapy, may have limited the full development of long-lived memory $CD4^+$ T lymphocyte populations.

Additional rounds of heterologous vaccination under the protection of ART, ideally directed against multiple drug targets, may allow for further reconstitution of $CD4^+$ T cell cellular memory and enhanced cellular immunity leading to greater restriction of rebound viremia.

Currently, cytokine chemotherapy utilizing IL-15 is being explored as an alternative treatment for HIV and SIV infections due to the potent immunoregulatory functions of this cytokine [165]. Studies have shown that the addition of exogenous IL-15 is capable of restoring functionality to anergic T lymphocytes, concurrent with IL-15 deficiencies present during chronic HIV infection [156, 168, 175, 176, 178]. We used an Ad-based delivery method for IL-15 immunotherapy, which was highly effective *in vitro* at inducing production of bioactive IL-15 in infected cells. Yet, we observed no significant differences in the induction of SIV-specific cellular immunity, the character of the cytokine profile, or the absolute numbers of $CD4^+$ or $CD8^+$ T memory cells as result of Ad-based IL-15 immunotherapy. In fact, although not statistically significant, we observed a trend towards reduced response to immunization and heightened viremia in animals receiving Ad-based IL-15. These negative results are consistent with recent findings by others using systemic IL-15 in a similar therapeutic setting in SIV-infected monkeys [376], and raise further doubts about the utility and safety of IL-15 as a therapeutic modality in HIV infection.

Our results highlight the relationship between maintaining immune function prior to ART initiation and having durable control of viremia. Perhaps not surprisingly, animals that had greater mucosal $CD4^+$ T cell counts and quantitatively and qualitatively stronger Ag-specific immunity in blood and mucosa during chronic infection were able to more effectively and durably suppress SIV viremia. This trend has been observed in individuals expressing various HLA molecules allowing for superior control of HIV infection [203, 392, 393]. Similarly, individuals receiving early ART have been shown to maintain healthy immunity and control HIV viremia [394]. It is likely that in our study the profound level of immunologic damage accrued during the first 12 wks, as evidenced by massive loss of mucosal $CD4^+$ T cells and peripheral blood $CD4^+$ Tcm cells, crippled host immunity to a point beyond transient therapeutic salvation. These findings would suggest that therapeutic intervention initiated early in infection while the host immune system is relatively intact may be more effective at controlling infection. In addition, combining different vaccine approaches such as DNA prime with Ad boost may enhance the quality of T cell response to infection, as has been shown in prophylactic SIV

vaccines [278], although the capacity to generate qualitatively superior cellular responses through combination immunotherapy remains to be tested.

4.7 CONCLUSIONS

Our findings illustrate the potent ability of Ad-based vaccination to enhance robust antiviral cellular immunity during chronic SIV infection. Immunization induced durable, high frequency T_H1 responses, and augmented $CD4^+$ Tcm populations, both implicated in viral containment. Regrettably, therapeutic vaccination failed to control rebound viremia following ART cessation, in spite of demonstrated immunogenicity. Previous studies have suggested that effective therapeutic augmentation of T cell-mediated immunity and T lymphocyte dynamics by vaccination is unfeasible in the absence of complete virologic containment during ART [181, 376, 395] (B. Felber, personal communication). This study demonstrates the potential for Ad-based immunization to enhance SIV-specific immunity in the presence of underlying viremia, without exacerbating disease state. Together these findings strongly support the further evaluation of Ad-based therapeutic vaccination in conjunction with improved ART treatment regimens.

5.0 CHAPTER THREE: EFFECTS OF IMMUNOTHERAPEUTIC VACCINATION UPON T LYMPHOCYTE DYNAMICS DURING CHRONIC $SIV_{MAC}251$ INFECTION

5.1 PREFACE

The following chapter is in fulfillment of specific aim 3. These findings represent a portion of the data currently being incorporated into a manuscript addressing pre-treatment SIV pathogenesis and responsiveness to therapeutic Ad-based vaccination. These findings will be submitted for publication by a peer-reviewed journal. Adam C. Soloff performed all experiments, data analysis, and statistical evaluation under the guidance of Simon M. Barratt-Boyes.

5.2 ABSTRACT

HIV pathogenesis is characterized by a massive elimination of $CD4^+$ T lymphocytes from the extralymphoid tissues, leading to deleterious T cell turnover and disruption of T lymphocyte homeostasis. Although ART inhibits $CD4^+$ T cell loss, ART alone is insufficient to continually restore mucosal and systemic T cell populations, naturally replenished through thymopoiesis and Ag-driven peripheral expansion. Therapeutic treatment with immunoregulatory IL-15 has been shown to enhance $CD4^+$ and $CD8^+$ Tem cell proliferation, and concurrent emigration to mucosal tissues. Using a rigorous model of chronic SIV infection in rhesus macaques, we evaluated whether therapeutic vaccination utilizing heterologous Ad5- and Ad35-based vectors administered during two separate cycles of ART was capable of enhancing cellular proliferation and promoting peripheral expansion of T cell populations. Vectors independently expressed SIV

gag, env, and *nef* genes, as well as bioactive rhesus *IL-15*, used here as a potential vaccine adjuvant. Ad-based vaccination, with or without IL-15 administration, was found to have negligible effects upon T cell phenotype or proliferative response either systemically or at the lung mucosa. Although progressive $CD4^+$ T cell depletion was seen in the peripheral blood in all animals, proportionate increases in both $CD4^+$ Tcm and Tem populations concomitant with steady loss of naïve $CD4^+$ T cells were observed. Alternatively, total peripheral blood $CD8^+$ T cells were dramatically augmented throughout infection, experiencing enhanced proportions of $CD8^+$ Tcm and Tem populations with gradual reductions of naïve $CD8^+$ T cells in all treatment groups. ART provided suppression of cellular proliferation, inhibiting dramatic expansion in PBMC. Additionally, ART transiently restored mucosal T cell balance, leading to increased $CD4^+$ T cell proportions and reduced proliferation. These findings suggest that therapeutic Ad-based vaccination, with or without IL-15, was incapable of further enhancing Ad-driven memory expansion, naïve T cell salvation, or proliferative responses above the effects mediated by ART during chronic SIV infection.

5.3 INTRODUCTION

The defining characteristic of HIV/SIV pathogenesis is the elimination of $CD4^+$ T cells within extralymphoid tissues leading to disruption of T cell function and homeostasis. In healthy individuals, T cell pools are established initially through thymic output of naïve pluripotent T lymphocytes capable of responding to neoantigens [396]. Thymopoiesis naturally declines with age, coinciding with a corrective increase in peripheral Ag-driven expansion of long-lived memory T cell populations necessary to maintain stable cellular immunity [397]. Although peripheral expansion supports all memory T cell populations, $CD4^+$ T cells are more reliant upon continual low-level thymopoeitic regeneration then their $CD8^+$ T cell counterparts [396]. During HIV infection, cellular immune exhaustion occurs only after years of disease progression, as progressive illness causes thymic atrophy, associated with inflammatory-induced increases in inhibitory cytokines and cellularity, particularly CTL infiltrates, within the perivascular space [398]. Further suppression of thymopoiesis during HIV infection may occur through direct viral-mediated killing of thymocytes or thymic dendritic cells, viral-induced damage to thymic

epithelial cells, or localized signaling inhibition [397, 399, 400]. In conjunction with viral-mediated reductions in the already age-limited capacity for thymopoiesis, viral- and inflammatory-mediated hyperactivation leads to rapid turnover of peripheral T cells, further burdening the ability to replenish systemic and mucosal T lymphocyte pools [92].

Inhibiting HIV/SIV pathogenesis, therapeutic intervention utilizing ART salvages the capacity for thymic and peripheral T cell regeneration. ART treatment during HIV infection has been shown to enhance *de novo* production of $TREC^+$, naïve T cells associated with increased thymic size and functional output as compared to untreated controls [91]. Effective ART restores the capacity of Ag-specific $CD4^+$ and $CD8^+$ T cells to proliferate in response to cognate Ag, essential to replenishing T lymphocyte pools [113, 126, 401]. In addition, ART has been shown to reduce the level of generalized systemic and mucosal cellular activation during infection, and is associated with reduced inflammation and bacterial translocation, inhibiting T cell pool turnover and alleviating supply pressure [100, 402]. Nevertheless, immune reconstitution following ART is incomplete, necessitating the development of immunoregulatory therapeutics capable of cellular regeneration.

Novel therapeutic approaches utilizing the common γ-chain cytokines IL-2, IL-7, IL-15, and IL-21 have been shown to expand $CD4^+$ and $CD8^+$ memory T cell populations both *in vitro* and *in vivo* in murine and nonhuman primate models [396, 403]. The most extensively tested cytokine, IL-2, has been utilized clinically to promote T cell regeneration and decrease latent viral reservoirs during HIV infection, but has been recently shown to establish regulatory T cell populations and peripheral tolerance through specific, non-redundant cytokine functioning [67, 404]. In contrast, IL-15 treatment reduces activation induced cell death, inhibits apoptosis, and expands and sustains $CD4^+$ and $CD8^+$ memory populations [374]. Although reports are conflicting as to the extent that IL-15 drives central and effector memory T cell proliferation, IL-15 definitively augments $CD4^+$ and $CD8^+$ T cells populations either through direct stimulated expansion or alleviating regenerative inhibition. In addition, systemic IL-15 delivery during ART-treated chronic SIV infection has been shown to potently induce $CD4^+$ and $CD8^+$ effector memory T cell expansion and emigration to extralymphoid tissues, strongly correlated with immunocompetence and delayed disease progression [177]. In the current study, we evaluated the potential of therapeutic vaccination, with or without Ad-mediated IL-15 support, to effect the

phenotype, proliferative capacity, and magnitude of T lymphocyte populations during ART-treated, chronic SIVmac251 infection in rhesus macaques.

5.4 MATERIALS AND METHODS

5.4.1 SIV infection and immunotherapy

The adult Indian rhesus macaques utilized presently were described in detail in Chapter 2. SIVmac251 infection, ART treatment with PMPA and FTC, and subsequent immunization utilizing Ad5- and Ad35-based vectors were performed as previously described in Materials and Methods sections 4.4.1, 4.4.2, and 4.4.3. Inclusion criteria were identical with fifteen macaques subsequently analyzed.

5.4.2 Absolute T lymphocyte determinations

Absolute counting of $CD4^+$ and $CD8^+$ T cell populations in the peripheral blood of rhesus macaques was performed as specified previously in Materials and Methods section 4.4.8.

5.4.3 FACS analysis of T cell phenotype and proliferation

Multiparametric FACS analysis was performed as described in Materials and Methods section 4.4.7 with minor alterations. Live T cells were identified through successive gating strategies based on size and viability using the blue LiveDead apoptotic stain (Molecular Probes/Invitrogen Life Technologies). Immunophenotyping was performed on frozen PBMC or fresh BAL samples using the following Abs (purchased from BD Pharmingen, unless otherwise noted): Pacific Blue (CD3-Pacblue; SP34-2), CD4-AmCyan (L200, NIH AIDS Research and Reference Reagent Program), CD8-PE-Cy7 (RPA-T8), CD28-APC (28.2), and CD95-PE (DX2). Cryopreserved cells were thawed, washed, and stained for surface markers for 1 h at 4^oC. Fixation and intracellular staining was performed with BD Biosciences Fix/Perm reagent kits according to the

BD Biosciences protocol. Upon permeabilization, cells were stained intracellularly for Ki-67-FITC (B56) for 30 min at 4°C. Appropriate isotype controls were included in all experiments. Data acquisition was performed on a LSRII cytometer with a minimum of 200,000 events collected from each sample. Data were analyzed using BD FACSDiva software (BD Biosciences). To examine alterations in cellular profiles, the change in absolute cell number of individual phenotypic and proliferating cells was compared to values measured at pre-infection baseline within each animal, except for naïve $CD4^+$ and $CD8^+$ T cell proliferation represented cell count/μl whole blood due to insufficient cell numbers.

5.4.4 Statistical analysis

Comparisons between treatment groups were carried out using the Mann-Whitney U test performed using SPSS 14.0 software (SPSS Inc., Chicago, IL). All *P* values are two-sided with significance considered to be $P < 0.05$.

5.5 RESULTS

5.5.1 Immunotherapy enhances $CD4^+$ T cell memory but not naïve $CD4^+$ T cell populations in peripheral blood

To determine the effects of ART and immunotherapeutic vaccination upon the size and proliferative capacity of peripheral blood T cell populations, we longitudinally measured $CD4^+$ T cells in PBMC utilizing multiparameter FACS analysis in conjunction with absolute cell quantitation. As depicted previously (Fig. 10a), live, size-gated $CD4^+$ and $CD8^+$ T cells were identified as Tcm, Tem, or naïve as described [384] and cellular proliferation was measured through intracellular detection of nuclear Ki-67 protein, present during all active phases of the cell cycle. Notably, no statistically significant differences were detected between immunized and controls groups for alterations in $CD4^+$ T cell phenotype or proliferative response. Immediately upon SIV infection (wk 1), all animals underwent a dramatic loss of absolute $CD4^+$

T cells from the peripheral blood, followed by varying degrees of restoration prior to ART initiation at wk 12 (Fig. 16a). Although $CD4^+$ T cell counts remained stable during periods of ART, modest decreases were observed during each of two STIs among all study animals. Interestingly, animals receiving Ad5-SIV with IL-15 had significantly increased $CD4^+$ T cell counts at wk 26 compared with animals immunized with Ad5-SIV alone, which were not different from controls (Fig. 16a). By wk 52 post-infection, all groups retained less than half of their original $CD4^+$ T cell numbers in peripheral blood.

We next determined the influence of immunotherapeutic vaccination upon the proportion of memory and naïve $CD4^+$ T cell populations in PBMC, measured as change from baseline, pre-infection levels. Interestingly, as the proportion of $CD4^+$ Tcm cells decreased during the first 12 wks post-infection, the percentage of Tem and naïve $CD4^+$ T cells increased dramatically in all treatment groups (Fig. 16b, c, d). Subsequent ART administration was found to inversely effect $CD4^+$ T cell memory populations, increasing the proportion of Tcm while decreasing Tem representation in PBMC during both treatment cycles (Fig. 16b, c). In contrast, naïve $CD4^+$ T cells were gradually lost from Ad-SIV and control groups, while animals receiving Ad-SIV/IL-15 immunizations maintained elevated naïve $CD4^+$ T cell proportions throughout the investigation (Fig. 16d). Notably, substantial expansion of the proportion of both $CD4^+$ Tcm and Tem memory populations occurred progressively over the course of investigation, with wk 52 levels of 157% and 391% of baseline values seen among all animals, respectively, whereas naïve $CD4^+$ T cells were reduced to 64% of baseline among all animals at this time.

To monitor Ag-driven peripheral expansion and potential naïve repopulation of $CD4^+$ T cells, Ki-67 levels were measured at timepoints of therapeutic intervention. Collectively, Tcm, Tem, and naïve $CD4^+$ T cells demonstrated consistent responses among groups to ART intervention, with or without Ad-based vaccination, in their level of proliferative activity. All $CD4^+$ T cell phenotypes underwent a burst of replication upon infection, sustained until ART initiation at wk 12, with Tcm and Tem cells doubling initial baseline proliferation levels (Fig. 16a). During ART cycle-1, all animals displayed inhibited rates of proliferation, abrogating increases or sustaining the pre-ART level of proliferation in total, Tcm, and naïve $CD4^+$ T cells (Fig. 16a, b, d). In contrast, $CD4^+$ Tem cells experienced reduced proliferation during the first ART cycle, suggesting inhibited generalized peripheral immune activation (Fig. 16c). Subsequently, during the first STI, all groups had a pronounced decrease in proliferation for all

phenotypes, potentially due to preferential elimination of activated $CD4^+$ T cells during heightened viremia. Reinstating ART plateaued $CD4^+$ T cell proliferation, with slight increases observed among Tem and total $CD4^+$ T cells (Fig. 16a, c). Interestingly, upon cessation of ART during the second STI, all treatment groups experienced increased a dramatic burst of proliferating $CD4^+$ T cells, irrespective of phenotype. Notably, at wk 52 post-infection, Ad-SIV and control groups, but not Ad-SIV/IL-15 immunized animals, had a greater proportion of total $CD4^+$ T cell proliferation than at pre-infection, baseline levels (Fig 16).

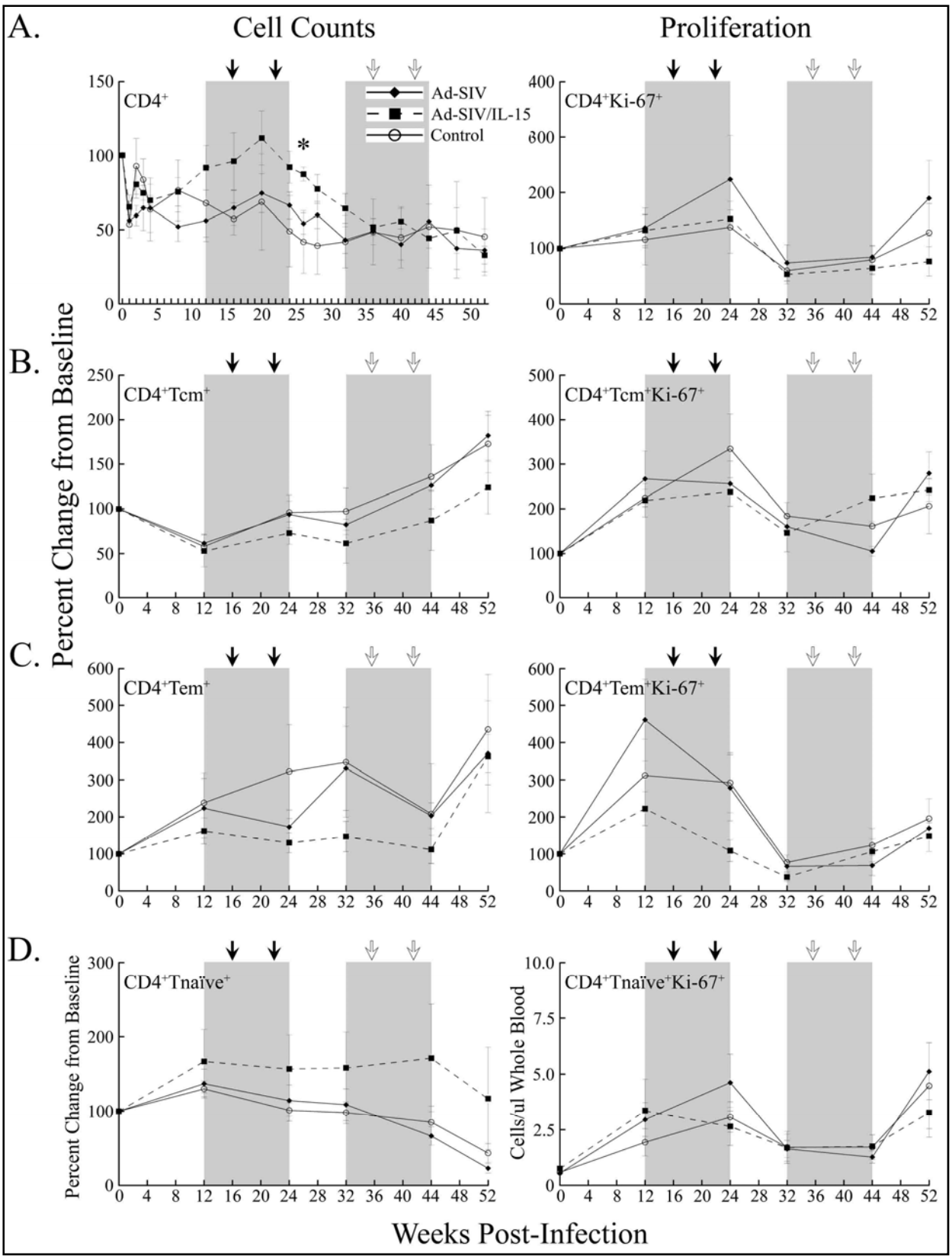
A.
Cell Counts
Proliferation
CD4+
Ad-SIV
Ad-SIV/IL-15
Control
*
CD4+Ki-67+
B.
CD4+Tcm+
CD4+Tcm+Ki-67+
C.
CD4+Tem+
CD4+Tem+Ki-67+
D.
CD4+Tnaïve+
CD4+Tnaïve+Ki-67+
Percent Change from Baseline
Cells/ul Whole Blood
Weeks Post-Infection

Figure 16: Peripheral expansion of memory but not naïve CD4$^+$ T cells throughout therapeutic intervention. Change in absolute CD4$^+$ T cells/μl whole blood (A, left and D, right) or proportion of CD4$^+$ T cells per pre-infection baseline values in PBMC for CD4$^+$ T cells at intervals after SIV infection in animals receiving Ad-SIV (closed diamonds), Ad-SIV with Ad-IL-15 (closed square) or mock (open circle) immunotherapy. Periods of ART are depicted as shaded boxes, and with Ad5- and Ad35-based immunizations shown as black and open arrows, respectively. Phenotype (Left) and proliferation (Right) illustrated for (A) total counts, (B) Tcm, (C) Tem, and (D) naïve CD4$^+$ T cells.*=P<.05.

5.5.2 Robust expansion of CD8$^+$ T cell memory with naïve CD8$^+$ T cell decline following therapeutic intervention in peripheral blood

To evaluate the ability of therapeutic vaccination to expand systemic CD8$^+$ T cell numbers, we monitored cellular phenotype and proliferation by FACS analysis of PBMC as described previously. Similar to observations of CD4$^+$ T cell populations, negligible differences were found between immunized and controls groups for any CD8$^+$ T cell phenotype or proliferative response. By 1 wk post-infection, peripheral blood CD8$^+$ T cell numbers had dramatically declined among all animals, likely representing the relocation and homing of these cells to extralymphoid tissues (Fig. 17a). This was followed by a robust expansion of the CD8$^+$ T cell population in all groups, reaching levels of roughly 200% baseline values. In contrast to the fluctuations of the CD4$^+$ T cell population, CD8$^+$ T cells were consistently elevated throughout the investigation, with negligible response to either ART or STI. Notably, animals receiving Ad5-SIV with IL-15 augmentation displayed a trend towards higher CD8$^+$ T cell counts than those of control or Ad5-SIV immunized groups during the first ART cycle. When immunized animals were evaluated irrespective of IL-15 administration, they had statistically significant increases in total CD8$^+$ T cell counts at wk 24 as compared to non-immunized controls (Fig 17a).

Next we sought to analyze the ability of immunotherapeutic vaccination to promote the Ad-driven peripheral expansion of naïve and memory CD8$^+$ T cell populations of the peripheral blood. Unlike the therapeutic augmentation seen in the CD4$^+$ Tcm population, CD8$^+$ Tcm proportions were largely unaffected by ART or vaccination, with control and Ad-SIV groups showing marginal increases and Ad-SIV/IL-15 animals remaining at baseline levels (Fig 17b). In contrast, a marked increase in the percentage of CD8$^+$ Tem cells represented was seen in all treatment groups, with wk 52 values reaching 238% of baseline levels among all study animals (Fig. 17c). Notably, as the proportion of CD8$^+$ memory T cells remained stable or elevated, all

treatment groups sustained a dramatic decrease in the proportion of naïve $CD8^+$ T cells in the peripheral blood, with immunized animals, irrespective of IL-15 administration, experiencing statistically reduced naïve $CD8^+$ T cells at wk32 compared to controls (Fig. 17d). Interestingly, ART intervention was capable of ameliorating naïve $CD8^+$ T cell declines during both treatment cycles.

To examine the ability of immunotherapeutic vaccination to augment the proliferative capacity of $CD8^+$ T cells in peripheral blood, we measured intracellular Ki-67 production at timepoints of therapeutic intervention. As seen in $CD4^+$ T cell populations, cellular proliferation profiles remained consistent between total $CD8^+$ T cells and individual memory and naïve phenotypes, regardless of immunization status. From pre-infection to ART initiation at wk 12, a proliferative burst was observed among all $CD8^+$ T cell phenotypes within the peripheral blood (Fig. 17). Subsequently, proliferation of $CD8^+$ T cell phenotypes from all treatment groups was found to gradually decline from wk 12 to wk 44, with values at the end of the second ART cycle approaching baseline levels (Fig. 17). During the second STI, robust increases in memory and naïve $CD8^+$ T cells were identified from wk 44 to wk 52 among all treatment groups (Fig. 17). Interestingly, in light of the continual reduction of naïve $CD8^+$ T cells proportions from pre-infection to wk 52 post-infection, naïve $CD8^+$ T cells experienced a roughly 15 fold increase in proliferation among all animals, suggesting functional differentiation, bystander killing, or heightened apoptotic susceptibility in eliminating naïve $CD8^+$ T cells (Fig. 17).

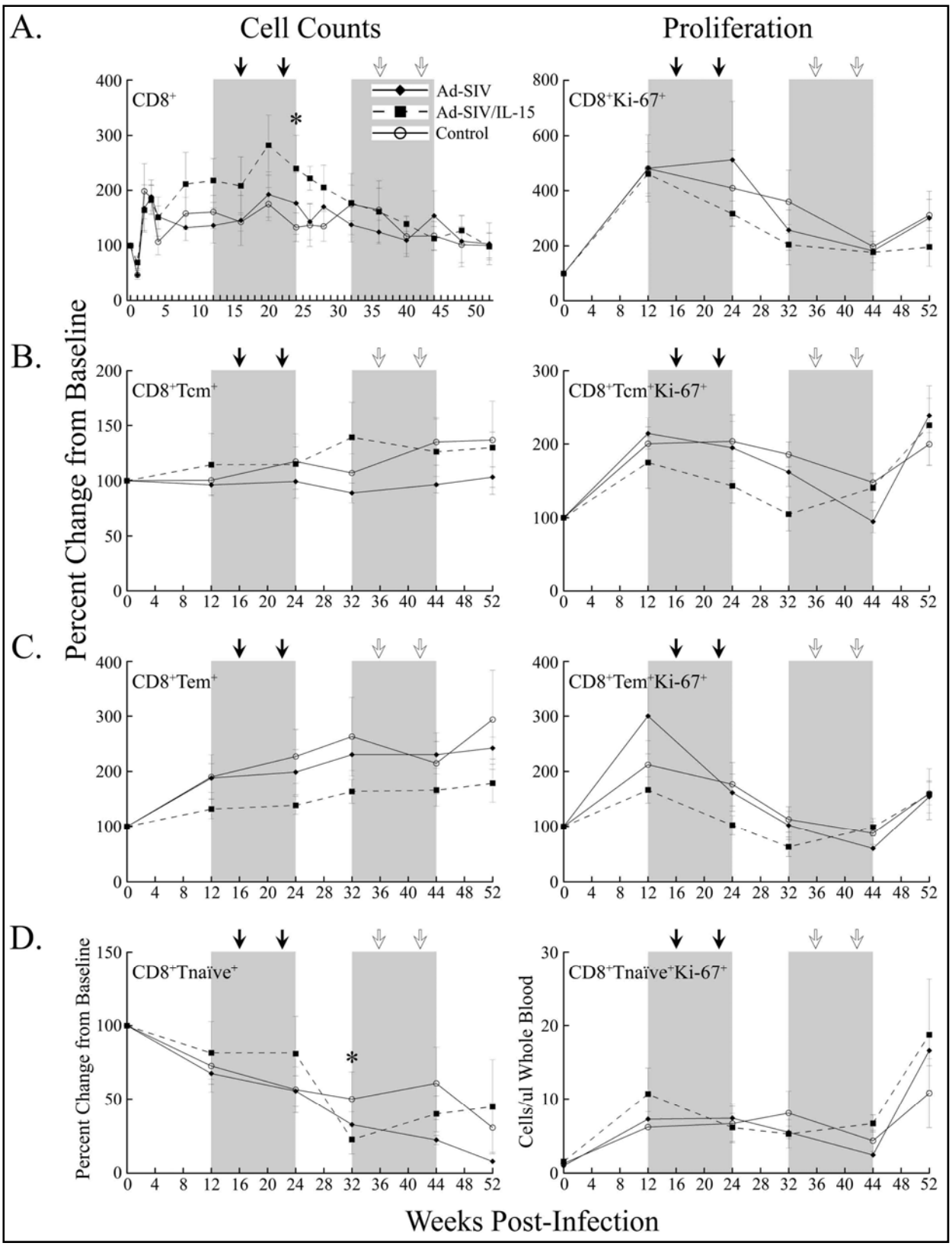
A.
Cell Counts
Proliferation
B.
C.
D.
CD8+
CD8+Ki-67+
CD8+Tcm+
CD8+Tcm+Ki-67+
CD8+Tem+
CD8+Tem+Ki-67+
CD8+Tnaïve+
CD8+Tnaïve+Ki-67+
Ad-SIV
Ad-SIV/IL-15
Control
Percent Change from Baseline
Cells/ul Whole Blood
Weeks Post-Infection

Figure 17: Therapeutic intervention enhances Tem but not naïve CD8$^+$ T cell populations in the peripheral blood.
Change in absolute CD8$^+$ T cells/μl whole blood (A, left and D, right) or proportion of CD8$^+$ T cells per pre-infection baseline values in PBMC for CD8$^+$ T cells at intervals after SIV infection in animals receiving Ad-SIV (closed diamonds), Ad-SIV with Ad-IL-15 (closed square) or mock (open circle) immunotherapy. Periods of ART are depicted as shaded boxes, and with Ad5- and Ad35-based immunizations shown as black and open arrows, respectively. Phenotype (Left) and proliferation (Right) illustrated for (A) total counts, (B) Tcm, (C) Tem, and (D) naïve CD8$^+$ T cells.*=P<.05.

