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Adjuvanting and delineating the mechanisms of induction of gut homing CD8 T cells elicited by candidate HIV vaccines

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Adjuvanting and delineating the mechanisms of induction of gut homing CD8 T cells elicited by candidate HIV vaccines

By

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MS, University of Pittsburgh, 2003

Advisor: Rama R. Amara, Ph.D.

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Abstract

Adjuvanting and delineating the mechanisms of induction of gut homing CD8 T cells elicited by candidate HIV vaccines

By Sumita Ganguly

An efficacious HIV (Human immunodeficiency Virus) vaccine is crucial to curb the global pandemic of the disease, AIDS. Gene-based (DNA) and viral-vector based vaccines are two vaccine design strategies that are in advanced stages of development. Here, we investigate the potential of co-stimulation through 4-1BB as an adjuvant for a HIV-1 DNA vaccine in mice. We designed plasmid DNAs expressing either the membrane bound or soluble form of 4-1BBL, and compared with the agonistic anti-4-1BB Ab for their ability to adjuvant the Gag DNA vaccine. Both, anti-4-1BB agonistic Ab as well as 4-1BBL DNA enhanced the Gag-specific cellular immune responses. However, in complete contrast to the agonistic Ab that suppressed humoral immunity to Gag, 4-1BBL DNA adjuvanted vaccines enhanced Gag-specific IgG responses. Importantly, the expression of Gag and 4-1BBL from the same plasmid was critical for the adjuvant activity. Collectively, our data suggest that for a HIV-1 vaccine, 4-1BBL expressed by a DNA vaccine is a superior adjuvant than anti-4-1BB agonistic Ab.

An effective HIV vaccine must also be able to confer protective immunity at the gut-associated mucosal tissue. Recent studies have shown that intramuscular immunization with some live viral vectors can prime antigen-specific CD8 T cells with gut homing potential. However, the mechanisms by which parenteral immunizations elicit antigen-specific CD8 T cells in the gut are not understood. Here we show that an adenovirus type 5 (Ad5) based HIV-1 vaccine primes a strong and durable antigen-specific CD8 T cell response in the gut following intramuscular immunization in mice. We also show that Ad5 rapidly induces expression of retinal dehydrogenase enzymes in splenic conventional DC (cDC) and enhances their ability to prime antigen-specific CD8 T cells with gut homing specificity *in vitro*. This effect of Ad5 did not require signaling through toll-like receptors, DNA-dependent activator of IRFs and several MAP kinases, or replication capacity of the virus, but was dependant on NF-κB. These results provide an innate mechanism through which Ad5 primes antigen-specific CD8 T cells with gut homing potential and have implications for the development of novel mucosal adjuvants for subunit vaccines administered *via* the intramuscular route.

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INTRODUCTION

Currently there are 33 million people living worldwide with HIV/AIDS (UNAIDS report). In 2008 alone, an estimated 2.7 million new HIV infections and 2 million deaths occurred globally because of AIDS-related illnesses. Though there has been an unprecedented increase in access to antiretroviral therapies in the last decade, AIDS still continues to be a major global health priority. Thus there is an urgent need to develop a safe and effective HIV vaccine.

A. Pathogenesis of the Human immunodeficiency virus-1 (HIV-1)

A.1. Basic Virology of HIV-1

HIV is a member of the lentivirus family of animal retroviruses. Lentiviruses are capable of either establishing long-term latency or inducing short-term cytopathic effects in infected cells, and they all produce slowly progressive, fatal diseases. Two closely related types of HIV, designated HIV-1 and HIV-2, have been identified. Phylogenetic analyses suggest that HIV-1 was transmitted to humans from the chimpanzee (the related strain is SIV_{cpz}) and HIV-2 from the sooty mangabey (the related strain is SIV_{sm}).

An infectious HIV particle consists of two identical strands of RNA, packaged within a core of viral proteins, and surrounded by a host cell membrane-derived phospholipid bilayer envelope that contains virally encoded Env glycoproteins. The HIV genome shares the basic structure of all known retroviruses, including nucleotide sequences called *gag* (encodes the core structural protein - matrix, capsid and the nucleocapsid); *env*

(encodes the envelope glycoproteins gp120 and gp41); and *pol* (encodes the reverse transcriptase, viral protease and integrase). In addition to these typical retrovirus genes, HIV also includes at least six accessory genes, namely *vpr*, *vif*, *tat*, *rev*, *nef* and *vpu* genes, whose products regulate viral replication in various ways.

Viral entry into target cells is mediated by the viral envelope glycoproteins, gp120 and gp41, which are organized into trimeric complexes on the virion surface (*1, 2*)**.** The first step is the high affinity binding of gp120 to CD4 molecules on the surface of a primate T cell or mononuclear phagocyte. This triggers conformational changes in gp120 that allow binding to one of the two chemokine receptors (coreceptors), CCR5 or CXCR4 (*3, 4*). Receptor binding leads to the exposure of gp41 transmembrane envelope glycoprotein and additional conformational changes that result in the fusion of the viral and cell membranes (*5, 6*). Upon entry into the target cell, the RNA genome of HIV is transcribed into a double stranded DNA form by viral reverse transcriptase (RT) in host cell cytoplasm (a process called *reverse transcription*). In the active mode of replication, the error-prone RT generates large number of mutations in the viral genomes, thus contributing directly to the genetic diversity of HIV. The viral integrase protein then catalyzes the integration of the viral DNA into the host cell genome, a process called *integration* that occurs in host cell nucleus. The integrated DNA form of HIV is called *provirus* and it may remain transcriptionally inactive for months or years, thus establishing a latent HIV infection. Transcription of the genes of the integrated provirus is regulated by long terminal repeat (*LTR*) sequences, which flank either side of the viral structural genes. Activation of $CD4^+$ T cells induces the transcription factor NF-κB, which binds to promoters in the viral LTR, thereby initiating the transcription of viral

RNA by the cellular RNA polymerase. After transcription of the viral genes, viral proteins are synthesized in the cytoplasm. Assembly of infectious virus particles takes place at the plasma membrane and virions are released by budding from the cell surface. HIV-1 assembly is controlled primarily by the Gag protein and this mode of assembly leads to the acquisition of host cell-derived lipid envelope.

A.2. Immunology of HIV-1 infection

Exposure to HIV-1 is primarily through the genital/ rectal mucosal routes, where the lamina propria resident $CCR5^+$ $CD4^+$ T cells are targets for establishment of infection and rapid dissemination over the next couple of weeks (acute stage of infection) (*7, 8*)**.** Studies have shown that naïve T cells that have been activated are sensitive to HIV-1 infection (*9*) and that cytokine signals in the absence of full TCR-mediated activation are sufficient for HIV-1 infection of resting naïve and memory $CD4^+$ T cells *in vitro* and *in vivo* (*10, 11*). Binding and internalization of intact virions via DC-SIGN also enhances infection *in trans* of $CD4^+$ Tcells (*12*). Although $CD4^+$ memory T cells are lost in all lymphoid-tissue compartments, the greatest losses are in the lamina propria of the gut-associated lymphoid tissue (GALT) (*13, 14*). The ongoing viral replication during the acute infection phase limits the renewal capacity of the $CD4^+$ T cell pool by impairing thymic output and inducing $CD4^+$ T cell activation that serves to propagate the virus, thus placing additional homeostatic strain on maintenance of the "resting" naïve and memory $CD4^+$ T cell pools.

During the chronic stage of infection, there is evidence of pathological changes in the bone marrow, disruption of lymph node architecture and decreased thymic output of naïve cells (*15, 16*). Both $CD4^+$ and $CD8^+$ T cells become chronically activated and thus become more susceptible to apoptosis. It is now widely accepted that chronic immune activation has a pivotal role in driving progression to AIDS (*17, 18*). Studies in sooty mangabeys have shown that, despite high levels of persisting viremia and depletion of mucosal $CD4^+$ T cells, they escape disease progression by downregulation of immune activation. In another study, it has been suggested that microbial translocation (due to damage of the gut mucosal barrier) may be the underlying cause of chronic immune activation during HIV-1 infection (*19*) and that plasma LPS (a quantitative indicator of microbial translocation) levels are elevated in people with chronic HIV-1 infection. Though there are high frequencies of persisting HIV-specific CTLs during the chronic asymptomatic phase of HIV infection (*20*), strong cytotoxic responses exert selection pressure on the virus, thus generating viral escape mutants. The emergence of CXCR4–tropic strains during the late stage of chronic infection also expands the repertoire of target cells and results in enhanced pathogenicity (*21*). Selection pressure by neutralizing antibody responses also results in rapid evolution of HIV-1 envelope possibly through point mutations, changes in glycosylation patterns, and insertions and deletions in the viral envelope thereby generating viral escape mutants. In summary, during the chronic phase of HIV infection, there is a constant interplay between T and B cell activation, death, renewal, emergence of viral escape variants and clearance of virus.

Primary infection (associated with acute viremia) with HIV causes an influenza-like illness in up to 80% of the cases and the diagnosis at this stage is usually missed. High

viral load drives the activation of HIV-specific $CD8^+$ T cells and subsequent antibody production (seroconversion). Since the start of the HIV epidemic, several antiretroviral drugs have been developed, most of which block the virus's ability to replicate. There are four main classes of these drugs – early inhibitors (blocking viral entry), nucleoside reverse transcriptase inhibitors (a nucleoside analog antiretroviral drug that cause premature termination of the proviral DNA chain), non-nucleoside reverse transcriptase inhibitors (binds to regions very close to the RT active site and induces detrimental conformational changes within the enzyme) and viral protease inhibitors (inhibits virion maturation). Current HAART (Highly Active Antiretroviral Therapy) regimens have been successfully used for immune reconstitution (decrease in HIV RNA levels and increase in CD4 count), thus delaying disease progression and onset of opportunistic infections. However, antiretroviral therapy has failed to eradicate long-lived latently infected cells despite apparent complete viral suppression.

With the emergence of drug resistant viruses, HIV-1 infection ultimately leads to progressive attrition in the number of circulating $CD4^+$ T cells, eventually culminating in AIDS (Acquired Immunodeficiency Syndrome) when blood $CD4^+$ Tells decline to less than 200 cells/ μl (the normal value is between 800 - 1200 cells/ μl). Genetic variation in HLA type of the host can modify the outcome of the disease: HLA-B57 and HLA-B27 are associated with better prognosis and HLA-B35 with more rapid disease progression. The CCR5-Δ32 mutation can confer resistance to HIV-1 infection.

B. HIV Vaccines

HIV-1/ AIDS is the fourth leading cause of death worldwide. Despite the intense international response to the pandemic, HIV transmission still continues to pose a global problem. With the advent of highly active antiretroviral therapy (HAART), it has been possible to increase the disease-free survival time of the patient (*22*). Unfortunately HAART has several limitations such as long-term toxicity, emergence of drug resistant viruses, poor adherence to complicated regimens, and high costs. Given the devastating nature of the disease and its impact on society, it is important to develop a safe and effective HIV vaccine that can save millions of lives worldwide.

B.1. Correlates of immune protection

An ideal HIV vaccine should have the potential to achieve "sterilizing immunity". However, such a task will be difficult to accomplish as the virus can escape neutralizing antibody responses by accumulating mutations in the envelope gene. Both humoral (antibody mediated) and cellular (T helper cells and CTL mediated) immune responses are crucial for lowering the viral load and increasing the absolute $CD4^+$T cell count in an infected individual. With regard to the humoral immune response, vaccines that can raise high titers of broadly cross-reactive neutralizing antibodies are of extreme value (passive immunization studies provide direct experimental proof for the protective role of neutralizing antibodies (*23*)). For cell-mediated immunity, a vaccine should stimulate polyfunctional (in terms of multiple cytokine production, proliferative capacity) antigen-specific $CD4^+$ and $CD8^+$ T cells to be effective against disease progression. Direct

evidence underscoring the functional significance of virus-specific CTL responses has been obtained from preclinical studies in which *in vivo* depletion of $CD8^+$ T cells in SIV-infected monkeys resulted in rapid and dramatic increases in viremia (*24, 25*). The fact that certain HLA alleles could better control viral replication (resulting in undetectable viremia without medication) and that CTL escape mutants are generated very early on during acute infection in humans, also argues strongly for the importance of CD8 responses to the virus. Since, it is now very well established that the primary site of HIV/ SIV replication immediately after infection is in the GALT, successful immunization strategies need to blunt the rapid amplification of HIV in the GALT during the acute stage of infection and the resulting substantial depletion of $CD4^+$ T lymphocytes.

B.2. Challenges in designing a HIV vaccine

Despite the considerable progress that has been made in the development of HIV vaccines over the past several years, key scientific challenges still remain. High antigenic variation of the envelope protein, shielding of the conserved V3 loop by two other variable loops (V1 and V2 loop) (*26*), and extensive glycosylation (*27*) limit the accessibility of neutralizing antibodies that are directed towards the envelope protein. Viral escape from CTL responses (by accumulating mutations in dominant CTL epitopes) can result in eventual progression towards AIDS and thus failure of CTL-based vaccine (*28, 29*). Due to the difficulty in eliciting broadly cross-reactive neutralizing activity by vaccines, the main focus of current vaccine candidates now is to elicit robust cellular immune responses capable of suppressing viral load and slowing the progression to AIDS

and also limiting the rate of transmission due to lowered plasma viremia. Preclinical vaccine studies in rhesus monkeys have shown that CTL-based vaccines effectively control viral replication and attenuate disease progression after challenge with simian-human immunodeficiency virus (SHIV 89.6P) (*30, 31*). It is critical to generate vaccine-induced $CD8^+$ T cell responses against those viral epitopes, mutation/s in which will impair *in vivo* viral fitness. Given the vast genetic diversity of HIV-1, it is also important to identify those CTL epitopes that can elicit cross-clade immune responses.

B.3. HIV Vaccine strategies

Recent data from a phase III vaccine trial (RV 144) has reported a thirty percent reduction in HIV-1 acquisition following prime/ boost vaccination with a recombinant canarypox-HIV vector and HIV envelope protein (*32*). Though the efficacy of protection is insufficient, yet, the results are very encouraging to the scientific community. Up until now, all four major HIV vaccine efficacy trials (Vaxgen,Inc. conducted AIDSVAX 003 and AIDSVAX 004; NIH-supported HIV Vaccines Trial Network conducted HVTN 502 or STEP and HVTN 503) failed to demonstrate any efficacy in terms of protection/ reduced infection. The recombinant HIV-1 envelope gp120 with alum adjuvant was used as the prototype immunogen in the AIDSVAX trials (*33*). Envelope gp120-specific antibody response raised by this vaccine could not neutralize HIV-1 isolates obtained from the blood of infected individuals (*34, 35*). Also, this vaccine failed to induce CTL responses and this can very well be the reason for its failure. The HVTN trials tested Merck's candidate vaccine MRKAd5 HIV-1 *gag*/ *pol*/ *nef* trivalent vaccine. The failure

of the STEP trial was based on the increased risk of HIV-1 acquisition in men that were baseline Ad5 seropositive (pre-existing immunity to the vector) and uncircumcised (*36*). Importantly, the vaccine also failed to confer any protection to individuals who had low Ad5 Ab titers (>18). It was hypothesized that the heightened risk of infection could be due to the increased frequency of virus target cells at the mucosa primed by Ad5. Currently, three modalities of vaccines that have the potential to elicit robust antigen-specific $CD8^+$ T cells are being extensively studied: live attenuated vaccines, live vectored vaccines (replication–competent or replication–defective) and DNA vaccines.

Live attenuated vaccine

Live, attenuated virus vaccines mimic natural exposure while avoiding pathogenicity. These vaccines can induce both cellular and humoral immunity and can provide long term immunity. Several vaccines of this prototype are licensed for use in humans (e.g. small pox, oral polio, MMR, yellow fever, varicella). However, with this mode of vaccination, there are chances of reversion to virulence and disease manifestations in immunocompromised individuals. Simultaneous mutation of multiple viral genes may decrease the possibility of reversion to a wild-type genotype, however, this strategy of attenuation may also compromise the immunogenicity of the live virus. The use of live attenuated SIV viruses (SIV Δnef, SIV Δnef Δvpr, Env SIV ΔV1-V2) as vaccines has provided complete or near-complete protection from homologous challenge by those same wild-type SIV strains, thus providing a critical proof of principal for the feasibility of HIV vaccine development (*37*). However, live attenuated vaccine strategy

faced a major setback when neonatal macaques became viremic by 2 weeks of age (and one-half died of AIDS within 6 months) upon oral administration with a SIV mutant vaccine (with deletions in *nef*, *vpr* and the negative regulatory element, called SIVmac293Δ3) (*38*).