5.5.3 Immunotherapy transiently restores mucosal T cell balance and reduces hyperactivation

To examine the capacity for immunotherapeutic vaccination to salvage mucosal immunity, we longitudinally monitored the phenotype and proliferative ability of CD4$^+$ and CD8$^+$ T cells present in BAL samples. Although CD4$^+$ T cell numbers were insufficient for detailed characterization, we examined the proportion of CD8$^+$ to CD4$^+$ T cells in BAL samples at timepoints throughout the investigation. Normal CD8$^+$ to CD4$^+$ ratios in uninfected macaques were found to be 2.57±0.37, providing a frame of reference to healthy cellular profiles in the lung. A substantial increase in CD8$^+$ to CD4$^+$ T cell proportion was observed following infection, prior to ART intervention at wk 12, attaining 22 and 23 fold increases for collective immunized and control groups, respectively, suggesting effector CD8$^+$ T cell homing to the lung in response to immune activation and inflammation (Fig. 18a). The first cycle of ART transiently restored cellular balance, potently increasing the percentage of CD4$^+$ T cells present compared to CD8$^+$ T cells among all treatment groups for the duration of ART administration (Fig. 18a). Upon ART cessation, during the first STI, all treatment groups again experienced a dramatic increase of mucosal CD8$^+$ to CD4$^+$ T cells. Subsequently, the second ART cycle was minimally effective at restricting skewed CD8$^+$ T cell levels, improving the mucosal CD4$^+$ ratio from initiation of ART in Ad-SIV immunized animals alone. Notably, CD8$^+$ to CD4$^+$ ratios at the end of the second ART cycle, wk 44, were considerably elevated compared to healthy levels, with Ad-SIV, Ad-SIV/IL-15, and mock immunized control groups displaying roughly 10, 30, and 30 fold increases in CD8$^+$ to CD4$^+$ cells compared to non-infected control animals, respectively (Fig. 18a).

To decipher the effects of therapeutic vaccination on CD8$^+$ T cell phenotype at the mucosa, we utilized FACS analysis to longitudinally measure cellular profiles. BAL samples

were nearly devoid of naïve $CD8^+$ T cells, consisting of predominantly $CD8^+$ Tem cells (Fig 18a, b, data not shown). As $CD8^+$ Tcm and Tem memory populations represented nearly 100% of $CD8^+$ T cells at the lung, the proportions of these populations were inversely related. Upon establishment of chronic infection at wk 12, $CD8^+$ Tcm percentages increased as Tem percentages decreased relative to values attained from uninfected controls (Fig. 18b, c). During the first ART cycle, $CD8^+$ Tem cell numbers were restored to those of pre-infection, healthy controls by wk 24, with concomitant decreases in Tcm seen in all treatment groups (Fig. 18b, c). $CD8^+$ T cell phenotypes were unaffected during STI regardless of treatment groups. Finally, slight decreases in $CD8^+$ Tem, with subsequent increases in Tcm were observed during the second ART cycle, leaving $CD8^+$ T cell profiles nearly identical to those identified in non-infected control animals (Fig. 18b, c).

To determine whether vaccination enhanced the proliferative activity and subsequent hyperactivation status of $CD8^+$ T cells at the lung mucosa, we measured intracellular Ki-67 production. As seen in peripheral blood T cell populations, cellular proliferative responses, regardless of immunization status, were consistent between total $CD8^+$ T cells and individual memory phenotypes. We observed marked increases in Ki-67^+ $CD8^+$ T cells over those seen in non-infected controls by wk 12 post-infection in all treatment groups (Fig. 18). Initiation of ART cycle-1 effectively reduced mucosal $CD8^+$ T cell proliferation during drug treatment, potentially due to decreased viral-induced activation and associated inflammation in the lung. Animals receiving Ad-based vaccination collectively, with or without IL-15, had significantly increased Ki-67^+ $CD8^+$ T cells at wk 17, one week post-Ad5-SIV vaccination, compared with controls, an effect mediated predominantly by the $CD8^+$ Tem population (Fig. 18a, c). Upon STI, all animals had gradual increases in $CD8^+$ T cell proliferation that were not restricted by re-initiation of ART.

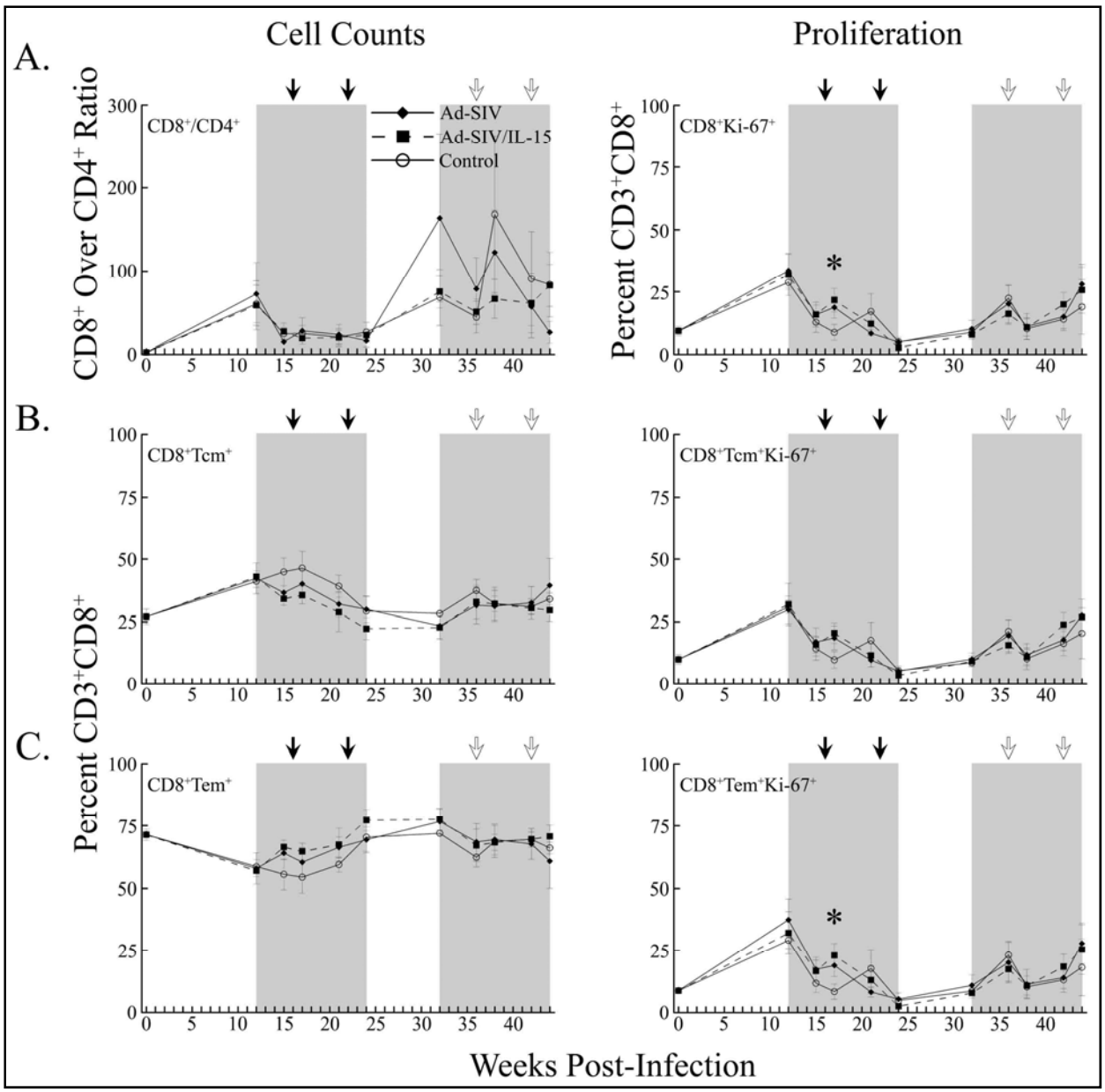

Figure 18: Immunotherapy transiently restores T cell balance and reduces hyperactivation at the lung mucosa.

Shown are the percentage change over time for animals receiving Ad-SIV (closed diamonds), Ad-SIV with Ad-IL-15 (closed square) or mock (open circle) immunotherapy from non-infected control animals used for baseline representations. Periods of ART are depicted as shaded boxes, and with Ad5- and Ad35-based immunizations shown as black and open arrows, respectively. Phenotypic (Left) and proliferative (Right) percentage changes are illustrated for (A) representative ratio of total $CD8^+$ to $CD4^+$ T cell counts, (B) $CD8^+$ Tcm, and (C) $CD8^+$ Tem cells.*=$P<.05$.

5.6 DISCUSSION

For the countless benefits afforded by ART, pharmacologic intervention against HIV is incapable of completely restoring the cellular immune system to an extent sufficient for perpetual self-regeneration and maintenance. Thus, we have evaluated the potential for therapeutic vaccination, with or without immunoregulatory IL-15 administration, to induce peripheral expansion of $CD4^+$ and $CD8^+$ memory T cell numbers both systemically and at the lung mucosa during ART-treated, chronic SIV infection. In addition, we proposed that effective immunization would support naïve T cell replenishment through alleviating virologic pressure, allowing for unmolested thymopoeitic supply of *de novo* $CD4^+$ T cells while inhibiting the heightened susceptibility of peripheral naïve T cell populations to apoptosis. Although Ad-based vaccination was previously shown to induce robust Ag-specific effector responses both peripherally and mucosally in our model, immunization failed to augment either T cell memory populations or enhance proliferation above levels seen in non-immunized controls. These findings suggest that although potently immunogenic, Ad-based vaccination has limited influence on global T cell dynamics in the presence of underlying SIV viremia during therapeutic intervention. Cellular maintenance and reductions in proliferation can be attributed primarily to ART, as no biologically significant differences were observed between animals immunized, with or without IL-15, and control animals throughout this investigation. Therefore, without attaining complete suppression of viral replication in our model, the results of Ad-based immunotherapeutic immunization were negligible and inconclusive.

As we have previously identified the role of early SIV-specific immunity and prolonged control of viremia in our model (Fig. 15), it is likely that the acute pathogenic insult sustained prior to immunotherapeutic intervention was insurmountable to completely restore T cell homeostasis. At the initiation of ART, all study animals presented gross $CD4^+$ T cell loss, consisting of reductions in both Tcm and naïve populations, factors strongly associated with response to immunization and virologic control [214, 388]. Concurrent with the significant loss of $CD4^+$ T cells, is the dramatic expansion of $CD8^+$ T cells, representing a near doubling of the effector arm of the $CD8^+$ T cell population. The dramatic expansion of systemic and mucosal $CD8^+$ T cells, sustained throughout the course of investigation, is indicative of a state of hyperactivation, potentially induced by continual exposure to viral Ag, bacterial translocation,

and cytokine deregulation [100, 401]. Finally, as ART failed to ubiquitously contain viral replication in our model (Fig. 13), gradual but progressive disruption of T lymphocyte dynamics ensued, leading to unrestricted and suboptimal $CD8^+$ effector T cell responses in the absence of $CD4^+$ T cell helper function, compounded by pathologic T cell turnover and lack of naïve T cell regeneration.

Given as an immunoregulatory adjuvant, animals receiving IL-15 administration demonstrated a trend towards enhanced absolute $CD4^+$ and $CD8^+$ T cell numbers throughout the first ART treatment cycle. Yet, in contrast to previous reports, animals receiving IL-15 had neither significantly increased $CD4^+$ or $CD8^+$ Tcm or Tem memory populations nor enhanced effector memory proliferation when compared to Ad-SIV or non-immunized control groups [177]. These results also conflict with studies suggesting that IL-15 is capable of peripheral memory $CD8^+$ T cell expansion, even in the absence of $CD4^+$ T cell helper functioning. Notably, animals receiving Ad-SIV with IL-15 demonstrated increased naïve $CD4^+$ T cell proportions, retaining roughly equivalent proportions, albeit with reduced absolute numbers, as seen prior to infection (Fig. 16). Although we previously demonstrated potent Ad-mediated production of IL-15 *in vitro* (Fig. 8), it is likely that the levels of vector-delivered cytokine are insufficient to effect systemic T cell dynamics, with IL-15 remaining largely localized to the site of vaccination. Further investigation into the use of high-dose, systemic IL-15 administration during Ad-based immunotherapeutic vaccination will be required to assess the global effects of cytokine augmentation in this model.

To the best of my knowledge, our findings represent the first report to examine cellular proliferation dynamics following therapeutic Ad-based vaccination of chronic SIV infection. Over the course of investigation, we observed a biphasic response to immunotherapeutic intervention, resulting in initial reductions of viral-induced hyperactivation both mucosally and in the peripheral blood, followed by enhanced proliferative rates during and immediately after ART cycle-2 in BAL and PBMC, respectively. Furthermore, it is possible that even as vaccination failed to enhance generalized, overall proliferation, Ag-specific stimulation and expansion of T cells may have occurred as seen following poxvirus-based therapeutic vaccination, but was not directly addressed in this investigation [282].

5.7 CONCLUSIONS

In the present study, immunotherapeutic vaccination had negligible influence upon the state of global T cell dynamics. As previously suggested, potential effects of immunization may be occluded by the persistent response to continual, low-level viremia present during suboptimal antiretroviral-mediated control of infection. Notably, trends in the expansion of $CD8^+$ T cell and $CD4^+$ Tcm cell populations were observed following immunotherapeutic vaccination. These findings support the further evaluation of therapeutic Ad-based vaccination in the presence of more compete antiretroviral-mediated control of viremia.

6.0 DISSERTATION RELEVANCE

HIV persists as one of the most devastating pathogens to coincide with modern humanity, representing a global challenge to not only biomedical researchers, clinicians, and public health experts, but individuals of compassion and conscience worldwide. Fortunately, with the knowledge of generations past and dedication of those at present, science has never been more prepared for such a challenge. The work presented in this document describes the utilization of novel, recombinant adenoviral vector-based vaccination regimens for the prevention and therapeutic intervention against –immunodeficiency virus infection in the nonhuman primate model. This work represents an incremental advancement in understanding the character of T cell-mediated immunity induced through the Ad-based vaccination platform, and the subsequent potential to inhibit SIV pathogenesis.

6.1 AIM 1: CHAPTER ONE - NOVEL VACCINE REGIMEN UTILIZING ADENOVIRUS SEROTYPE 5- AND 35-BASED VECTORS

At the turn of the 21st century, the field of HIV vaccine development had shifted its attention towards the development of vaccines capable of inducing robust CTL-mediated immunity in an attempt to curtail the HIV pandemic. Failures to create conformationally representative antigenic targets for humoral immune induction, in conjunction with a growing body of evidence supporting the role of cellular immunity in restricting disease progression, led to interest and development of viral vectors as immunogens. In 2002, a hallmark study by Shiver et al. demonstrated the ability of Ad5-based vectors to control SHIV89.6 infection in nonhuman primates, albeit utilizing genetically optimal Mamu-A*01-expressing animals and a challenge virus of attenuated pathogenesis, encouraging further investigation of Ad-based vectors and

infusing optimism into a demoralized field that vaccine-induced control of HIV infection was possible [211]. With the revelation that vector-specific humoral immunity limited the potential of homologous Ad-based vaccination regimens, development of Ad vectors based on rare human or nonhuman species was advanced. Studies by our group were among the first to develop and explore the utility of Ad35-based vectors as potential HIV vaccine candidates due to their low seroprevalence in the United States and regions of Africa [305, 310]. Furthermore, the data presented in Chapter 1 represents the first reported study utilizing heterologous Ad5- and Ad35-based vaccination of nonhuman primates against SIV infection [405]. This study provided the preliminary immunogenicity results necessary to support the continued development of heterologous Ad-based vaccination regimens for the prevention and therapeutic inhibition of HIV infection.

6.1.1 Immunogenicity of Prophylactic Dual-Serotype Adenoviral-Based Vaccination

Our laboratory's demonstration that novel Ad35-based vectors effectively enhanced immunity previously induced through Ad5-based immunization of nonhuman primates expanded the field of analysis of heterologous Ad-based prime/boost vaccine regimens, building upon previous reports illustrating the effective use of the Ad5/Ad35 prime-boost combination in mice, or Ad5/AdC6 interspecies prime-boost models in nonhuman primates [311, 319]. Notably, as a proof-of-concept, we demonstrated the ability of sequential heterologous Ad5/Ad35-based immunization to augment the magnitude of Gag-specific IFN-γ production over that induced following homologous Ad5-based vaccination, coinciding with lack of Ad5-specific NAb to cross-neutralize Ad35-based vectors in nonhuman primates (Fig. 1). Following pathogenic SIV challenge, we showed that vaccine induced IFN-γ-mediated responses were rapidly recalled, demonstrating the ability of this novel immunization regimen to promote durable Ag-specific memory responses, a goal of any effective preventative immunization strategy. Detailed characterization of vaccine-induced and post-challenge cellular immunity revealed a plethora of epitope targets with slightly varied profiles, suggesting that efforts to maximize T cell targeting such as the inclusion of heterologous vaccine Ags may be necessary to optimize protective immunity. The discovery of the novel $CD4^+$- and $CD8^+$-restricted T cell epitopes described in

this work adds to the burgeoning library of known nonhuman primate epitopes within SIV, and will allow for a more precise characterization of the ability of vaccine induced immunity to translate into effective anamnestic responses. Taken together, these findings have confirmed the ability of the Ad5/Ad35-based vaccine regimen to induced robust Ag-specific cellular immunity in a pre-clinical nonhuman primate trial against SIV infection and support the advancement of heterologous over homologous Ad-based vaccine regimens for further investigation as immunogens against HIV infection.

6.1.2 Viral Escape from Cellular Immune Pressure

Although clinical observation of HIV viral escape following the development of cellular immunity have been reported, the nonhuman primate model of infection provides the opportunity to study the mechanisms and consequences, for both host and virus, of T cell-driven viral escape through the use of molecularly cloned viral strains, specific timing of infection, and defined genetic models. In chapter 1, we utilized IFN-γ ELISPOT matrices to provide detailed characterization of the precise T cell-mediated Gag-specific responses following Ad-based immunization against the molecular isolate SIVmac239 and recalled upon heterologous viral challenge utilizing SIV/DeltaB670. As cellular immune responses conserved between vaccination and challenge should be capable of applying immunologic pressure and influencing viral escape, we elucidated precise T cell epitopes and their associated viral sequences to evaluate sustained viral immune evasion. Notably, the SIV/DeltaB670 challenge virus consists of an uncloned, pathogenic viral swarm and has seldom been examined in infection models, allowing for the primary characterization of T cell-driven immune evasion mechanisms. Our findings demonstrated that $CD8^+$ T cell-mediated responses directed against the Mamu-A*01-restricted CM9 epitope were capable of driving viral escape, with rapid selection of position 2 anchor residue viral variants following SIV/DeltaB670 challenge, consistent with previous reports [357]. Yet, further investigation revealed that SIV/DeltaB670 developed sustained CM9 escape variants in the absence of extraepitopic flanking mutations, shown to be necessary for persistent CM9 escape in SIVmac239 and SIVmac251 [361, 362]. Notably, we found the extraepitopic regions of the CM9 epitope to be hypervariable in SIV/DeltaB670, suggesting a flexible Gag structure, unique to SIV/DeltaB670, which may allow for viral escape without

concurrent reductions in viral fitness, posing a new mechanism for SIV CTL escape. In contrast to the rapid viral escape identified from CM9-driven CTL pressure, potent vaccine induced, $CD8^+$ T cell-mediated responses targeting the novel GL9/GS9 ($Gag_{141-150}$), GY9 ($Gag_{270-278}$), and QP9 ($Gag_{272-280}$) in the SIV capsid, as well as $CD4^+$ T cell-mediated targeting of the novel GT15 (Gag_{56-70}) epitope found in the viral matrix protein failed to induce sustained alterations in concomitant viral sequences (Fig. 7). Interestingly, two of three animals who developed $CD8^+$ T cell-mediated responses to the novel GY9/QP9 epitope regions (p68/p69) following vaccination demonstrated superior control of viremia upon SIV/DeltaB670 challenge for over 30 wks post-infection. These findings suggest the presence of an evolutionarily conserved viral sequence within the SIV/DeltaB670 capsid protein that is under severe structural or functional constraints, thus limiting its capacity to sustain mutations and inhibiting viral escape, as demonstrated by in vitro analysis of HIV capsid and population wide assessments of HLA effect and HIV evolution [406, 407]. Although the MHC allele or alleles responsible for binding these peptide epitopes could not be identified at this time, further investigation into the mechanisms of the viral restriction associated with cellular immune responses directed against this region would advance our current understanding of the qualitative role of immunologic targeting, potentially elucidating the most effective targets for vaccination.

6.1.3 Vaccine-mediated Restriction of Heterologous SIV Challenge

The recent failure of the phase IIb STEP trial performed by Merck and the NIH of a homologous Ad5-based vaccine candidate including HIV clade B *Gag*, *Nef*, and *Pol* genes to either prevent HIV infection or suppress post-infection viremia has raised the questions of what vaccine-induced antiviral cellular immune responses will be efficacious, and how much can we extrapolate from current nonhuman primate models of HIV infection [408]. The STEP Ad5-based vaccine demonstrated limited immunogenicity among a human population heterogeneous in pre-existing Ad5-specific NAb titres, with responses per Ag determined by IFN-γ ELISPOT detected in roughly 38-76% of subjects depending upon their anti-Ad5 NAb titres [409]. By comparison, the magnitude of these cellular immune responses represent roughly 10-20% of those seen in HIV infected individuals who innately control infection [410]. In contrast, our study (Chapter 1) showed that macaques ubiquitously developed IFN-γ-mediated responses

following Ad5-Gag priming immunization that were further augmented in those animals receiving Ad35-Gag administration (Fig 1). Although animals were initially devoid of anti-Ad5 NAb titres, we observed potent enhancement of Gag-specific IFN-γ production during four rounds of homologous Ad5-based immunization in all animals following the development of Ad5-specific NAb titres >100 and in 7/8 animals with Ad5-specific NAb titres >200, the STEP delineation level for elevated NAb titre. Notably, animals receiving Ad5/Ad35-Gag vaccination demonstrated significantly lower viral loads over the course of investigation and extended survival rates compared with controls, with two vaccinees attaining undetectable viremia following challenge with heterologous and pathogenic SIV/DeltaB670 (Fig. 5). Remarkably, our immunization targeted the related but distinct Gag protein of SIVmac239 alone, suggesting that the failure of the STEP trial may under represent the potential utility of Ad-based vaccination against HIV. Unfortunately, Ad5/Ad35-based vaccination in our model failed to achieve the 1.5 log reduction in post-infection chronic viremia that is the current benchmark for an altruistic vaccination to inhibit HIV transmission and disease progression [322, 323, 411-413]. Potentially, a higher dosage of Ad-based vaccine or continued rounds of heterologous Ad-based immunization may have been necessary for the optimal induction of *de novo* cellular immunity and subsequent expansion of antiviral cellular memory.

6.1.4 Insights into Nonhuman Primate Models of HIV Infection

The SIV challenge model of rhesus macaques remains inadequately characterized, prohibiting comparison of preclinical vaccine modalities and obscuring the potential for direct clinical translation of results. Once the gold-standard for pathogenic challenge, SHIV-89.6P viruses have been shown to inaccurately reflect disease course in humans, demonstrating nearly ubiquitous and aberrant depletion of both memory and naïve peripheral $CD4^+$ T cells through CXCR4-mediated entry [414]. In contrast to natural HIV or pathogenic SIV infection, SHIV-89.6P viruses are exquisitely sensitive to Ab-mediated neutralization, and have been controlled repeatedly through a multitude of immunization strategies. This discrepancy was highlighted by the results of the STEP trial, failing to demonstrate clinical efficacy following protection against SHIV-89.6P challenge [211]. Our findings examining the ability of Ad5/Ad35-based vaccination to elicit T cell-mediated protection against the relatively unutilized SIV/DeltaB670

quasispecies support the use of uncloned, pathogenic SIV species as a stringent model of HIV infection. Although vaccinated animals sustained lower viral loads compared to control animals, statistically significant reductions in viremia were not observed at any specific timepoint after heterologous SIV/DeltaB670 infection (Fig. 5). As the standardization of challenge models will be necessary for the preclinical evaluation of next-generation vaccines, our findings are a notable addition to the field, supporting the use of uncloned, pathogenic SIV to realistically model HIV infection.

6.2 AIM 2: CHAPTER 2 - THERAPEUTIC ADENOVIRUS-BASED VACCINATION DURING ART-TREATED CHRONIC $SIV_{MAC}251$ INFECTION

6.2.1 Therapeutic Ad-Based Vaccination Enhances the Breadth and Magnitude of Antiviral Cellular Immunity

With an estimated 33.2 million people living with HIV in 2007, therapeutic intervention strategies aimed at augmenting dormant antiviral immunity, decreasing latent viral reservoirs, and salvaging crucial $CD4^+$ T cell memory populations are being examined to alleviate the pathogenic effects of ongoing HIV infection and potentially promote a state of immunologic balance capable of durable containment of viremia in the absence of continuous pharmacologic treatment. Building upon the success of preventative Ad-based vaccination regimens, we utilized Ad5- and Ad35-based vectors encoding the SIVmac239 *gag*, *env*, and *nef* genes, with or without Ad-mediated IL-15 delivery, in a therapeutic vaccination strategy during ART-treated, chronic SIVmac251 infection of rhesus macaques, extending the use of Ad-based vectors for the first time beyond prophylactic vaccination in this model. As hypothesized, Ad5/Ad35-based vaccination was capable of augmenting cumulative antiviral cellular immunity over innate responses seen in mock immunized control animals during chronic SIVmac251 infection, as determined through IFN-γ ELISPOT (Fig. 9). Furthermore, vaccination expanded peak Ag-specific cellular immunity during immunotherapy against the individual Gag, Env, and Nef proteins to a significant degree (Fig. 9). Notably, two rounds of ART followed by STI failed to induce heighted T cell-mediated immunity in mock immunized control animals, demonstrating

that immune induction was due to vaccination. As conventional theories supported by years of preclinical observations suggest that enhancing the overall magnitude of Ag-specific IFN-γ-mediated cellular immunity is necessary for effective control of all stages of HIV/SIV viremia, our findings support the development of Ad-based vaccination strategies for the treatment of chronic –immunodeficiency virus infection.

6.2.2 Therapeutic Vaccination Induces $CD8^+$ T cell-Biased Monofunctional Immunity

Prior to the development of multiparametric FACS analysis, T cell functionality and presumptive antiviral immunity at the single cell level was predicted by the quantitation of IFN-γ, TNF-α or IL-2 individually in responses to viral Ag. As the sensitivity and scope of cellular cytokine detection has expanded, so too has our understanding of the effective antiviral mediators associated with protection from disease [292]. Studies comparing the T cell effector profiles of individuals who control HIV infection, with or without ART, compared to those with progressive infection have suggested that functional heterogeneity consisting of polyfunctional IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a/b responses are essential in disease containment [219, 222]. Therefore, we utilized multiparameter FACS analysis to characterize the $CD4^+$ and $CD8^+$ T cell-mediated production of IFN-γ, TNF-α, and IL-2 simultaneously during peak responses subsequent to Ad-based vaccination. Within the peripheral blood $CD8^+$ T cell population, therapeutic Ad5-based vaccination enhanced predominantly monofunctional production of both IFN-γ and TNF-α, as well as IL-2^+ IFN-γ^+, IFN-γ^+ TNF-α^+, and triple positive cytokine profiles to a lesser degree (Fig. 10). Additionally, increased Ag-specific IL-2 was detected from $CD8^+$ T cells in BAL samples following Ad5-SIV administration (Fig. 13). In contrast, Ad5-SIV immunization induced limited $CD4^+$ T cell responses, consisting of Ag-specific IFN-γ production alone (Fig. 10). Subsequent boosting with heterologous Ad35-SIV maintained IFN-γ-mediated production from $CD8^+$ T cells in PBMC and enhanced IL-2^+ IFN-γ^+ in BAL samples in responses to peptide stimulation, failing to support previously induced $CD4^+$ effector responses. The predominance of monofunctional $CD8^+$ T cell-mediated immunity following vaccination suggests that Ad-based immunization serves to establish terminal differentiation of Ag-specific effector T cells, leading to cytokine dysfunction. The conspicuous absence of IL-2

production from either $CD4^+$ or $CD8^+$ T cells of the peripheral blood further supports the idea that therapeutic Ad-based immunization fails to induce a regenerative cytokine profile. These findings are in agreement with recent immunization studies suggesting that Ad5-based vaccination induces a $CD8^+$ T cell-skewed, qualitatively restricted immune profile when compared to less overtly immunogenic serotyped vectors such as Ad26 and Ad48 [294]. Collectively, these findings suggest that the unique immunogenicity of Ad5-based vectors may promote less efficacious immunity against complex intracellular pathogens such as HIV.

6.2.3 Vaccination Transiently Restores $CD4^+$ Tcm Populations

Therapeutic interventions capable of restoring depleted $CD4^+$ Tcm populations will allow for the regeneration of $CD4^+$ Tem cells and provide support for memory $CD8^+$ T cells necessary to maintain effective antiviral cellular immunity. In addition, the maintenance of $CD4^+$ Tcm cells in animals receiving DNA-prime/Ad-boost immunizations strongly correlated with survival following pathogenic SIVmac251 challenge [214]. We currently demonstrate that administration of Ad5/Ad35-SIV administration significantly augmented peripheral blood $CD4^+$ Tcm populations from levels detected at ART initiation (Fig. 12). Unfortunately, increases in $CD4^+$ Tcm cells were not reflected in the $CD4^+$ Tem cell populations or the overall $CD4^+$ T cell populations of the peripheral blood or lung mucosa (Fig. 12 and 13). Augmentation of peripheral $CD4^+$ Tcm populations may be indicative of peripheral Ag-driven memory expansion, suggesting that further immunization under restricted viremic conditions afforded by effective ART may serve to fully restore homeostasis within the $CD4^+$ T cell memory compartment.

6.2.4 Therapeutic Vaccination Fails to Control Rebound Viremia upon ART Cessation

The primary goal of the Ad-based immunotherapy described in chapter 2 was to promote a SIV-specific cellular immune response of sufficient quality and magnitude that would be capable of containing rebound viremia upon cessation of ART. Although therapeutic Ad5/Ad35-SIV administration induced robust $CD8^+$ and moderate $CD4^+$ T cell responses, immunization failed to further contain SIVmac251 viremia beyond the effects seen with ART alone (Fig. 14).

Although disappointing, these results are not surprising given that the combination of PMPA and FTC utilized in our study failed to suppress chronic viremia in 11 of 15 animals selected for median control of chronic viral load (Fig. 14). Previous therapeutic intervention trials and personal communications (B. Felber, National Cancer Institute) strongly suggest that in the absence of ART-controlled SIV viremia the effects of therapeutic vaccination will be undetectable due to continual low-level antigenic stimulation of effector responses [376, 395]. Taken together, the full potential of the current heterologous Ad-based vaccine regimen may yet to be recognized, as the underlying viremia present in our study confounds interpretation, providing SIV-mediated Ag-stimulation, homeostatic T cell disruption, and ongoing disease pathogenesis.

6.2.5 Effects of IL-15 Augmentation

Within Aim 2, we addressed whether Ad-mediated delivery of the immunoregulatory cytokine IL-15 as a vaccine adjuvant would augment SIV-specific immunity allowing for further suppression of viremia throughout the investigation. Co-administration of Ad-IL-15 with Ad-SIV vectors failed to enhance cumulative or individual Ag-specific cellular immune responses, with animals receiving Ad-IL-15 adjuvantation displaying reduced reaction to immunization during both ART cycles (Fig. 9). In addition, no distinguishable differences were identified between animals, with or without IL-15 augmentation, in the Ag-specific $CD4^+$ and $CD8^+$ T cell T_H1 cytokine profiles induced through vaccination (Fig. 10). Furthermore, although not statistically significant, animals receiving Ad-SIV with Ad-IL-15 had median viral loads 1 log greater than either Ad-SIV or control groups at 52 wks post-infection. While not detected systemically, IL-15-induced activation and proliferation of SIV-specific $CD4^+$ Tem at the local site of vaccination and compartmentally in lymph nodes may enhance the susceptibility of these cells to SIV-mediated elimination, subsequently removing essential Ag-specific $CD4^+$ T cell support functions necessary to propagate antiviral cellular immunity while increasing SIV replication.

6.2.6 Immunologic Correlates to SIV Control

The diverse range of viral control demonstrated by study animals in Chapter 2 in conjunction with the detailed analysis of cellular immunity performed during this investigation provided the opportunity to identify the immunologic factors that correlate with pathogenic inhibition throughout therapeutic intervention. When animals were analyzed, irrespective of treatment group, we found significant correlations between the magnitude of peak Gag- and Env-specific IFN-γ ELISPOT responses in PBMC during both ART cycles and reduced viral load (Fig. 15). Additionally, systemic IFN-γ^+ TNF-α^+ producing $CD8^+$ T cells and polyfunctional $CD4^+$ T cells in PBMC producing two or three cytokines were negatively correlated with viremia (Fig. 15). As moderators of cellular dynamics, the absolute number of $CD4^+$ Tcm cells in peripheral blood were negatively associated with both early (wk 12) and late (wk 44) viremia, and positively associated with the maintenance of polyfunctional Ag-specific $CD8^+$ Tcm populations in the periphery. Maintenance of gross $CD4^+$ T cell populations at the lung mucosa were found to correlate with reduced plasma viral loads both early and late during infection, an association which was not identified with peripheral blood $CD4^+$ T cells, illustrating the sensitivity of mucosal $CD4^+$ T cell analysis. As the precise correlates of protective immunity against HIV infection have yet to be defined, our findings provide insight into the mechanisms of immunologic control of chronic SIVmac251 infection, and corroborate the role of polyfunctional Ag-specific T cell-mediated immunity, peripheral $CD4^+$ Tcm, and mucosal $CD4^+$ T cells in the inhibition of SIVmac251 pathogenesis.

6.3 AIM 3: CHAPTER 3 - EFFECTS OF IMMUNOTHERAPEUTIC VACCINATION ON SYSTEMIC AND MUCOSAL T CELL DYNAMICS

6.3.1 Limitations of Ad-Based Vaccination During Chronic SIV Infection

The results presented in chapter 3 detailing the effects of immunotherapeutic Ad-based vaccination during ART-treated, chronic SIVmac251 infection suggest the inability of

parenterally administered Ad-based vectors, with or without IL-15, to influence T cell population phenotypes or proliferative activity. Regrettably, statistically significant differences between treatment groups were practically absent, leaving general trends of phenotypic evolution and proliferative response throughout the course of investigation. In contrast, administration of ART altered both systemic and mucosal T cell phenotypes and proliferative response, and was the primary influence on T cell dynamics within our treatment model. Moreover, as ART failed to suppress viremia, the sustained viral loads of greater than 10^4 RNA copies per ml seen in all treatment groups throughout investigation may have been capable of inducing homeostatic T cell dysfunction, as evidenced by loss of peripheral and mucosal $CD4^+$ T cells, naïve T cell populations, and lack of proliferation.

6.3.2 Immunotherapy Promotes $CD4^+$ T Cell Memory But Not Naïve Populations in PBMC

Throughout the course of infection, we observed a stark contrast between the dramatic expansion of memory $CD4^+$ T cell populations and the gradual and near complete elimination of naïve $CD4^+$ T cells in the peripheral blood (Fig. 16). Initial declines in $CD4^+$ Tcm cells, potentially due to Ag-driven differentiation into Tem cells, were reversed with the initiation of ART at wk 12, subsequently demonstrating continued expansion throughout observation. Similarly, $CD4^+$ Tem populations experienced a roughly 400% increase among all treatment groups from pre-infection baseline levels. Interestingly, ART had contradictory effects upon $CD4^+$ T cell memory populations, promoting Tcm cell numbers while causing moderate declines in Tem populations, potentially due to suppressed peripheral activation. Conversely, naïve $CD4^+$ T cells underwent a limited increase prior to the initiation of ART at wk 12, which was followed by progressive depletion of these cells in the peripheral blood. Interestingly, although not statistically significant, animals receiving Ad-IL-15 administration maintained heighted naïve and lower memory $CD4^+$ T cell numbers throughout investigation. Collectively, immunotherapy had limited effects on $CD4^+$ T cell phenotype, as ART-mediated control of viremia was incomplete, and therapeutic vaccination failed to expand memory $CD4^+$ T cells at any timepoint over controls.

6.3.3 Expansion of $CD8^+$ Tem Cells and Loss of Naïve $CD8^+$ T Cells Throughout Progressive Infection

Upon the establishment of chronic infection, we observed a rapid expansion of absolute $CD8^+$ T cell numbers in peripheral blood, characteristic of pathogenic SIV infection. Phenotypic analysis identified the majority of this burgeoning $CD8^+$ T cell compartment to be composed $CD8^+$ Tem cells, indicative of the adaptive response to viral infection and activation induced through systemic hyperactivation. In addition, a marginal collective increase in $CD8^+$ Tcm cells was identified among all groups over the course of infection (Fig. 17). Interestingly, expansion of the $CD8^+$ T cell memory populations coincided with a progressive loss of peripheral naïve $CD8^+$ T cell population over the period of observation. As neither ART nor Ad-based vaccination were found to significantly effect the $CD8^+$ T cell compartment, it can be presumed that the observed T cell dynamics represent a close to natural state of progressive viral pathogenesis. These findings suggest that overrepresentation of $CD8^+$ Tem populations and depletion of naïve $CD8^+$ T cells in the peripheral blood were due to continual Ag-driven peripheral expansion of $CD8^+$ T cells in the presence of ongoing viremia.

6.3.4 Immunotherapy Transiently Restores Mucosal $CD8^+$ to $CD4^+$ T cell balance

The effects of HIV/SIV infection upon the T cell dynamics of mucosal surfaces are the most predictive indicators of systemic pathogenesis and disease progression. In as much, we evaluated the potential for therapeutic vaccination to promote functional cell-mediated immunity in the lung mucosa either through alleviating virologic pressure or through the expansion and recruitment of $CD4^+$ Tem cells to the extralymphoid tissue [177, 215]. As normal macaque T lymphocytes in BAL samples are predominantly $CD8^+$ T cells, we monitored the ratio of $CD8^+$ to $CD4^+$ T cells, providing baseline values of healthy mucosal T cell homeostasis. SIV infection resulted in a rapid augmentation of $CD8^+$ to $CD4^+$ T cells percentages, likely due to both Ag-specific effector $CD8^+$ T cell recruitment and mucosal $CD4^+$ Tem depletion (Fig. 17). Notably, initiation of ART restored $CD8^+$ T cell-skewed populations to levels approaching pre-infection baseline, maintaining this ratio for the duration of ART. A $CD8^+$ T cell-skewed profile was again established during the first STI. Interestingly, although the second ART cycle transiently

supported cellular balance within all treatment groups, only animals receiving Ad-SIV achieved cellular ratios comparable to those established during the first ART cycle. While the effects of therapeutic vaccination, with or without Ad-mediated IL-15 delivery were negligible, our findings illustrate the clinical benefit of ART upon T cell dynamics at sites of the mucosa, and provide insight into the cellular pathogenesis of SIVmac251 infection at the lung.

6.3.5 Immunotherapy Suppresses Systemic and Mucosal Proliferation

As ART has been shown to reduce cellular activation, we characterized non-specific cellular proliferation through the detection of Ki-67 in T lymphocyte populations of the peripheral blood and lung mucosa. Regrettably, therapeutic vaccination was ineffective at promoting peripheral or mucosal proliferation of T cell memory populations, as no statistically significant differences were observed between treatment groups (Fig. 16-18). Interestingly, memory and naïve phenotypes of both $CD4^+$ and $CD8^+$ T cell populations in PBMC and BAL samples demonstrated a consistent pattern of cellular proliferation following SIV infection, ART treatments, and STIs (Fig. 16-18). Although T cell proliferation was suppressed for the duration of ART intervention, proliferative rates were elevated above pre-infection baseline values for all T cell phenotypes found in the peripheral blood or lung mucosa at study endpoint, wk 52 and wk 44, respectively. Collectively, these findings suggest that although ART was capable of transiently suppressing the hyperactivation associated with chronic SIV infection through inhibiting viral replication, transient immunotherapeutic intervention was incapable of durably controlling rapid T cell turnover and subsequent cellular exhaustion.

7.0 CURRENT STATE OF HIV VACCINE DEVELOPMENT

7.1 THE FAILURE OF THE STEP TRIAL

To date, only two vaccine candidates have completed human efficacy trials for the prevention of HIV infection. The first, VaxGen's gp120-specific, subunit protein-based vaccine failed to elicit NAb responses and subsequently protect from infection. The inability of this vaccine to elicit Ab-mediated sterilizing immunity was among several factors leading to a shift in research emphasis towards the pursuit of vaccines capable of promoting antiviral cellular immunity. Theoretically, T cell-mediated immunity may limit peak HIV viral loads, resulting in reduced acute pathogenesis, as well as inhibit chronic viremia through eliminating infected cells, allowing for durable immune control in the absence of sterilizing immunity. Over ten years after the failure of VaxGen's vaccine, clinical evaluation of the first T cell-mediated vaccine candidate was performed by Merck and the US National Institutes of Health with the testing of a recombinant replication-defective Ad5-based immunogen in the STEP trial. Regrettably, this study was abruptly terminated when interim analysis showed that vaccination failed to prevent HIV infection or reduce post-infection, set-point viral loads at three months following HIV seroconversion. The results of this phase IIb clinical efficacy trial represent an invaluable tool to shape the future of HIV vaccine development, and provide insight into the small successes that accompany this unfortunate failure.

The Step trial consisted of a double-blind, phase IIb, test-of-concept vaccine study involving 3000 HIV seronegative volunteers within North America, South America, the Caribbean, and Australia who were at high risk of HIV acquisition. Participants received three immunizations at 0, 1, and 6.5 months of replication-defective Ad5-based vectors individually encoding the *gag, nef,* and *pol* genes from HIV clade B strains [328]. Notably, HIV *env* transgenes were not included within vaccination vectors, tailoring immunization for the

induction of T cell-mediated immunity specifically. Study participants were randomized to receive Ad5-based vaccination or immunization diluent as a placebo based on gender, pre-existing Ad5-specific Ab titres, and testing site. Interim analysis of individuals who acquired HIV infection, all but one male, revealed that immunization was incapable of preventing HIV infection, as 49 of 914 male vaccine recipients became infected as compared to 33 of 922 men receiving placebo controls [415]. Furthermore, no reduction in mean set-point viral-loads following seroconversion were observed among vaccine recipients as compared to placebo-treated control subjects [415]. Unexpectedly, the STEP trial identified higher rates of HIV acquisition among men who had either greater Ad5-specific NAb titres, who were uncircumcised, or both [415]. In addition to the absence of a protective effect afforded by vaccination, the increased rates of HIV acquisition among vaccine recipients are alarming, raising concerns over the safety as well as utility of Ad-based and other viral vector-based immunizations against HIV infection. Collectively, these results highlight the need to further expand basic research into the specific mechanisms of viral-vector induced immunity and potential vaccine-induced immunologic phenomenon capable of enhancing sensitivity to HIV infection.

Historically touted for their ability to induce robust IFN-γ-mediated cellular immune responses, the STEP trial demonstrated that the Ad5-based vaccine candidate utilized induced T cell-mediated responses which were incapable of containing acute or chronic HIV viremia. First, IFN-γ-mediated responses to HIV Ags targeted by immunization were detected by ELISPOT in only 77% of vaccine recipients after completing the full vaccination regimen, demonstrating limited immunogenicity in a genetically diverse population with variable pre-existing Ad5-specific NAb titres [416]. The STEP Ad5-based vaccination resulted in a narrow breadth of response to targeted Ags, as a mere 62% of vaccinees mounted IFN-γ responses against two or more of the three targeted proteins [416]. As expected, the level of pre-existing Ad5-specific NAb titres were correlated with reduced vaccine immunogenicity. Furthermore, Ad5-based vaccination resulted in predominantly $CD8^+$ T cell-mediated responses consisting of primarily IFN-γ and TNF-α with limited polyfunctional capacity. Finally, Ad5-based vaccination established a $CD8^+$ T cell-biased Ag-specific response, with only 41% of vaccine recipients mounting $CD4^+$ T cell-mediated responses and 31% of vaccinees developing both $CD4^+$ and $CD8^+$ T cell response against HIV antigens [416]. Notably, there were no differences in the

magnitude, breadth, or character of HIV-specific vaccine-induced immunity identified between vaccine recipients who remained HIV negative or subsequently contracted HIV, suggesting that the Ad5-based STEP immunization promoted a functionally deficient immune response [416].

With only preliminary data available for interpretation, it is difficult to explain the failure of the Ad5-based STEP vaccination to effect HIV transmission rates or the dynamics of early infection. Potentially, Ad5-induced immunity was of insufficient magnitude, as only three fourths of vaccine recipients developed significant ELISPOT responses, measured between 1:1500 and 1:6000 IFN-γ-producing cells within PBMC [416]. The magnitude of this response is dramatically lower than T cell-mediated responses attained through similar homologous Ad5-based immunizations, heterologous Ad-based regimens, or vaccination strategies employing DNA priming prior to Ad- or vaccinia-based boosting in both preclinical and clinical trials [211, 212, 267, 276, 288, 289, 321]. Additionally, STEP researchers noted that vaccine-induced $CD8^+$ T cell-mediated cytokine responses were 43% lower than those observed in HIV long-term non-progressors in a North American cohort [416]. Although not empirically evaluated in the STEP trial, pre-existing Ad5-specific humoral immunity may have limited vector immunogenicity, rendering immunization ineffective at the administered dose. Furthermore, detailed characterization of Ad5-mediated cellular immunity demonstrated that vaccination resulted in predominantly $CD8^+$ T cell-biased responses of restricted breadth and polyfunctional capacity. Thus, in addition to the quantitative shortcomings of vaccination, a qualitative deficiency may have been present, as immunization failed to promote HIV-specific T cell-mediated responses against all targeted viral Ags, $CD4^+$ T cell responses of a significant nature, and functional heterogeneity within the antiviral cytokine response. As the precise correlates of T cell-mediated inhibition of HIV disease progression are yet undefined, it is imperative that novel vaccination strategies are rigorously tested in the preclinical setting to further elucidate the functional characteristics and subsequent mechanisms of T cell-mediated control of HIV infection prior to their advancement into clinical trials.

It has been proposed that individuals with pre-existing Ad5-specific immunity would undergo Ag-specific expansion of T cell populations following Ad5-based vaccination with subsequent redistribution of Ad5-specific effector memory $CD4^+$ T cells to the mucosa, resulting in an increased availability of cellular targets susceptible to HIV infection. This would effectively increase the vulnerability of a vaccine recipient to HIV infection, due to the elevated

density of permissive target cells within the reproductive and gastrointestinal mucosa. Recent analysis from STEP trial participants has demonstrated that pre-existing Ad5-specific NAb titers were not correlated with increased Ad5-specific $CD4^+$ and $CD8^+$ T cell responses [417]. In fact, the opposite has been shown, with trends of lower Ad5-specific $CD4^+$ and $CD8^+$ T cell responses detected among STEP vaccine recipients with higher Ad5-specific NAb titers, presumably due to Ab-mediated opsonization and innate immune clearance of Ad5 vectors prior to the induction of T cell-mediated immunity [417]. Notably, vaccine recipients who contracted HIV during the STEP trial had lower Ad5-specific $CD4^+$ T cell-mediated responses when compared to vaccinees who remained seronegative, indicating that the frequency of Ad5-specific $CD4^+$ T cell in the peripheral blood did not correlate with infection. However, these studies examined Ad5-specific cellular immunity in the peripheral blood alone. As HIV is largely transmitted across the mucosal tissues, systemic analysis may not accurately represent the dynamics of Ad5-specific effector memory $CD4^+$ T cell infection, as has been previously shown in HIV-specific effector memory T cells of the GALT and lymph nodes [39, 50]. Therefore, further analysis is necessary to define the mechanism, if any, through which Ad5-based vaccination enhanced the susceptibility to HIV infection among STEP vaccine recipients. Remarkably, STEP participants in the placebo group did not receive empty (Ad-ψ5) vectors to control for the delivery of Ad particles, eliminating the potential to compare the effects of Ad5-specific $CD4^+$ T cell-mediated immune responses with enhanced susceptibility to HIV infection. Therefore, future evaluation of viral vector-based vaccination strategies against HIV must account for the induction of deleterious cellular or humoral immune responses through the inclusion of appropriate mock controls.

7.2 FUTURE DIRECTIONS IN HIV VACCINE DEVELOPMENT

In the brief period I have been involved in the field of HIV vaccine development, I have been continually surprised by the ideologic dichotomy between those investigators who emphasize humoral immunity and the believers of T cell-mediated responses for the control of HIV infection. The apparent resistance of researchers and funding institutions to empirically evaluate intervention strategies encompassing innate, humoral, as well as cellular immune

responses may needlessly postpone the discovery of effective correlates of immune protection against progressive HIV infection. This is exemplified by the two major vaccine failures to date testing gp120- and Ad5-based vaccine candidates aimed at inducing isolated humoral and cellular immunity, respectively. Additionally, the association of Ad5-specific NAb responses with higher rates of HIV acquisition in STEP trial vaccine recipients further emphasizes the need to critically evaluate the complex interactions of innate and adaptive immunity to non-conventional immunogens to fully assess vaccine safety and the mechanisms of immunologic protection. This is not to suggest that immunogens inducing humoral or T cell-mediated immunity alone are without merit; merely that their utility lies in the preclinical identification of the correlates of protection against HIV infection, furthering our understanding of the precise nature of adaptive immunity necessary to prevent or durably contain disease progression.

In as much, it seems imperative that future HIV vaccine development focuses on immunogens or regimens capable of promoting a combined HIV-specific humoral as well as T cell-mediated immune response. Within hours to days following infection, HIV has seeded the cells of the mucosa and lymph nodes, creating a cell-associated reservoir sufficient for continued viral generation and immune evasion. As anamnestic T cell responses will take several days to develop following HIV exposure, the presence of vaccine-induced mucosal Ab would confer the most effective means of preventing or limiting the initial establishment of viral infection. Conversely, if a pre-existing humoral immune barrier fails to provide sterilizing immunity, Ab-mediated responses will likely be incapable of eliminating quickly established cell-associated viral reservoirs due to the lack of exposed antigenic targets during latency. At this point, T cell-mediated responses capable of eliminating virally-infected host cells and thus limiting cellular pathogenesis and viral replication will be of greater benefit. Furthermore, humoral and cellular immune responses will function in concert during chronic HIV infection, effectively inhibiting the *de novo* infection of susceptible host targets while clearing latent cell-associated reservoirs, respectively. In conjunction, activated innate immunity will enhance Ag-specific adaptive responses, supplementing antiviral function through non-specific viral inhibition and clearance of opsonized viral particles and infected host cells, and facilitating the induction of optimized adaptive immunity. Although individual Ab- or T cell-mediated responses have been shown to prevent or inhibit SIV replication alone under optimal experimental conditions, a dynamic

immune response combining both compartments of the adaptive immune system will likely be necessary to contain clinical HIV infection.

As a preventative intervention remains unavailable, immunotherapeutic strategies to prolong asymptomatic infection and augment antiviral immunity must be explored. Advances in our understanding of the $CD4^+$ T cells dynamics within cellular homeostasis and HIV pathogenesis suggest that methods to therapeutically regenerate $CD4^+$ T helper lymphocyte populations during chronic HIV infection may lead to durable containment of disease. As the gold standard clinical indicator for progression to AIDS, $CD4^+$ T cells have long been acknowledged to play the crucial role in the development of immunodeficiency. Superior ART regimens reduce the rate of disease pathogenesis, but are unable to completely prevent viral replication and subsequent exhaustion and destruction of the $CD4^+$ T cell compartment. Although 32 drugs have been approved to interrupt the HIV life cycle, no pharmacologic agent has been developed which effectively promotes $CD4^+$ T cell regeneration. In support of this idea, findings in the nonhuman primate have demonstrated that adoptive transfer of autologous $CD4^+$ T cells during established SIV infection is sufficient to promote immunocompetence and long-term nonprogressor status in the absence of ART [418]. Furthermore, techniques for the efficient *ex vivo* propagation of autologous $CD4^+$ T cells are currently being explored, as therapeutic $CD4^+$ T cell delivery may provide the support necessary to continually immunologically suppress disease regardless of the development of viral immune and drug evasion mutations [419]. In conjunction, preclinical immunogenicity studies have demonstrated that during experimental $CD4^+$ T cell depletion, immunization utilizing a DNA-prime, MVA-boost fails to promote dynamic, polyfunctional cellular immunity as compared to vaccination in the presence of T lymphocyte assistance, suggesting that any therapeutic vaccination may be insufficient in the absence of appropriate $CD4^+$ T cell-mediated support [420]. Therefore, pharmacologic or immunologic strategies aimed at enhancing thymopoiesis and the peripheral expansion of the $CD4^+$ T cell compartment must be actively pursued, as restoring cellular T cell homeostasis may provide greater clinical benefit then augmenting HIV-specific immunity alone.

7.3 CONCLUDING REMARKS

For over a quarter of a century, dedicated researchers of unmatched creativity have struggled tirelessly to develop a cure for HIV infection. The current lack of a preventative or therapeutic vaccine reflects greatly upon the monumental challenge presented by the biology and pathology of the human immunodeficiency virus itself. Nevertheless, adversity has inspired ingenuity, as the pursuit of a cure has lead to remarkable advancements in the fields of immunology, virology, and vaccine development. In the wake of agonizing vaccine failures and collective disappointments, incremental progress continues to being made in our understanding of the nature of HIV-associated disease and our technical ability to intervene therein. It is upon this foundation of knowledge established by those investigators past and present that the cure will eventually be found.

PUBLICATION LIST

1. Gao W, Tamin A, **Soloff A**, D'Aiuto L, Nwanegbo E, Robbins PD, Bellini WJ, Barratt-Boyes SM, Gambotto A: Effects of a SARS-associated coronavirus vaccine in monkeys. Lancet, 362(9399):1895-1896, 2003.

2. Barratt-Boyes SM, **Soloff AC**, Gao W, Nwanegbo E, Liu X, Rajakumar PA, Brown KN, Robbins PD, Murphey-Corb M, Day RD, and Gambotto A: Broad Cellular Immunity with Robust Memory Responses to Simian Immunodeficiency Virus Following Serial Vaccination with Adenovirus 5- and 35-Based Vectors. Journal of General Virology, 87:139-49, 2006.