Live vector vaccine

Many viruses are now being used as live vectors and these have shown promise in heterologous prime-boost regimens. The two most popular replication defective/ attenuated (in humans) viral vectors are adenovirus 5 (Ad5) and Modified Vaccinia Ankara (MVA). High transduction efficiency in mammalian cells, rapid growth to high titers in culture, and genetic stability has made adenovirus an attractive vector for gene delivery. For enhanced safety, recombinant Ad (rAd) vaccine vectors are typically made replication defective by deletion of the E1 region (first generation adenoviral vectors), which encodes proteins critical for viral replication. The second generation adenoviral vectors were made by either deleting or modifying E2A, E3 and E4 genes. Furthermore, adenoviral vectors with deletion of all viral gene products, called 'gutless vectors' have also been generated. Studies have shown that both rAd5 vaccines as well as DNA prime/ rAd5 boost regimens have provided partial protection in rhesus macaques against multiple AIDS virus isolates, including SHIV 89.6P (*39*), SIVmac239 and SIVmac251. However, despite preclinical success with rAd5 vectors, the STEP trial (HVTN 502) that tested the efficacy of the replication-defective rAd5 HIV-1 *gag*/ *pol*/ *nef* trivalent vaccine failed to confer any protection in humans. It is believed that the high prevalence of pre-

existing immunity to Ad5 in human population blunt the immunogenicity of Ad5 vaccines, thus failing to induce an optimal cell-mediated immunity. To circumvent this problem, non-human adenoviral vectors (*40*), and adenoviral vectors from rare human serotypes are being developed (*41, 42*). Also, a recent study has shown that following co-culture of $CD4^+$ T cells with Ad5-pulsed autologous DCs, there is a preferential expansion of HIV-1 susceptible $CD4^+$ T cells with gut homing specificity, thereby having the potential to increase the target cell population and compounding the risk of HIV infection *in vivo* (*43*).

Modified Vaccinia Ankara (MVA) was originally developed as an attenuated small pox vaccine by more than 500 passages in chick embryo fibroblasts, during which it suffered multiple deletions and lost the ability to replicate in human and most other mammalian cells (*44*). Because of a late stage block in its replication cycle (at the virus assembly stage), viral or recombinant gene expression is unimpaired, making MVA an efficient and also a safe live viral vector (*45*). Unlike the adenovirus, pre-existing immunity to MVA is largely restricted to individuals who have been vaccinated for small pox, which was stopped in 1970s. Preclinical data suggest that rMVA is a particularly effective agent for boosting T-cell responses (*46, 47*). It is an increasingly promising viral vector due to its marked immunogenicity when used as a boosting agent, its excellent safety profile in an immunocompromised macaque study (*48*) and its safety in humans (*49, 50*). This DNA/ MVA mode of vaccination strategy for HIV and AIDS is currently undergoing phase II human clinical trials conducted by Geovax Inc. in the United States.

The heterologous prime-boost (DNA prime followed by viral vector boost or two different viral vectors to prime and boost, that encode the same antigen) immunization

strategy raises higher magnitude and breadth of immune responses in comparison to corresponding homologous prime-boost modality. The good quality memory cells (mostly central memory phenotype) raised by a DNA prime, is further expanded by a viral vector boost. In preclinical studies, this vaccination modality has been successfully used for AIDS (*30*), malaria (*46*), tuberculosis (*47*) and cancer (*51*). Using challenge model in primates, it has been shown that though DNA prime/ MVA boost vaccination did not prevent infection, it did lower the viral load setpoints, increased CD4 counts, and reduced morbidity and mortality in vaccinated animals, compared to controls (*30, 52*).

DNA vaccines

Vaccination with purified plasmid DNA involves injection of the plasmid into rodents/ non-human primates/ humans to elicit an immune response to a protein that is encoded on the plasmid (*53*). These plasmids remain episomal and act as expression vectors. In contrast to viral vectors, protein production in response to DNA vaccines can focus the immune response more narrowly on the recombinant insert sequences. This modality of vaccination is relatively safe (there is little chance of causing any detrimental side-effects to the host since vaccine plasmids are non-replicating), can induce a broad spectrum of cellular and humoral immnune responses and do not have problems of pre-existing immunity, as seen with viral vectors like adenovirus. Because of the endogenous production of viral antigens following DNA immunization, the proteins are presented in their “native” form to the immune system, thus mimicking antigen presentation during natural infection. However, to elicit an optimal immune response, DNA vaccines had to

be given in large doses in higher animals (*54*), raising the concern of potential plasmid DNA integration into host genomic DNA. It is evident from various studies that DNA vaccines do not show any relevant levels of integration into host cellular DNA (*55, 56*). Neither is there any evidence of development of autoimmunity (anti-nuclear or anti-DNA antibodies) in association with DNA vaccines (*57*). Since DNA vaccination by itself did not prove to be as efficient in humans as it did in rodents (*58*), several optimization strategies have been undertaken to improve expression, potency and immunogenicity of DNA vaccines. Different delivery strategies (gene gun, electroporation, particle-mediated epidermal delivery etc.) are currently being optimized to improve the transfection efficiency of target cells. DNA alone is a weak immunogen (even in the presence of unmethylated CpG motifs that can signal through TLR9) and therefore it is important to adjuvant DNA vaccines to increase their potency and clinical utility.

B.3.i. Strategies to adjuvant DNA vaccines

Several strategies are currently under investigation to modulate the immune response induced by a DNA vaccine. One approach is to use codon-optimized genes to enhance protein expression (*59, 60*). Other strategies aim to modulate and enhance host immune response by including genetic adjuvant such as cytokines, chemokines and T-cell costimulatory molecules as a component of the vaccine itself. Immunogenicity of a DNA vaccine can be fine-tuned by several factors: (i) by recruiting of professional APCs (resulting in a higher *in vivo* transfection rate and consequently enhanced antigen presentation), (ii) inducing of “danger signals”, (iii) providing additional costimulation,

or (iv) by delivering greater survival signals to the responding antigen-specific T cells. Different adjuvants stimulate unique cytokine profile and driven by the differential cytokine expression profile by T cell subsets, the immune response is directed either towards cell-mediated, T-helper 1 (Th1) – type response, or an antibody-mediated, Th2-type response.

Many cytokine – encoding plasmids have been studied as potential adjuvants for DNA vaccines. Co-inoculation of a plasmid expressing GM-CSF (granulocyte macrophage – colony stimulating factor) with DNA vaccine for HIV-1 antigens boosted antibody responses and Th-proliferative responses as well as $CD8^+$ T cell responses (*61, 62*). Studies in rhesus macaques have also shown that adjuvanting a SHIV-89.6 DNA/MVA vaccine with GM-CSF DNA broadened the neutralizing Ab response, augmented avidity maturation of anti-Env IgG and conferred greater protection against challenge infection (*63*), (*64*)**.** The mechanism of adjuvant properties of plasmid encoding GM-CSF involve recruitment of professional APCs at the site of inoculation and also induce their maturation. Coimmunization of IFN-γ plasmid with HIV *env* and SIV *gag*/ *pol* expressing plasmids in rhesus macaques enhanced the antigen-specific antibody responses (*65, 66*). IFN-γ, a typical Th1 type cytokine, is produced by Th1, $CD8^+$ and NK cells, and has been shown to have antiviral effects as well as immunomodulatory properties such as up regulation of MHC class I and II antigens. The efficacy of IL-2 as an adjuvant for HIV DNA vaccine has also been evaluated. In one study it has been shown that immune responses elicited in rhesus macaques by DNA vaccines could be augmented approximately two-fold by the administration of IL-2/Ig fusion protein or the plasmid encoding IL-2/Ig and that cytokine-augmented DNA

vaccine immunized macaques could control viral replication to a greater extent than DNA-only immunized animals (*31*). Other cytokine-encoding plasmids (like IL-12, IL-15 and IL-18) have also been evaluated for their ability to act as an adjuvant for DNA vaccines. Studies have shown that co-administration of IL-12 with DNA plasmids encoding HIV-1 antigens resulted in a dramatic increase in CTL responses, T-cell proliferation and in some instances induced Ag-specific antibodies (*67, 68*).

There are several chemokine-encoding plasmids (IL-8, MIP-1α, RANTES) that have been tested as adjuvants for HIV-1 DNA vaccines (*69, 70*). Chemokines act primarily as chemoattractants and are important regulators of lymphocyte trafficking. Since HIV entry is dependent on chemokine receptors, co-administration of β-chemokines as adjuvants would exert antiviral effects through receptor competition, particularly during initial establishment of infection. Molecular adjuvants like mutant caspases (*71*) and interferon regulatory factors (IRFs) (*72*) were also used to adjuvant influenza DNA vaccines in mice.

B.3.ii. Costimulatory molecules as an adjuvant for DNA vaccines

Efficient T cell activation requires both MHC:peptide – TCR recognition (signal 1) as well as costimulatory signals (signal 2). Co-delivery of plasmids expressing costimulatory molecules is a strategy to enhance the antigen presentation capacity of transfected host cells (*73*). This is particularly true for intramuscular immunization as myocytes do not express costimulatory molecules (*74*). Also, studies have shown that optimal costimulation can decrease the activation threshold of responding T cells and this

can be of paramount importance in the context of chronic infections where T cell exhaustion is rampant.

Costimulatory molecules belong to either of the two classes: the immunoglobulin superfamily or the tumor-necrosis factor receptor (TNFR) superfamily. Dramatic increase in antigen-specific CTL activity and high level of proliferation was observed when CD86 plasmid was coimmunized with HIV-env (*73, 75*). Other than the classical costimulatory molecules, there are other additional cell surface molecules (such as adhesion molecules on APCs) that play critical roles both in cell migration and cell-cell contact during immune activation and expansion. It has been shown that coexpression of ICAM-1 or LFA-3 molecules along with HIV DNA immunogens resulted in a significant enhancement of antigen-specific T cell proliferative responses and when coinjected, ICAM-1 encoding plasmid resulted in dramatic enhancement of CTL responses (*76*).

Some of the costimulatory molecules belonging to the TNFR superfamily have also been tested as an adjuvant for DNA vaccines. Multimeric soluble CD40L-adjuvanted HIV-1 DNA vaccine could induce strong $CD8^+$ T cell response, although it did not elicit significant $CD4^+$ T cell or antibody response (*77*). In the same study, it was also shown that multimeric soluble form of GITRL (glucocorticoid-induced TNF-family-related receptor ligand) could enhance $CD4^+$ T cell proliferative responses as well as antibody responses to Gag DNA vaccines (*77*). In another study, the RANK-L gene was used as an adjuvant to enhance the induction of *T. cruzi* and malaria Ag-specific $CD8^+$ T cells by vaccination with plasmid DNA or recombinant influenza virus expressing the parasite antigens (*78*).

B.3.iii. The 4-1BB / 4-1BBL pathway

4-1BB (CD137), a type I transmembrane protein, is a member of the TNFR superfamily (TNFSF9) with several extracellular cysteine-rich domains. Though it is up-regulated primarily on activated $CD4^+$ and $CD8^+$ T cells, it is also present on activated NK cells, NK T cells (*79*), mast cells (*80*) and eosinophils (*81*). It is also found on a subset of splenic DCs and bone-marrow derived DCs in mice, and triggering through cell surface 4-1BB increased the secretion of IL-6 and IL-12 from DCs (*82*). 4-1BB can either exist as a monomer in the cell membrane or can self-oligomerize to form trimeric signaling complexes when interacting with its ligand. 4-1BB ligand (4-1BBL) is a type II transmembrane protein containing a TNF-homology domain that interacts with the cysteine-rich domain of 4-1BB (*83*). It is expressed on activated APCs such as DCs, macrophages and B cells (*79*) and also exists as self-assembling trimers (*84*). Surface expression of 4-1BB is transient, reaching the peak at about 48 hr post primary activation and declining by 4-5 days (*85, 86*). Since the expression of 4-1BB/ 4-1BBL are inducible upon activation, these interactions probably do not contribute much during initial antigen encounter, but play a more predominant role later to sustain the ongoing immune response. 4-1BB signaling occurs downstream of CD28 signaling and is mediated by an adapter protein TRAF2 (TNF receptor associated factor 2) (*87*). The N-terminal RING domain of the TRAF proteins is critical for downstream effector functions (*88*). Cross-linking of 4-1BB on T cell surface increased tyrosine phosphorylation of TCR-signaling molecules like CD3ε, CD3ζ, Lck, LAT (linker for activation of T cells) and SLP-76 (*89*). As seen with other TNFR family members, the 4-BB /4-1BBL system shares the ability of bi-directional signal transduction. It has been shown that interaction between 4-1BB

on monocytes and 4-1BBL on B cells, induces apoptosis in B cells (*90*). Though TCR: MHC interaction is mandatory, studies have shown that 4-1BB signaling can be achieved even in the absence of CD28 (*91, 92*), which can be very valuable in the context of chronic infections (like HIV) where CD28 negative T cells accumulate over time. Co-stimulation through 4-1BB results in the activation of NF-κB, JNK and p38 MAPK pathways, ultimately leading to the up-regulation of anti-apoptotic (*93*) and cytokine genes (*94*). The pro-survival signal delivered upon 4-1BB costimulation at the peak of effector phase during an immune response is critical for preventing activation induced cell death (AICD) of the effector population and thus generating a larger memory pool. There is evidence that 4-1BB /4-1BBL interaction interferes with AICD and promotes survival of T cells by inducing Bcl-x_L (an anti-apoptotic Bcl-2 family member that inhibits intrinsic pathway of apoptosis at the mitochondrial level), and cellular FLICE-inhibitory protein (c-FLIP that directly inhibits caspase-8 activity) (*95*). *In vivo* and *in vitro* studies in mice have shown that 4-1BB co-stimulated $CD8^+$ T cells secrete large amounts of IFN-γ and can develop into antigen-specific CTLs (*96*). Several studies have also suggested that 4-1BB /4-1BBL interactions are critical for generating memory CTL responses (*97-99*), thus making this costimulatory pathway an attractive target for immunotherapy and use as a vaccine adjuvant. The fact that autologous adherent monocytes could be converted into efficient APCs only after overnight incubation with recombinant adenovirus expressing 4-1BBL (thus bypassing the need to generate dendritic cells, a process that would take approx. 7 – 10 days) and that 4-1BB/4-1BBL mediated costimulation lowered the effective antigen dose by >100-fold for both influenza and EBV-specific responses, clearly underscores the potency of this

costimulatory pathway (*100*). Though several studies have suggested a more prominent role of 4-1BB on $CD8^+$ T cell activation, other studies have suggested that 4-1BB / 4-1BBL sustain both $CD4^+$ and $CD8^+$ T cell responses with similar efficacy (*91, 101*).

In preclinical cancer immunotherapy, eradication of established tumors has been successfully achieved by delivering costimulatory signals through 4-1BB (*102-105*). In antiviral immunity, 4-1BB mediated costimulation has been shown to broaden primary influenza-specific $CD8^+$ T cell response (*106*) and also enhance secondary responses to the same (*107*). Several studies indicate that anti-4-1BB or 4-1BBL therapy is likely to be most effective during the boost phase of a prime-boost vaccination strategy (*100, 107*). Timing of administration of agonist anti-4-1BB Abs also seems to be an important factor in determining the outcome of an antiviral response. In the LCMV Armstrong infection model (acute infection that is normally cleared within 14 days), when anti-4-1BB mAb was given at the time of infection, there was a complete suppression of antiviral T cell responses and persistence of LCMV Armstrong in mice. However, when the antibody treatment was initiated 3 days after infection, antiviral immunity was preserved and the virus was cleared (*108*).

The involvement of 4-1BB in HIV infection has also been investigated. HIV+ individuals had a greater percentage of 4-1BB expressing T cells (particularly $CD8^+$ T cells) in comparison to HIV negative controls (*109*). Also, the level of 4-1BB expression was inversely correlated with $CD4^+$ T cell counts. In addition, crosslinking of 4-1BB with agonistic mAb enhanced HIV replication (as measured by the reverse transcriptase activity), both in primary and secondary stimulation of $CD4^+$ T cells from HIV-1+ individuals. Thus, 4-1BB may be involved in activation of latently infected $CD4^+$ T cells.