3. **Soloff AC**[*]**,** Gao W[*], Lu X[*], Montecalvo A, Nguyen DC, Matsuoka Y, Robbins PD, Swayne DE, Donis RO, Katz JM, Barratt-Boyes SM, and Gambotto A: Protection of Mice and Poultry from Lethal H5N1 Avian Influenza Virus Through Adenoviral-Based Immunization. Journal of Virology, 80(4):1959-1964, 2006. * Authors contributed equally to this work.

4. Schito ML, **Soloff AC**, Slovitz D, Trichel A, Inman JK, Appella E, Turpin JA, and Barratt-Boyes SM: Preclinical Evaluation of a Zinc Finger Inhibitor Targeting Lentivirus Nucleocapsid Protein in SIV-Infected Monkeys. Current HIV Research, Jul; 4(3):379-86, 2006.

5. McBurney SP, Young KR, Nwaigwe CI, **Soloff AC**, Stefano-Cole KA, Ross TM: Lentivirus-Like Particles Without Reverse Transcriptase Elicit Efficient Immune Responses. Current HIV Research, Oct;4(4):475-84, 2006.

6. Barratt-Boyes SM, Brown KN, Melhem NM, **Soloff AC**, and Gleason SM: Understanding and Exploiting Dendritic Cells in Human Immunodeficiency Virus Infection Using the Nonhuman Primate Model. Immunology Research, 36(1-3):265-74, 2006.

7. Qin S, Sui Y, **Soloff AC**, Junecko BA, Kirschner DE, Murphey-Corb MA, Watkins SC, Tarwater PM, Pease JP, Barratt-Boyes SM, and Reinhart TA: Chemokine and Cytokine Mediated Loss of Regulatory T Cells in Lymphoid Tissues During Pathogenic Simian Immunodeficiency Virus Infection. Journal of Immunology, 180: 5530-5536, 2008.

8. **Soloff AC**, Liu X, Gao W, Day RD, Gambotto A, and Barratt-Boyes SM: Adenovirus-Based Immunotherapy Induces Primarily Monofunctional $CD8^+$ T cell Responses in Blood and Mucosa and Fails to Control Viremia in Chronic SIV Infection. *Manuscript submitted for Publication to the European Journal of Immunology*, 2008.

BIBLIOGRAPHY

1 **McCutchan, F. E.,** Understanding the genetic diversity of HIV-1. *AIDS* 2000. **14 Suppl 3**: S31-44.

2 **Gentile, M., Adrian, T., Scheidler, A., Ewald, M., Dianzani, F., Pauli, G. and Gelderblom, H. R.,** Determination of the size of HIV using adenovirus type 2 as an internal length marker. *J Virol Methods* 1994. **48**: 43-52.

3 **Moore, J. P., Kitchen, S. G., Pugach, P. and Zack, J. A.,** The CCR5 and CXCR4 coreceptors--central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. *AIDS Res Hum Retroviruses* 2004. **20**: 111-126.

4 **Schrager, L. K. and D'Souza, M. P.,** Cellular and anatomical reservoirs of HIV-1 in patients receiving potent antiretroviral combination therapy. *JAMA* 1998. **280**: 67-71.

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

6 **Letvin, N. L., Daniel, M. D., Sehgal, P. K., Desrosiers, R. C., Hunt, R. D., Waldron, L. M., MacKey, J. J., Schmidt, D. K., Chalifoux, L. V. and King, N. W.,** Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. *Science* 1985. **230**: 71-73.

7 **Bibollet-Ruche, F., Bailes, E., Gao, F., Pourrut, X., Barlow, K. L., Clewley, J. P., Mwenda, J. M., Langat, D. K., Chege, G. K., McClure, H. M., Mpoudi-Ngole, E., Delaporte, E., Peeters, M., Shaw, G. M., Sharp, P. M. and Hahn, B. H.,** New simian immunodeficiency virus infecting De Brazza's monkeys (Cercopithecus neglectus): evidence for a cercopithecus monkey virus clade. *J Virol* 2004. **78**: 7748-7762.

8 **Hahn, B. H., Shaw, G. M., De Cock, K. M. and Sharp, P. M.,** AIDS as a zoonosis: scientific and public health implications. *Science* 2000. **287**: 607-614.

9 **Gao, F., Bailes, E., Robertson, D. L., Chen, Y., Rodenburg, C. M., Michael, S. F., Cummins, L. B., Arthur, L. O., Peeters, M., Shaw, G. M., Sharp, P. M. and Hahn, B. H.,** Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* 1999. **397**: 436-441.

10 **Corbet, S., Muller-Trutwin, M. C., Versmisse, P., Delarue, S., Ayouba, A., Lewis, J., Brunak, S., Martin, P., Brun-Vezinet, F., Simon, F., Barre-Sinoussi, F. and Mauclere, P.,** env sequences of simian immunodeficiency viruses from chimpanzees in Cameroon are strongly related to those of human immunodeficiency virus group N from the same geographic area. *J Virol* 2000. **74**: 529-534.

11 **Bailes, E., Gao, F., Bibollet-Ruche, F., Courgnaud, V., Peeters, M., Marx, P. A., Hahn, B. H. and Sharp, P. M.,** Hybrid origin of SIV in chimpanzees. *Science* 2003. **300**: 1713.

12 **Wain, L. V., Bailes, E., Bibollet-Ruche, F., Decker, J. M., Keele, B. F., Van Heuverswyn, F., Li, Y., Takehisa, J., Ngole, E. M., Shaw, G. M., Peeters, M., Hahn, B. H. and Sharp, P. M.,** Adaptation of HIV-1 to its human host. *Mol Biol Evol* 2007. **24**: 1853-1860.

13 **Murphey-Corb, M., Martin, L. N., Rangan, S. R., Baskin, G. B., Gormus, B. J., Wolf, R. H., Andes, W. A., West, M. and Montelaro, R. C.,** Isolation of an HTLV-III-related retrovirus from macaques with simian AIDS and its possible origin in asymptomatic mangabeys. *Nature* 1986. **321**: 435-437.

14 **Daniel, M. D., Letvin, N. L., King, N. W., Kannagi, M., Sehgal, P. K., Hunt, R. D., Kanki, P. J., Essex, M. and Desrosiers, R. C.,** Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. *Science* 1985. **228**: 1201-1204.

15 **Vidal, N., Peeters, M., Mulanga-Kabeya, C., Nzilambi, N., Robertson, D., Ilunga, W., Sema, H., Tshimanga, K., Bongo, B. and Delaporte, E.,** Unprecedented degree of human immunodeficiency virus type 1 (HIV-1) group M genetic diversity in the Democratic Republic of Congo suggests that the HIV-1 pandemic originated in Central Africa. *J Virol* 2000. **74**: 10498-10507.

16 **Simon, F., Mauclere, P., Roques, P., Loussert-Ajaka, I., Muller-Trutwin, M. C., Saragosti, S., Georges-Courbot, M. C., Barre-Sinoussi, F. and Brun-Vezinet, F.,** Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nat Med* 1998. **4**: 1032-1037.

17 **Peeters, M., Gueye, A., Mboup, S., Bibollet-Ruche, F., Ekaza, E., Mulanga, C., Ouedrago, R., Gandji, R., Mpele, P., Dibanga, G., Koumare, B., Saidou, M., Esu-Williams, E., Lombart, J. P., Badombena, W., Luo, N., Vanden Haesevelde, M. and Delaporte, E.,** Geographical distribution of HIV-1 group O viruses in Africa. *AIDS* 1997. **11**: 493-498.

18 **Zhu, T., Korber, B. T., Nahmias, A. J., Hooper, E., Sharp, P. M. and Ho, D. D.,** An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. *Nature* 1998. **391**: 594-597.

19 **Korber, B., Muldoon, M., Theiler, J., Gao, F., Gupta, R., Lapedes, A., Hahn, B. H., Wolinsky, S. and Bhattacharya, T.,** Timing the ancestor of the HIV-1 pandemic strains. *Science* 2000. **288**: 1789-1796.

20 **MMWR,** Kaposi's sarcoma and pneumocystis pneumonia among homosexual men - New York City and California. *MMWR Weekly* 1981. **30**: 305-308.

21 **Barre-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W. and Montagnier, L.,** Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 1983. **220**: 868-871.

22 **Gallo, R. C., Salahuddin, S. Z., Popovic, M., Shearer, G. M., Kaplan, M., Haynes, B. F., Palker, T. J., Redfield, R., Oleske, J., Safai, B. and et al.,** Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* 1984. **224**: 500-503.

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

24 **Schupbach, J., Popovic, M., Gilden, R. V., Gonda, M. A., Sarngadharan, M. G. and Gallo, R. C.,** Serological analysis of a subgroup of human T-lymphotropic retroviruses (HTLV-III) associated with AIDS. *Science* 1984. **224**: 503-505.

25 **Levy, J. A., Hoffman, A. D., Kramer, S. M., Landis, J. A., Shimabukuro, J. M. and Oshiro, L. S.,** Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* 1984. **225**: 840-842.

26 **Gottlieb, M. S., Schroff, R., Schanker, H. M., Weisman, J. D., Fan, P. T., Wolf, R. A. and Saxon, A.,** Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N Engl J Med* 1981. **305**: 1425-1431.

27 **UNAIDS,** AIDS epidemic update: December 2007. Joint United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organization (WHO), Geneva 2007.

28 **Galvin, S. R. and Cohen, M. S.,** The role of sexually transmitted diseases in HIV transmission. *Nat Rev Microbiol* 2004. **2**: 33-42.

29 **Al Jabri, A. A.,** HLA and in vitro susceptibility to HIV infection. *Mol Immunol* 2002. **38**: 959-967.

30 **Lehner, T.,** The role of CCR5 chemokine ligands and antibodies to CCR5 coreceptors in preventing HIV infection. *Trends Immunol* 2002. **23**: 347-351.

31 **Cohen, M. S., Hellmann, N., Levy, J. A., DeCock, K. and Lange, J.,** The spread, treatment, and prevention of HIV-1: evolution of a global pandemic. *J Clin Invest* 2008. **118**: 1244-1254.

32 **Vajdy, M., Veazey, R. S., Knight, H. K., Lackner, A. A. and Neutra, M. R.,** Differential effects of simian immunodeficiency virus infection on immune inductive and effector sites in the rectal mucosa of rhesus macaques. *Am J Pathol* 2000. **157**: 485-495.

33 **Veazey, R. S., DeMaria, M., Chalifoux, L. V., Shvetz, D. E., Pauley, D. R., Knight, H. L., Rosenzweig, M., Johnson, R. P., Desrosiers, R. C. and Lackner, A. A.,** Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* 1998. **280**: 427-431.

34 **Zhang, Z., Schuler, T., Zupancic, M., Wietgrefe, S., Staskus, K. A., Reimann, K. A., Reinhart, T. A., Rogan, M., Cavert, W., Miller, C. J., Veazey, R. S., Notermans, D., Little, S., Danner, S. A., Richman, D. D., Havlir, D., Wong, J., Jordan, H. L., Schacker, T. W., Racz, P., Tenner-Racz, K., Letvin, N. L., Wolinsky, S. and Haase, A. T.,** Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. *Science* 1999. **286**: 1353-1357.

35 **Reimann, K. A., Tenner-Racz, K., Racz, P., Montefiori, D. C., Yasutomi, Y., Lin, W., Ransil, B. J. and Letvin, N. L.,** Immunopathogenic events in acute infection of rhesus monkeys with simian immunodeficiency virus of macaques. *J Virol* 1994. **68**: 2362-2370.

36 **Douek, D. C., Picker, L. J. and Koup, R. A.,** T cell dynamics in HIV-1 infection. *Annu Rev Immunol* 2003. **21**: 265-304.

37 **Cheynier, R., Henrichwark, S., Hadida, F., Pelletier, E., Oksenhendler, E., Autran, B. and Wain-Hobson, S.,** HIV and T cell expansion in splenic white pulps is accompanied by infiltration of HIV-specific cytotoxic T lymphocytes. *Cell* 1994. **78**: 373-387.

38 **Grossman, Z., Feinberg, M. B. and Paul, W. E.,** Multiple modes of cellular activation and virus transmission in HIV infection: a role for chronically and latently infected cells in sustaining viral replication. *Proc Natl Acad Sci U S A* 1998. **95**: 6314-6319.

39 **Douek, D. C., Brenchley, J. M., Betts, M. R., Ambrozak, D. R., Hill, B. J., Okamoto, Y., Casazza, J. P., Kuruppu, J., Kunstman, K., Wolinsky, S., Grossman, Z., Dybul, M., Oxenius, A., Price, D. A., Connors, M. and Koup, R. A.,** HIV preferentially infects HIV-specific CD4+ T cells. *Nature* 2002. **417**: 95-98.

40 **Pitcher, C. J., Quittner, C., Peterson, D. M., Connors, M., Koup, R. A., Maino, V. C. and Picker, L. J.,** HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat Med* 1999. **5**: 518-525.

41 **Schnittman, S. M., Lane, H. C., Greenhouse, J., Justement, J. S., Baseler, M. and Fauci, A. S.,** Preferential infection of CD4+ memory T cells by human immunodeficiency virus type 1: evidence for a role in the selective T-cell functional defects observed in infected individuals. *Proc Natl Acad Sci U S A* 1990. **87**: 6058-6062.

42 **Zaunders, J. J., Munier, M. L., Kaufmann, D. E., Ip, S., Grey, P., Smith, D., Ramacciotti, T., Quan, D., Finlayson, R., Kaldor, J., Rosenberg, E. S., Walker, B. D., Cooper, D. A. and Kelleher, A. D.,** Early proliferation of CCR5(+) CD38(+++) antigen-specific CD4(+) Th1 effector cells during primary HIV-1 infection. *Blood* 2005. **106**: 1660-1667.

43 **Kewenig, S., Schneider, T., Hohloch, K., Lampe-Dreyer, K., Ullrich, R., Stolte, N., Stahl-Hennig, C., Kaup, F. J., Stallmach, A. and Zeitz, M.,** Rapid mucosal CD4(+) T-cell depletion and enteropathy in simian immunodeficiency virus-infected rhesus macaques. *Gastroenterology* 1999. **116**: 1115-1123.

44 **Stahl-Hennig, C., Steinman, R. M., Tenner-Racz, K., Pope, M., Stolte, N., Matz-Rensing, K., Grobschupff, G., Raschdorff, B., Hunsmann, G. and Racz, P.,** Rapid infection of oral mucosal-associated lymphoid tissue with simian immunodeficiency virus. *Science* 1999. **285**: 1261-1265.

45 **Li, Q., Duan, L., Estes, J. D., Ma, Z. M., Rourke, T., Wang, Y., Reilly, C., Carlis, J., Miller, C. J. and Haase, A. T.,** Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. *Nature* 2005. **434**: 1148-1152.

46 **Okoye, A., Meier-Schellersheim, M., Brenchley, J. M., Hagen, S. I., Walker, J. M., Rohankhedkar, M., Lum, R., Edgar, J. B., Planer, S. L., Legasse, A., Sylwester, A. W., Piatak, M., Jr., Lifson, J. D., Maino, V. C., Sodora, D. L., Douek, D. C., Axthelm, M. K., Grossman, Z. and Picker, L. J.,** Progressive CD4+ central memory T cell decline results in CD4+ effector memory insufficiency and overt disease in chronic SIV infection. *J Exp Med* 2007. **204**: 2171-2185.

47 **Mowat, A. M. and Viney, J. L.,** The anatomical basis of intestinal immunity. *Immunol Rev* 1997. **156**: 145-166.

48 **Brenchley, J. M., Schacker, T. W., Ruff, L. E., Price, D. A., Taylor, J. H., Beilman, G. J., Nguyen, P. L., Khoruts, A., Larson, M., Haase, A. T. and Douek, D. C.,** CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* 2004. **200**: 749-759.

49 **Mehandru, S., Poles, M. A., Tenner-Racz, K., Horowitz, A., Hurley, A., Hogan, C., Boden, D., Racz, P. and Markowitz, M.,** Primary HIV-1 infection is associated with

preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* 2004. **200**: 761-770.

50 **Mattapallil, J. J., Douek, D. C., Hill, B., Nishimura, Y., Martin, M. and Roederer, M.,** Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* 2005. **434**: 1093-1097.

51 **Smit-McBride, Z., Mattapallil, J. J., McChesney, M., Ferrick, D. and Dandekar, S.,** Gastrointestinal T lymphocytes retain high potential for cytokine responses but have severe CD4(+) T-cell depletion at all stages of simian immunodeficiency virus infection compared to peripheral lymphocytes. *J Virol* 1998. **72**: 6646-6656.

52 **Ling, B., Veazey, R. S., Hart, M., Lackner, A. A., Kuroda, M., Pahar, B. and Marx, P. A.,** Early restoration of mucosal CD4 memory CCR5 T cells in the gut of SIV-infected rhesus predicts long term non-progression. *AIDS* 2007. **21**: 2377-2385.

53 **Verhoeven, D., Sankaran, S., Silvey, M. and Dandekar, S.,** Antiviral therapy during primary simian immunodeficiency virus infection fails to prevent acute loss of CD4+ T cells in gut mucosa but enhances their rapid restoration through central memory T cells. *J Virol* 2008. **82**: 4016-4027.

54 **Chun, T. W., Finzi, D., Margolick, J., Chadwick, K., Schwartz, D. and Siliciano, R. F.,** In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. *Nat Med* 1995. **1**: 1284-1290.

55 **McLean, A. R. and Michie, C. A.,** In vivo estimates of division and death rates of human T lymphocytes. *Proc Natl Acad Sci U S A* 1995. **92**: 3707-3711.

56 **Sonza, S., Mutimer, H. P., Oelrichs, R., Jardine, D., Harvey, K., Dunne, A., Purcell, D. F., Birch, C. and Crowe, S. M.,** Monocytes harbour replication-competent, non-latent HIV-1 in patients on highly active antiretroviral therapy. *AIDS* 2001. **15**: 17-22.

57 **Hlavacek, W. S., Stilianakis, N. I., Notermans, D. W., Danner, S. A. and Perelson, A. S.,** Influence of follicular dendritic cells on decay of HIV during antiretroviral therapy. *Proc Natl Acad Sci U S A* 2000. **97**: 10966-10971.

58 **Wong, J. K., Hezareh, M., Gunthard, H. F., Havlir, D. V., Ignacio, C. C., Spina, C. A. and Richman, D. D.,** Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science* 1997. **278**: 1291-1295.

59 **Chun, T. W., Stuyver, L., Mizell, S. B., Ehler, L. A., Mican, J. A., Baseler, M., Lloyd, A. L., Nowak, M. A. and Fauci, A. S.,** Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc Natl Acad Sci U S A* 1997. **94**: 13193-13197.

60 **Chun, T. W., Carruth, L., Finzi, D., Shen, X., DiGiuseppe, J. A., Taylor, H., Hermankova, M., Chadwick, K., Margolick, J., Quinn, T. C., Kuo, Y. H., Brookmeyer, R., Zeiger, M. A., Barditch-Crovo, P. and Siliciano, R. F.,** Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature* 1997. **387**: 183-188.

61 **Finzi, D., Hermankova, M., Pierson, T., Carruth, L. M., Buck, C., Chaisson, R. E., Quinn, T. C., Chadwick, K., Margolick, J., Brookmeyer, R., Gallant, J., Markowitz, M., Ho, D. D., Richman, D. D. and Siliciano, R. F.,** Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* 1997. **278**: 1295-1300.

62 **Chun, T. W., Engel, D., Berrey, M. M., Shea, T., Corey, L. and Fauci, A. S.,** Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. *Proc Natl Acad Sci U S A* 1998. **95**: 8869-8873.

63 **Finzi, D., Blankson, J., Siliciano, J. D., Margolick, J. B., Chadwick, K., Pierson, T., Smith, K., Lisziewicz, J., Lori, F., Flexner, C., Quinn, T. C., Chaisson, R. E., Rosenberg, E., Walker, B., Gange, S., Gallant, J. and Siliciano, R. F.,** Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat Med* 1999. **5**: 512-517.

64 **Geeraert, L., Kraus, G. and Pomerantz, R. J.,** Hide-and-seek: the challenge of viral persistence in HIV-1 infection. *Annu Rev Med* 2008. **59**: 487-501.

65 **Bartlett, J. A., Miralles, G. D., Sevin, A. D., Silberman, M., Pruitt, S. K., Ottinger, J., Gryszowska, V., Fiscus, S. A. and Bucy, R. P.,** Addition of cyclophosphamide to antiretroviral therapy does not diminish the cellular reservoir in HIV-infected persons. *AIDS Res Hum Retroviruses* 2002. **18**: 535-543.

66 **Lehrman, G., Hogue, I. B., Palmer, S., Jennings, C., Spina, C. A., Wiegand, A., Landay, A. L., Coombs, R. W., Richman, D. D., Mellors, J. W., Coffin, J. M., Bosch, R. J. and Margolis, D. M.,** Depletion of latent HIV-1 infection in vivo: a proof-of-concept study. *Lancet* 2005. **366**: 549-555.

67 **Chun, T. W., Engel, D., Mizell, S. B., Hallahan, C. W., Fischette, M., Park, S., Davey, R. T., Jr., Dybul, M., Kovacs, J. A., Metcalf, J. A., Mican, J. M., Berrey, M. M., Corey, L., Lane, H. C. and Fauci, A. S.,** Effect of interleukin-2 on the pool of latently infected, resting CD4+ T cells in HIV-1-infected patients receiving highly active anti-retroviral therapy. *Nat Med* 1999. **5**: 651-655.

68 **Lafeuillade, A., Poggi, C., Chadapaud, S., Hittinger, G., Chouraqui, M., Pisapia, M. and Delbeke, E.,** Pilot study of a combination of highly active antiretroviral therapy and cytokines to induce HIV-1 remission. *J Acquir Immune Defic Syndr* 2001. **26**: 44-55.

69 **van Praag, R. M., Prins, J. M., Roos, M. T., Schellekens, P. T., Ten Berge, I. J., Yong, S. L., Schuitemaker, H., Eerenberg, A. J., Jurriaans, S., de Wolf, F., Fox, C. H., Goudsmit, J., Miedema, F. and Lange, J. M.,** OKT3 and IL-2 treatment for purging of the latent HIV-1 reservoir in vivo results in selective long-lasting CD4+ T cell depletion. *J Clin Immunol* 2001. **21**: 218-226.

70 **Rey-Cuille, M. A., Berthier, J. L., Bomsel-Demontoy, M. C., Chaduc, Y., Montagnier, L., Hovanessian, A. G. and Chakrabarti, L. A.,** Simian immunodeficiency virus replicates to high levels in sooty mangabeys without inducing disease. *J Virol* 1998. **72**: 3872-3886.

71 **Bostik, P., Noble, E. S., Mayne, A. E., Gargano, L., Villinger, F. and Ansari, A. A.,** Central memory CD4 T cells are the predominant cell subset resistant to anergy in SIV disease resistant sooty mangabeys. *AIDS* 2006. **20**: 181-188.

72 **Barry, A. P., Silvestri, G., Safrit, J. T., Sumpter, B., Kozyr, N., McClure, H. M., Staprans, S. I. and Feinberg, M. B.,** Depletion of CD8+ cells in sooty mangabey monkeys naturally infected with simian immunodeficiency virus reveals limited role for immune control of virus replication in a natural host species. *J Immunol* 2007. **178**: 8002-8012.

73 **Milush, J. M., Reeves, J. D., Gordon, S. N., Zhou, D., Muthukumar, A., Kosub, D. A., Chacko, E., Giavedoni, L. D., Ibegbu, C. C., Cole, K. S., Miamidian, J. L., Paiardini, M., Barry, A. P., Staprans, S. I., Silvestri, G. and Sodora, D. L.,** Virally induced CD4+ T cell depletion is not sufficient to induce AIDS in a natural host. *J Immunol* 2007. **179**: 3047-3056.

74 **Silvestri, G., Fedanov, A., Germon, S., Kozyr, N., Kaiser, W. J., Garber, D. A., McClure, H., Feinberg, M. B. and Staprans, S. I.,** Divergent host responses during primary simian immunodeficiency virus SIVsm infection of natural sooty mangabey and nonnatural rhesus macaque hosts. *J Virol* 2005. **79**: 4043-4054.

75 **Giorgi, J. V., Hultin, L. E., McKeating, J. A., Johnson, T. D., Owens, B., Jacobson, L. P., Shih, R., Lewis, J., Wiley, D. J., Phair, J. P., Wolinsky, S. M. and Detels, R.,** Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *J Infect Dis* 1999. **179**: 859-870.

76 **De Luca, A., Teofili, L., Antinori, A., Iovino, M. S., Mencarini, P., Visconti, E., Tamburrini, E., Leone, G. and Ortona, L.,** Haemopoietic CD34+ progenitor cells are not infected by HIV-1 in vivo but show impaired clonogenesis. *Br J Haematol* 1993. **85**: 20-24.

77 **Haase, A. T.,** Population biology of HIV-1 infection: viral and CD4+ T cell demographics and dynamics in lymphatic tissues. *Annu Rev Immunol* 1999. **17**: 625-656.

78 **Schacker, T. W., Nguyen, P. L., Beilman, G. J., Wolinsky, S., Larson, M., Reilly, C. and Haase, A. T.,** Collagen deposition in HIV-1 infected lymphatic tissues and T cell homeostasis. *J Clin Invest* 2002. **110**: 1133-1139.

79 **Cao, J., Park, I. W., Cooper, A. and Sodroski, J.,** Molecular determinants of acute single-cell lysis by human immunodeficiency virus type 1. *J Virol* 1996. **70**: 1340-1354.

80 **Stewart, S. A., Poon, B., Jowett, J. B. and Chen, I. S.,** Human immunodeficiency virus type 1 Vpr induces apoptosis following cell cycle arrest. *J Virol* 1997. **71**: 5579-5592.

81 **Gandhi, R. T., Chen, B. K., Straus, S. E., Dale, J. K., Lenardo, M. J. and Baltimore, D.,** HIV-1 directly kills CD4+ T cells by a Fas-independent mechanism. *J Exp Med* 1998. **187**: 1113-1122.

82 **Mohri, H., Bonhoeffer, S., Monard, S., Perelson, A. S. and Ho, D. D.,** Rapid turnover of T lymphocytes in SIV-infected rhesus macaques. *Science* 1998. **279**: 1223-1227.

83 **Hakim, F. T., Cepeda, R., Kaimei, S., Mackall, C. L., McAtee, N., Zujewski, J., Cowan, K. and Gress, R. E.,** Constraints on CD4 recovery postchemotherapy in adults: thymic insufficiency and apoptotic decline of expanded peripheral CD4 cells. *Blood* 1997. **90**: 3789-3798.

84 **Yue, F. Y., Kovacs, C. M., Dimayuga, R. C., Gu, X. X., Parks, P., Kaul, R. and Ostrowski, M. A.,** Preferential apoptosis of HIV-1-specific CD4+ T cells. *J Immunol* 2005. **174**: 2196-2204.

85 **Ferreira, C., Barthlott, T., Garcia, S., Zamoyska, R. and Stockinger, B.,** Differential survival of naive CD4 and CD8 T cells. *J Immunol* 2000. **165**: 3689-3694.

86 **Watanabe, N., De Rosa, S. C., Cmelak, A., Hoppe, R., Herzenberg, L. A. and Roederer, M.,** Long-term depletion of naive T cells in patients treated for Hodgkin's disease. *Blood* 1997. **90**: 3662-3672.

87 **Steffens, C. M., Smith, K. Y., Landay, A., Shott, S., Truckenbrod, A., Russert, M. and Al-Harthi, L.,** T cell receptor excision circle (TREC) content following maximum HIV suppression is equivalent in HIV-infected and HIV-uninfected individuals. *AIDS* 2001. **15**: 1757-1764.

88 **Teixeira, L., Valdez, H., McCune, J. M., Koup, R. A., Badley, A. D., Hellerstein, M. K., Napolitano, L. A., Douek, D. C., Mbisa, G., Deeks, S., Harris, J. M., Barbour, J. D., Gross, B. H., Francis, I. R., Halvorsen, R., Asaad, R. and Lederman, M. M.,** Poor

CD4 T cell restoration after suppression of HIV-1 replication may reflect lower thymic function. *AIDS* 2001. **15**: 1749-1756.

89 **Smith, K. Y., Valdez, H., Landay, A., Spritzler, J., Kessler, H. A., Connick, E., Kuritzkes, D., Gross, B., Francis, I., McCune, J. M. and Lederman, M. M.,** Thymic size and lymphocyte restoration in patients with human immunodeficiency virus infection after 48 weeks of zidovudine, lamivudine, and ritonavir therapy. *J Infect Dis* 2000. **181**: 141-147.

90 **Franco, J. M., Rubio, A., Martinez-Moya, M., Leal, M., Merchante, E., Sanchez-Quijano, A. and Lissen, E.,** T-cell repopulation and thymic volume in HIV-1-infected adult patients after highly active antiretroviral therapy. *Blood* 2002. **99**: 3702-3706.

91 **Douek, D. C., McFarland, R. D., Keiser, P. H., Gage, E. A., Massey, J. M., Haynes, B. F., Polis, M. A., Haase, A. T., Feinberg, M. B., Sullivan, J. L., Jamieson, B. D., Zack, J. A., Picker, L. J. and Koup, R. A.,** Changes in thymic function with age and during the treatment of HIV infection. *Nature* 1998. **396**: 690-695.

92 **Douek, D. C., Betts, M. R., Hill, B. J., Little, S. J., Lempicki, R., Metcalf, J. A., Casazza, J., Yoder, C., Adelsberger, J. W., Stevens, R. A., Baseler, M. W., Keiser, P., Richman, D. D., Davey, R. T. and Koup, R. A.,** Evidence for increased T cell turnover and decreased thymic output in HIV infection. *J Immunol* 2001. **167**: 6663-6668.

93 **Homann, D., Teyton, L. and Oldstone, M. B.,** Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nat Med* 2001. **7**: 913-919.

94 **Opferman, J. T., Ober, B. T. and Ashton-Rickardt, P. G.,** Linear differentiation of cytotoxic effectors into memory T lymphocytes. *Science* 1999. **283**: 1745-1748.

95 **Foulds, K. E., Zenewicz, L. A., Shedlock, D. J., Jiang, J., Troy, A. E. and Shen, H.,** Cutting edge: CD4 and CD8 T cells are intrinsically different in their proliferative responses. *J Immunol* 2002. **168**: 1528-1532.

96 **Whitmire, J. K. and Ahmed, R.,** The economy of T-cell memory: CD4+ recession in times of CD8+ stability? *Nat Med* 2001. **7**: 892-893.

97 **Wu, C. Y., Kirman, J. R., Rotte, M. J., Davey, D. F., Perfetto, S. P., Rhee, E. G., Freidag, B. L., Hill, B. J., Douek, D. C. and Seder, R. A.,** Distinct lineages of T(H)1 cells have differential capacities for memory cell generation in vivo. *Nat Immunol* 2002. **3**: 852-858.

98 **Tham, E. L. and Mescher, M. F.,** The poststimulation program of CD4 versus CD8 T cells (death versus activation-induced nonresponsiveness). *J Immunol* 2002. **169**: 1822-1828.

99 **Brenchley, J. M., Price, D. A., Schacker, T. W., Asher, T. E., Silvestri, G., Rao, S., Kazzaz, Z., Bornstein, E., Lambotte, O., Altmann, D., Blazar, B. R., Rodriguez, B., Teixeira-Johnson, L., Landay, A., Martin, J. N., Hecht, F. M., Picker, L. J., Lederman, M. M., Deeks, S. G. and Douek, D. C.,** Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med* 2006. **12**: 1365-1371.

100 **Brenchley, J. M., Price, D. A. and Douek, D. C.,** HIV disease: fallout from a mucosal catastrophe? *Nat Immunol* 2006. **7**: 235-239.

101 **Mackay, C. R., Marston, W. L. and Dudler, L.,** Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J Exp Med* 1990. **171**: 801-817.

102 **Butcher, E. C. and Picker, L. J.,** Lymphocyte homing and homeostasis. *Science* 1996. **272**: 60-66.

103 **Rodrigo Mora, J. and Von Andrian, U. H.,** Specificity and plasticity of memory lymphocyte migration. *Curr Top Microbiol Immunol* 2006. **308**: 83-116.

104 **Phillips, A. N.,** Reduction of HIV concentration during acute infection: independence from a specific immune response. *Science* 1996. **271**: 497-499.

105 **Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., Farthing, C. and Ho, D. D.,** Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 1994. **68**: 4650-4655.

106 **Borrow, P., Lewicki, H., Hahn, B. H., Shaw, G. M. and Oldstone, M. B.,** Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* 1994. **68**: 6103-6110.

107 **Kuroda, M. J., Schmitz, J. E., Charini, W. A., Nickerson, C. E., Lifton, M. A., Lord, C. I., Forman, M. A. and Letvin, N. L.,** Emergence of CTL coincides with clearance of virus during primary simian immunodeficiency virus infection in rhesus monkeys. *J Immunol* 1999. **162**: 5127-5133.

108 **McNeil, A. C., Shupert, W. L., Iyasere, C. A., Hallahan, C. W., Mican, J. A., Davey, R. T., Jr. and Connors, M.,** High-level HIV-1 viremia suppresses viral antigen-specific CD4(+) T cell proliferation. *Proc Natl Acad Sci U S A* 2001. **98**: 13878-13883.

109 **Brenchley, J. M., Karandikar, N. J., Betts, M. R., Ambrozak, D. R., Hill, B. J., Crotty, L. E., Casazza, J. P., Kuruppu, J., Migueles, S. A., Connors, M., Roederer, M., Douek, D. C. and Koup, R. A.,** Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. *Blood* 2003. **101**: 2711-2720.

110 **Gamberg, J. C., Bowmer, M. I., Trahey, J. C., Campbell, C. M., Pardoe, I. and Grant, M. D.,** Functional and genetic integrity of the CD8 T-cell repertoire in advanced HIV infection. *AIDS* 1999. **13**: 2043-2053.

111 **Trimble, L. A., Shankar, P., Patterson, M., Daily, J. P. and Lieberman, J.,** Human immunodeficiency virus-specific circulating CD8 T lymphocytes have down-modulated CD3zeta and CD28, key signaling molecules for T-cell activation. *J Virol* 2000. **74**: 7320-7330.

112 **Gorochov, G., Neumann, A. U., Kereveur, A., Parizot, C., Li, T., Katlama, C., Karmochkine, M., Raguin, G., Autran, B. and Debre, P.,** Perturbation of CD4+ and CD8+ T-cell repertoires during progression to AIDS and regulation of the CD4+ repertoire during antiviral therapy. *Nat Med* 1998. **4**: 215-221.

113 **Connors, M., Kovacs, J. A., Krevat, S., Gea-Banacloche, J. C., Sneller, M. C., Flanigan, M., Metcalf, J. A., Walker, R. E., Falloon, J., Baseler, M., Feuerstein, I., Masur, H. and Lane, H. C.,** HIV infection induces changes in CD4+ T-cell phenotype and depletions within the CD4+ T-cell repertoire that are not immediately restored by antiviral or immune-based therapies. *Nat Med* 1997. **3**: 533-540.

114 **Pantaleo, G., Soudeyns, H., Demarest, J. F., Vaccarezza, M., Graziosi, C., Paolucci, S., Daucher, M., Cohen, O. J., Denis, F., Biddison, W. E., Sekaly, R. P. and Fauci, A. S.,** Evidence for rapid disappearance of initially expanded HIV-specific CD8+ T cell clones during primary HIV infection. *Proc Natl Acad Sci U S A* 1997. **94**: 9848-9853.

115 **Appay, V., Nixon, D. F., Donahoe, S. M., Gillespie, G. M., Dong, T., King, A., Ogg, G. S., 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., HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. *J Exp Med* 2000. **192**: 63-75.

116 **Migueles, S. A., Laborico, A. C., Shupert, W. L., Sabbaghian, M. S., Rabin, R., Hallahan, C. W., Van Baarle, D., Kostense, S., Miedema, F., McLaughlin, M., Ehler, L., Metcalf, J., Liu, S. and Connors, M.,** HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol* 2002. **3**: 1061-1068.

117 **McKay, P. F., Barouch, D. H., Schmitz, J. E., Veazey, R. S., Gorgone, D. A., Lifton, M. A., Williams, K. C. and Letvin, N. L.,** Global dysfunction of CD4 T-lymphocyte cytokine expression in simian-human immunodeficiency virus/SIV-infected monkeys is prevented by vaccination. *J Virol* 2003. **77**: 4695-4702.

118 **Fuller, M. J., Hildeman, D. A., Sabbaj, S., Gaddis, D. E., Tebo, A. E., Shang, L., Goepfert, P. A. and Zajac, A. J.,** Cutting edge: emergence of CD127high functionally competent memory T cells is compromised by high viral loads and inadequate T cell help. *J Immunol* 2005. **174**: 5926-5930.

119 **Champagne, P., Ogg, G. S., King, A. S., Knabenhans, C., Ellefsen, K., Nobile, M., Appay, V., Rizzardi, G. P., Fleury, S., Lipp, M., Forster, R., Rowland-Jones, S., Sekaly, R. P., McMichael, A. J. and Pantaleo, G.,** Skewed maturation of memory HIV-specific CD8 T lymphocytes. *Nature* 2001. **410**: 106-111.

120 **Chen, G., Shankar, P., Lange, C., Valdez, H., Skolnik, P. R., Wu, L., Manjunath, N. and Lieberman, J.,** CD8 T cells specific for human immunodeficiency virus, Epstein-Barr virus, and cytomegalovirus lack molecules for homing to lymphoid sites of infection. *Blood* 2001. **98**: 156-164.

121 **Day, C. L., Kaufmann, D. E., Kiepiela, P., Brown, J. A., Moodley, E. S., Reddy, S., Mackey, E. W., Miller, J. D., Leslie, A. J., DePierres, C., Mncube, Z., Duraiswamy, J., Zhu, B., Eichbaum, Q., Altfeld, M., Wherry, E. J., Coovadia, H. M., Goulder, P. J., Klenerman, P., Ahmed, R., Freeman, G. J. and Walker, B. D.,** PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006. **443**: 350-354.

122 **Petrovas, C., Casazza, J. P., Brenchley, J. M., Price, D. A., Gostick, E., Adams, W. C., Precopio, M. L., Schacker, T., Roederer, M., Douek, D. C. and Koup, R. A.,** PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J Exp Med* 2006. **203**: 2281-2292.

123 **Trautmann, L., Janbazian, L., Chomont, N., Said, E. A., Gimmig, S., Bessette, B., Boulassel, M. R., Delwart, E., Sepulveda, H., Balderas, R. S., Routy, J. P., Haddad, E. K. and Sekaly, R. P.,** Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med* 2006. **12**: 1198-1202.

124 **Pomerantz, R. J. and Horn, D. L.,** Twenty years of therapy for HIV-1 infection. *Nat Med* 2003. **9**: 867-873.

125 **Li, T. S., Tubiana, R., Katlama, C., Calvez, V., Ait Mohand, H. and Autran, B.,** Long-lasting recovery in CD4 T-cell function and viral-load reduction after highly active antiretroviral therapy in advanced HIV-1 disease. *Lancet* 1998. **351**: 1682-1686.

126 **Autran, B., Carcelain, G., Li, T. S., Blanc, C., Mathez, D., Tubiana, R., Katlama, C., Debre, P. and Leibowitch, J.,** Positive effects of combined antiretroviral therapy on

CD4+ T cell homeostasis and function in advanced HIV disease. *Science* 1997. **277**: 112-116.

127 **Dornadula, G., Zhang, H., VanUitert, B., Stern, J., Livornese, L., Jr., Ingerman, M. J., Witek, J., Kedanis, R. J., Natkin, J., DeSimone, J. and Pomerantz, R. J.,** Residual HIV-1 RNA in blood plasma of patients taking suppressive highly active antiretroviral therapy. *JAMA* 1999. **282**: 1627-1632.

128 **Lifson, J. D., Piatak, M., Jr., Cline, A. N., Rossio, J. L., Purcell, J., Pandrea, I., Bischofberger, N., Blanchard, J. and Veazey, R. S.,** Transient early post-inoculation anti-retroviral treatment facilitates controlled infection with sparing of CD4+ T cells in gut-associated lymphoid tissues in SIVmac239-infected rhesus macaques, but not resistance to rechallenge. *J Med Primatol* 2003. **32**: 201-210.

129 **Flexner, C.,** HIV drug development: the next 25 years. *Nat Rev Drug Discov* 2007. **6**: 959-966.

130 **De Clercq, E.,** The design of drugs for HIV and HCV. *Nat Rev Drug Discov* 2007. **6**: 1001-1018.

131 **Lori, F., Lewis, M. G., Xu, J., Varga, G., Zinn, D. E., Jr., Crabbs, C., Wagner, W., Greenhouse, J., Silvera, P., Yalley-Ogunro, J., Tinelli, C. and Lisziewicz, J.,** Control of SIV rebound through structured treatment interruptions during early infection. *Science* 2000. **290**: 1591-1593.

132 **Lafeuillade, A., Poggi, C., Hittinger, G., Counillon, E. and Emilie, D.,** Predictors of plasma human immunodeficiency virus type 1 RNA control after discontinuation of highly active antiretroviral therapy initiated at acute infection combined with structured treatment interruptions and immune-based therapies. *J Infect Dis* 2003. **188**: 1426-1432.

133 **Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Boswell, S. L., Sax, P. E., Kalams, S. A. and Walker, B. D.,** Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* 1997. **278**: 1447-1450.

134 **Younes, S. A., Yassine-Diab, B., Dumont, A. R., Boulassel, M. R., Grossman, Z., Routy, J. P. and Sekaly, R. P.,** HIV-1 viremia prevents the establishment of interleukin 2-producing HIV-specific memory CD4+ T cells endowed with proliferative capacity. *J Exp Med* 2003. **198**: 1909-1922.

135 **Rinaldo, C. R., Jr., Huang, X. L., Fan, Z., Margolick, J. B., Borowski, L., Hoji, A., Kalinyak, C., McMahon, D. K., Riddler, S. A., Hildebrand, W. H., Day, R. B. and Mellors, J. W.,** Anti-human immunodeficiency virus type 1 (HIV-1) CD8(+) T-lymphocyte reactivity during combination antiretroviral therapy in HIV-1-infected patients with advanced immunodeficiency. *J Virol* 2000. **74**: 4127-4138.

136 **Waldmann, T. A. and Tagaya, Y.,** The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Annu Rev Immunol* 1999. **17**: 19-49.

137 **Tagaya, Y., Bamford, R. N., DeFilippis, A. P. and Waldmann, T. A.,** IL-15: a pleiotropic cytokine with diverse receptor/signaling pathways whose expression is controlled at multiple levels. *Immunity* 1996. **4**: 329-336.

138 **Meazza, R., Verdiani, S., Biassoni, R., Coppolecchia, M., Gaggero, A., Orengo, A. M., Colombo, M. P., Azzarone, B. and Ferrini, S.,** Identification of a novel interleukin-15 (IL-15) transcript isoform generated by alternative splicing in human small cell lung cancer cell lines. *Oncogene* 1996. **12**: 2187-2192.

139 **Onu, A., Pohl, T., Krause, H. and Bulfone-Paus, S.,** Regulation of IL-15 secretion via the leader peptide of two IL-15 isoforms. *J Immunol* 1997. **158**: 255-262.

140 **Bamford, R. N., DeFilippis, A. P., Azimi, N., Kurys, G. and Waldmann, T. A.,** The 5' untranslated region, signal peptide, and the coding sequence of the carboxyl terminus of IL-15 participate in its multifaceted translational control. *J Immunol* 1998. **160**: 4418-4426.

141 **Dubois, S., Mariner, J., Waldmann, T. A. and Tagaya, Y.,** IL-15Ralpha recycles and presents IL-15 In trans to neighboring cells. *Immunity* 2002. **17**: 537-547.

142 **Neely, G. G., Epelman, S., Ma, L. L., Colarusso, P., Howlett, C. J., Amankwah, E. K., McIntyre, A. C., Robbins, S. M. and Mody, C. H.,** Monocyte surface-bound IL-15 can function as an activating receptor and participate in reverse signaling. *J Immunol* 2004. **172**: 4225-4234.

143 **Fehniger, T. A. and Caligiuri, M. A.,** Interleukin 15: biology and relevance to human disease. *Blood* 2001. **97**: 14-32.

144 **Judge, A. D., Zhang, X., Fujii, H., Surh, C. D. and Sprent, J.,** Interleukin 15 controls both proliferation and survival of a subset of memory-phenotype CD8(+) T cells. *J Exp Med* 2002. **196**: 935-946.

145 **Zhang, X., Sun, S., Hwang, I., Tough, D. F. and Sprent, J.,** Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. *Immunity* 1998. **8**: 591-599.

146 **Ku, C. C., Murakami, M., Sakamoto, A., Kappler, J. and Marrack, P.,** Control of homeostasis of CD8+ memory T cells by opposing cytokines. *Science* 2000. **288**: 675-678.

147 **Marks-Konczalik, J., Dubois, S., Losi, J. M., Sabzevari, H., Yamada, N., Feigenbaum, L., Waldmann, T. A. and Tagaya, Y.,** IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. *Proc Natl Acad Sci U S A* 2000. **97**: 11445-11450.

148 **Schluns, K. S., Klonowski, K. D. and Lefrancois, L.,** Transregulation of memory CD8 T-cell proliferation by IL-15Ralpha+ bone marrow-derived cells. *Blood* 2004. **103**: 988-994.

149 **Becker, T. C., Wherry, E. J., Boone, D., Murali-Krishna, K., Antia, R., Ma, A. and Ahmed, R.,** Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J Exp Med* 2002. **195**: 1541-1548.

150 **Dubois, S., Shou, W., Haneline, L. S., Fleischer, S., Waldmann, T. A. and Muller, J. R.,** Distinct pathways involving the FK506-binding proteins 12 and 12.6 underlie IL-2- versus IL-15-mediated proliferation of T cells. *Proc Natl Acad Sci U S A* 2003. **100**: 14169-14174.

151 **Schluns, K. S., Williams, K., Ma, A., Zheng, X. X. and Lefrancois, L.,** Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. *J Immunol* 2002. **168**: 4827-4831.

152 **Alves, N. L., Hooibrink, B., Arosa, F. A. and van Lier, R. A.,** IL-15 induces antigen-independent expansion and differentiation of human naive CD8+ T cells in vitro. *Blood* 2003. **102**: 2541-2546.

153 **Kanegane, H. and Tosato, G.,** Activation of naive and memory T cells by interleukin-15. *Blood* 1996. **88**: 230-235.

154 **Quinn, L. S., Anderson, B. G., Drivdahl, R. H., Alvarez, B. and Argiles, J. M.,** Overexpression of interleukin-15 induces skeletal muscle hypertrophy in vitro: implications for treatment of muscle wasting disorders. *Exp Cell Res* 2002. **280**: 55-63.

155 **Bulfone-Paus, S., Ungureanu, D., Pohl, T., Lindner, G., Paus, R., Ruckert, R., Krause, H. and Kunzendorf, U.,** Interleukin-15 protects from lethal apoptosis in vivo. *Nat Med* 1997. **3**: 1124-1128.

156 **Naora, H. and Gougeon, M. L.,** Interleukin-15 is a potent survival factor in the prevention of spontaneous but not CD95-induced apoptosis in CD4 and CD8 T lymphocytes of HIV-infected individuals. Correlation with its ability to increase BCL-2 expression. *Cell Death Differ* 1999. **6**: 1002-1011.

157 **Berard, M., Brandt, K., Bulfone-Paus, S. and Tough, D. F.,** IL-15 promotes the survival of naive and memory phenotype CD8+ T cells. *J Immunol* 2003. **170**: 5018-5026.

158 **Bulfone-Pau, S. S., Bulanova, E., Pohl, T., Budagian, V., Durkop, H., Ruckert, R., Kunzendorf, U., Paus, R. and Krause, H.,** Death deflected: IL-15 inhibits TNF-alpha-mediated apoptosis in fibroblasts by TRAF2 recruitment to the IL-15Ralpha chain. *FASEB J* 1999. **13**: 1575-1585.

159 **Rappl, G., Abken, H., Hasselmann, D. O., Tilgen, W., Ugurel, S. and Reinhold, U.,** The CD7(-) subset of CD4(+) memory T cells is prone to accelerated apoptosis that is prevented by interleukin-15 (IL-15). *Cell Death Differ* 2001. **8**: 395-402.

160 **Qin, J. Z., Zhang, C. L., Kamarashev, J., Dummer, R., Burg, G. and Dobbeling, U.,** Interleukin-7 and interleukin-15 regulate the expression of the bcl-2 and c-myb genes in cutaneous T-cell lymphoma cells. *Blood* 2001. **98**: 2778-2783.

161 **Wilkinson, P. C. and Liew, F. Y.,** Chemoattraction of human blood T lymphocytes by interleukin-15. *J Exp Med* 1995. **181**: 1255-1259.

162 **McInnes, I. B., al-Mughales, J., Field, M., Leung, B. P., Huang, F. P., Dixon, R., Sturrock, R. D., Wilkinson, P. C. and Liew, F. Y.,** The role of interleukin-15 in T-cell migration and activation in rheumatoid arthritis. *Nat Med* 1996. **2**: 175-182.

163 **McInnes, I. B. and Liew, F. Y.,** Interleukin 15: a proinflammatory role in rheumatoid arthritis synovitis. *Immunol Today* 1998. **19**: 75-79.

164 **Agostini, C., Trentin, L., Sancetta, R., Facco, M., Tassinari, C., Cerutti, A., Bortolin, M., Milani, A., Siviero, M., Zambello, R. and Semenzato, G.,** Interleukin-15 triggers activation and growth of the CD8 T-cell pool in extravascular tissues of patients with acquired immunodeficiency syndrome. *Blood* 1997. **90**: 1115-1123.

165 **Ahmad, A., Ahmad, R., Iannello, A., Toma, E., Morisset, R. and Sindhu, S. T.,** IL-15 and HIV infection: lessons for immunotherapy and vaccination. *Curr HIV Res* 2005. **3**: 261-270.

166 **d'Ettorre, G., Forcina, G., Lichtner, M., Mengoni, F., D'Agostino, C., Massetti, A. P., Mastroianni, C. M. and Vullo, V.,** Interleukin-15 in HIV infection: immunological and virological interactions in antiretroviral-naive and -treated patients. *AIDS* 2002. **16**: 181-188.

167 **Amicosante, M., Poccia, F., Gioia, C., Montesano, C., Topino, S., Martini, F., Narciso, P., Pucillo, L. P. and D'Offizi, G.,** Levels of interleukin-15 in plasma may predict a favorable outcome of structured treatment interruption in patients with chronic human immunodeficiency virus infection. *J Infect Dis* 2003. **188**: 661-665.

168 **Chehimi, J., Marshall, J. D., Salvucci, O., Frank, I., Chehimi, S., Kawecki, S., Bacheller, D., Rifat, S. and Chouaib, S.,** IL-15 enhances immune functions during HIV infection. *J Immunol* 1997. **158**: 5978-5987.

169 **d'Ettorre, G., Forcina, G., Andreotti, M., Sarmati, L., Palmisano, L., Andreoni, M., Vella, S., Mastroianni, C. M. and Vullo, V.,** Interleukin-15 production by monocyte-derived dendritic cells and T cell proliferation in HIV-infected patients with discordant response to highly active antiretroviral therapy. *Clin Exp Immunol* 2004. **135**: 280-285.

170 **Patki, A. H., Quinones-Mateu, M. E., Dorazio, D., Yen-Lieberman, B., Boom, W. H., Thomas, E. K. and Lederman, M. M.,** Activation of antigen-induced lymphocyte proliferation by interleukin-15 without the mitogenic effect of interleukin-2 that may induce human immunodeficiency virus-1 expression. *J Clin Invest* 1996. **98**: 616-621.

171 **Lucey, D. R., Pinto, L. A., Bethke, F. R., Rusnak, J., Melcher, G. P., Hashemi, F. N., Landay, A. L., Kessler, H. A., Paxton, R. J., Grabstein, K. and Shearer, G. M.,** In vitro immunologic and virologic effects of interleukin 15 on peripheral blood mononuclear cells from normal donors and human immunodeficiency virus type 1-infected patients. *Clin Diagn Lab Immunol* 1997. **4**: 43-48.

172 **Kutzler, M. A., Robinson, T. M., Chattergoon, M. A., Choo, D. K., Choo, A. Y., Choe, P. Y., Ramanathan, M. P., Parkinson, R., Kudchodkar, S., Tamura, Y., Sidhu, M., Roopchand, V., Kim, J. J., Pavlakis, G. N., Felber, B. K., Waldmann, T. A., Boyer, J. D. and Weiner, D. B.,** Coimmunization with an optimized IL-15 plasmid results in enhanced function and longevity of CD8 T cells that are partially independent of CD4 T cell help. *J Immunol* 2005. **175**: 112-123.

173 **Pulle, G., Vidric, M. and Watts, T. H.,** IL-15-dependent induction of 4-1BB promotes antigen-independent CD8 memory T cell survival. *J Immunol* 2006. **176**: 2739-2748.

174 **Sato, N., Patel, H. J., Waldmann, T. A. and Tagaya, Y.,** The IL-15/IL-15Ralpha on cell surfaces enables sustained IL-15 activity and contributes to the long survival of CD8 memory T cells. *Proc Natl Acad Sci U S A* 2007. **104**: 588-593.

175 **Mueller, Y. M., Bojczuk, P. M., Halstead, E. S., Kim, A. H., Witek, J., Altman, J. D. and Katsikis, P. D.,** IL-15 enhances survival and function of HIV-specific CD8+ T cells. *Blood* 2003. **101**: 1024-1029.

176 **Forcina, G., D'Ettorre, G., Mastroianni, C. M., Carnevalini, M., Scorzolini, L., Ceccarelli, G., D'Agostino, C., Lichtner, M., Massetti, A. P. and Vullo, V.,** Interleukin-15 modulates interferon-gamma and beta-chemokine production in patients with HIV infection: implications for immune-based therapy. *Cytokine* 2004. **25**: 283-290.

177 **Picker, L. J., Reed-Inderbitzin, E. F., Hagen, S. I., Edgar, J. B., Hansen, S. G., Legasse, A., Planer, S., Piatak, M., Jr., Lifson, J. D., Maino, V. C., Axthelm, M. K. and Villinger, F.,** IL-15 induces CD4 effector memory T cell production and tissue emigration in nonhuman primates. *J Clin Invest* 2006. **116**: 1514-1524.

178 **Mueller, Y. M., Petrovas, C., Bojczuk, P. M., Dimitriou, I. D., Beer, B., Silvera, P., Villinger, F., Cairns, J. S., Gracely, E. J., Lewis, M. G. and Katsikis, P. D.,** Interleukin-15 increases effector memory CD8+ t cells and NK Cells in simian immunodeficiency virus-infected macaques. *J Virol* 2005. **79**: 4877-4885.

179 **Villinger, F., Miller, R., Mori, K., Mayne, A. E., Bostik, P., Sundstrom, J. B., Sugimoto, C. and Ansari, A. A.,** IL-15 is superior to IL-2 in the generation of long-lived antigen specific memory CD4 and CD8 T cells in rhesus macaques. *Vaccine* 2004. **22**: 3510-3521.

180 **Oh, S., Berzofsky, J. A., Burke, D. S., Waldmann, T. A. and Perera, L. P.,** Coadministration of HIV vaccine vectors with vaccinia viruses expressing IL-15 but not IL-2 induces long-lasting cellular immunity. *Proc Natl Acad Sci U S A* 2003. **100**: 3392-3397.

181 **Boyer, J. D., Robinson, T. M., Kutzler, M. A., Vansant, G., Hokey, D. A., Kumar, S., Parkinson, R., Wu, L., Sidhu, M. K., Pavlakis, G. N., Felber, B. K., Brown, C., Silvera, P., Lewis, M. G., Monforte, J., Waldmann, T. A., Eldridge, J. and Weiner, D. B.,** Protection against simian/human immunodeficiency virus (SHIV) 89.6P in macaques after coimmunization with SHIV antigen and IL-15 plasmid. *Proc Natl Acad Sci U S A* 2007. **104**: 18648-18653.