A distinctive feature of 4-1BB mediated costimulation is that it can occur even in the absence of CD28 signaling. This is very crucial in the context of HIV infection where a predominance of CD28 negative anti-HIV CTLs has been seen to parallel disease progression. Synergising costimulation through 4-1BB and B7.1 (CD80) resulted in similar levels of T cell responses when long-term infected subjects were compared to those early in infection (*110*), thus representing a promising combination for therapeutic vaccination for HIV. Since 4-1BB seems to be a critical regulator of $CD8^+$ T cells responses, this molecule has been targeted in various vaccination strategies. When agonistic anti-4-1BB Ab was given at the time of boost in a DNA prime/ MVA boost vaccination strategy in mice, there was a 2 - 4 fold increase in Ag-specific $CD8^+$ T cells both at the peak and memory phase of the response (*111*). Another study in BALB/c mice has shown significantly enhanced anti- HIV $CD8^+$ T cell response when immunized with a rFPV (recombinant fowlpox virus) expressing murine 4-1BBL (*112*). However neither HIV-specific CD4 nor antibody responses were detected in these studies.

In contradiction to its positive regulatory role on T cells and its anti-tumor activity, 4-1BB mediated costimulation has also been shown to ameliorate or prevent various autoimmune conditions in mice (*113*). Administration of agonistic 4-1BB mAb significantly reduced the incidence of experimental autoimmune encephalomyelitis (EAE) by accelerating the activation-induced cell death (AICD) of Ag-specific $CD4^+$ T cells (*114*). In systemic lupus erythematosus (SLE), a $CD4^+$ T cell dependent autoimmune disease, treatment with agonist 4-1BB Ab suppressed autoantibody production by inducing anergy in $CD4^+$ T cells, thus resulting in withdrawal of CD4 help (*115*). In the arthritis model, immunotherapy with anti 41BB mAb suppressed serum

antibodies and antigen-specific $CD4^+$ T cell recall responses by an IDO-dependent mechanism that suppressed Ag-specific $CD4^+$ T cells (*116*).

B.4. Importance of targeting vaccine-raised HIV-specific T cells to the gut mucosa

Because of the abundance of activated memory T cells ($CD4^+$ $CCR5^+$) in the gastrointestinal tract, the gut is one of the major target organs for viral replication in HIV infection (*117*). DCs can further amplify the infection by binding and internalizing the virus through DC-SIGN, and carrying the virus to activated T cells (*118*). It is estimated that approximately 80% of $CD4^+$ T cells in the GALT (gut-associated lymphoid tissue) can be depleted in the first three weeks of HIV-1 infection. Though studies have shown that $CD4^+$ T cell numbers can return to near normal levels in blood at later time points due to redistribution from other tissues and new thymic emigrants, yet $CD4^+$ T cell reconstitution in the gut mucosa remains insufficient (*119, 120*). As a consequence of massive destruction of various cell types in the gut during early HIV-1 infection, development of gut germinal centers (critical for gut B cell responses) is also impaired (*121*).

Several studies (in macaques and humans) have shown that mucosal $CD8^+$ CTLs as well as antibody responses are important for protection against mucosal transmission of HIV. Passive transfer of potent neutralizing IgG monoclonal Abs could confer protection against oral and vaginal challenges in macaques (*122, 123*). From studies that show an inverse correlation between $CD8^+$ T cell count and viral titer, it can be inferred that control of both early and late-stage SIV infection is dependent on $CD8^+$ T cells (*124,*

125). In three cohorts of HIV-uninfected individuals who were repeatedly exposed to HIV through sexual intercourse, resistance to infection was associated with anti-gp160 IgA antibodies in urogenital tract secretions (*126-128*). Also, HIV-specific $CD8^+$ CTLs were found in the cervical mucosa of HIV-exposed seronegative prostitutes in Nairobi (*129*). Thus all evidences support the fact that for an effective control of the dissemination of the founder virus population at the portal of entry, it is absolutely critical to design vaccine strategies that will generate gut/ mucosa-resident or rapidly responding mucosal effector T and B cell population.

B.4.i. Induction of mucosal immune response

Organized mucosal inductive sites are concentrated at the portal of entry of pathogens (oral and nasopharynx) and at sites of high microbial density (lower intestinal tract). The best studied of the mucous membranes is that lining the gastrointestinal tract. The outer mucosal epithelial layer contains the intraepithelial lymphocytes (IELs). The lamina propria (LP), which lies under the epithelial layer, contains large numbers of B cells, plasma cells, activated T_H cells and macrophages in loose clusters. Finally, within the submucosal layer of the intestinal lining are nodules consisting of aggregates of organized lymphoid follicles called the Peyer's patches. The follicle-associated epithelium (FAE) of Peyer's patches can be differentiated from the nearby villous epithelium by the presence of antigen-sampling cells called the M cells. Foreign antigens (from the intestinal lumen) are delivered by vesicular transport directly into the intraepithelial pockets formed by the M cells and to the underlying DCs. The FAE of

Peyer's patches produce chemokines like MIP-3α and MIP-1γ that attract DCs and lymphocytes expressing CCR6 and CCR1, respectively. Accumulation of DCs in the FAE results in high phagocytic activity and thus elimination of pathogens at entry sites. DCs can also capture particles and live bacteria in the subepithelial dome (SED) regions of Peyer's patches and transport them to the adjacent interfollicular T-cell zones. Some Peyer's patch DCs can carry antigen to draining lymph nodes, where they interface with the systemic immune system.

B.4.ii. Adjuvants for mucosal vaccines and mucosal vaccination strategies

Natural transmission of many viruses occurs at a mucosal surface (genital, gastrointestinal or respiratory) and thus, prevention of mucosal transmission and dissemination to the regional lymph nodes is an important goal for any successful vaccine. Furthermore, an additional rationale for generating mucosal CTLs in the case of HIV/ SIV infection is to target the eradication of viral reservoir in the gut.

Mucosal immune responses and protection are highly dependent on mucosal adjuvants. Non-adjuvanted vaccines delivered to the mucosal surface fail to stimulate any immunogenic responses and are known to induce tolerance. Currently, various truncated and mutated forms of two bacterial toxins, namely, cholera toxin (CT) and *E.coli* heat-labile toxin (LT) are being used as mucosal adjuvants. In macaques, intranasal immunization with HIV-Env protein and adjuvant CTA1-DD (a combination of the enzymatically active A1 subunit of CT with a dimer of an Ig-binding element from *Staphylococcus aureus* protein A) induced significant humoral and cell-mediated

immunity against HIV-1 Env (*130*). In addition, peptides linked to heat-shock proteins (HSP70), delivered through a targeted iliac lymph node route, have been found to be effective mucosal adjuavnts (*131*). Various TLR ligands including CpG-containing oligodeoxynucleotides (ODN), flagellin and bacterial porins also manifest effective mucosal adjuvant function. In mice, intranasal coadministration of CpG (a TLR9 agonist) with SIV VLPs (virus like particles), showed promising mucosal adjuvant activity by enhancing both cellular and humoral immune responses (*132*). Intranasal immunization in mice with inactivated gp120-depleted HIV-1 Ag along with CpG ODN also induced a strong mucosal T cell response and conferred cross-clade protection against intravaginal challenge (*133*). Paradoxically, intravaginal application of imiquimod (a TLR7 agonist) and CpG ODN did not prevent SIV transmission in macaques due to the inflammation induced (thus resulting in more target cell availability) by these TLR agonists (*134*). Synergistic combination of cytokines such as IL-12 and GM-CSF (delivered intrarectally along with a HIV-1 IIIB peptide vaccine and CT) has also been shown to adjuvant mucosal immunity in mice. (*135*).

Mucosal vaccines and microbicides (agents that hinder HIV entry at mucosal surfaces) may act in synergy to confer mucosal protection against HIV/ SIV challenge. Though several promising microbicide candidates have failed to demonstrate efficacy in clinical trials (*136, 137*), yet, microbicide-mediated protection can boost vaccine-induced immune responses. Microbicides that include HIV entry inhibitors and broadly neutralizing human monoclonal Ab to HIV-1gp120 (b12), have shown promising results in nonhuman primate models in terms of reducing infectivity (*138, 139*). A more recent study with glycerol monolaurate (an antimicrobial compound with inhibitory activity

against MIP3α and other proinflammatory cytokines) has been shown to protect macaques from repeated intravaginal high dose exposure to SIV (*140*). A novel live microbcide using *Lactobacillus* has been developed by Osel Inc. (CA, USA). *Lactobacillus jensenii* 1153 (known to have good mucoadherence) was genetically modified to secrete two-domain CD4 (2D-CD4, a secreted form of CD4 that binds HIV-1gp120 to prevent viral entry) and cyanovirin-N (CVN, a protein-based HIV-microbicide that blocks membrane fusion and entry) (*141, 142*). Studies in macaques have demonstrated successful colonization with the CV-N-expressing construct, leading the way to future SHIV challenge experiments.

Delivery of mucosal vaccines is a challenge and thus vaccines that specifically target mucosal sites with particle-based systems are now being designed. As a proof-of concept, researchers have developed a rice based oral vaccine expressing cholera toxin B subunit (CTB) and when administered orally (in mice), these genetically modified rice seeds were taken up by the M cells (in Payer's patches) and could induce CTB-specific serum IgG and mucosal neutralizing IgA Abs (*143*). Another study in mice has also shown that by selectively targeting the M cells (by using mAbs that binds M cell-specific carbohydrate moieties) and directing antigen uptake by these cells, it is possible to induce a highly protective mucosal immune response (*144*).

B.4.iii. Mucosal trafficking of lymphocytes

Several studies in murine models have underscored the vital role played by mucosal APCs in imprinting gut homing phenotype on antigen-specific lymphocytes. A

series of studies have demonstrated that rodents deficient in vitamin A have reduced IgA-producing antibody secreting cells (ASC) and effector/ memory T cells in the intestinal mucosa (*145, 146*). A seminal study in murine model has shown that addition of retinoic acid (RA) can induce expression of the integrin α4β7 and chemokine receptor CCR9 on anti-CD3 and anti-CD28 stimulated $CD4^+$ and $CD8^+$ T cells (*146*). Both α4β7 and CCR9 are implicated in lymphocyte homing to the intestinal mucosa. The ligand for α4β7 is MADCAM1 (mucosal vascular addressin cell-adhesion molecule 1), which is expressed under steady-state as well as inflammatory conditions by endothelial cells in the intestinal tract and associated lymphoid tissue (*147, 148*). The ligand for CCR9, CC-chemokine ligand 25 (CCL25), is constitutively and selectively expressed by epithelial cells of the human and mouse small intestine and facilitates effector T cell recruitment to the small intestinal lamina propria and epithelium (*149, 150*). Intriguingly, it has also been reported that the α4β7 heterodimer expressed on gut homing T cells (as they transit through blood) can bind to HIV gp120 on infected CD^+ T cells, thus in turn facilitating cell-to-cell spread of the virus (*151*).

DCs mediate the generation of gut-homing T/ B cells in the draining intestinal lymph nodes. $CD8^+$ T cells, stimulated with peptide-pulsed mesenteric lymph node cells depleted of DCs, failed to express any gut-homing marker, thus emphasizing the necessity of DCs to imprint gut-homing potential on lymphocytes (*152*). Synthesis of retinoic acid depends on the oxidative metabolism of retinol to retinal (which requires alcohol dehydrogenases) and then conversion of retinal to retinoic acid (which requires retinal dehydrogenases (RALDH)). Though alcohol dehydrogenases are ubiquitously expressed in all DCs (peripheral as well as gut-resident), only Peyer's patch and

mesenteric lymph node DCs constitutively express RALDH and thus can synthesize retinoic acid. This unique property of gut-resident DCs enables them to imprint gut-homing specificity on the lymphocytes.

B.4.iv. Influence of route of immunization to induce mucosal immunity

Several studies have argued that a protective mucosal immune response can only be achieved by mucosal immunization through oral, intranasal (i.n.), intrarectal (i.r.) or intravaginal routes (*153-156*). Intranasal administration of vaccines in mice, macaques and humans has been shown to induce mucosal IgA antibody responses in the salivary glands, upper and lower respiratory tracts, and the male and female genital tracts (*157-159*). Interestingly, the nasal immunization route has also generated more impressive systemic immune responses than other mucosal routes. In intranasally vaccinated mice and macaques, splenic anti-viral CTL activity and levels of specific serum IgG have been reported comparable to those induced by intramuscular, intradermal, intraperitoneal, or intravenous injection (*160-163*). More importantly, nasal vaccination of macaques with Sabin poliovirus vectors expressing SIV *env*, *gag*, *pol* or a non-pathogenic *nef*-deleted SHIV has prevented vaginal transmission of pathogenic SIVmac251 or SHIV89.6P (*163, 164*). Oral and rectal immunization routes have also elicited $\alpha 4\beta 7$+ antigen-specific B cells in the circulation, IgA plasma cells in intestinal mucosa, and IgA antibodies in intestinal secretions (*165, 166*). In a macaque study, rectal immunization with a HIV peptide vaccine was shown to induce antiviral CTL responses in the rectal mucosa and also confer protection from a rectal challenge with pathogenic SHIV (*167*).

Designing mucosal vaccines (either given orally or deposited directly on mucosal surfaces) has been challenging in many aspects. Relatively large doses of antigen are required as vaccines administered *via.* this route can get diluted in mucous secretions, attacked by proteases and nucleases, and excluded by epithelial barriers. Also, orally delivered antigens are generally known to induce tolerance in the gut, resulting in a subdued immune response (*168*), and often adjuvants are necessary for mucosal vaccines to mount an effective innate and adaptive immune response.

More recent studies in murine and macaque models have shown that intramuscular immunizations with replication deficient/ attenuated viral vectors can elicit immune responses in the gut, suggesting that gut mucosal immunity is also achievable with a parenteral route of immunization (*169-171*). Intramuscular immunization with ALVAC-HIV-1 recombinant vaccine not only decreased the viral set point in blood as well as in mucosal sites, but also protected macaques from peripheral $CD4^+$ T cell depletion (*172*). However comparative studies in macaques have shown that mucosal delivery of vaccines was more efficacious than systemic in conferring protection. In one study with HIV/SIV peptide vaccine, unlike the intrarectally immunized animals that had undetectable plasma virus levels, subcutaneously immunized animals had residual viremia (*167*). Similarly, nasal but not intravenous administration of non-pathogenic SHIV in macaques was found to confer protection after vaginal challenge with a pathogenic virus (SHIV 89.6P) (*163*). Furthermore, some studies have also demonstrated that prime-boost combinations that involved both mucosal and systemic delivery of the vaccine, was most effective in generating a mucosal immune response (*173, 174*).

Optimal protection against most pathogens requires both mucosal as well as systemic immune response. It is crucial to consider various factors like the choice of vaccine vector, adjuvant and the route of immunization while designing a HIV vaccine to ensure long-term systemic and mucosal immunity to the virus as well as an effective control of the virus at the portal of entry.

My research addressed two aspects of HIV vaccine design: (a) Enhancing immunogenicity of a HIV-1 Gag DNA vaccine using a costimulatory molecule (discussed in Chapter 2)

and (b) Understanding the mechanism of how Ad5 vector-based vaccine can imprint gut homing specificity on antigen-specific $CD8^+$ T cells (discussed in Chapter 3).

Chapter 2

Adjuvantive effects of anti-4-1BB agonist Ab and 4-1BBL DNA for a HIV-1 Gag DNA vaccine: Different effects on cellular and humoral immunity

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2.1. Abstract

Plasmid DNA immunizations induce low levels but a broad spectrum of cellular and humoral immune responses. Here, we investigate the potential of co-stimulation through 4-1BB as an adjuvant for a HIV-1 DNA vaccine in mice. We designed plasmid DNAs expressing either the membrane bound or soluble form of 4-1BBL, and compared with the agonistic anti-4-1BB Ab for their ability to adjuvant the Gag DNA vaccine. Both, anti-4-1BB agonistic Ab as well as 4-1BBL DNA enhanced the Gag-specific cellular immune responses. However, in complete contrast to the agonistic Ab that suppressed humoral immunity to Gag, 4-1BBL DNA adjuvanted vaccines enhanced Gag-specific IgG responses. Importantly, the expression of Gag and 4-1BBL from the same plasmid was critical for the adjuvant activity. Collectively, our data suggest that for a HIV-1 vaccine where both antigen-specific cellular and humoral immunity are desirable, 4-1BBL expressed by a DNA vaccine is a superior adjuvant than anti-4-1BB agonistic Ab.