182 **Chong, S. Y., Egan, M. A., Kutzler, M. A., Megati, S., Masood, A., Roopchard, V., Garcia-Hand, D., Montefiori, D. C., Quiroz, J., Rosati, M., Schadeck, E. B., Boyer, J. D., Pavlakis, G. N., Weiner, D. B., Sidhu, M., Eldridge, J. H. and Israel, Z. R.,** Comparative ability of plasmid IL-12 and IL-15 to enhance cellular and humoral immune responses elicited by a SIVgag plasmid DNA vaccine and alter disease progression following SHIV(89.6P) challenge in rhesus macaques. *Vaccine* 2007. **25**: 4967-4982.

183 **Halwani, R., Boyer, J. D., Yassine-Diab, B., Haddad, E. K., Robinson, T. M., Kumar, S., Parkinson, R., Wu, L., Sidhu, M. K., Phillipson-Weiner, R., Pavlakis, G. N., Felber, B. K., Lewis, M. G., Shen, A., Siliciano, R. F., Weiner, D. B. and Sekaly, R. P.,** Therapeutic Vaccination with Simian Immunodeficiency Virus (SIV)-DNA+IL-12 or IL-15 Induces Distinct CD8 Memory Subsets in SIV-Infected Macaques. *J Immunol* 2008. **180**: 7969-7979.

184 **Richman, D. D., Wrin, T., Little, S. J. and Petropoulos, C. J.,** Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proc Natl Acad Sci U S A* 2003. **100**: 4144-4149.

185 **Frost, S. D., Wrin, T., Smith, D. M., Kosakovsky Pond, S. L., Liu, Y., Paxinos, E., Chappey, C., Galovich, J., Beauchaine, J., Petropoulos, C. J., Little, S. J. and Richman, D. D.,** Neutralizing antibody responses drive the evolution of human immunodeficiency virus type 1 envelope during recent HIV infection. *Proc Natl Acad Sci U S A* 2005. **102**: 18514-18519.

186 **Wei, X., Decker, J. M., Wang, S., Hui, H., Kappes, J. C., Wu, X., Salazar-Gonzalez, J. F., Salazar, M. G., Kilby, J. M., Saag, M. S., Komarova, N. L., Nowak, M. A., Hahn, B. H., Kwong, P. D. and Shaw, G. M.,** Antibody neutralization and escape by HIV-1. *Nature* 2003. **422**: 307-312.

187 **Klasse, P. J. and Sattentau, Q. J.,** Occupancy and mechanism in antibody-mediated neutralization of animal viruses. *J Gen Virol* 2002. **83**: 2091-2108.

188 **Pantophlet, R. and Burton, D. R.,** GP120: target for neutralizing HIV-1 antibodies. *Annu Rev Immunol* 2006. **24**: 739-769.

189 **Huber, M. and Trkola, A.,** Humoral immunity to HIV-1: neutralization and beyond. *J Intern Med* 2007. **262**: 5-25.

190 **Johnson, W. E. and Desrosiers, R. C.,** Viral persistance: HIV's strategies of immune system evasion. *Annu Rev Med* 2002. **53**: 499-518.

191 **Parren, P. W., Marx, P. A., Hessell, A. J., Luckay, A., Harouse, J., Cheng-Mayer, C., Moore, J. P. and Burton, D. R.,** Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. *J Virol* 2001. **75**: 8340-8347.

192 **Mascola, J. R., Stiegler, G., VanCott, T. C., Katinger, H., Carpenter, C. B., Hanson, C. E., Beary, H., Hayes, D., Frankel, S. S., Birx, D. L. and Lewis, M. G.,** Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med* 2000. **6**: 207-210.

193 **Veazey, R. S., Shattock, R. J., Pope, M., Kirijan, J. C., Jones, J., Hu, Q., Ketas, T., Marx, P. A., Klasse, P. J., Burton, D. R. and Moore, J. P.,** Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. *Nat Med* 2003. **9**: 343-346.

194 **Gauduin, M. C., Parren, P. W., Weir, R., Barbas, C. F., Burton, D. R. and Koup, R. A.,** Passive immunization with a human monoclonal antibody protects hu-PBL-SCID mice against challenge by primary isolates of HIV-1. *Nat Med* 1997. **3**: 1389-1393.

195 **Trkola, A., Kuster, H., Rusert, P., Joos, B., Fischer, M., Leemann, C., Manrique, A., Huber, M., Rehr, M., Oxenius, A., Weber, R., Stiegler, G., Vcelar, B., Katinger, H., Aceto, L. and Gunthard, H. F.,** Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. *Nat Med* 2005. **11**: 615-622.

196 **Baba, T. W., Liska, V., Hofmann-Lehmann, R., Vlasak, J., Xu, W., Ayehunie, S., Cavacini, L. A., Posner, M. R., Katinger, H., Stiegler, G., Bernacky, B. J., Rizvi, T. A., Schmidt, R., Hill, L. R., Keeling, M. E., Lu, Y., Wright, J. E., Chou, T. C. and Ruprecht, R. M.,** Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nat Med* 2000. **6**: 200-206.

197 **Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B. J., Ghrayeb, J., Forman, M. A., Montefiori, D. C., Rieber, E. P., Letvin, N. L. and Reimann, K. A.,** Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 1999. **283**: 857-860.

198 **Matano, T., Shibata, R., Siemon, C., Connors, M., Lane, H. C. and Martin, M. A.,** Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. *J Virol* 1998. **72**: 164-169.

199 **Jin, X., Bauer, D. E., Tuttleton, S. E., Lewin, S., Gettie, A., Blanchard, J., Irwin, C. E., Safrit, J. T., Mittler, J., Weinberger, L., Kostrikis, L. G., Zhang, L., Perelson, A. S. and Ho, D. D.,** Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* 1999. **189**: 991-998.

200 **Hel, Z., Venzon, D., Poudyal, M., Tsai, W. P., Giuliani, L., Woodward, R., Chougnet, C., Shearer, G., Altman, J. D., Watkins, D., Bischofberger, N., Abimiku, A., Markham, P., Tartaglia, J. and Franchini, G.,** Viremia control following antiretroviral treatment and therapeutic immunization during primary SIV251 infection of macaques. *Nat Med* 2000. **6**: 1140-1146.

201 **Tryniszewska, E., Nacsa, J., Lewis, M. G., Silvera, P., Montefiori, D., Venzon, D., Hel, Z., Parks, R. W., Moniuszko, M., Tartaglia, J., Smith, K. A. and Franchini, G.,** Vaccination of macaques with long-standing SIVmac251 infection lowers the viral set point after cessation of antiretroviral therapy. *J Immunol* 2002. **169**: 5347-5357.

202 **Carrington, M., Nelson, G. W., Martin, M. P., Kissner, T., Vlahov, D., Goedert, J. J., Kaslow, R., Buchbinder, S., Hoots, K. and O'Brien, S. J.,** HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. *Science* 1999. **283**: 1748-1752.

203 **Kaslow, R. A., Carrington, M., Apple, R., Park, L., Munoz, A., Saah, A. J., Goedert, J. J., Winkler, C., O'Brien, S. J., Rinaldo, C., Detels, R., Blattner, W., Phair, J., Erlich, H. and Mann, D. L.,** Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat Med* 1996. **2**: 405-411.

204 **Goulder, P. J., Bunce, M., Krausa, P., McIntyre, K., Crowley, S., Morgan, B., Edwards, A., Giangrande, P., Phillips, R. E. and McMichael, A. J.,** Novel, cross-restricted, conserved, and immunodominant cytotoxic T lymphocyte epitopes in slow progressors in HIV type 1 infection. *AIDS Res Hum Retroviruses* 1996. **12**: 1691-1698.

205 **Carrington, M. and O'Brien, S. J.,** The influence of HLA genotype on AIDS. *Annu Rev Med* 2003. **54**: 535-551.

206 **Russell, J. H. and Ley, T. J.,** Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol* 2002. **20**: 323-370.

207 **Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C. and Lusso, P.,** Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* 1995. **270**: 1811-1815.

208 **Guidotti, L. G. and Chisari, F. V.,** Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu Rev Immunol* 2001. **19**: 65-91.

209 **Wang, R., Rogers, A. M., Ratliff, T. L. and Russell, J. H.,** CD95-dependent bystander lysis caused by CD4+ T helper 1 effectors. *J Immunol* 1996. **157**: 2961-2968.

210 **Barouch, D. H., Santra, S., Schmitz, J. E., Kuroda, M. J., Fu, T. M., Wagner, W., Bilska, M., Craiu, A., Zheng, X. X., Krivulka, G. R., Beaudry, K., Lifton, M. A., Nickerson, C. E., Trigona, W. L., Punt, K., Freed, D. C., Guan, L., Dubey, S., Casimiro, D., Simon, A., Davies, M. E., Chastain, M., Strom, T. B., Gelman, R. S., Montefiori, D. C., Lewis, M. G., Emini, E. A., Shiver, J. W. and Letvin, N. L.,** Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* 2000. **290**: 486-492.

211 **Shiver, J. W., Fu, T. M., Chen, L., Casimiro, D. R., Davies, M. E., Evans, R. K., Zhang, Z. Q., Simon, A. J., Trigona, W. L., Dubey, S. A., Huang, L., Harris, V. A., Long, R. S., Liang, X., Handt, L., Schleif, W. A., Zhu, L., Freed, D. C., Persaud, N. V., Guan, L., Punt, K. S., Tang, A., Chen, M., Wilson, K. A., Collins, K. B., Heidecker, G. J., Fernandez, V. R., Perry, H. C., Joyce, J. G., Grimm, K. M., Cook, J. C., Keller, P. M., Kresock, D. S., Mach, H., Troutman, R. D., Isopi, L. A., Williams, D. M., Xu, Z., Bohannon, K. E., Volkin, D. B., Montefiori, D. C., Miura, A., Krivulka, G. R., Lifton, M. A., Kuroda, M. J., Schmitz, J. E., Letvin, N. L., Caulfield, M. J., Bett, A. J., Youil, R., Kaslow, D. C. and Emini, E. A.,** Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* 2002. **415**: 331-335.

212 **Hel, Z., Nacsa, J., Tryniszewska, E., Tsai, W. P., Parks, R. W., Montefiori, D. C., Felber, B. K., Tartaglia, J., Pavlakis, G. N. and Franchini, G.,** Containment of simian immunodeficiency virus infection in vaccinated macaques: correlation with the magnitude of virus-specific pre- and postchallenge CD4+ and CD8+ T cell responses. *J Immunol* 2002. **169**: 4778-4787.

213 **Barouch, D. H., Craiu, A., Santra, S., Egan, M. A., Schmitz, J. E., Kuroda, M. J., Fu, T. M., Nam, J. H., Wyatt, L. S., Lifton, M. A., Krivulka, G. R., Nickerson, C. E., Lord, C. I., Moss, B., Lewis, M. G., Hirsch, V. M., Shiver, J. W. and Letvin, N. L.,** Elicitation of high-frequency cytotoxic T-lymphocyte responses against both dominant and subdominant simian-human immunodeficiency virus epitopes by DNA vaccination of rhesus monkeys. *J Virol* 2001. **75**: 2462-2467.

214 **Letvin, N. L., Mascola, J. R., Sun, Y., Gorgone, D. A., Buzby, A. P., Xu, L., Yang, Z. Y., Chakrabarti, B., Rao, S. S., Schmitz, J. E., Montefiori, D. C., Barker, B. R., Bookstein, F. L. and Nabel, G. J.,** Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys. *Science* 2006. **312**: 1530-1533.

215 **Picker, L. J., Hagen, S. I., Lum, R., Reed-Inderbitzin, E. F., Daly, L. M., Sylwester, A. W., Walker, J. M., Siess, D. C., Piatak, M., Jr., Wang, C., Allison, D. B., Maino, V. C., Lifson, J. D., Kodama, T. and Axthelm, M. K.,** Insufficient production and tissue delivery of CD4+ memory T cells in rapidly progressive simian immunodeficiency virus infection. *J Exp Med* 2004. **200**: 1299-1314.

216 **Lichterfeld, M., Kaufmann, D. E., Yu, X. G., Mui, S. K., Addo, M. M., Johnston, M. N., Cohen, D., Robbins, G. K., Pae, E., Alter, G., Wurcel, A., Stone, D., Rosenberg, E. S., Walker, B. D. and Altfeld, M.,** Loss of HIV-1-specific CD8+ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4+ T cells. *J Exp Med* 2004. **200**: 701-712.

217 **Rolland, M., Heckerman, D., Deng, W., Rousseau, C. M., Coovadia, H., Bishop, K., Goulder, P. J., Walker, B. D., Brander, C. and Mullins, J. I.,** Broad and Gag-biased HIV-1 epitope repertoires are associated with lower viral loads. *PLoS ONE* 2008. **3**: e1424.

218 **Frahm, N., Kiepiela, P., Adams, S., Linde, C. H., Hewitt, H. S., Sango, K., Feeney, M. E., Addo, M. M., Lichterfeld, M., Lahaie, M. P., Pae, E., Wurcel, A. G., Roach, T., St John, M. A., Altfeld, M., Marincola, F. M., Moore, C., Mallal, S., Carrington, M., Heckerman, D., Allen, T. M., Mullins, J. I., Korber, B. T., Goulder, P. J., Walker, B. D. and Brander, C.,** Control of human immunodeficiency virus replication by cytotoxic T lymphocytes targeting subdominant epitopes. *Nat Immunol* 2006. **7**: 173-178.

219 **Betts, M. R., Nason, M. C., West, S. M., De Rosa, S. C., Migueles, S. A., Abraham, J., Lederman, M. M., Benito, J. M., Goepfert, P. A., Connors, M., Roederer, M. and Koup, R. A.,** HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 2006. **107**: 4781-4789.

220 **Lichterfeld, M., Yu, X. G., Waring, M. T., Mui, S. K., Johnston, M. N., Cohen, D., Addo, M. M., Zaunders, J., Alter, G., Pae, E., Strick, D., Allen, T. M., Rosenberg, E. S., Walker, B. D. and Altfeld, M.,** HIV-1-specific cytotoxicity is preferentially mediated by a subset of CD8(+) T cells producing both interferon-gamma and tumor necrosis factor-alpha. *Blood* 2004. **104**: 487-494.

221 **Almeida, J. R., Price, D. A., Papagno, L., Arkoub, Z. A., Sauce, D., Bornstein, E., Asher, T. E., Samri, A., Schnuriger, A., Theodorou, I., Costagliola, D., Rouzioux, C., Agut, H., Marcelin, A. G., Douek, D., Autran, B. and Appay, V.,** Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J Exp Med* 2007. **204**: 2473-2485.

222 **Kannanganat, S., Kapogiannis, B. G., Ibegbu, C., Chennareddi, L., Goepfert, P., Robinson, H. L., Lennox, J. and Amara, R. R.,** Human immunodeficiency virus type 1 controllers but not noncontrollers maintain CD4 T cells coexpressing three cytokines. *J Virol* 2007. **81**: 12071-12076.

223 **Coffin, J. M.,** HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. *Science* 1995. **267**: 483-489.

224 **Goulder, P. J. and Watkins, D. I.,** HIV and SIV CTL escape: implications for vaccine design. *Nat Rev Immunol* 2004. **4**: 630-640.

225 **Price, D. A., Goulder, P. J., Klenerman, P., Sewell, A. K., Easterbrook, P. J., Troop, M., Bangham, C. R. and Phillips, R. E.,** Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. *Proc Natl Acad Sci U S A* 1997. **94**: 1890-1895.

226 **Evans, D. T., O'Connor, D. H., Jing, P., Dzuris, J. L., Sidney, J., da Silva, J., Allen, T. M., Horton, H., Venham, J. E., Rudersdorf, R. A., Vogel, T., Pauza, C. D., Bontrop, R. E., DeMars, R., Sette, A., Hughes, A. L. and Watkins, D. I.,** Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef. *Nat Med* 1999. **5**: 1270-1276.

227 **Allen, T. M., O'Connor, D. H., Jing, P., Dzuris, J. L., Mothe, B. R., Vogel, T. U., Dunphy, E., Liebl, M. E., Emerson, C., Wilson, N., Kunstman, K. J., Wang, X., Allison, D. B., Hughes, A. L., Desrosiers, R. C., Altman, J. D., Wolinsky, S. M., Sette, A. and Watkins, D. I.,** Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. *Nature* 2000. **407**: 386-390.

228 **O'Connor, D. H., Allen, T. M., Vogel, T. U., Jing, P., DeSouza, I. P., Dodds, E., Dunphy, E. J., Melsaether, C., Mothe, B., Yamamoto, H., Horton, H., Wilson, N., Hughes, A. L. and Watkins, D. I.,** Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. *Nat Med* 2002. **8**: 493-499.

229 **Koenig, S., Conley, A. J., Brewah, Y. A., Jones, G. M., Leath, S., Boots, L. J., Davey, V., Pantaleo, G., Demarest, J. F., Carter, C. and et al.,** Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. *Nat Med* 1995. **1**: 330-336.

230 **Yoshiyama, H., Mo, H., Moore, J. P. and Ho, D. D.,** Characterization of mutants of human immunodeficiency virus type 1 that have escaped neutralization by a monoclonal antibody to the gp120 V2 loop. *J Virol* 1994. **68**: 974-978.

231 **Reitz, M. S., Jr., Wilson, C., Naugle, C., Gallo, R. C. and Robert-Guroff, M.,** Generation of a neutralization-resistant variant of HIV-1 is due to selection for a point mutation in the envelope gene. *Cell* 1988. **54**: 57-63.

232 **Watkins, B. A., Buge, S., Aldrich, K., Davis, A. E., Robinson, J., Reitz, M. S., Jr. and Robert-Guroff, M.,** Resistance of human immunodeficiency virus type 1 to neutralization by natural antisera occurs through single amino acid substitutions that cause changes in antibody binding at multiple sites. *J Virol* 1996. **70**: 8431-8437.

233 **Park, E. J., Vujcic, L. K., Anand, R., Theodore, T. S. and Quinnan, G. V., Jr.,** Mutations in both gp120 and gp41 are responsible for the broad neutralization resistance of variant human immunodeficiency virus type 1 MN to antibodies directed at V3 and non-V3 epitopes. *J Virol* 1998. **72**: 7099-7107.

234 **Burns, D. P. and Desrosiers, R. C.,** Selection of genetic variants of simian immunodeficiency virus in persistently infected rhesus monkeys. *J Virol* 1991. **65**: 1843-1854.

235 **Bradney, A. P., Scheer, S., Crawford, J. M., Buchbinder, S. P. and Montefiori, D. C.,** Neutralization escape in human immunodeficiency virus type 1-infected long-term nonprogressors. *J Infect Dis* 1999. **179**: 1264-1267.

236 **Zhang, P. F., Chen, X., Fu, D. W., Margolick, J. B. and Quinnan, G. V., Jr.,** Primary virus envelope cross-reactivity of the broadening neutralizing antibody response during early chronic human immunodeficiency virus type 1 infection. *J Virol* 1999. **73**: 5225-5230.

237 **Derdeyn, C. A., Decker, J. M., Bibollet-Ruche, F., Mokili, J. L., Muldoon, M., Denham, S. A., Heil, M. L., Kasolo, F., Musonda, R., Hahn, B. H., Shaw, G. M., Korber, B. T., Allen, S. and Hunter, E.,** Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission. *Science* 2004. **303**: 2019-2022.

238 **Goulder, P. J., Brander, C., Tang, Y., Tremblay, C., Colbert, R. A., Addo, M. M., Rosenberg, E. S., Nguyen, T., Allen, R., Trocha, A., Altfeld, M., He, S., Bunce, M., Funkhouser, R., Pelton, S. I., Burchett, S. K., McIntosh, K., Korber, B. T. and Walker, B. D.,** Evolution and transmission of stable CTL escape mutations in HIV infection. *Nature* 2001. **412**: 334-338.

239 **Peyerl, F. W., Bazick, H. S., Newberg, M. H., Barouch, D. H., Sodroski, J. and Letvin, N. L.,** Fitness costs limit viral escape from cytotoxic T lymphocytes at a structurally constrained epitope. *J Virol* 2004. **78**: 13901-13910.

240 **Friedrich, T. C., Dodds, E. J., Yant, L. J., Vojnov, L., Rudersdorf, R., Cullen, C., Evans, D. T., Desrosiers, R. C., Mothe, B. R., Sidney, J., Sette, A., Kunstman, K., Wolinsky, S., Piatak, M., Lifson, J., Hughes, A. L., Wilson, N., O'Connor, D. H. and Watkins, D. I.,** Reversion of CTL escape-variant immunodeficiency viruses in vivo. *Nat Med* 2004. **10**: 275-281.

241 **Daniel, M. D., Kirchhoff, F., Czajak, S. C., Sehgal, P. K. and Desrosiers, R. C.,** Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* 1992. **258**: 1938-1941.

242 **Baba, T. W., Jeong, Y. S., Pennick, D., Bronson, R., Greene, M. F. and Ruprecht, R. M.,** Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. *Science* 1995. **267**: 1820-1825.

243 **Baba, T. W., Liska, V., Khimani, A. H., Ray, N. B., Dailey, P. J., Penninck, D., Bronson, R., Greene, M. F., McClure, H. M., Martin, L. N. and Ruprecht, R. M.,** Live attenuated, multiply deleted simian immunodeficiency virus causes AIDS in infant and adult macaques. *Nat Med* 1999. **5**: 194-203.

244 **Whitney, J. B. and Ruprecht, R. M.,** Live attenuated HIV vaccines: pitfalls and prospects. *Curr Opin Infect Dis* 2004. **17**: 17-26.

245 **Gorry, P. R., McPhee, D. A., Verity, E., Dyer, W. B., Wesselingh, S. L., Learmont, J., Sullivan, J. S., Roche, M., Zaunders, J. J., Gabuzda, D., Crowe, S. M., Mills, J., Lewin, S. R., Brew, B. J., Cunningham, A. L. and Churchill, M. J.,** Pathogenicity and immunogenicity of attenuated, nef-deleted HIV-1 strains in vivo. *Retrovirology* 2007. **4**: 66.

246 **Learmont, J. C., Geczy, A. F., Mills, J., Ashton, L. J., Raynes-Greenow, C. H., Garsia, R. J., Dyer, W. B., McIntyre, L., Oelrichs, R. B., Rhodes, D. I., Deacon, N. J.**

and Sullivan, J. S., Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1. A report from the Sydney Blood Bank Cohort. *N Engl J Med* 1999. **340**: 1715-1722.

247 **Marthas, M. L., Sutjipto, S., Higgins, J., Lohman, B., Torten, J., Luciw, P. A., Marx, P. A. and Pedersen, N. C.,** Immunization with a live, attenuated simian immunodeficiency virus (SIV) prevents early disease but not infection in rhesus macaques challenged with pathogenic SIV. *J Virol* 1990. **64**: 3694-3700.

248 **Murphey-Corb, M., Martin, L. N., Davison-Fairburn, B., Montelaro, R. C., Miller, M., West, M., Ohkawa, S., Baskin, G. B., Zhang, J. Y., Putney, S. D. and et al.,** A formalin-inactivated whole SIV vaccine confers protection in macaques. *Science* 1989. **246**: 1293-1297.

249 **Billich, A.,** AIDSVAX VaxGen. *Curr Opin Investig Drugs* 2004. **5**: 214-221.

250 **Berman, P. W., Gregory, T. J., Riddle, L., Nakamura, G. R., Champe, M. A., Porter, J. P., Wurm, F. M., Hershberg, R. D., Cobb, E. K. and Eichberg, J. W.,** Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature* 1990. **345**: 622-625.

251 **VaxGen,** VaxGen announces initial results of its Phase III AIDS vaccine trial. 2003.

252 **Cohen, J.,** Public health. AIDS vaccine trial produces disappointment and confusion. *Science* 2003. **299**: 1290-1291.

253 **Gurunathan, S., Klinman, D. M. and Seder, R. A.,** DNA vaccines: immunology, application, and optimization*. *Annu Rev Immunol* 2000. **18**: 927-974.

254 **Kwissa, M., Amara, R. R., Robinson, H. L., Moss, B., Alkan, S., Jabbar, A., Villinger, F. and Pulendran, B.,** Adjuvanting a DNA vaccine with a TLR9 ligand plus Flt3 ligand results in enhanced cellular immunity against the simian immunodeficiency virus. *J Exp Med* 2007. **204**: 2733-2746.

255 **Egan, M. A., Charini, W. A., Kuroda, M. J., Schmitz, J. E., Racz, P., Tenner-Racz, K., Manson, K., Wyand, M., Lifton, M. A., Nickerson, C. E., Fu, T., Shiver, J. W. and Letvin, N. L.,** Simian immunodeficiency virus (SIV) gag DNA-vaccinated rhesus monkeys develop secondary cytotoxic T-lymphocyte responses and control viral replication after pathogenic SIV infection. *J Virol* 2000. **74**: 7485-7495.

256 **Muthumani, K., Bagarazzi, M., Conway, D., Hwang, D. S., Manson, K., Ciccarelli, R., Israel, Z., Montefiori, D. C., Ugen, K., Miller, N., Kim, J., Boyer, J. and Weiner, D. B.,** A Gag-Pol/Env-Rev SIV239 DNA vaccine improves CD4 counts, and reduce viral loads after pathogenic intrarectal SIV(mac)251 challenge in rhesus Macaques. *Vaccine* 2003. **21**: 629-637.

257 **Eller, M. A., Eller, L. A., Opollo, M. S., Ouma, B. J., Oballah, P. O., Galley, L., Karnasuta, C., Kim, S. R., Robb, M. L., Michael, N. L., Kibuuka, H., Wabwire-Mangen, F., Graham, B. S., Birx, D. L., de Souza, M. S. and Cox, J. H.,** Induction of HIV-specific functional immune responses by a multiclade HIV-1 DNA vaccine candidate in healthy Ugandans. *Vaccine* 2007. **25**: 7737-7742.

258 **Seth, A., Ourmanov, I., Kuroda, M. J., Schmitz, J. E., Carroll, M. W., Wyatt, L. S., Moss, B., Forman, M. A., Hirsch, V. M. and Letvin, N. L.,** Recombinant modified vaccinia virus Ankara-simian immunodeficiency virus gag pol elicits cytotoxic T lymphocytes in rhesus monkeys detected by a major histocompatibility complex class I/peptide tetramer. *Proc Natl Acad Sci U S A* 1998. **95**: 10112-10116.

259 **Hu, S. L., Fultz, P. N., McClure, H. M., Eichberg, J. W., Thomas, E. K., Zarling, J., Singhal, M. C., Kosowski, S. G., Swenson, R. B., Anderson, D. C. and et al.,** Effect of immunization with a vaccinia-HIV env recombinant on HIV infection of chimpanzees. *Nature* 1987. **328**: 721-723.

260 **Hu, S. L., Zarling, J. M., Chinn, J., Travis, B. M., Moran, P. A., Sias, J., Kuller, L., Morton, W. R., Heidecker, G. and Benveniste, R. E.,** Protection of macaques against simian AIDS by immunization with a recombinant vaccinia virus expressing the envelope glycoproteins of simian type D retrovirus. *Proc Natl Acad Sci U S A* 1989. **86**: 7213-7217.

261 **Amara, R. R., Villinger, F., Altman, J. D., Lydy, S. L., O'Neil, S. P., Staprans, S. I., Montefiori, D. C., Xu, Y., Herndon, J. G., Wyatt, L. S., Candido, M. A., Kozyr, N. L., Earl, P. L., Smith, J. M., Ma, H. L., Grimm, B. D., Hulsey, M. L., Miller, J., McClure, H. M., McNicholl, J. M., Moss, B. and Robinson, H. L.,** Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* 2001. **292**: 69-74.

262 **Seth, A., Ourmanov, I., Schmitz, J. E., Kuroda, M. J., Lifton, M. A., Nickerson, C. E., Wyatt, L., Carroll, M., Moss, B., Venzon, D., Letvin, N. L. and Hirsch, V. M.,** Immunization with a modified vaccinia virus expressing simian immunodeficiency virus (SIV) Gag-Pol primes for an anamnestic Gag-specific cytotoxic T-lymphocyte response and is associated with reduction of viremia after SIV challenge. *J Virol* 2000. **74**: 2502-2509.

263 **Ourmanov, I., Brown, C. R., Moss, B., Carroll, M., Wyatt, L., Pletneva, L., Goldstein, S., Venzon, D. and Hirsch, V. M.,** Comparative efficacy of recombinant modified vaccinia virus Ankara expressing simian immunodeficiency virus (SIV) Gag-Pol and/or Env in macaques challenged with pathogenic SIV. *J Virol* 2000. **74**: 2740-2751.

264 **Hirsch, V. M., Fuerst, T. R., Sutter, G., Carroll, M. W., Yang, L. C., Goldstein, S., Piatak, M., Jr., Elkins, W. R., Alvord, W. G., Montefiori, D. C., Moss, B. and Lifson, J. D.,** Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. *J Virol* 1996. **70**: 3741-3752.

265 **Precopio, M. L., Betts, M. R., Parrino, J., Price, D. A., Gostick, E., Ambrozak, D. R., Asher, T. E., Douek, D. C., Harari, A., Pantaleo, G., Bailer, R., Graham, B. S., Roederer, M. and Koup, R. A.,** Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J Exp Med* 2007. **204**: 1405-1416.

266 **Gupta, K., Hudgens, M., Corey, L., McElrath, M. J., Weinhold, K., Montefiori, D. C., Gorse, G. J., Frey, S. E., Keefer, M. C., Evans, T. G., Dolin, R., Schwartz, D. H., Harro, C., Graham, B., Spearman, P. W., Mulligan, M. and Goepfert, P.,** Safety and immunogenicity of a high-titered canarypox vaccine in combination with rgp120 in a diverse population of HIV-1-uninfected adults: AIDS Vaccine Evaluation Group Protocol 022A. *J Acquir Immune Defic Syndr* 2002. **29**: 254-261.

267 **Evans, T. G., Keefer, M. C., Weinhold, K. J., Wolff, M., Montefiori, D., Gorse, G. J., Graham, B. S., McElrath, M. J., Clements-Mann, M. L., Mulligan, M. J., Fast, P., Walker, M. C., Excler, J. L., Duliege, A. M. and Tartaglia, J.,** A canarypox vaccine expressing multiple human immunodeficiency virus type 1 genes given alone or with

rgp120 elicits broad and durable CD8+ cytotoxic T lymphocyte responses in seronegative volunteers. *J Infect Dis* 1999. **180**: 290-298.

268 **Doan, L. X., Li, M., Chen, C. and Yao, Q.,** Virus-like particles as HIV-1 vaccines. *Rev Med Virol* 2005. **15**: 75-88.

269 **Yamshchikov, G. V., Ritter, G. D., Vey, M. and Compans, R. W.,** Assembly of SIV virus-like particles containing envelope proteins using a baculovirus expression system. *Virology* 1995. **214**: 50-58.

270 **Haffar, O., Garrigues, J., Travis, B., Moran, P., Zarling, J. and Hu, S. L.,** Human immunodeficiency virus-like, nonreplicating, gag-env particles assemble in a recombinant vaccinia virus expression system. *J Virol* 1990. **64**: 2653-2659.

271 **Yao, Q., Compans, R. W. and Chen, C.,** HIV envelope proteins differentially utilize CXCR4 and CCR5 coreceptors for induction of apoptosis. *Virology* 2001. **285**: 128-137.

272 **Yao, Q., Bu, Z., Vzorov, A., Yang, C. and Compans, R. W.,** Virus-like particle and DNA-based candidate AIDS vaccines. *Vaccine* 2003. **21**: 638-643.

273 **Quan, F. S., Sailaja, G., Skountzou, I., Huang, C., Vzorov, A., Compans, R. W. and Kang, S. M.,** Immunogenicity of virus-like particles containing modified human immunodeficiency virus envelope proteins. *Vaccine* 2007. **25**: 3841-3850.

274 **Mossman, S. P., Pierce, C. C., Watson, A. J., Robertson, M. N., Montefiori, D. C., Kuller, L., Richardson, B. A., Bradshaw, J. D., Munn, R. J., Hu, S. L., Greenberg, P. D., Benveniste, R. E. and Haigwood, N. L.,** Protective immunity to SIV challenge elicited by vaccination of macaques with multigenic DNA vaccines producing virus-like particles. *AIDS Res Hum Retroviruses* 2004. **20**: 425-434.

275 **Young, K. R., McBurney, S. P., Karkhanis, L. U. and Ross, T. M.,** Virus-like particles: designing an effective AIDS vaccine. *Methods* 2006. **40**: 98-117.

276 **Casimiro, D. R., Wang, F., Schleif, W. A., Liang, X., Zhang, Z. Q., Tobery, T. W., Davies, M. E., McDermott, A. B., O'Connor, D. H., Fridman, A., Bagchi, A., Tussey, L. G., Bett, A. J., Finnefrock, A. C., Fu, T. M., Tang, A., Wilson, K. A., Chen, M., Perry, H. C., Heidecker, G. J., Freed, D. C., Carella, A., Punt, K. S., Sykes, K. J., Huang, L., Ausensi, V. I., Bachinsky, M., Sadasivan-Nair, U., Watkins, D. I., Emini, E. A. and Shiver, J. W.,** Attenuation of simian immunodeficiency virus SIVmac239 infection by prophylactic immunization with dna and recombinant adenoviral vaccine vectors expressing Gag. *J Virol* 2005. **79**: 15547-15555.

277 **Wilson, N. A., Reed, J., Napoe, G. S., Piaskowski, S., Szymanski, A., Furlott, J., Gonzalez, E. J., Yant, L. J., Maness, N. J., May, G. E., Soma, T., Reynolds, M. R., Rakasz, E., Rudersdorf, R., McDermott, A. B., O'Connor, D. H., Friedrich, T. C., Allison, D. B., Patki, A., Picker, L. J., Burton, D. R., Lin, J., Huang, L., Patel, D., Heindecker, G., Fan, J., Citron, M., Horton, M., Wang, F., Liang, X., Shiver, J. W., Casimiro, D. R. and Watkins, D. I.,** Vaccine-induced cellular immune responses reduce plasma viral concentrations after repeated low-dose challenge with pathogenic simian immunodeficiency virus SIVmac239. *J Virol* 2006. **80**: 5875-5885.

278 **Mattapallil, J. J., Douek, D. C., Buckler-White, A., Montefiori, D., Letvin, N. L., Nabel, G. J. and Roederer, M.,** Vaccination preserves CD4 memory T cells during acute simian immunodeficiency virus challenge. *J Exp Med* 2006. **203**: 1533-1541.

279 **Santra, S., Barouch, D. H., Korioth-Schmitz, B., Lord, C. I., Krivulka, G. R., Yu, F., Beddall, M. H., Gorgone, D. A., Lifton, M. A., Miura, A., Philippon, V., Manson, K., Markham, P. D., Parrish, J., Kuroda, M. J., Schmitz, J. E., Gelman, R. S., Shiver, J.**

W., Montefiori, D. C., Panicali, D. and Letvin, N. L., Recombinant poxvirus boosting of DNA-primed rhesus monkeys augments peak but not memory T lymphocyte responses. *Proc Natl Acad Sci U S A* 2004. **101**: 11088-11093.

280 **Lu, W., Wu, X., Lu, Y., Guo, W. and Andrieu, J. M.,** Therapeutic dendritic-cell vaccine for simian AIDS. *Nat Med* 2003. **9**: 27-32.

281 **Lu, W., Arraes, L. C., Ferreira, W. T. and Andrieu, J. M.,** Therapeutic dendritic-cell vaccine for chronic HIV-1 infection. *Nat Med* 2004. **10**: 1359-1365.

282 **Hel, Z., Nacsa, J., Tsai, W. P., Thornton, A., Giuliani, L., Tartaglia, J. and Franchini, G.,** Equivalent immunogenicity of the highly attenuated poxvirus-based ALVAC-SIV and NYVAC-SIV vaccine candidates in SIVmac251-infected macaques. *Virology* 2002. **304**: 125-134.

283 **Nacsa, J., Radaelli, A., Edghill-Smith, Y., Venzon, D., Tsai, W. P., Morghen Cde, G., Panicali, D., Tartaglia, J. and Franchini, G.,** Avipox-based simian immunodeficiency virus (SIV) vaccines elicit a high frequency of SIV-specific CD4+ and CD8+ T-cell responses in vaccinia-experienced SIVmac251-infected macaques. *Vaccine* 2004. **22**: 597-606.

284 **Lore, K., Adams, W. C., Havenga, M. J., Precopio, M. L., Holterman, L., Goudsmit, J. and Koup, R. A.,** Myeloid and plasmacytoid dendritic cells are susceptible to recombinant adenovirus vectors and stimulate polyfunctional memory T cell responses. *J Immunol* 2007. **179**: 1721-1729.

285 **Boshart, M., Weber, F., Jahn, G., Dorsch-Hasler, K., Fleckenstein, B. and Schaffner, W.,** A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* 1985. **41**: 521-530.

286 **Gao, W., Rzewski, A., Sun, H., Robbins, P. D. and Gambotto, A.,** UpGene: Application of a web-based DNA codon optimization algorithm. *Biotechnol Prog* 2004. **20**: 443-448.

287 **Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y. and Phipps, M. L.,** Construction of adenovirus vectors through Cre-lox recombination. *J Virol* 1997. **71**: 1842-1849.

288 **Casimiro, D. R., Chen, L., Fu, T. M., Evans, R. K., Caulfield, M. J., Davies, M. E., Tang, A., Chen, M., Huang, L., Harris, V., Freed, D. C., Wilson, K. A., Dubey, S., Zhu, D. M., Nawrocki, D., Mach, H., Troutman, R., Isopi, L., Williams, D., Hurni, W., Xu, Z., Smith, J. G., Wang, S., Liu, X., Guan, L., Long, R., Trigona, W., Heidecker, G. J., Perry, H. C., Persaud, N., Toner, T. J., Su, Q., Liang, X., Youil, R., Chastain, M., Bett, A. J., Volkin, D. B., Emini, E. A. and Shiver, J. W.,** Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene. *J Virol* 2003. **77**: 6305-6313.

289 **Barratt-Boyes, S. M., Soloff, A. C., Gao, W., Nwanegbo, E., Liu, X., Rajakumar, P. A., Brown, K. N., Robbins, P. D., Murphey-Corb, M., Day, R. D. and Gambotto, A.,** Broad cellular immunity with robust memory responses to simian immunodeficiency virus following serial vaccination with adenovirus 5- and 35-based vectors. *J Gen Virol* 2006. **87**: 139-149.

290 **Wille-Reece, U., Flynn, B. J., Lore, K., Koup, R. A., Miles, A. P., Saul, A., Kedl, R. M., Mattapallil, J. J., Weiss, W. R., Roederer, M. and Seder, R. A.,** Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates. *J Exp Med* 2006. **203**: 1249-1258.

291 **Darrah, P. A., Patel, D. T., De Luca, P. M., Lindsay, R. W., Davey, D. F., Flynn, B. J., Hoff, S. T., Andersen, P., Reed, S. G., Morris, S. L., Roederer, M. and Seder, R. A.,** Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat Med* 2007. **13**: 843-850.

292 **Seder, R. A., Darrah, P. A. and Roederer, M.,** T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* 2008. **8**: 247-258.

293 **Yang, T. C., Dayball, K., Wan, Y. H. and Bramson, J.,** Detailed analysis of the CD8+ T-cell response following adenovirus vaccination. *J Virol* 2003. **77**: 13407-13411.

294 **Liu, J., Ewald, B. A., Lynch, D. M., Denholtz, M., Abbink, P., Lemckert, A. A., Carville, A., Mansfield, K. G., Havenga, M. J., Goudsmit, J. and Barouch, D. H.,** Magnitude and Phenotype of Cellular Immune Responses Elicited by Recombinant Adenovirus Vectors and Heterologous Prime-Boost Regimens in Rhesus Monkeys. *J Virol* 2008.

295 **Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L. and Finberg, R. W.,** Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 1997. **275**: 1320-1323.

296 **Gaggar, A., Shayakhmetov, D. M. and Lieber, A.,** CD46 is a cellular receptor for group B adenoviruses. *Nat Med* 2003. **9**: 1408-1412.

297 **Vogels, R., Zuijdgeest, D., van Rijnsoever, R., Hartkoorn, E., Damen, I., de Bethune, M. P., Kostense, S., Penders, G., Helmus, N., Koudstaal, W., Cecchini, M., Wetterwald, A., Sprangers, M., Lemckert, A., Ophorst, O., Koel, B., van Meerendonk, M., Quax, P., Panitti, L., Grimbergen, J., Bout, A., Goudsmit, J. and Havenga, M.,** Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity. *J Virol* 2003. **77**: 8263-8271.

298 **Sumida, S. M., Truitt, D. M., Lemckert, A. A., Vogels, R., Custers, J. H., Addo, M. M., Lockman, S., Peter, T., Peyerl, F. W., Kishko, M. G., Jackson, S. S., Gorgone, D. A., Lifton, M. A., Essex, M., Walker, B. D., Goudsmit, J., Havenga, M. J. and Barouch, D. H.,** Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. *J Immunol* 2005. **174**: 7179-7185.

299 **Sumida, S. M., Truitt, D. M., Kishko, M. G., Arthur, J. C., Jackson, S. S., Gorgone, D. A., Lifton, M. A., Koudstaal, W., Pau, M. G., Kostense, S., Havenga, M. J., Goudsmit, J., Letvin, N. L. and Barouch, D. H.,** Neutralizing antibodies and CD8+ T lymphocytes both contribute to immunity to adenovirus serotype 5 vaccine vectors. *J Virol* 2004. **78**: 2666-2673.

300 **Molinier-Frenkel, V., Gahery-Segard, H., Mehtali, M., Le Boulaire, C., Ribault, S., Boulanger, P., Tursz, T., Guillet, J. G. and Farace, F.,** Immune response to recombinant adenovirus in humans: capsid components from viral input are targets for vector-specific cytotoxic T lymphocytes. *J Virol* 2000. **74**: 7678-7682.

301 **Gahery-Segard, H., Farace, F., Godfrin, D., Gaston, J., Lengagne, R., Tursz, T., Boulanger, P. and Guillet, J. G.,** Immune response to recombinant capsid proteins of adenovirus in humans: antifiber and anti-penton base antibodies have a synergistic effect on neutralizing activity. *J Virol* 1998. **72**: 2388-2397.

302 **Heemskerk, B., Veltrop-Duits, L. A., van Vreeswijk, T., ten Dam, M. M., Heidt, S., Toes, R. E., van Tol, M. J. and Schilham, M. W.,** Extensive cross-reactivity of CD4+ adenovirus-specific T cells: implications for immunotherapy and gene therapy. *J Virol* 2003. **77**: 6562-6566.

303 **Varnavski, A. N., Zhang, Y., Schnell, M., Tazelaar, J., Louboutin, J. P., Yu, Q. C., Bagg, A., Gao, G. P. and Wilson, J. M.,** Preexisting immunity to adenovirus in rhesus monkeys fails to prevent vector-induced toxicity. *J Virol* 2002. **76**: 5711-5719.

304 **Shiver, J. W. and Emini, E. A.,** Recent advances in the development of HIV-1 vaccines using replication-incompetent adenovirus vectors. *Annu Rev Med* 2004. **55**: 355-372.

305 **Nwanegbo, E., Vardas, E., Gao, W., Whittle, H., Sun, H., Rowe, D., Robbins, P. D. and Gambotto, A.,** Prevalence of neutralizing antibodies to adenoviral serotypes 5 and 35 in the adult populations of The Gambia, South Africa, and the United States. *Clin Diagn Lab Immunol* 2004. **11**: 351-357.

306 **Kostense, S., Koudstaal, W., Sprangers, M., Weverling, G. J., Penders, G., Helmus, N., Vogels, R., Bakker, M., Berkhout, B., Havenga, M. and Goudsmit, J.,** Adenovirus types 5 and 35 seroprevalence in AIDS risk groups supports type 35 as a vaccine vector. *AIDS* 2004. **18**: 1213-1216.

307 **Thorner, A. R., Vogels, R., Kaspers, J., Weverling, G. J., Holterman, L., Lemckert, A. A., Dilraj, A., McNally, L. M., Jeena, P. M., Jepsen, S., Abbink, P., Nanda, A., Swanson, P. E., Bates, A. T., O'Brien, K. L., Havenga, M. J., Goudsmit, J. and Barouch, D. H.,** Age dependence of adenovirus-specific neutralizing antibody titers in individuals from sub-Saharan Africa. *J Clin Microbiol* 2006. **44**: 3781-3783.

308 **Brown, K., Gao, W., Alber, S., Trichel, A., Murphey-Corb, M., Watkins, S. C., Gambotto, A. and Barratt-Boyes, S. M.,** Adenovirus-transduced dendritic cells injected into skin or lymph node prime potent simian immunodeficiency virus-specific T cell immunity in monkeys. *J Immunol* 2003. **171**: 6875-6882.

309 **Abbink, P., Lemckert, A. A., Ewald, B. A., Lynch, D. M., Denholtz, M., Smits, S., Holterman, L., Damen, I., Vogels, R., Thorner, A. R., O'Brien, K. L., Carville, A., Mansfield, K. G., Goudsmit, J., Havenga, M. J. and Barouch, D. H.,** Comparative seroprevalence and immunogenicity of six rare serotype recombinant adenovirus vaccine vectors from subgroups B and D. *J Virol* 2007. **81**: 4654-4663.

310 **Gao, W., Robbins, P. D. and Gambotto, A.,** Human adenovirus type 35: nucleotide sequence and vector development. *Gene Ther* 2003. **10**: 1941-1949.

311 **Pinto, A. R., Fitzgerald, J. C., Giles-Davis, W., Gao, G. P., Wilson, J. M. and Ertl, H. C.,** Induction of CD8+ T cells to an HIV-1 antigen through a prime boost regimen with heterologous E1-deleted adenoviral vaccine carriers. *J Immunol* 2003. **171**: 6774-6779.

312 **Reyes-Sandoval, A., Fitzgerald, J. C., Grant, R., Roy, S., Xiang, Z. Q., Li, Y., Gao, G. P., Wilson, J. M. and Ertl, H. C.,** Human immunodeficiency virus type 1-specific immune responses in primates upon sequential immunization with adenoviral vaccine carriers of human and simian serotypes. *J Virol* 2004. **78**: 7392-7399.

313 **Fitzgerald, J. C., Gao, G. P., Reyes-Sandoval, A., Pavlakis, G. N., Xiang, Z. Q., Wlazlo, A. P., Giles-Davis, W., Wilson, J. M. and Ertl, H. C.,** A simian replication-defective adenoviral recombinant vaccine to HIV-1 gag. *J Immunol* 2003. **170**: 1416-1422.

314 **Hofmann, C., Loser, P., Cichon, G., Arnold, W., Both, G. W. and Strauss, M.,** Ovine adenovirus vectors overcome preexisting humoral immunity against human adenoviruses in vivo. *J Virol* 1999. **73**: 6930-6936.

315 **Reddy, P. S., Idamakanti, N., Chen, Y., Whale, T., Babiuk, L. A., Mehtali, M. and Tikoo, S. K.,** Replication-defective bovine adenovirus type 3 as an expression vector. *J Virol* 1999. **73**: 9137-9144.

316 **Reddy, P. S., Idamakanti, N., Babiuk, L. A., Mehtali, M. and Tikoo, S. K.,** Porcine adenovirus-3 as a helper-dependent expression vector. *J Gen Virol* 1999. **80 (Pt 11)**: 2909-2916.

317 **Farina, S. F., Gao, G. P., Xiang, Z. Q., Rux, J. J., Burnett, R. M., Alvira, M. R., Marsh, J., Ertl, H. C. and Wilson, J. M.,** Replication-defective vector based on a chimpanzee adenovirus. *J Virol* 2001. **75**: 11603-11613.

318 **Lemckert, A. A., Sumida, S. M., Holterman, L., Vogels, R., Truitt, D. M., Lynch, D. M., Nanda, A., Ewald, B. A., Gorgone, D. A., Lifton, M. A., Goudsmit, J., Havenga, M. J. and Barouch, D. H.,** Immunogenicity of heterologous prime-boost regimens involving recombinant adenovirus serotype 11 (Ad11) and Ad35 vaccine vectors in the presence of anti-ad5 immunity. *J Virol* 2005. **79**: 9694-9701.

319 **Barouch, D. H., Pau, M. G., Custers, J. H., Koudstaal, W., Kostense, S., Havenga, M. J., Truitt, D. M., Sumida, S. M., Kishko, M. G., Arthur, J. C., Korioth-Schmitz, B., Newberg, M. H., Gorgone, D. A., Lifton, M. A., Panicali, D. L., Nabel, G. J., Letvin, N. L. and Goudsmit, J.,** Immunogenicity of recombinant adenovirus serotype 35 vaccine in the presence of pre-existing anti-Ad5 immunity. *J Immunol* 2004. **172**: 6290-6297.

320 **Thorner, A. R., Lemckert, A. A., Goudsmit, J., Lynch, D. M., Ewald, B. A., Denholtz, M., Havenga, M. J. and Barouch, D. H.,** Immunogenicity of heterologous recombinant adenovirus prime-boost vaccine regimens is enhanced by circumventing vector cross-reactivity. *J Virol* 2006. **80**: 12009-12016.

321 **Fattori, E., Zampaglione, I., Arcuri, M., Meola, A., Ercole, B. B., Cirillo, A., Folgori, A., Bett, A., Cappelletti, M., Sporeno, E., Cortese, R., Nicosia, A. and Colloca, S.,** Efficient immunization of rhesus macaques with an HCV candidate vaccine by heterologous priming-boosting with novel adenoviral vectors based on different serotypes. *Gene Ther* 2006. **13**: 1088-1096.

322 **Quinn, T. C., Wawer, M. J., Sewankambo, N., Serwadda, D., Li, C., Wabwire-Mangen, F., Meehan, M. O., Lutalo, T. and Gray, R. H.,** Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group. *N Engl J Med* 2000. **342**: 921-929.

323 **Gray, R. H., Li, X., Wawer, M. J., Gange, S. J., Serwadda, D., Sewankambo, N. K., Moore, R., Wabwire-Mangen, F., Lutalo, T. and Quinn, T. C.,** Stochastic simulation of the impact of antiretroviral therapy and HIV vaccines on HIV transmission; Rakai, Uganda. *AIDS* 2003. **17**: 1941-1951.

324 **Letvin, N. L., Barouch, D. H. and Montefiori, D. C.,** Prospects for vaccine protection against HIV-1 infection and AIDS. *Annu Rev Immunol* 2002. **20**: 73-99.

325 **Barouch, D. H., Santra, S., Kuroda, M. J., Schmitz, J. E., Plishka, R., Buckler-White, A., Gaitan, A. E., Zin, R., Nam, J. H., Wyatt, L. S., Lifton, M. A., Nickerson, C. E., Moss, B., Montefiori, D. C., Hirsch, V. M. and Letvin, N. L.,** Reduction of

simian-human immunodeficiency virus 89.6P viremia in rhesus monkeys by recombinant modified vaccinia virus Ankara vaccination. *J Virol* 2001. **75**: 5151-5158.

326 **Patterson, L. J., Malkevitch, N., Venzon, D., Pinczewski, J., Gomez-Roman, V. R., Wang, L., Kalyanaraman, V. S., Markham, P. D., Robey, F. A. and Robert-Guroff, M.,** Protection against mucosal simian immunodeficiency virus SIV(mac251) challenge by using replicating adenovirus-SIV multigene vaccine priming and subunit boosting. *J Virol* 2004. **78**: 2212-2221.

327 **Rose, N. F., Marx, P. A., Luckay, A., Nixon, D. F., Moretto, W. J., Donahoe, S. M., Montefiori, D., Roberts, A., Buonocore, L. and Rose, J. K.,** An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. *Cell* 2001. **106**: 539-549.

328 **Priddy, F. H., Brown, D., Kublin, J., Monahan, K., Wright, D. P., Lalezari, J., Santiago, S., Marmor, M., Lally, M., Novak, R. M., Brown, S. J., Kulkarni, P., Dubey, S. A., Kierstead, L. S., Casimiro, D. R., Mogg, R., Dinubile, M. J., Shiver, J. W., Leavitt, R. Y., Robertson, M. N., Mehrotra, D. V. and Quirk, E.,** Safety and Immunogenicity of a Replication-Incompetent Adenovirus Type 5 HIV-1 Clade B gag/pol/nef Vaccine in Healthy Adults. *Clin Infect Dis* 2008.

329 **Sullivan, N. J., Geisbert, T. W., Geisbert, J. B., Xu, L., Yang, Z. Y., Roederer, M., Koup, R. A., Jahrling, P. B. and Nabel, G. J.,** Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. *Nature* 2003. **424**: 681-684.

330 **Gao, W., Tamin, A., Soloff, A., D'Aiuto, L., Nwanegbo, E., Robbins, P. D., Bellini, W. J., Barratt-Boyes, S. and Gambotto, A.,** Effects of a SARS-associated coronavirus vaccine in monkeys. *Lancet* 2003. **362**: 1895-1896.

331 **Gao, W., Soloff, A. C., Lu, X., Montecalvo, A., Nguyen, D. C., Matsuoka, Y., Robbins, P. D., Swayne, D. E., Donis, R. O., Katz, J. M., Barratt-Boyes, S. M. and Gambotto, A.,** Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. *J Virol* 2006. **80**: 1959-1964.

332 **Yang, Y., Li, Q., Ertl, H. C. and Wilson, J. M.,** Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol* 1995. **69**: 2004-2015.

333 **DeMatteo, R. P., Yeh, H., Friscia, M., Caparrelli, D., Burke, C., Desai, N., Chu, G., Markmann, J. F., Raper, S. E. and Barker, C. F.,** Cellular immunity delimits adenoviral gene therapy strategies for the treatment of neoplastic diseases. *Ann Surg Oncol* 1999. **6**: 88-94.

334 **Juillard, V., Villefroy, P., Godfrin, D., Pavirani, A., Venet, A. and Guillet, J. G.,** Long-term humoral and cellular immunity induced by a single immunization with replication-defective adenovirus recombinant vector. *Eur J Immunol* 1995. **25**: 3467-3473.

335 **Santra, S., Seaman, M. S., Xu, L., Barouch, D. H., Lord, C. I., Lifton, M. A., Gorgone, D. A., Beaudry, K. R., Svehla, K., Welcher, B., Chakrabarti, B. K., Huang, Y., Yang, Z. Y., Mascola, J. R., Nabel, G. J. and Letvin, N. L.,** Replication-defective adenovirus serotype 5 vectors elicit durable cellular and humoral immune responses in nonhuman primates. *J Virol* 2005. **79**: 6516-6522.

336 **Seshidhar Reddy, P., Ganesh, S., Limbach, M. P., Brann, T., Pinkstaff, A., Kaloss, M., Kaleko, M. and Connelly, S.,** Development of adenovirus serotype 35 as a gene transfer vector. *Virology* 2003. **311**: 384-393.

337 **Amedee, A. M., Lacour, N., Gierman, J. L., Martin, L. N., Clements, J. E., Bohm, R., Jr., Harrison, R. M. and Murphey-Corb, M.,** Genotypic selection of simian immunodeficiency virus in macaque infants infected transplacentally. *J Virol* 1995. **69**: 7982-7990.

338 **Trichel, A. M., Rajakumar, P. A. and Murphey-Corb, M.,** Species-specific variation in SIV disease progression between Chinese and Indian subspecies of rhesus macaque. *J Med Primatol* 2002. **31**: 171-178.

339 **Trichel, A. M., Roberts, E. D., Wilson, L. A., Martin, L. N., Ruprecht, R. M. and Murphey-Corb, M.,** SIV/DeltaB670 transmission across oral, colonic, and vaginal mucosae in the macaque. *J Med Primatol* 1997. **26**: 3-10.

340 **Palmowski, M. J., Choi, E. M., Hermans, I. F., Gilbert, S. C., Chen, J. L., Gileadi, U., Salio, M., Van Pel, A., Man, S., Bonin, E., Liljestrom, P., Dunbar, P. R. and Cerundolo, V.,** Competition between CTL narrows the immune response induced by prime-boost vaccination protocols. *J Immunol* 2002. **168**: 4391-4398.