2.2. Introduction

Despite intense multifarious efforts over the past several years, a successful HIV vaccine still remains elusive. Of the many current vaccination strategies, DNA vaccination is relatively safe and can induce a broad spectrum of cellular and humoral immnune responses. Also, problems like pre-existing immunity, as seen with other viral vectors such as Adenovirus type 5 (Ad5), Adeno Associated Virus (AAV) and Modified Vaccinia Ankara (MVA) can be averted with DNA based immunizations. However, DNA alone is a weak immunogen and several strategies are currently under investigation to adjuvant DNA vaccines to increase their potency and clinical utility. One such approach is to modulate and enhance the host immune response by using genetic adjuvants such as cytokines, chemokines and T-cell co-stimulatory molecules as a component of the vaccine.

4-1BB (CD137), a type I transmembrane protein, is a member of the TNFR superfamily (TNFSF9) with several extracellular cysteine-rich domains. 4-1BB expression can be induced on CD4 and CD8 T cells upon activation (*175, 176*) and is also found on a subset of splenic and bone-marrow derived DCs, mast cells, natural killer (NK) cells and on human monocytes and eosinophills (*177*). Surface expression of 4-1BB on activated T cells is transient, reaching the peak at about 48 hrs post activation and declining by 4-5 days (*175, 178*). Co-stimulation through 4-1BB delivers pro-survival signals during the peak effector phase of an immune response and is important for preventing activation induced cell death of the effector population, thus generating a larger memory pool. Consistent with this, several studies have indicated that 4-1BB and 4-1BB ligand (4-1BBL) interactions are important for inducing robust CTL responses

and for the establishment of long-lived memory CTLs (*179-182*). All of these favorable effects on the final outcome of an immune response make this co-stimulatory pathway an attractive target for immunotherapy and adjuvanticity.

Because of its crucial role in the generation and sustenance of CD4 and CD8 T cell responses, the 4-1BB/ 4-1BBL co-stimulatory pathway has been exploited both for anti-tumor and anti-viral immunity (*183-186*). However, an interesting and important feature of this co-stimulatory pathway is that signaling through 4-1BB can also suppress the immune response. In contrast to its positive regulatory role on T cells and anti-tumor/ anti-viral activity, 4-1BB co-stimulation has also been shown to ameliorate or prevent various autoimmune conditions in mice by suppressing immune responses (*187-189*). Thus, given its conflicting role on immune regulation, it is important to modulate this pathway appropriately for the purpose of vaccine development where enhancement, and not suppression, of the immune response is desirable.

In this study, we investigated if the form of adjuvant could influence antigen-specific T and B cell responses elicited by a HIV-1 Gag DNA vaccine in mice. We designed plasmid DNAs expressing either membrane bound or soluble 4-1BBL, and compared with the agonistic anti-4-1BB Ab in their ability to adjuvant the DNA vaccine. Our results demonstrate differential adjuvantive effects on the cellular and humoral immunity by the agonistic Ab and ligand DNA. Our results also highlight that by changing the form (agonistic Ab vs. ligand DNA), the same molecular adjuvant can be exploited either to enhance or suppress the humoral arm of adaptive immunity and thus can have varied applications in anti-tumor or anti-viral vaccines or in autoimmune diseases.

2.3. Material and Methods

2.3.1. Immunizations

Female BALB/c mice of 6-8 weeks of age were purchased from Charles River Laboratories (Wilmington, Mass.). The DNA immunogen, pGA1/JS8 (*190*), expresses clade B consensus Gag. The MVA immunogen, MVA/HIV62 expresses HIV-1 clade B Gag-Pol and Env (*191*). Both DNA and MVA vaccines were administered intramuscularly. Agonistic anti-4-1BB Ab (clone 3H3) was given intraperitoneally at a dose of 200μg per mouse. Unless otherwise stated, for experiments with agonistic Ab, DNA vaccine was used at a dose of 100μg per mouse. For *in vivo* depletion of CD4 T cells, mice (n=3) were injected i.p. with 200μg of GK1.5 antibody one day before and after DNA prime along with or without the agonistic anti-4-1BBAb. Gag-tetramer specific CD8 T cells were evaluated in blood for both wild type and CD4 depleted mice at different time points following MVA boost. For experiments with ligand DNA, Gag, m4-1BBL and SPD-4-1BBL DNAs were used at a dose of 20μg per mouse, and the Gag-m4-1BBL DNA was used at a dose of 40μg per mouse. MVA vaccine was used at a dose of 10^6 pfu. All immunizations were performed in sterile PBS in a final volume of 100μl and 50μl was injected in each of the hind legs. Mice were cared for under guidelines established by the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals" using protocols approved by the Emory University Institutional Animal Care and Use Committee.

2.3.2. Construction of 4-1BBL DNA plasmids

cDNA encoding murine 4-1BBL was PCR amplified using specific primers. The oligonucleotide primers were designed to include NheI site in the sense primer (TATCGCTAGCATGGACCAGCACACACTTGATG) and AvrII site in the antisense primer (TATCCCTAGGTCATTCCCATGGGTTGTCGGG). The restriction enzyme (RE) -digested PCR fragment was ligated to the pGA1 expression vector at NheI and AvrII sites to create the m4-1BBL plasmid (GenBank Accession#: GQ258348). Both the m4-1BBL and the pGA1/JS8 plasmids were digested with NotI and PvuI and the 4-1BBL gene along with the CMV-IA promoter (insert) was ligated to the pGA1/JS8 expression vector to create the 4-1BBL and Gag co-expressing plasmid (Gag-m4-1BBL plasmid, GenBank Accession#: GQ258349). The insertion and orientation of the 4-1BBL cDNA in the expression vectors were confirmed by RE analysis. The SP-D (lung surfactant protein D) fragment was amplified from pSP-D-CD40L (*192*) using the following PCR primers: sense GGGGGCTAGCGAATTCCACCAGGAAGC; antisense CTCGGTGCGGCCATCAGGGAACAATGCAGC. The extracellular domain (ECD) of 4-1BBL was PCR amplified using CCCTGATGGCCGCACCGAGCCTCGGC (sense) and TATCCCTAGGTCATTCCCATGGGTTGTCGGG (antisense). The PCR products were gel purified and an overlap PCR was done to create the junction between SP-D on the 5' end and ECD of 4-1BBL on 3' end. The amino acid sequence at the junction between SP-D and murine 4-1BBL was KAALFPDG/ RTEPRPAL, where the N-terminal portion is from SP-D (amino acids 1 to 256 of GenBank protein sequence no. NP_033186) and the C-terminal portion is the extracellular sequence of murine 4-1BBL (amino acids 104 to 310 of GenBank protein sequence no. NP_033430.1). The overlap

PCR product (SPD-4-1BBL form) was ligated to the pGA1 expression vector between NheI and AvrII sites (GenBank Accession#: GQ258350).

2.3.3. Protein expression analysis of 4-1BBL

293T cells (human embryonic kidney cell line) were transfected with plasmid DNA using Lipofectamine 2000 following the manufacturer's guidelines (Life Technologies). Intracellullar expression of 4-1BBL and Gag were analyzed by flow cytometry using flurochrome-conjugated mAbs 4-1BBL-FITC (clone 19H3) and KC-57-PE (Beckman Coulter), respectively. To isolate virus-like particles (VLPs), supernatants from plasmid DNA transfected 293T cells were overlayed on a 20% sucrose layer and centrifuged in a Beckman SW41 rotor at 32,000rpm for 2hrs and pellet was resuspended in PBS. VLPs were diluted with sample buffer and were run on 4-15% acrylamide gels (Biorad). Gels were transferred to nitrocellulose membranes and probed sequentially with anti-4-1BBL polyclonal antibody (clone D-20, Santa Cruz Biotechnology), and anti-goat IgG-HRP (Santa Cruz Biotechnology). Western blots were developed using the chemiluminescence reagent (GE Healthcare). For detecting Gag, H12.5C mAb (NIH AIDS reagent resource) was used as the primary antibody and anti-mouse IgG-HRP (Sigma) was used as the secondary antibody. For detecting SPD-4-1BBL in 293T cell culture supernatants, purified anti-mouse 4-1BBL mAb (clone TKS-1) was used as the primary antibody and anti-rat IgG-HRP (Southern Biotech) was used as the secondary antibody. The polyclonal Ab was not used for this purpose, as this Ab was raised against a peptide mapping near the N-terminus of the 4-1BBL protein that had been deleted in the SPD-4-1BBL form.

Plasmid DNAs were grown in *E.coli* DH5α and purified using Endotoxin-free Gigaprep kits (Qiagen).

2.3.4. *In vitro* activity assay for 4-1BBL

Total splenocytes (7 X 10^6 cells/well) were stimulated with soluble anti-CD3 (clone 145.2C11, 1μg/ml) for 72 hrs at 37°C in 6-well plates. After 72hrs, cells were washed and surface expression of 4-1BB was confirmed by flow cytometry. 96 well plates were coated overnight with anti-CD3 (clone 145.2C11, 0.003μg/ml) and mouse IgG (1ug/ml). The 72hr stimulated splenocytes (0.5 x10^6 cells/well) were cultured with 50μl of 293T culture supernatants containing either membrane bound 4-1BBL (m4-1BBL) on VLP or SPD-4-1BBL for an additional 48hrs. Cells were pulsed with [^{3}H]thymidine (1 μCi/well) for the last 8 -10 hours before harvesting and proliferation was measured by scintillation counting.

2.3.5. Tetramer assays

For tetramer analyses, cells were stained using allophycocyanin (APC)-conjugated Gag-tetramer (NIH tetramer core facility), CD4-FITC, CD19-FITC, CD11a-PE and CD8-PerCP in 100μl of complete RPMI at 4°C for 30 min. Cells were washed twice with wash buffer (PBS with 2% FBS) and acquired (approx. 200,000 lymphocytes) on the FACS Calibur (Becton Dickinson) and analyzed using Flowjo software (Tree Star, San Carlos,

California). All antibodies were purchased from BD Pharmingen. CD8+ CD11a+ CD4- CD19- and Gag Tetramer+ cells were scored as tetramer positive cells.

2.3.6. Intracellular Cytokine Staining

Approximately $2x10^6$ splenocytes were stimulated in complete RPMI in a final volume of 200µl. 5 peptide pools specific for HIV-1 Gag HXB2, each containing 25 peptides (15mers overlapping by 11aa) at a final concentration 1µg/ml were used for stimulations. The stimulation medium was supplemented with 1µg/ml of anti-mouse CD28 and anti-mouse CD49d. Cells were cultured for 6 hours (at 37^oC) and Golgi stop (10µg/ml, BD Biosciences Pharmingen) was included for the last 4 hours. At the end of stimulation, cells were surface stained with anti-CD3-FITC and anti-CD8-PerCP at 4°C for 20 mins, washed with 2% PBS-FBS, and fixed and permeabilized with Cytofix-Cytoperm solution (Pharmingen). Cells were then washed twice with Perm wash and incubated with anti-mouse IFN-γ-APC and IL-2-PE at 4°C for 30 mins. Following incubation, cells were washed once with Perm wash and once with 2% PBS-FBS and resuspended in PBS with 1% paraformaldehyde (PFA) and analyzed by flow cytometry. All antibodies were purchased from BD Pharmingen.

2.3.7. Gag-specific ELISA

Sera from immunized mice were assayed by ELISA to measure Gag-specific Ab. Ninety-six well ELISA plates were coated overnight with HIV-1 HXB2-Pr55 (Chiron Corp.) at a concentration of 1μg/ml. Wells were blocked using non-fat dry milk and test sera were assayed at four-fold dilutions in duplicate wells. A standard curve was generated using anti-mouse IgG (2μg/ml, Sigma) followed by a two-fold dilution series of mouse IgG (Sigma) starting from 800ng/ml. Peroxidase conjugated anti-mouse IgG (Southern Biotech) was used to detect bound IgG for both the test sera and the standard curve. ELISA plates were read on a SPECTRAmax PLUS (Molecular Devices, CA). Standard curves were fitted and total Gag-specific IgG was determined using SOFTmax 2.3 software (Molecular Devices, CA).

2.3.8. Statistical analyses

Statistical analyses were done using Student's *t* -test or wilcoxon rank-sum test using S-PLUS software. Results are expressed as the means $\pm$ SEM. Each experiment was repeated at least twice (n = 3–10).

2.4. Results

2.4.1. Agonistic anti-4-1BB Ab adjuvants Gag-specific CD4 but not CD8 T cell responses following HIV-1 DNA prime

To evaluate the potential of agonistic anti-4-1BB Ab as an adjuvant for a DNA vaccine, we primed mice with 100μg of DNA vaccine expressing HIV-1 Clade B Gag either in the presence or absence of an agonistic anti-4-1BB Ab (Fig.2.1). The Gag-specific CD4 and CD8 T cell responses were analyzed by measuring IFN-γ production after *ex vivo* stimulation of splenocytes with a Gag peptide pool that encompasses the entire Gag protein (Fig.2.1A, lower panel). The Gag-specific CD8 responses in blood were also evaluated using a H-2K^d restricted Gag tetramer (Fig.2.1A, top panel). Since the agonist Ab treatment resulted in a marginally enlarged spleen during the peak immune response, we are reporting the absolute numbers and not frequencies of the antigen-specific cells in the anti-4-1BB agonist Ab adjuvanted and non-adjuvanted groups. As expected, the DNA prime induced low levels of Gag-specific CD4 as well as CD8 T cell responses (Fig.2.1B and 2.1C). However, the Gag-specific CD4 T cell responses were 6-fold higher in the adjuvant group compared to the non-adjuvant group (Fig.2.1B). This enhancement was observed both at the peak (week 2) as well as memory (week 12) time points following prime. In contrast to the CD4 responses, the Gag-specific CD8 responses (evaluated both by tetramer in blood as well as by intracellular IFN-γ staining in spleen) were comparable between the two groups following prime (Fig. 2.1C). These results demonstrate that co-administration of agonistic anti-4-1BB Ab enhances the antigen-specific CD4 but not CD8 T cell response elicited by a DNA vaccine following prime.

To further understand whether the observed preferential adjuvanticity of CD4 T cell response *in vivo* was due to a faster/higher expression of 4-1BB by these cells, we studied the kinetics of 4-1BB expression on CD4 and CD8 T cells following anti-CD3 stimulation *in vitro* (Fig.2.1D). Following stimulation, expression of 4-1BB peaked at 48hrs on both CD4 as well as CD8 T cells, and was marginally higher on CD8 than on CD4 T cells suggesting that the lack of adjuvant effect on CD8 T cells *in vivo* may not be due to their inability to upregulate the 4-1BB receptor.

2.4.2. Anti-4-1BB agonistic Ab lowers the DNA dose required for a DNA/MVA HIV vaccine

Since the agonistic anti-4-1BB Ab failed to shown any adjuvant effects on Gag-specific CD8 responses following a relatively high dose (100μg) DNA prime (Fig.2.1), we next investigated whether the DNA priming dose had any influence on the adjuvant effects of the agonist Ab. Three doses (1, 10 and 100μg) of Gag DNA were used for priming either in the absence or presence of anti-4-1BB agonistic Ab (Fig.2.2). We measured the Gag-specific CD8 T cell response in blood after the prime using the H-2K^d restricted Gag tetramer (Fig.2.2A). Following DNA prime, low levels of tetramer-specific CD8 T cells were detectable in the 10 and 100μg dose groups with no difference in magnitude between the adjuvanted and non-adjuvanted groups.

To better assess the differences in immune responses following DNA prime, we boosted these mice with a recombinant MVA expressing HIV-1 clade B Gag, that is known to boost DNA primed responses by more than 10-20 fold (*193*). We did not use

the DNA to boost because a homologous DNA prime/ DNA boost for 1 and 10 μg priming doses could have yielded very low levels of Gag-specific T cells. The Gag-specific CD4 and CD8 T cells were analyzed by intracellular IFN-γ staining following the boost (Fig.2.2B, 2.2C and 2.2D). At 1 week following the MVA boost, the Gag-specific IFN-γ producing CD4 T cell responses were about 5-fold higher at all doses tested in the adjuvanted groups compared to the respective non-adjuvanted group (Fig.2.2C). However, for Gag-specific CD8 responses, enhancement was observed only at lower doses (1 and 10 μg) and not at the highest dose (100μg) when evaluated both by Gag-tetramer specific as well as by Gag-specific IFNγ producing CD8 responses (Fig.2.2D). Interestingly, the frequency of Gag-specific CD8 T cells in the adjuvanted group that received 1μg of DNA was similar to the frequency of Gag-specific CD8 T cells in the non-adjuvanted group that received 100 μg of DNA suggesting that 4-1BB co-stimulation lowered the DNA dose required for priming by 100-fold in CD8 T cells. These results demonstrate that agonistic anti-4-1BB Ab when given during a DNA prime can enhance both antigen-specific CD4 as well as CD8 T cell responses after a MVA boost and suggest that the enhanced CD4 help primed in the adjuvanted group could have contributed for enhanced CD8 T cell response/survival following the boost. Consistent with this hypothesis, transient depletion of CD4 T cells during DNA prime abrogated the adjuvant effects of agonistic anti-4-1BB Ab following the MVA boost (Fig.2.2E).