341 **Fuller, D. H., Rajakumar, P. A., Wilson, L. A., Trichel, A. M., Fuller, J. T., Shipley, T., Wu, M. S., Weis, K., Rinaldo, C. R., Haynes, J. R. and Murphey-Corb, M.,** Induction of mucosal protection against primary, heterologous simian immunodeficiency virus by a DNA vaccine. *J Virol* 2002. **76**: 3309-3317.

342 **Day, R., Ganz, P. A., Costantino, J. P., Cronin, W. M., Wickerham, D. L. and Fisher, B.,** Health-related quality of life and tamoxifen in breast cancer prevention: a report from the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Clin Oncol* 1999. **17**: 2659-2669.

343 **Fisher, E. R. and van Belle, G.,** *Biostatistics: a Methodology for the Health Sciences*. Wiley, New York, NY: 1993.

344 **Miller, M. D., Yamamoto, H., Hughes, A. L., Watkins, D. I. and Letvin, N. L.,** Definition of an epitope and MHC class I molecule recognized by gag-specific cytotoxic T lymphocytes in SIVmac-infected rhesus monkeys. *J Immunol* 1991. **147**: 320-329.

345 **Barouch, D. H., Kunstman, J., Glowczwskie, J., Kunstman, K. J., Egan, M. A., Peyerl, F. W., Santra, S., Kuroda, M. J., Schmitz, J. E., Beaudry, K., Krivulka, G. R., Lifton, M. A., Gorgone, D. A., Wolinsky, S. M. and Letvin, N. L.,** Viral escape from dominant simian immunodeficiency virus epitope-specific cytotoxic T lymphocytes in DNA-vaccinated rhesus monkeys. *J Virol* 2003. **77**: 7367-7375.

346 **Barouch, D. H. and Nabel, G. J.,** Adenovirus vector-based vaccines for human immunodeficiency virus type 1. *Hum Gene Ther* 2005. **16**: 149-156.

347 **Mei, Y. F., Skog, J., Lindman, K. and Wadell, G.,** Comparative analysis of the genome organization of human adenovirus 11, a member of the human adenovirus species B, and the commonly used human adenovirus 5 vector, a member of species C. *J Gen Virol* 2003. **84**: 2061-2071.

348 **Vanniasinkam, T. and Ertl, H. C.,** Adenoviral gene delivery for HIV-1 vaccination. *Curr Gene Ther* 2005. **5**: 203-212.

349 **Vogel, T. U., Horton, H., Fuller, D. H., Carter, D. K., Vielhuber, K., O'Connor, D. H., Shipley, T., Fuller, J., Sutter, G., Erfle, V., Wilson, N., Picker, L. J. and Watkins, D. I.,** Differences between T cell epitopes recognized after immunization and after infection. *J Immunol* 2002. **169**: 4511-4521.

350 **Horton, H., Vogel, T. U., Carter, D. K., Vielhuber, K., Fuller, D. H., Shipley, T., Fuller, J. T., Kunstman, K. J., Sutter, G., Montefiori, D. C., Erfle, V., Desrosiers, R.**

C., Wilson, N., Picker, L. J., Wolinsky, S. M., Wang, C., Allison, D. B. and Watkins, D. I., Immunization of rhesus macaques with a DNA prime/modified vaccinia virus Ankara boost regimen induces broad simian immunodeficiency virus (SIV)-specific T-cell responses and reduces initial viral replication but does not prevent disease progression following challenge with pathogenic SIVmac239. *J Virol* 2002. **76**: 7187-7202.

351 **Letvin, N. L., Huang, Y., Chakrabarti, B. K., Xu, L., Seaman, M. S., Beaudry, K., Korioth-Schmitz, B., Yu, F., Rohne, D., Martin, K. L., Miura, A., Kong, W. P., Yang, Z. Y., Gelman, R. S., Golubeva, O. G., Montefiori, D. C., Mascola, J. R. and Nabel, G. J.,** Heterologous envelope immunogens contribute to AIDS vaccine protection in rhesus monkeys. *J Virol* 2004. **78**: 7490-7497.

352 **Hel, Z., Tsai, W. P., Tryniszewska, E., Nacsa, J., Markham, P. D., Lewis, M. G., Pavlakis, G. N., Felber, B. K., Tartaglia, J. and Franchini, G.,** Improved vaccine protection from simian AIDS by the addition of nonstructural simian immunodeficiency virus genes. *J Immunol* 2006. **176**: 85-96.

353 **Koopman, G., Mortier, D., Hofman, S., Niphuis, H., Fagrouch, Z., Norley, S., Sutter, G., Liljestrom, P. and Heeney, J. L.,** Vaccine protection from CD4+ T-cell loss caused by simian immunodeficiency virus (SIV) mac251 is afforded by sequential immunization with three unrelated vaccine vectors encoding multiple SIV antigens. *J Gen Virol* 2004. **85**: 2915-2924.

354 **Peyerl, F. W., Barouch, D. H. and Letvin, N. L.,** Structural constraints on viral escape from HIV- and SIV-specific cytotoxic T-lymphocytes. *Viral Immunol* 2004. **17**: 144-151.

355 **Mothe, B. R., Horton, H., Carter, D. K., Allen, T. M., Liebl, M. E., Skinner, P., Vogel, T. U., Fuenger, S., Vielhuber, K., Rehrauer, W., Wilson, N., Franchini, G., Altman, J. D., Haase, A., Picker, L. J., Allison, D. B. and Watkins, D. I.,** Dominance of CD8 responses specific for epitopes bound by a single major histocompatibility complex class I molecule during the acute phase of viral infection. *J Virol* 2002. **76**: 875-884.

356 **Gitti, R. K., Lee, B. M., Walker, J., Summers, M. F., Yoo, S. and Sundquist, W. I.,** Structure of the amino-terminal core domain of the HIV-1 capsid protein. *Science* 1996. **273**: 231-235.

357 **Barouch, D. H., Kunstman, J., Kuroda, M. J., Schmitz, J. E., Santra, S., Peyerl, F. W., Krivulka, G. R., Beaudry, K., Lifton, M. A., Gorgone, D. A., Montefiori, D. C., Lewis, M. G., Wolinsky, S. M. and Letvin, N. L.,** Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. *Nature* 2002. **415**: 335-339.

358 **Rue, S. M., Roos, J. W., Amzel, L. M., Clements, J. E. and Barber, S. A.,** Hydrogen bonding at a conserved threonine in lentivirus capsid is required for virus replication. *J Virol* 2003. **77**: 8009-8018.

359 **Goulder, P. J., Phillips, R. E., Colbert, R. A., McAdam, S., Ogg, G., Nowak, M. A., Giangrande, P., Luzzi, G., Morgan, B., Edwards, A., McMichael, A. J. and Rowland-Jones, S.,** Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat Med* 1997. **3**: 212-217.

360 **Yeh, W. W., Cale, E. M., Jaru-Ampornpan, P., Lord, C. I., Peyerl, F. W. and Letvin, N. L.,** Compensatory substitutions restore normal core assembly in simian

immunodeficiency virus isolates with Gag epitope cytotoxic T-lymphocyte escape mutations. *J Virol* 2006. **80**: 8168-8177.

361 **Friedrich, T. C., Frye, C. A., Yant, L. J., O'Connor, D. H., Kriewaldt, N. A., Benson, M., Vojnov, L., Dodds, E. J., Cullen, C., Rudersdorf, R., Hughes, A. L., Wilson, N. and Watkins, D. I.,** Extraepitopic compensatory substitutions partially restore fitness to simian immunodeficiency virus variants that escape from an immunodominant cytotoxic-T-lymphocyte response. *J Virol* 2004. **78**: 2581-2585.

362 **Friedrich, T. C., McDermott, A. B., Reynolds, M. R., Piaskowski, S., Fuenger, S., De Souza, I. P., Rudersdorf, R., Cullen, C., Yant, L. J., Vojnov, L., Stephany, J., Martin, S., O'Connor, D. H., Wilson, N. and Watkins, D. I.,** Consequences of cytotoxic T-lymphocyte escape: common escape mutations in simian immunodeficiency virus are poorly recognized in naive hosts. *J Virol* 2004. **78**: 10064-10073.

363 **Mothe, B. R., Weinfurter, J., Wang, C., Rehrauer, W., Wilson, N., Allen, T. M., Allison, D. B. and Watkins, D. I.,** Expression of the major histocompatibility complex class I molecule Mamu-A*01 is associated with control of simian immunodeficiency virus SIVmac239 replication. *J Virol* 2003. **77**: 2736-2740.

364 **Muhl, T., Krawczak, M., Ten Haaft, P., Hunsmann, G. and Sauermann, U.,** MHC class I alleles influence set-point viral load and survival time in simian immunodeficiency virus-infected rhesus monkeys. *J Immunol* 2002. **169**: 3438-3446.

365 **Simon, V., Ho, D. D. and Abdool Karim, Q.,** HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. *Lancet* 2006. **368**: 489-504.

366 **Chun, T. W., Davey, R. T., Jr., Ostrowski, M., Shawn Justement, J., Engel, D., Mullins, J. I. and Fauci, A. S.,** Relationship between pre-existing viral reservoirs and the re-emergence of plasma viremia after discontinuation of highly active anti-retroviral therapy. *Nat Med* 2000. **6**: 757-761.

367 **Davey, R. T., Jr., Bhat, N., Yoder, C., Chun, T. W., Metcalf, J. A., Dewar, R., Natarajan, V., Lempicki, R. A., Adelsberger, J. W., Miller, K. D., Kovacs, J. A., Polis, M. A., Walker, R. E., Falloon, J., Masur, H., Gee, D., Baseler, M., Dimitrov, D. S., Fauci, A. S. and Lane, H. C.,** HIV-1 and T cell dynamics after interruption of highly active antiretroviral therapy (HAART) in patients with a history of sustained viral suppression. *Proc Natl Acad Sci U S A* 1999. **96**: 15109-15114.

368 **Ogg, G. S., Jin, X., Bonhoeffer, S., Moss, P., Nowak, M. A., Monard, S., Segal, J. P., Cao, Y., Rowland-Jones, S. L., Hurley, A., Markowitz, M., Ho, D. D., McMichael, A. J. and Nixon, D. F.,** Decay kinetics of human immunodeficiency virus-specific effector cytotoxic T lymphocytes after combination antiretroviral therapy. *J Virol* 1999. **73**: 797-800.

369 **Luzuriaga, K., McManus, M., Catalina, M., Mayack, S., Sharkey, M., Stevenson, M. and Sullivan, J. L.,** Early therapy of vertical human immunodeficiency virus type 1 (HIV-1) infection: control of viral replication and absence of persistent HIV-1-specific immune responses. *J Virol* 2000. **74**: 6984-6991.

370 **Letvin, N. L. and Walker, B. D.,** Immunopathogenesis and immunotherapy in AIDS virus infections. *Nat Med* 2003. **9**: 861-866.

371 **Kiepiela, P., Ngumbela, K., Thobakgale, C., Ramduth, D., Honeyborne, I., Moodley, E., Reddy, S., de Pierres, C., Mncube, Z., Mkhwanazi, N., Bishop, K., van der Stok, M., Nair, K., Khan, N., Crawford, H., Payne, R., Leslie, A., Prado, J., Prendergast, A., Frater, J., McCarthy, N., Brander, C., Learn, G. H., Nickle, D., Rousseau, C.,**

Coovadia, H., Mullins, J. I., Heckerman, D., Walker, B. D. and Goulder, P., CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat Med* 2007. **13**: 46-53.

372 **Sankaran, S., Guadalupe, M., Reay, E., George, M. D., Flamm, J., Prindiville, T. and Dandekar, S.,** Gut mucosal T cell responses and gene expression correlate with protection against disease in long-term HIV-1-infected nonprogressors. *Proc Natl Acad Sci U S A* 2005. **102**: 9860-9865.

373 **Ahmad, R., Sindhu, S. T., Toma, E., Morisset, R. and Ahmad, A.,** Studies on the production of IL-15 in HIV-infected/AIDS patients. *J Clin Immunol* 2003. **23**: 81-90.

374 **Waldmann, T. A.,** The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat Rev Immunol* 2006. **6**: 595-601.

375 **Chitnis, V., Pahwa, R. and Pahwa, S.,** Determinants of HIV-specific CD8 T-cell responses in HIV-infected pediatric patients and enhancement of HIV-gag-specific responses with exogenous IL-15. *Clin Immunol* 2003. **107**: 36-45.

376 **Hryniewicz, A., Price, D. A., Moniuszko, M., Boasso, A., Edghill-Spano, Y., West, S. M., Venzon, D., Vaccari, M., Tsai, W. P., Tryniszewska, E., Nacsa, J., Villinger, F., Ansari, A. A., Trindade, C. J., Morre, M., Brooks, D., Arlen, P., Brown, H. J., Kitchen, C. M., Zack, J. A., Douek, D. C., Shearer, G. M., Lewis, M. G., Koup, R. A. and Franchini, G.,** Interleukin-15 but not interleukin-7 abrogates vaccine-induced decrease in virus level in simian immunodeficiency virus mac251-infected macaques. *J Immunol* 2007. **178**: 3492-3504.

377 **Mueller, Y. M., Do, D. H., Altork, S. R., Artlett, C. M., Gracely, E. J., Katsetos, C. D., Legido, A., Villinger, F., Altman, J. D., Brown, C. R., Lewis, M. G. and Katsikis, P. D.,** IL-15 treatment during acute simian immunodeficiency virus (SIV) infection increases viral set point and accelerates disease progression despite the induction of stronger SIV-specific CD8+ T cell responses. *J Immunol* 2008. **180**: 350-360.

378 **Fernandez-Cruz, E., Moreno, S., Navarro, J., Clotet, B., Bouza, E., Carbone, J., Pena, J. M., Perez Molina, J., Podzamczer, D., Rubio, R., Ocana, I., Pulido, F., Viciana, P., Maradona, J. A., Blazquez, R., Barros, C., Quereda, C., Rodriguez-Sainz, C., Gil, J., Abad, M. L., Diaz, L., Canto, C., Munoz, M. A., Ferrer, E., Jou, A., Sirera, G., Diaz, M., Lopez, F., Gatell, J. M. and Gonzalez-Lahoz, J.,** Therapeutic immunization with an inactivated HIV-1 Immunogen plus antiretrovirals versus antiretroviral therapy alone in asymptomatic HIV-infected subjects. *Vaccine* 2004. **22**: 2966-2973.

379 **Halwani, R., Boyer, J. D., Yassine-Diab, B., Haddad, E. K., Robinson, T. M., Kumar, S., Parkinson, R., Wu, L., Sidhu, M. K., Phillipson-Weiner, R., Pavlakis, G. N., Felber, B. K., Lewis, M. G., Shen, A., Siliciano, R. F., Weiner, D. B. and Sekaly, R. P.,** Therapeutic vaccination with simian immunodeficiency virus (SIV)-DNA+IL-12 or IL-15 induces distinct CD8 memory subsets in SIV-infected macaques. *J Immunol* 2008. **180**: 7969-7979.

380 **Cohen, J.,** AIDS research. Promising AIDS vaccine's failure leaves field reeling. *Science* 2007. **318**: 28-29.

381 **McCoy, K., Tatsis, N., Korioth-Schmitz, B., Lasaro, M. O., Hensley, S. E., Lin, S. W., Li, Y., Giles-Davis, W., Cun, A., Zhou, D., Xiang, Z., Letvin, N. L. and Ertl, H. C.,** Effect of preexisting immunity to adenovirus human serotype 5 antigens on the

immune responses of nonhuman primates to vaccine regimens based on human- or chimpanzee-derived adenovirus vectors. *J Virol* 2007. **81**: 6594-6604.

382 **Schito, M. L., Soloff, A. C., Slovitz, D., Trichel, A., Inman, J. K., Appella, E., Turpin, J. A. and Barratt-Boyes, S. M.,** Preclinical evaluation of a zinc finger inhibitor targeting lentivirus nucleocapsid protein in SIV-infected monkeys. *Curr HIV Res* 2006. **4**: 379-386.

383 **Daucher, M., Price, D. A., Brenchley, J. M., Lamoreaux, L., Metcalf, J. A., Rehm, C., Nies-Kraske, E., Urban, E., Yoder, C., Rock, D., Gumkowski, J., Betts, M. R., Dybul, M. R. and Douek, D. C.,** Virological outcome after structured interruption of antiretroviral therapy for human immunodeficiency virus infection is associated with the functional profile of virus-specific CD8+ T cells. *J Virol* 2008. **82**: 4102-4114.

384 **Pitcher, C. J., Hagen, S. I., Walker, J. M., Lum, R., Mitchell, B. L., Maino, V. C., Axthelm, M. K. and Picker, L. J.,** Development and homeostasis of T cell memory in rhesus macaque. *J Immunol* 2002. **168**: 29-43.

385 **Sun, Y., Schmitz, J. E., Buzby, A. P., Barker, B. R., Rao, S. S., Xu, L., Yang, Z. Y., Mascola, J. R., Nabel, G. J. and Letvin, N. L.,** Virus-specific cellular immune correlates of survival in vaccinated monkeys after simian immunodeficiency virus challenge. *J Virol* 2006. **80**: 10950-10956.

386 **Malkevitch, N., Patterson, L. J., Aldrich, K., Richardson, E., Alvord, W. G. and Robert-Guroff, M.,** A replication competent adenovirus 5 host range mutant-simian immunodeficiency virus (SIV) recombinant priming/subunit protein boosting vaccine regimen induces broad, persistent SIV-specific cellular immunity to dominant and subdominant epitopes in Mamu-A*01 rhesus macaques. *J Immunol* 2003. **170**: 4281-4289.

387 **Pantaleo, G. and Koup, R. A.,** Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. *Nat Med* 2004. **10**: 806-810.

388 **Letvin, N. L.,** Correlates of immune protection and the development of a human immunodeficiency virus vaccine. *Immunity* 2007. **27**: 366-369.

389 **Markowitz, M., Jin, X., Hurley, A., Simon, V., Ramratnam, B., Louie, M., Deschenes, G. R., Ramanathan, M., Jr., Barsoum, S., Vanderhoeven, J., He, T., Chung, C., Murray, J., Perelson, A. S., Zhang, L. and Ho, D. D.,** Discontinuation of antiretroviral therapy commenced early during the course of human immunodeficiency virus type 1 infection, with or without adjunctive vaccination. *J Infect Dis* 2002. **186**: 634-643.

390 **Kinloch-de Loes, S., Hoen, B., Smith, D. E., Autran, B., Lampe, F. C., Phillips, A. N., Goh, L. E., Andersson, J., Tsoukas, C., Sonnerborg, A., Tambussi, G., Girard, P. M., Bloch, M., Battegay, M., Carter, N., El Habib, R., Theofan, G., Cooper, D. A. and Perrin, L.,** Impact of therapeutic immunization on HIV-1 viremia after discontinuation of antiretroviral therapy initiated during acute infection. *J Infect Dis* 2005. **192**: 607-617.

391 **Lefrancois, L. and Puddington, L.,** Intestinal and pulmonary mucosal T cells: local heroes fight to maintain the status quo. *Annu Rev Immunol* 2006. **24**: 681-704.

392 **Migueles, S. A., Sabbaghian, M. S., Shupert, W. L., Bettinotti, M. P., Marincola, F. M., Martino, L., Hallahan, C. W., Selig, S. M., Schwartz, D., Sullivan, J. and Connors, M.,** HLA B*5701 is highly associated with restriction of virus replication in a

subgroup of HIV-infected long term nonprogressors. *Proc Natl Acad Sci U S A* 2000. **97**: 2709-2714.

393 **Scherer, A., Frater, J., Oxenius, A., Agudelo, J., Price, D. A., Gunthard, H. F., Barnardo, M., Perrin, L., Hirschel, B., Phillips, R. E. and McLean, A. R.,** Quantifiable cytotoxic T lymphocyte responses and HLA-related risk of progression to AIDS. *Proc Natl Acad Sci U S A* 2004. **101**: 12266-12270.

394 **Rosenberg, E. S., Altfeld, M., Poon, S. H., Phillips, M. N., Wilkes, B. M., Eldridge, R. L., Robbins, G. K., D'Aquila, R. T., Goulder, P. J. and Walker, B. D.,** Immune control of HIV-1 after early treatment of acute infection. *Nature* 2000. **407**: 523-526.

395 **von Gegerfelt, A. S., Rosati, M., Alicea, C., Valentin, A., Roth, P., Bear, J., Franchini, G., Albert, P. S., Bischofberger, N., Boyer, J. D., Weiner, D. B., Markham, P., Israel, Z. R., Eldridge, J. H., Pavlakis, G. N. and Felber, B. K.,** Long-lasting decrease in viremia in macaques chronically infected with simian immunodeficiency virus SIVmac251 after therapeutic DNA immunization. *J Virol* 2007. **81**: 1972-1979.

396 **Nikolich-Zugich, J.,** Ageing and life-long maintenance of T-cell subsets in the face of latent persistent infections. *Nat Rev Immunol* 2008. **8**: 512-522.

397 **Sempowski, G. D. and Haynes, B. F.,** Immune reconstitution in patients with HIV infection. *Annu Rev Med* 2002. **53**: 269-284.

398 **Haynes, B. F., Hale, L. P., Weinhold, K. J., Patel, D. D., Liao, H. X., Bressler, P. B., Jones, D. M., Demarest, J. F., Gebhard-Mitchell, K., Haase, A. T. and Bartlett, J. A.,** Analysis of the adult thymus in reconstitution of T lymphocytes in HIV-1 infection. *J Clin Invest* 1999. **103**: 921.

399 **Bonyhadi, M. L., Rabin, L., Salimi, S., Brown, D. A., Kosek, J., McCune, J. M. and Kaneshima, H.,** HIV induces thymus depletion in vivo. *Nature* 1993. **363**: 728-732.

400 **Valentin, H., Nugeyre, M. T., Vuillier, F., Boumsell, L., Schmid, M., Barre-Sinoussi, F. and Pereira, R. A.,** Two subpopulations of human triple-negative thymic cells are susceptible to infection by human immunodeficiency virus type 1 in vitro. *J Virol* 1994. **68**: 3041-3050.

401 **Grossman, Z., Meier-Schellersheim, M., Paul, W. E. and Picker, L. J.,** Pathogenesis of HIV infection: what the virus spares is as important as what it destroys. *Nat Med* 2006. **12**: 289-295.

402 **Lempicki, R. A., Kovacs, J. A., Baseler, M. W., Adelsberger, J. W., Dewar, R. L., Natarajan, V., Bosche, M. C., Metcalf, J. A., Stevens, R. A., Lambert, L. A., Alvord, W. G., Polis, M. A., Davey, R. T., Dimitrov, D. S. and Lane, H. C.,** Impact of HIV-1 infection and highly active antiretroviral therapy on the kinetics of CD4+ and CD8+ T cell turnover in HIV-infected patients. *Proc Natl Acad Sci U S A* 2000. **97**: 13778-13783.

403 **Gu, X. X., Yue, F. Y., Kovacs, C. M. and Ostrowski, M. A.,** The role of cytokines which signal through the common gamma chain cytokine receptor in the reversal of HIV specific CD4+ and CD8+ T cell anergy. *PLoS ONE* 2007. **2**: e300.

404 **Malek, T. R. and Bayer, A. L.,** Tolerance, not immunity, crucially depends on IL-2. *Nat Rev Immunol* 2004. **4**: 665-674.

405 **Soloff, A. C., Liu, X. D., Rajakumar, P. A., Nwanegbo, E., Brown, K., Gao, W., Murphey-Corb, M., Robbins, P. D., Gambotto, A. and Barratt-Boyes, S.,** Dual Adenoviral-Based Vaccination Using Serotypes 5 and 35 Induces Broad T Cell

Responses to SIV Which Are Recalled With Heterologous Viral Challenge *Keystone Symposia: HIV Vaccine Development*, Whistler, British Columbia, Canada 2004.

406 **Moore, C. B., John, M., James, I. R., Christiansen, F. T., Witt, C. S. and Mallal, S. A.,** Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science* 2002. **296**: 1439-1443.

407 **Martinez-Picado, J., Prado, J. G., Fry, E. E., Pfafferott, K., Leslie, A., Chetty, S., Thobakgale, C., Honeyborne, I., Crawford, H., Matthews, P., Pillay, T., Rousseau, C., Mullins, J. I., Brander, C., Walker, B. D., Stuart, D. I., Kiepiela, P. and Goulder, P.,** Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. *J Virol* 2006. **80**: 3617-3623.

408 **Steinbrook, R.,** One step forward, two steps back--will there ever be an AIDS vaccine? *N Engl J Med* 2007. **357**: 2653-2655.

409 **Sekaly, R. P.,** The failed HIV Merck vaccine study: a step back or a launching point for future vaccine development? *J Exp Med* 2008. **205**: 7-12.

410 **Watkins, D. I., Burton, D. R., Kallas, E. G., Moore, J. P. and Koff, W. C.,** Nonhuman primate models and the failure of the Merck HIV-1 vaccine in humans. *Nat Med* 2008. **14**: 617-621.

411 **Pilcher, C. D., Tien, H. C., Eron, J. J., Jr., Vernazza, P. L., Leu, S. Y., Stewart, P. W., Goh, L. E. and Cohen, M. S.,** Brief but efficient: acute HIV infection and the sexual transmission of HIV. *J Infect Dis* 2004. **189**: 1785-1792.

412 **Gray, R. H., Wawer, M. J., Brookmeyer, R., Sewankambo, N. K., Serwadda, D., Wabwire-Mangen, F., Lutalo, T., Li, X., vanCott, T. and Quinn, T. C.,** Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda. *Lancet* 2001. **357**: 1149-1153.

413 **Leynaert, B., Downs, A. M. and de Vincenzi, I.,** Heterosexual transmission of human immunodeficiency virus: variability of infectivity throughout the course of infection. European Study Group on Heterosexual Transmission of HIV. *Am J Epidemiol* 1998. **148**: 88-96.

414 **Feinberg, M. B. and Moore, J. P.,** AIDS vaccine models: challenging challenge viruses. *Nat Med* 2002. **8**: 207-210.

415 **Buchbinder, S. P., Mehrotra, D. V., Duerr, A., Fitzgerald, D. W., Mogg, R., Li, D., Gilbert, P. B., Lama, J. R., Marmor, M., Del Rio, C., McElrath, M. J., Casimiro, D. R., Gottesdiener, K. M., Chodakewitz, J. A., Corey, L. and Robertson, M. N.,** Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. *Lancet* 2008. **372**: 1881-1893.

416 **McElrath, M. J., De Rosa, S. C., Moodie, Z., Dubey, S., Kierstead, L., Janes, H., Defawe, O. D., Carter, D. K., Hural, J., Akondy, R., Buchbinder, S. P., Robertson, M. N., Mehrotra, D. V., Self, S. G., Corey, L., Shiver, J. W. and Casimiro, D. R.,** HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. *Lancet* 2008. **372**: 1894-1905.

417 **IAVI-Report,** AIDS vaccine researchers STEP up to the challenge. *IAVI Report* 2008. **12**: 1-7.

418 **Villinger, F., Brice, G. T., Mayne, A. E., Bostik, P., Mori, K., June, C. H. and Ansari, A. A.,** Adoptive transfer of simian immunodeficiency virus (SIV) naive

autologous CD4(+) cells to macaques chronically infected with SIV is sufficient to induce long-term nonprogressor status. *Blood* 2002. **99**: 590-599.

419 **Onlamoon, N., Plagman, N., Rogers, K. A., Mayne, A. E., Bostik, P., Pattanapanyasat, K., Ansari, A. A. and Villinger, F.,** Anti-CD3/28 mediated expansion of macaque CD4+ T cells is polyclonal and provides extended survival after adoptive transfer. *J Med Primatol* 2007. **36**: 206-218.

420 **Vaccari, M., Mattapallil, J., Song, K., Tsai, W. P., Hryniewicz, A., Venzon, D., Zanetti, M., Reimann, K. A., Roederer, M. and Franchini, G.,** Reduced protection from simian immunodeficiency virus SIVmac251 infection afforded by memory CD8+ T cells induced by vaccination during CD4+ T-cell deficiency. *J Virol* 2008. **82**: 9629-9638.