2.4.3. Construction and *in vitro* characterization of DNA plasmids expressing different forms of 4-1BBL

DNA vaccines are more practical than Ab based treatments in terms of affordability for the purpose of mass vaccination. So, we explored the possibility of using 4-1BBL expressed by DNA as an adjuvant for HIV-1 DNA vaccine. *In vivo*, 4-1BBL exists as a trimer and can be either membrane bound or secreted. Trimerization of the ligand is important for its co-stimulatory activity (*194*). We constructed DNA plasmids that express the full-length cell membrane bound 4-1BBL (m4-1BBL) or the secreted multimeric form of 4-1BBL. The m4-1BBL was expressed both in *trans* (on separate plasmids; m4-1BBL-*trans*) as well as in *cis* (on the same plasmid; Gag-m4-1BBL-*cis*) with respect to Gag expression. For the secreted, multimeric form of the ligand, we fused the extracellular domain of 4-1BBL with the multimerization domain of the lung surfactant protein D (SPD-4-1BBL). This construct is expected to produce a dodecameric form (4 molecules of 4-1BBL trimers covalently linked to each other) of 4-1BBL and will be secreted into the extracellular milieu as has been shown for CD40L previously (*192*) (Fig.2.3A).

The intracellular expression of Gag and 4-1BBL was confirmed using antibodies specific to respective proteins (Fig.2.3B). As expected, the Gag plasmid expressed only Gag, the m4-1BBL and SPD-4-1BBL plasmids expressed only 4-1BBL, and the Gag-m4-1BBL plasmid expressed both Gag and 4-1BBL. In Gag-m4-1BBL plasmid DNA transfected cultures, about fifty-percent of the Gag-positive cells also co-expressed 4-1BBL. To test for *in vitro* biological activity of the membrane bound 4-1BBL (m4-1BBL) and SPD-4-1BBL forms, we performed a thymidine proliferation assay that

measures the co-stimulatory potential of 4-1BBL following anti-CD3 stimulation (Fig. 2.3C). We observed a 2.2-fold and 5-fold enhancement in T cell proliferation with the supernatant from 293T cells transfected with Gag-m4-1BBL and SPD-4-1BBL DNAs respectively. As expected, enhancement in proliferation was not observed with supernatant from cells transfected with the m4-1BBL plasmid. The m4-1BBL plasmid expresses only the membrane bound ligand and not the Gag, and thus the adjuvant remains cell-associated. In the Gag-m4-1BBL construct, we co-expressed the membrane bound form of 4-1BBL with HIV-1 Gag that is known to form virus-like particles (VLP) (*190*) (Fig.2.3A). This construct is expected to produce VLPs that incorporate multiple trimers of 4-1BBL on their surface and are secreted into the extracellular milieu.

To further confirm the incorporation of 4-1BBL in the Gag VLPs, virus-like particles from cell culture supernatants were purified over a sucrose cushion and tested for expression of 4-1BBL and Gag by Western blot analyses. Both the 4-1BBL and Gag proteins were detected in the VLPs that were generated upon transfection of 293T cells with Gag-m4-1BBL DNA, indicating the co-localization of the antigen and adjuvant (Fig.2.3D). As expected, the Gag protein was detected in VLPs made from cells transfected with the Gag plasmid. Also, only the Gag protein was detected in VLPs that were purified from cells co-transfected with DNA plasmids expressing the Gag and SPD-4-1BBL (data not shown). Since the SPD-4-1BBL plasmid expresses a secreted form of the ligand, the Gag VLPs are not expected to incorporate any ligand as it buds from the cell membrane. However, 4-1BBL protein was detected in 293T cell supernatant that was transfected with the DNA plasmid expressing SPD-4-1BBL (Fig.2.3E).

2.4.4. Membrane bound 4-1BBL expressed in *cis* with Gag enhances the magnitude of Gag-specific CD8 T cell responses

To test for the *in vivo* adjuvant potential of DNA vaccines expressing the two forms of 4-1BBL, BALB/c mice were primed and boosted with DNA plasmids expressing either the membrane bound 4-1BBL (m4-1BBL) or soluble SPD-4-1BBL (Fig.2.4). Immunizations with the m4-1BBL form compared 4-1BBL expression in *cis* (Gag and 4-1BBL on the same plasmid) as well as in *trans* (Gag and 4-1BBL on separate plasmids but co-injected) with respect to co-expression with Gag. Similarly, we also performed immunizations with SPD-4-1BBL form expressed in *cis* as well as in *trans* with respect to Gag. However, the expression of SPD-4-1BBL was significantly lower in the *cis* form compared to the *trans* form. Thus we are not reporting the immunogenicity data for the *cis* form in this study. The Gag, m4-1BBL (in *trans*) and SPD-4-1BBL DNAs were used at a dose of 20μg per mouse, and the Gag-m4-1BBL DNA (in *cis*) was used at a dose of 40μg per mouse. Since a high Gag DNA priming dose (100μg) failed to adjuvant Gag-specific CD8 responses (as evident from the *in vivo* data with the agonist antibody), we chose a low Gag DNA dose (20μg) for immunizations along with the ligand DNAs.

Following DNA prime, we measured the frequency of Gag tetramer-specific CD8 T cells in the blood. These analyses demonstrated marginally higher frequencies of these cells in the m4-1BBL-*cis* group but not in m4-1BBL-*trans* and SPD-4-1BBL-*trans* groups (data not shown). At one week after the DNA boost, Gag tetramer-specific CD8 T cell responses were higher in the m4-1BBL-*cis* (7-fold) and SPD-4-1BBL-*trans* (4.6-fold) groups than in the non-adjuvanted controls (Fig.2.4A). However, at 2 weeks after

boost, only the m4-1BBL-*cis* group maintained higher (2.2-fold) frequencies of Gag tetramer-specific CD8 T cells. In contrast to the m-4-1BBL-*cis* group, adjuvancity was not observed in the m4-1BBL-*trans* or the SPD-4-1BBL-*trans* group. Consistent with tetramer analyses, the frequency of IFN-γ producing CD8 T cells was 2.5-fold higher in the m4-1BBL-*cis* group than in the non-adjuvanted controls (Fig.2.4B). In addition, the frequency of Gag-specific CD8 T cells that co-produce IFN-γ and IL-2 were also 2-fold greater (Fig.2.4C, D) in the m4-1BBL-*cis* group. As observed with the tetramer analyses, the SPD-4-1BBL-*trans* and m4-1BBL-*trans* groups failed to show any augmented cytokine response. These results demonstrate that the membrane bound 4-1BBL (m4-1BBL) expressed in *cis* but not in *trans* with Gag can augment antigen-specific CD8 T cell responses elicited by a DNA vaccine. Interestingly, though the SPD-4-1BBL protein showed the highest level of adjuvancity in an *in vitro* proliferation assay (Fig.2.3C), yet, it failed to augment any antigen-specific CD8 T cell responses *in vivo.*

2.4.5. Rapid expansion of Gag-specific CD4 T cells in the 4-1BBL adjuvanted groups

As seen with the Gag-specific CD8 T cell response, the Gag-specific CD4 T cell responses were also augmented by 4-1BBL mediated co-stimulation. At one week following the DNA boost, the frequency of IFN-γ producing CD4 T cells in the m4-1BBL-*cis* (4-fold) and the secreted SPD-4-1BBL-*trans* form (2.3-fold) adjuvanted groups were higher compared to the non-adjuvanted controls (Fig.2.5A). Similarly, the frequency of Gag-specific CD4 T cells that co-produced IFN-γ and IL-2 (Fig.2.5B) was also 4-fold and 2.4-fold higher in the m4-1BBL-*cis* and SPD-4-1BBL-*trans* form

adjuvanted groups, respectively. However, by 2 weeks post boost, these responses further expanded in the SPD-4-1BBL-*trans* form adjuvanted and non-adjuvanted groups but not in the m4-1BBL-*cis* adjuvanted group. As a result, the frequency of Gag-specific CD4 T cells was similar between the adjuvanted and non-adjuvanted groups at 2 weeks post boost. Also, at 2 weeks post boost, there was no difference in the magnitude of antigen-specific CD4 responses between the non-adjuvanted controls and the m4-1BBL-*trans* group. These results demonstrate that adjuvanting DNA with the 4-1BBL results in a more rapid expansion of vaccine-specific CD4 T cells rather than an overall increase in the magnitude of this response.

2.4.6. Gag-specific IgG response: Suppression with agonistic anti-4-1BB Ab and augmentation with membrane bound 4-1BBL DNA expressed in *cis*

To investigate whether 4-1BB/ 4-1BBL-mediated co-stimulation could also adjuvant the humoral immune response, sera from vaccinated mice were assayed for titer of Gag-specific binding Ab. Mice that were primed with DNA and boosted with MVA generated Gag-specific Ab response with a mean titer of 176 μg/ml of serum at 1 week after the boost (Fig.2.6A). However, administration of anti-4-1BB agonistic Ab during the DNA prime resulted in a near complete loss of Gag-specific Ab response. These results demonstrate that co-delivery of anti-4-1BB agonistic Ab during DNA prime enhances the magnitude of cellular immune response but diminishes the humoral immune response. In contrast to the agonistic Ab, co-delivery of 4-1BBL DNA during DNA prime not only preserved the Gag-specific Ab response but also resulted in 1.8-fold

enhancement following the MVA boost (Fig.2.6B). Similarly, a 6-fold increase in Gag-specific IgG response was also observed when mice were primed and boosted with DNA co-expressing Gag and 4-1BBL (Fig.2.6C). Unlike the enhanced humoral immune responses seen in the m4-1BBL-*cis* adjuvanted group, no augmentation in the Gag-specific Ab responses was observed in the m4-1BBL-*trans* or SPD-4-1BBL-*trans* form adjuvanted groups (Fig.2.6C). In fact, a trend towards diminished Ab response was observed in the SPD-4-1BBL-*trans* group compared to the non-adjuvanted control group. These results clearly demonstrate that the anti-4-1BB agonistic Ab and 4-1BBL DNA have opposing effects on the humoral immunity, the former suppressing the response while the latter enhancing the response.

2.5 Discussion

The principal goal of this study was to target the 4-1BB/4-1BBL co-stimulatory pathway to adjuvant a HIV DNA vaccine. Several pre-clinical studies have suggested that a strong CTL response is the basis for 4-1BB mediated anti-tumor activity and currently humanized anti-4-1BB monoclonal Abs are also in clinical trials in patients with solid tumors. Given the fact that a strong CTL response is also desirable for an effective anti-HIV immunity, we hypothesized that modulating this co-stimulatory pathway could be a viable option for designing HIV vaccines. In this study, we compared and contrasted the antigen-specific cellular and humoral immune responses raised when 4-1BB

co-stimulation is either delivered by an agonistic antibody or by 4-1BBL expressed by a DNA vaccine. Our results demonstrate that while both forms augment the Gag-specific CD8 and CD4 T cell responses, only the ligand form augments the Gag-specific Ab response. In fact, the agonistic Ab suppressed the Gag-specific humoral immune response. These results demonstrate that 4-1BBL rather than anti-4-1BB agonistic Ab is an ideal adjuvant for DNA vaccines for enhancing their immunogenicity.

One of the important findings of our study is that the m4-1BBL-*cis* group but not m4-1BBL-*trans* group enhances immunogenicity *in vivo* demonstrating that for the membrane bound form of the ligand, it is important that both adjuvant and antigen are expressed by the same antigen presenting cell for enhancing vaccine-specific cellular immune responses. *In vitro*, we observed similar levels of co-expression of Gag and 4-1BBL when 293T cells were either co-transfected with both m4-1BBL and Gag plasmids (4-1BBL in *trans*) or with the Gag-m4-1BBL plasmid (4-1BBL in *cis*) (data not shown). Since *in vivo* transfection efficiency is much lower than *in vitro* transfection efficiency,

one possibility for the reduced *in vivo* immunogenicity with the *trans* form could be due to the fact that though the antigen and the adjuvant plasmids were pre-mixed and co-injected in mice, yet *in vivo*, they were not taken up by the same APC simultaneously. To study this phenomenon, we attempted to detect CD11c positive dendritic cells (DC) co-expressing Gag (intracellular) and 41BBL (surface) in the draining lymph nodes at day 3 following vaccination using antibodies specific to respective proteins. We were successful in detecting 4-1BBL positive cells but not Gag positive cells (data not shown). However, the frequency of 4-1BBL positive DCs were higher in mice that were vaccinated with either of the membrane bound form of 4-1BBL expressing plasmids (in *cis* or in *trans*) compared to mice vaccinated with Gag DNA alone suggesting that the failure to enhance the immunogenicity by m4-1BBL plasmid when delivered in *trans* with respect to Gag was not due to the presence of lower levels of 4-1BBL positive DCs in the draining lymph nodes following vaccination.

Previous studies using other members of the TNF superfamily such as FasL, CD40L and GITRL demonstrated that multimerization is an important aspect for their biological activity (*192, 195, 196*). We created the SPD-4-1BBL plasmid to investigate if multimerization had any additive effect on the adjuvant potential of 4-1BBL. In addition, we anticipated that since it is a secreted form of the ligand, co-expression along with Gag antigen (*cis* form) may not be a requirement. Unexpectedly, though the soluble SPD-4-1BBL form demonstrated higher levels of *in vitro* co-stimulatory activity in comparison to the m4-1BBL-*cis* form, yet *in vivo*, the latter had a greater adjuvant effect. The reason for this disagreement between *in vitro* and *in vivo* activity is unclear. However, our data suggest that both for the membrane bound form as well as the multimeric secreted form

of the ligand, it may be crucial to co-express the antigen and the adjuvant in *cis* to achieve any *in vivo* adjuvancity.

One of the important findings in our study is that 4-1BB mediated co-stimulation, in particular with the agonistic Ab, augments Ag-specific CD4 T cell responses primed by a DNA vaccine and this enhanced CD4 help contributes to the enhanced magnitude of Ag-specific CD8 T cell responses following the boost. *In vitro,* both CD4 and CD8 T cells can respond to 4-1BB mediated co-stimulation (*178, 197, 198*). However, *in vivo* studies in murine antiviral models have shown that 4-1BB mediated co-stimulation primarily augments CTL responses (*185, 199, 200*). Consistently, studies in mice using either the agonistic anti-4-1BB Ab or the 4-1BBL along with poxvirus vectors expressing HIV-1 immunogens also demonstrated an enhancement in antigen-specific CD8 T cell responses but not in CD4 T cell responses (*201, 202*). Collectively, these results suggest that the 4-1BB-mediated co-stimulation may be more effective in adjuvanting Ag-specific CD4 T cell responses when administered along with DNA but not with viral vectors. However, in a recent study in macaques that administered three doses of agonistic anti-4-1BB Ab during prime along with a SIV Gag DNA vaccine, enhancement was observed for Gag-specific CD8 and not for CD4 T cell responses (*203*). Unlike in our study, where the adjuvant was given at the same time as the DNA prime, the agonistic anti-4-1BB Ab treatments were administered between 12-19 days following prime in the macaque study. We thus suggest that it may be important to deliver the agonistic Ab or 4-1BBL simultaneously with the DNA prime to be able to augment Ag-specific CD4 T cell responses.

Suppressive effects of anti-4-1BB agonistic Ab on antibody responses are well documented and this phenomenon have been therapeutically used in several autoimmune disease models to inhibit immune responses (*188, 204*). B cells do not express 4-1BB and effects of 4-1BB co-stimulation on humoral immunity have been shown to be mediated through other cell types. Studies have shown that this suppression of humoral immunity can be mediated by either diminished CD4 helper responses or by CD4-independent mechanisms. The proposed CD4-dependent mechanisms include, anergizing the CD4 T cells during antigen priming (*188*), induction of suppressor CD8 T cells (*189*), expansion of CD4+ regulatory T cells (*205*) and generation of TGFβ secreting effector CD8 T cells (*206*). The CD4-independent mechanisms include, diminished dendritic cell networks in B cell follicles (*207*) and direct killing of B cells by activated monocytes (*208*), although the latter has only been shown for human B cells. In our study, we augmented the Gag-specific Th1 CD4 T cell responses upon anti-4-1BB agonistic Ab treatment suggesting that the suppression of Gag-specific IgG responses was mediated through some CD4-independent mechanisms.

To further investigate whether the agonistic Ab mediated suppressive effect on IgG responses was due to a block in isotype switching, we evaluated the titer of Gag-specific IgM in serum. Serum IgM levels after a single DNA immunization was low in both the agonistic Ab treated and untreated mice, but were similar between adjuvanted and control groups (data not shown) suggesting that impairment in isotype class switching of Ag-specific B cells could have contributed to the suppressive effect of agonistic Ab on humoral immune response.

In conclusion, our results demonstrate that both anti-4-1BB agonistic Ab as well as 4-1BBL DNA can enhance the antigen-specific cellular immune responses elicited by a DNA vaccine. Our results also demonstrate that administration of agonistic Ab but not the ligand DNA results in suppression of antigen-specific humoral immunity. Collectively, our data suggest that for a HIV-1 vaccine, 4-1BBL expressed by a DNA vaccine is a superior adjuvant than the anti-4-1BB agonistic Ab. These results highlight that by changing the form (agonistic Ab vs. ligand DNA), the same molecular adjuvant can be exploited either to enhance or suppress the humoral arm of adaptive immunity and thus can have varied applications in anti-tumor or anti-viral vaccines or in autoimmune diseases.

2.6. Legends to the Figures

Figure 2.1: Agonistic anti-4-1BB Ab adjuvants the Gag-specific CD4 but not the CD8 T cell responses following HIV-1 DNA prime. BALB/c mice (n=3) were primed with HIV-1 Gag DNA vaccine in the presence or absence of agonistic anti-4-1BB Ab. ***A***, Representative FACS plot showing the frequency of Gag-tetramer specific CD8 T cells in blood (top panel) and Gag-specific CD4 and CD8 T cell responses in spleen (lower panel) at 2 weeks following the DNA prime. In the lower panel representative plot, the left quadrants represent CD4 T cells (CD3+, CD8-) and right quadrants represent CD8 T cells (CD3+, CD8+). Numbers indicate Gag-specific IFN-γ^+ CD4 or CD8 T cells expressed as a percent of respective total cells. ***B and C***, Summary of Gag-specific CD4 and CD8 T cells respectively following DNA prime. Bars represent the mean absolute number ± SEM per spleen. ***D***, Kinetics of 4-1BB expression on CD4 and CD8 T cells. Total splenocytes were activated *in vitro* with soluble anti-CD3 for the indicated time periods. Cells were harvested and 4-1BB expression on CD4 (CD3+ CD4+) and CD8 (CD3+ CD8+) T cells were analyzed by flow cytometry. Open histograms correspond to cells stained with anti-4-1BBAb and shaded histograms correspond to cells stained with anti-rat IgG2a isotype control. Percentage of CD4+ 4-1BB+ or CD8+ 4-1BB+ T cells are indicated on the plot. *, $p < 0.05$.

Figure 2.2: Anti-4-1BB agonistic Ab lowers the DNA dose required for a DNA/MVA HIV vaccine. BALB/c mice (n=5) were primed with three different doses of HIV-1 Gag DNA vaccine in the presence or absence of agonistic anti-4-1BB Ab at week 0 and

boosted at week 12. ***A*,** Gag-tetramer specific CD8 T cells in blood were analyzed at 2 weeks following the DNA prime. The bars represent the means ± SEM. ***B***, Representative FACS plot showing Gag-specific CD4 and CD8 T cell responses at one week following the MVA boost. Left quadrants represent CD4 T cells (CD3+, CD8-) and right quadrants represent CD8 T cells (CD3+, CD8+). Numbers indicate Gag-specific IFN-γ^+ CD4 or CD8 T cells expressed as a percent of respective total cells. Gag ID: Gag immunodominant peptide; PP3: Peptide Pool 3; PP4: Peptide Pool 4. ***C***, Summary of Gag-specific CD4 T cells at 1 week following the MVA boost. Data from two independent experiments (Exp.1 and Exp.2) with the 10μg and 100μg DNA priming doses are shown. Bars represent the mean absolute number per spleen (spleens from each group were pooled for analyses except for Exp.2 with 100μg DNA priming dose). ***D***, Summary of Gag-specific CD8 T cells analyzed at 1 week following the MVA boost. *Left panel*, Gag-tetramer specific CD8 T cells in blood (pooled data). *Right panel*, Mean absolute number of IFN-γ producing CD8 T cells per spleen is plotted (spleens from each group were pooled for analyses except for Exp.2 with 100μg DNA priming dose). Data from two independent experiments (Exp.1 and Exp.2) with the 10μg and 100μg of DNA priming dose are shown. ***E***, Adjuvant effect of anti-4-1BB agonistic Ab is CD4 dependent. For *in vivo* depletion of CD4 T cells, mice (n=3) were injected intraperitoneally with 200μg of GK1.5 antibody one day before and after DNA prime along with or without the agonistic anti-4-1BBAb. Gag-tetramer specific CD8 T cells were evaluated in blood for both WT and CD4 depleted mice at the indicated time points following MVA boost. The results are represented as means ± SEM. *, $p < 0.05$.

Figure 2.3: Construction and *in vitro* characterization of plasmid DNAs expressing different forms of 4-1BBL. ***A***, Schematic of the plasmid DNA constructs. The Gag expression vector consists of a HIV-1 Clade B consensus Gag sequence. The m4-1BBL plasmid consists of the membrane-bound (m) 4-1BBL. The Gag-m4-1BBL plasmid consists of the membrane-bound (m) 4-1BBL and the HIV-1 Clade B Gag genes. The SPD-4-1BBL plasmid consists of the extracellular domain of 4-1BBL fused to the multimerization domain of SP-D. CMV-IA: cytomegalovirus immediate-early promoter plus intron A; BGH polyA: bovine growth hormone polyadenylation signal; Kan^r: Kanamycin resistance. ***B***, *In vitro* expression of Gag and 4-1BBL. 293T cells were transfected with the respective plasmid DNA constructs and intracellular expression of 4-1BBL and Gag was analyzed by flow cytometry at 36 hrs post transfection. No DNA was added to the mock well. ***C***, *In vitro* biological activity of different forms of 4-1BBL. Activated T cells were stimulated with immobilized anti-CD3 (0.003μg/ml) and 50ul of supernatant from 293T cells transfected with respective DNA plasmids. *In vitro* proliferation was measured at 48 hrs using [^{3}H] incorporation. This experiment was repeated thrice. *, $p < 0.05$. ***D***, Western blot analysis of 4-1BBL expression in VLPs. VLPs, from the culture supernatant of DNA transfected 293T cells, were purified by ultracentrifugation and western blotting was performed using a polyclonal anti-4-1BBL Ab and H12.5C mAb to detect 4-1BBL and Gag proteins, respectively. Positive Control for 4-1BBL gel: Gag-m4-1BBL cell lysate. Positive control for Gag gel: Gag cell lysate. ***E***, Western blot analysis of 4-1BBL in culture supernatant from 293T cells transfected with SPD-4-1BBL plasmid. For detecting soluble 4-1BBL, purified anti-mouse 4-1BBL mAb was used as the primary antibody.

Figure 2.4: Enhanced magnitude of Gag-specific CD8 T cell responses in the m4-1BBL-*cis* adjuvanted group following DNA vaccination. BALB/c mice (n=10) were primed and boosted with indicated DNAs at week 0 and week 4 respectively. ***A***, Gag-tetramer specific CD8 T cells in spleen. ***B***, Gag-specific IFN-γ producing CD8 T cells in spleen as evaluated by intracellular cytokine staining. ***C***, Representative FACS plots showing Gag-specific CD8 T cells that co-produce IFN-γ and IL-2 (cells were gated on CD3 and CD8). Numbers indicate Gag-specific CD8 T cells either secreting IFN-γ or IFN-γ and IL-2 expressed as a percent of total CD8 T cells. ***D***, Summary of the frequency of IFN-γ^+ IL-2^+ CD8 T cells in spleen. The bars represent mean frequency ± SEM. The Gag group was immunized with 20μg of Gag plasmid and the m4-1BBL-*cis* group with 40μg of Gag-m4-1BBL plasmid. The m4-1BBL-*trans* and SPD-4-1BBL-*trans* groups were immunized with 20μg of the respective plasmids pre-mixed with 20μg of Gag plasmid. *, $p < 0.05$; and **, $p < 0.001$. NA, Not available.

Figure 2.5: Rapid expansion of Gag-specific CD4 T cells in the m4-1BBL-*cis* and SPD-4-1BBL adjuvanted groups. BALB/c mice (n=5) were primed and boosted with indicated DNAs at weeks 0 and 4, respectively. ***A***, Summary of Gag-specific IFN-γ producing CD4 T cells in spleen as evaluated by intracellular cytokine staining. ***B***, Summary of Gag-specific IFN-γ^+ IL-2^+ CD4 T cells in spleen. The bars represent mean frequency ± SEM. *, $p < 0.05$. NA, Not available.

Figure 2.6: Gag-specific IgG response: Suppression with agonistic anti- 4-1BB Ab, while preservation/augmentation with membrane bound 4-1BBL DNA expressed in *cis*. *A*, BALB/c mice (n=3) were primed with 100μg of HIV-1 Gag DNA vaccine with or without the agonistic anti-4-1BB Ab at week 0 and boosted with MVA-Gag at week 12. Sera were collected at 1 week after the boost and total Gag-specific IgG was determined by ELISA. ***B***, Mice (n=10) were primed with plasmid DNA (20μg) at week 0 and boosted with MVA-Gag at week 4. Gag-specific IgG was determined by ELISA at one week following the boost. ***C***, Mice (n=10) were primed and boosted with plasmid DNA (20μg) at week 0 and at week 4 respectively. Total Gag-specific IgG was evaluated at two weeks after the DNA boost. Sera from unimmunized naïve mice were used as negative control. The bars represent mean ± SEM of Gag-specific IgG titer in serum. *, p= 0.05. NA, Not available.

2.7. Figures

Fig.2.1. Agonistic anti-4-1BB Ab adjuvants the Gag-specific CD4 but not CD8 T cell responses following HIV-1 DNA prime

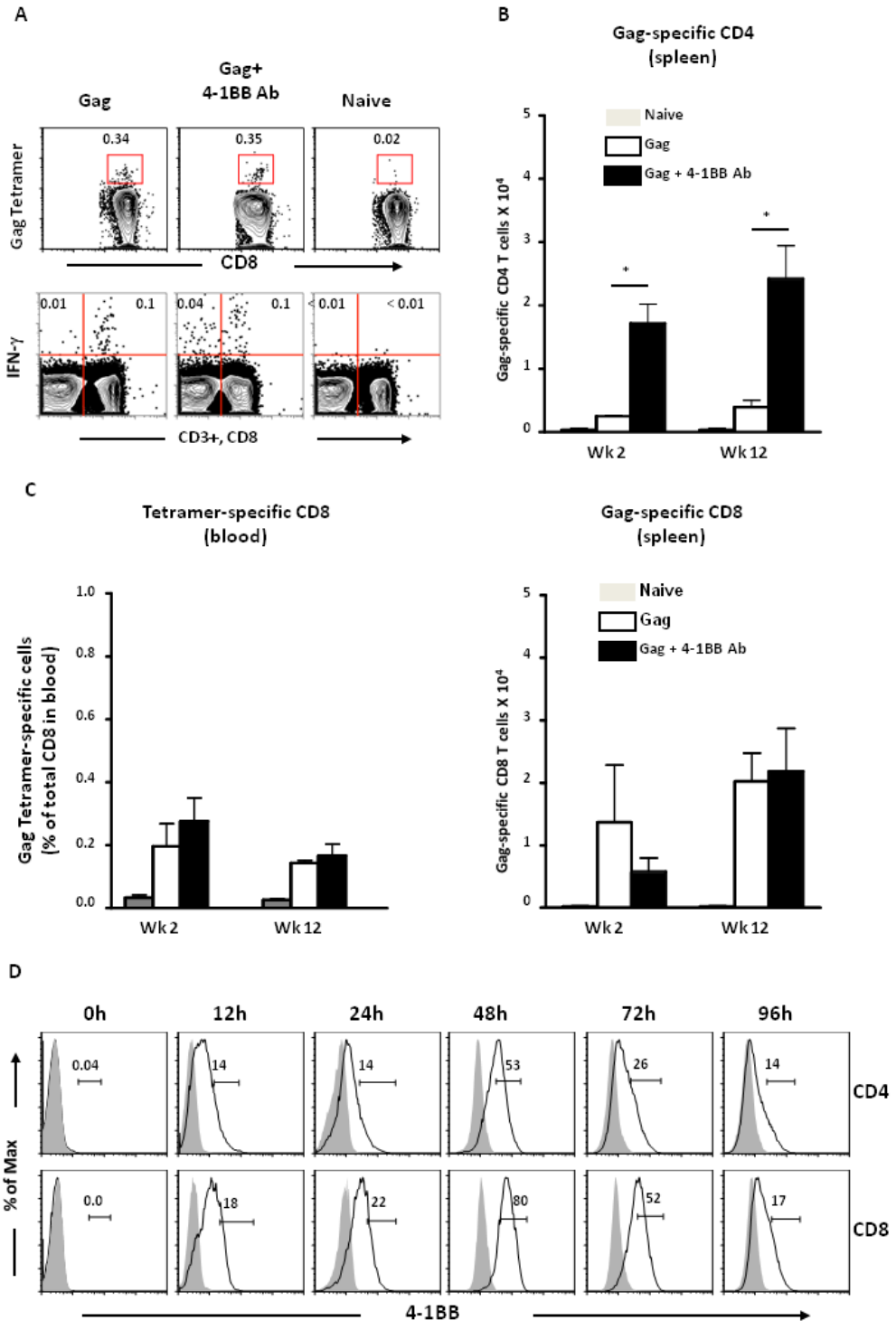


Fig.2.2. Anti-4-1BB agonistic Ab lowers the DNA dose required for a DNA/MVA HIV vaccine

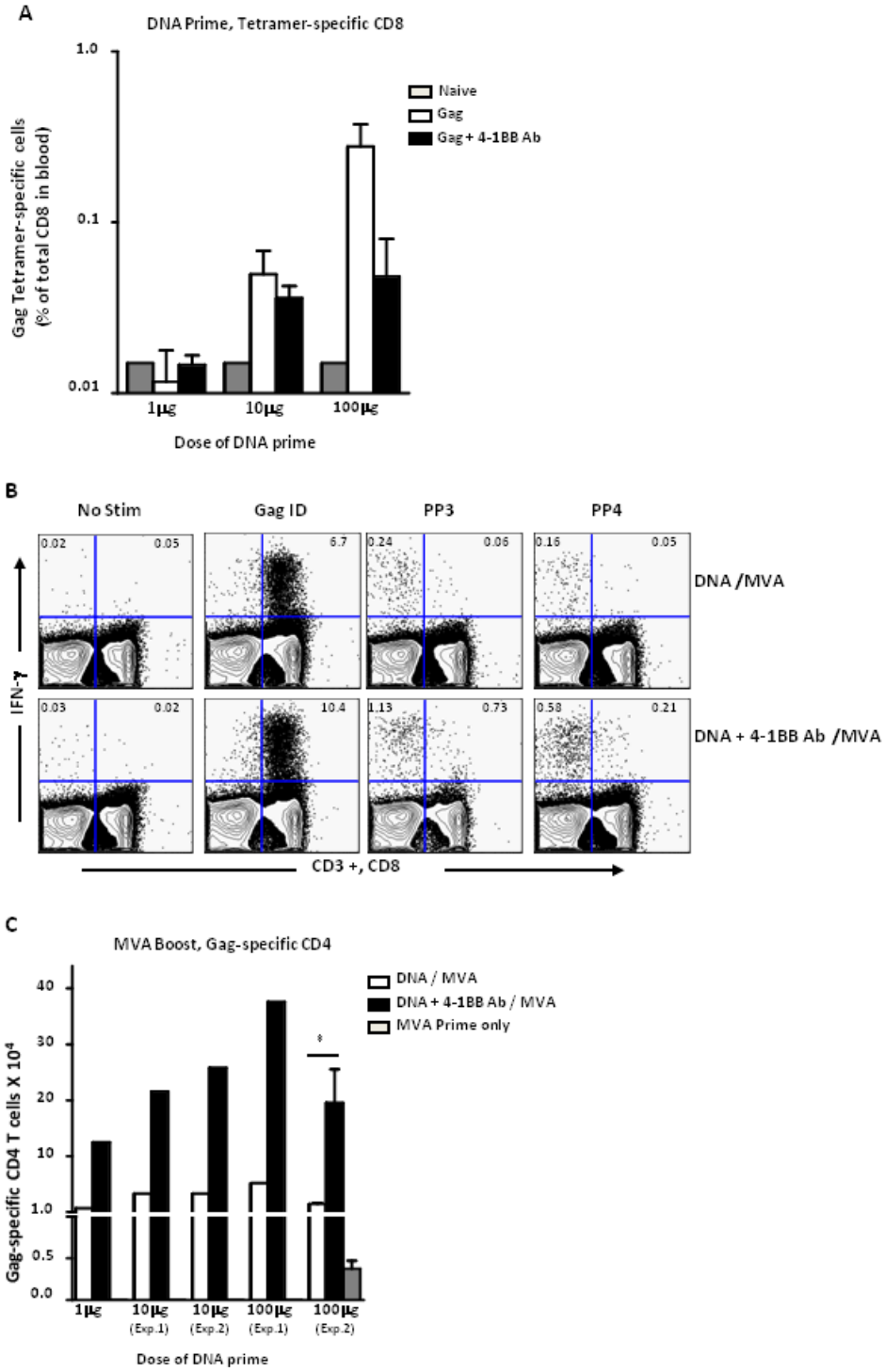


Fig.2.2. Continued

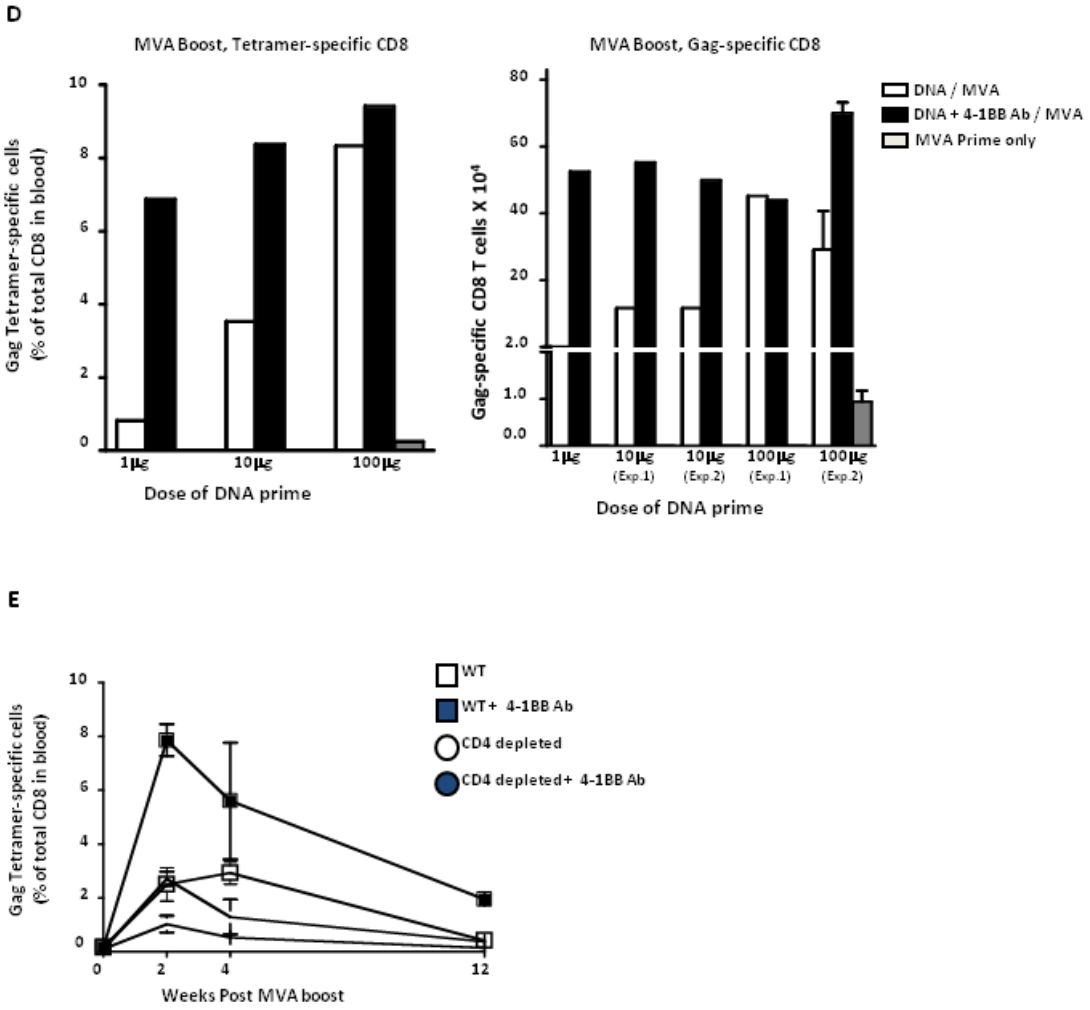


Fig.2.3. Construction and *in vitro* characterization of DNA plasmids expressing different forms of 4-1BBL

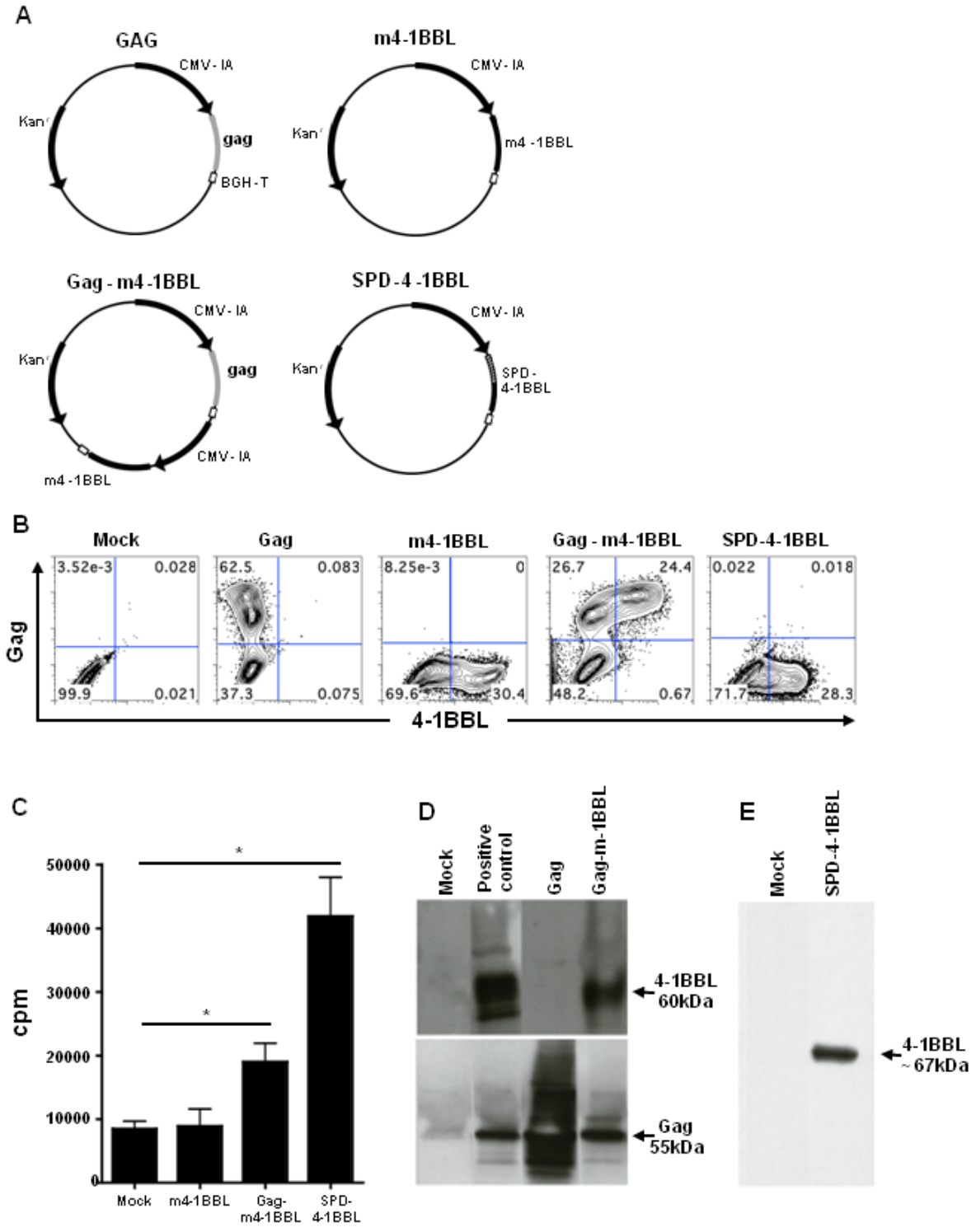


Fig.2.4. Membrane bound 4-1BBL expressed in *cis* with Gag enhances the magnitude of Gag-specific CD8 T cell responses

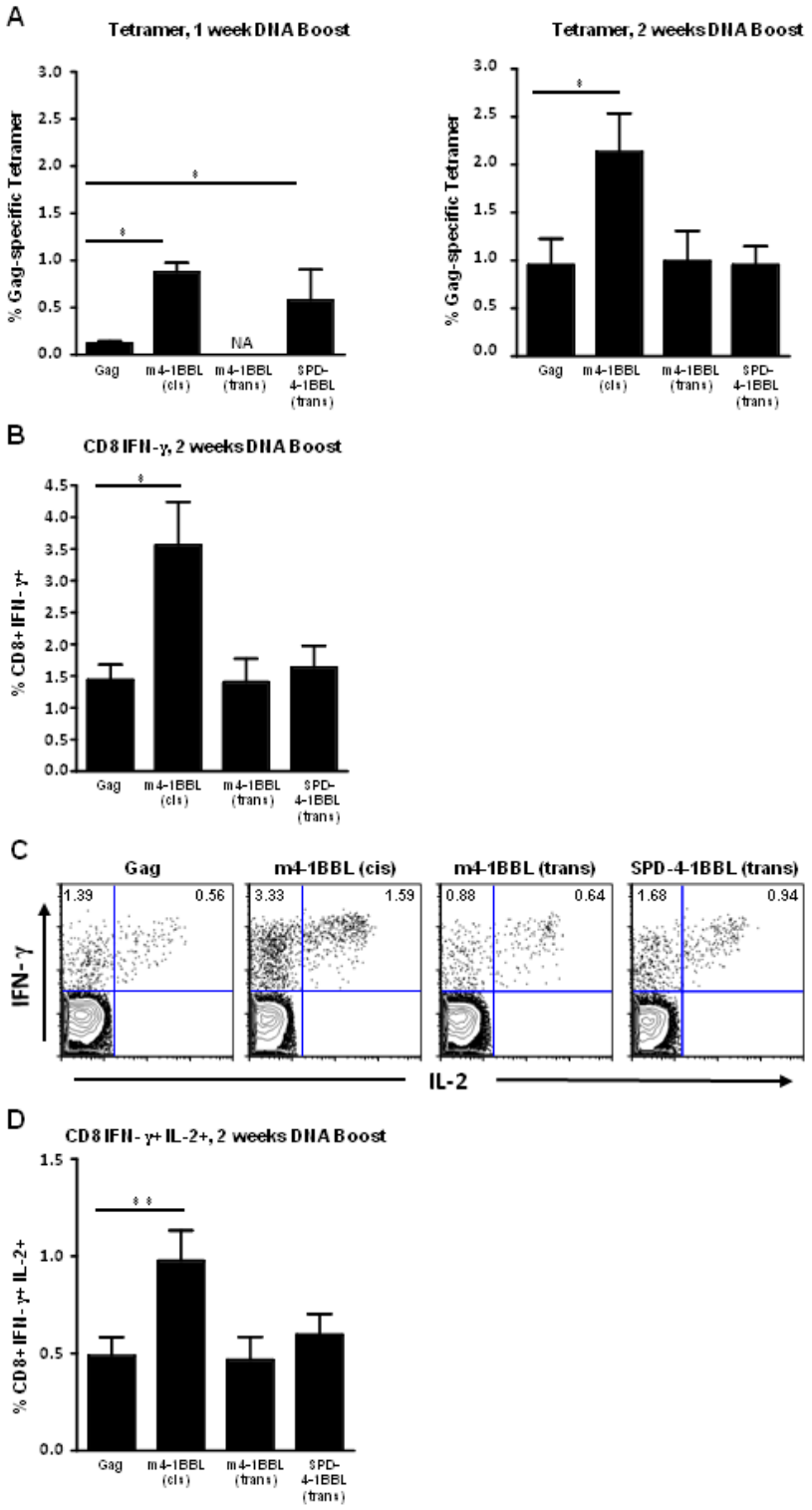


Fig.2.5. Rapid expansion of Gag-specific CD4 T cells in the 4-1BBL adjuvanted groups

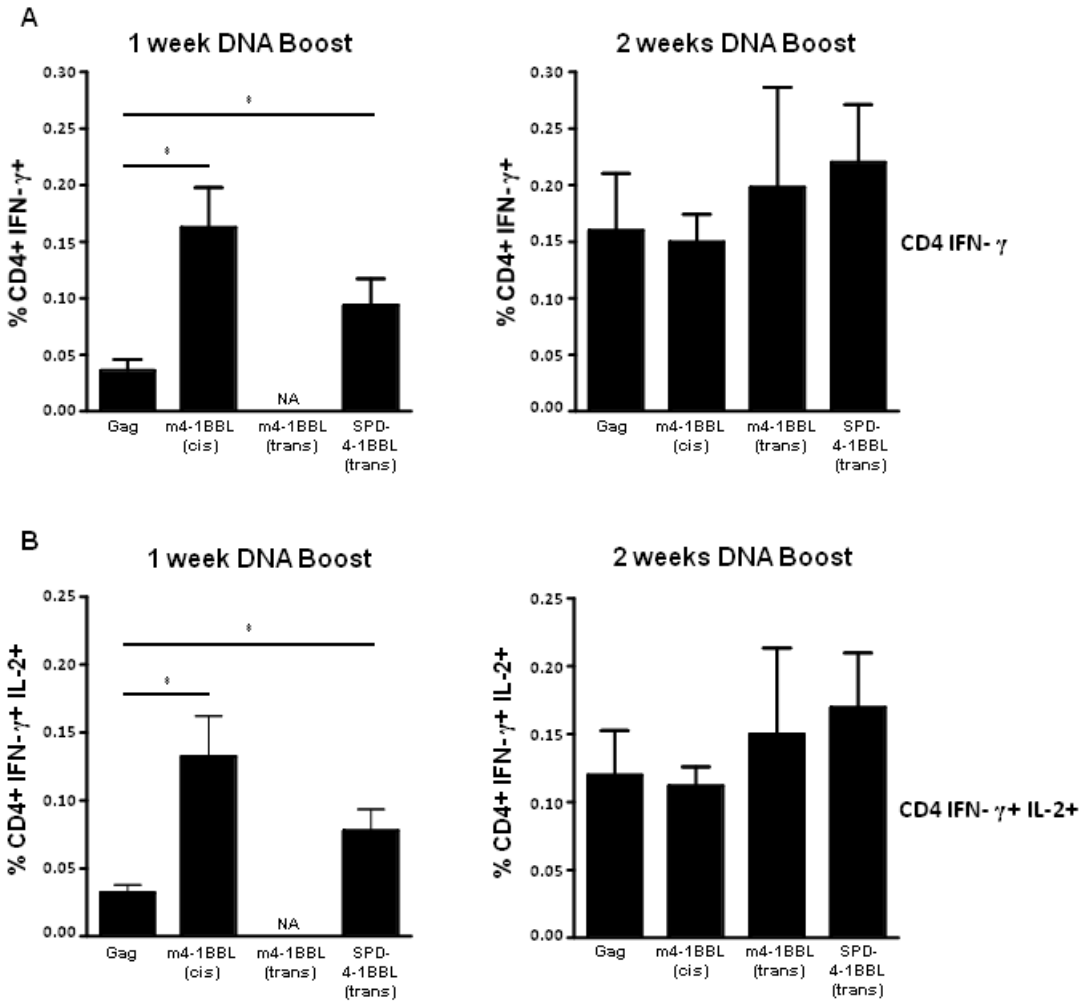
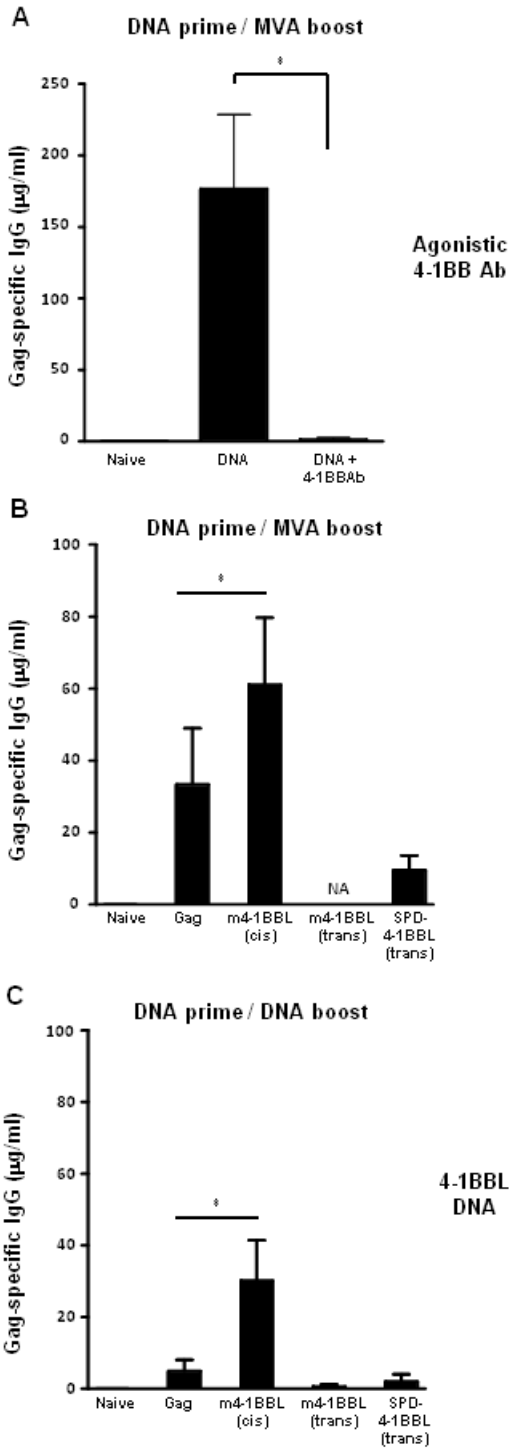


Fig.2.6. Gag-specific IgG response: Suppression with agonistic anti-4-1BB Ab and augmentation with membrane bound 4-1BBL DNA expressed in *cis*



2.8. Design of recombinant MVA expressing murine 4-1BBL

Our data suggests that 4-1BBL when co-expressed by HIV-1 Gag DNA vaccine, can augment both cellular and humoral immune responses. To further test the adjuvant potential of 4-1BBL when included during the boost of a heterologous DNA prime/ MVA boost immunization schedule, we constructed the single recombinant MVA expressing murine 4-1BBL (m4-1BBL). A double recombinant MVA expressing both HIV-1 Gag and 4-1BBL will be generated by transfecting the 4-1BBL containing transfer vector (*see below*) into single recombinant MVA expressing the Gag gene. The 4-1BBL and the Gag gene will be inserted in del II and del III region of the MVA respectively. *In vivo* immunogenicity studies with rMVA will be done in the future. Following is the protocol for generating the single recombinant MVA expressing murine 4-1BBL:

2.8.1. Subcloning of 4-1BBL gene into plasmid vector pLW-1

pLW-17 (*209*) is a transfer vector which contains the modified H5 promoter (*210*) to express recombinant foreign gene and inserts within the natural deletion site (del II) of MVA. Insertion is mediated by the sequences in the flanking regions of the plasmid (fl1 and fl2) that are homologous to the MVA DNA. The 4-1BBL gene was inserted into the unique *Sma I* site of the plasmid and transcription was initiated upon binding of the viral polymerase to the H5 promoter (Fig.2.7). Recombinant virus was selected by live immunostain using anti-4-1BBL mAb (clone 19H3).

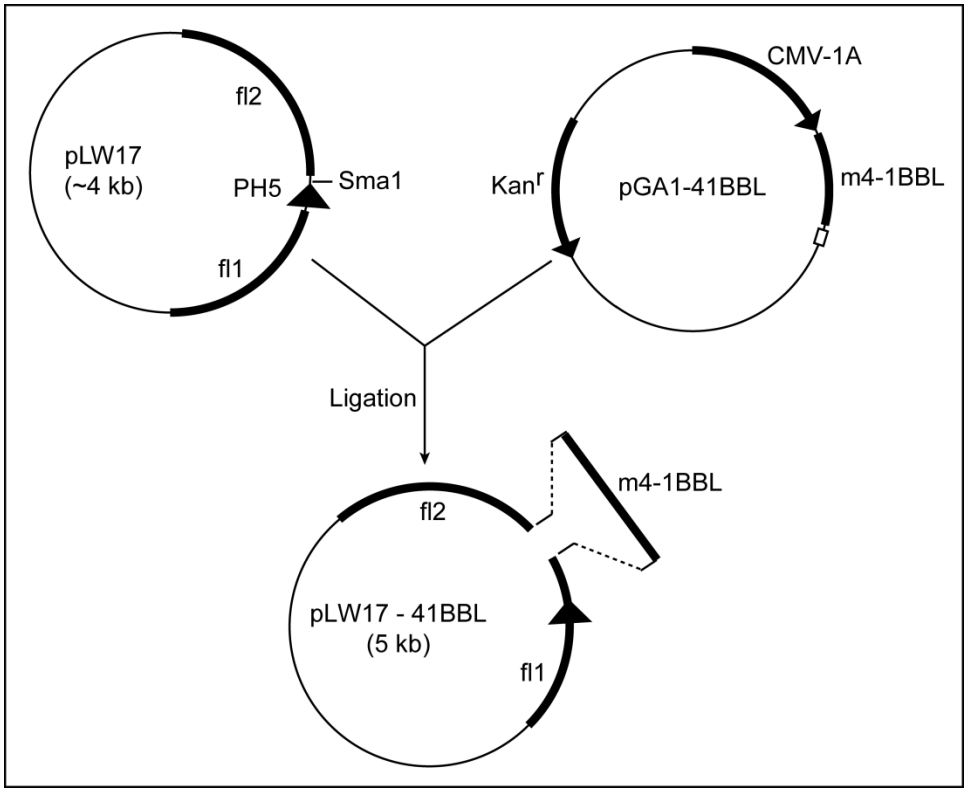
2.8.2. Transfection in HeLa cells to verify protein expression

HeLa cells were infected with MVA at a multiplicity of infection (MOI) of 10 in a six-well plate. At 90 min post-infection, the virus inoculum was removed and infected cells were transfected with 2μg of pLW-17/4-1BBL per well using Lipofectamine 2000. 4-1BBL-FITC (clone 19H3) was used to check surface expression by flow cytometry (Fig.2.8).

2.8.3. Recombinant MVA-4-1BBL and Live immunostain

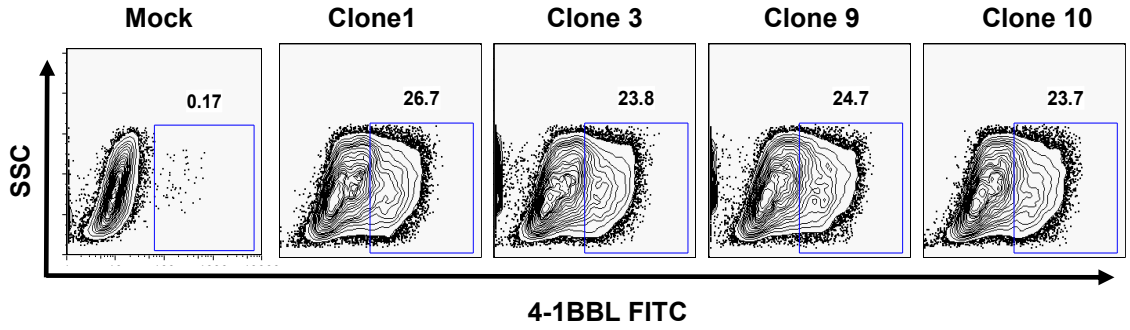
CEF cells (chicken embryo fibroblasts) in six-well culture plates were infected with 0.05 plaque forming units (PFU) of MVA per cell and transfected with 2μg of pLW-17/4-1BBL. The recombinant virus was generated by homologous recombination. After 48 hrs of incubation at 37°C, the infected cells were harvested, lysed (freeze/thaw) and different dilutions of the lysate were used to inoculate fresh CEF cells in concanavalin coated six-well plates. After two days of incubation at 37°C, live cells were immunostained for picking recombinant foci. Unconjugated anti-4-1BBL mAb (clone 19H3) was used as primary antibody and HRP conjugated anti-mouse IgG was used as the secondary antibody. The plaques that stained brown after adding HRP substrate were picked and replaqued on CEFs (Fig.2.9). After five rounds of plaque purification, the recombinant virus was amplified in CEFs.

Fig.2.7. Cloning strategy of recombinant MVA-4-1BBL using transfer vector pLW-17



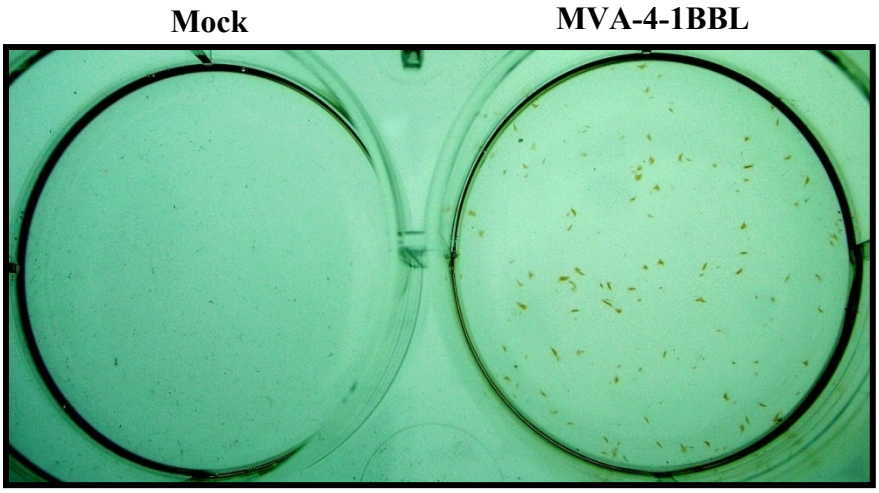
The plasmid pLW-17 *(see section 2.8.1)* was digested with *SmaI* (unique site) that created blunt ends. pGA1-41BBL that expresses full-length membrane-bound murine 4-1BBL was digested with *NheI* and *AvrII* to release the 4-1BBL fragment (approx. 1kb) with overhangs that were blunted with Klenow. The SAP-treated pLW-17 and 4-1BBL were then ligated (blunt end ligation) with T4 ligase (at room temperature for 16 hrs) to create the recombinant plasmid pLW-17-41BBL (approx. 5kb). It was then transformed into supercompetent cells (XL1 Blue) and recombinant clones were selected using ampicillin as the selectable marker.

Fig.2.8. Transfection of HeLa cells with pLW17-4-1BBL to verify surface expression of 4-1BBL



HeLa cells were infected with wtMVA at a MOI of 10 in a six-well plate. At 90 minutes post infection, the virus inoculum was removed and cells were transfected with 2µg of pLW-17-41BBL *(see Fig. 2.7)* per well using Lipofectamine 2000. 4-1BBL-FITC (clone 19H3) was used to check surface expression by flow cytometry. Percentage of HeLa cells staining positive for 4-1BBL in each well are very similar for all the 4 clones (1, 3, 9 and 10). The mock well was infected with the wtMVA but not transfected with pLW-17-41BBL.

Fig.2.9. Live Immunostain of recombinant MVA expressing murine 4-1BBL



CEF cells were infected with wtMVA (MOI of 0.05) and transfected with 2μg of each of the positive clones in a 6-well plate *(see Fig. 2.8)*. After 48hrs of incubation at 37°C, CEF cells were harvested, lysed (freeze/ thaw) and different dilutions of the lysate (containing recombinant and wtMVA) were used to inoculate fresh CEF cells in concanavalin coated six-well plates for the purpose of purifying recombinant clones by live immunostaining. Unconjugated anti-4-1BBL mAb (clone 19H3) was used as 1° antibody and HRP-conjugated anti-mouse IgG was used as the 2° antibody. The recombinant plaques stained brown after adding the substrate dianisidine (right well). The mock (left well) was treated similarly except that it was not infected with the virus.

Chapter 3

Adenovirus Type 5 Induces Vitamin A–Metabolizing Enzymes in Dendritic Cells and Enhances Priming of Gut Homing CD8 T Cells

Adapted from Ganguly,*et.al*. (manuscript submitted)

3.1. Abstract

Protective immunity at the gut-associated mucosal tissue is induced primarily by oral/ rectal immunization owing to the need for targeting antigen to the gut-resident dendritic cells (DC). Recent studies have shown that intramuscular immunization with some live viral vectors can prime antigen-specific CD8 T cells that home to the gut. However, the mechanisms by which parenteral immunizations elicit antigen-specific CD8 T cells in the gut are not understood. Here we show that an adenovirus type 5 (Ad5) based HIV-1 vaccine primes a strong and durable antigen-specific CD8 T cell response in the gut following an intramuscular immunization in mice. We also show that Ad5 rapidly induces expression of retinal dehydrogenase (RALDH) enzymes in splenic conventional DC (cDC) and enhances their ability to prime antigen-specific CD8 T cells with gut homing potential *in vitro*. This effect of Ad5 did not require signaling through toll-like receptors, DNA-dependent activator of IRFs and several MAP kinases, or replication capacity of the virus, but was dependant on NF-κB. These results provide an innate mechanism through which Ad5-stimulated DCs prime antigen-specific CD8 T cells with gut homing potential and have implications for the development of novel mucosal adjuvants for subunit vaccines administered *via* the intramuscular route.

3.2. Introduction

Pathogenic HIV/SIV infections are characterized by the rapid depletion of CD4 T cells in the gastrointestinal tract within 2 weeks following infection and vaccination strategies that induce high levels of anti-viral immunity at the gut-associated mucosal tissue can significantly enhance protection. Oral /rectal routes of vaccination is the best way to induce immunity at the gut-associated mucosal tissue (*211, 212*). However, both routes of vaccinations are limited by requiring multiple high doses of the vaccine and the need to use adjuvants for induction of an optimal immune response.

The need for oral/ rectal immunizations to elicit protective immunity at gut-associated mucosal tissue was attributed to the findings that only gut-resident dendritic cells (DCs) have an intrinsic capacity of metabolizing vitamin A to Retinoic Acid (RA) that is required for imprinting gut-homing potential on T and B lymphocytes (*146, 213*). Synthesis of RA depends on the oxidative metabolism of retinol to retinal that requires alcohol dehydrogenases and then conversion of retinal to RA that requires retinal dehydrogenases (RALDH). The gut-resident DCs possess this unique property to synthesize RA because they constitutively express RALDH enzymes (*146*). However, unlike the gut DCs, peripheral DCs do not constitutively express RALDH enzymes and thus are incapable of imprinting gut-homing phenotype on T and B cells.

More recent studies have demonstrated that intramuscular immunizations with live replication defective/attenuated recombinant viral vectors such as adenovirus type 5 (Ad5) and modified vaccinia Ankara (MVA) can elicit immune responses in the gut-associated mucosal tissue in the murine (*169, 170*) and macaque (*171*) models suggesting

that a potent gut mucosal immunity is achievable with a parenteral route of immunization. Similarly, acute lymphocytic choriomeningitis virus (LCMV) infection of mice has been shown to induce anti-viral CD8 T cells capable of trafficking to gut within days after infection (*214*). However, the mechanisms by which parenteral immunizations induce antigen-specific CD8 T cells with gut homing potential are not understood. It is possible that these viruses modulate the function of peripheral DCs such that they acquire the capacity to induce gut homing potential on antigen-specific CD8 T cells. Furthermore, recently the STEP trial evaluating the efficacy of an Ad5 based HIV-1 vaccine was halted because of the presumed increased risk of HIV-1 acquisition in men who were baseline Ad5 seropositive and uncircumcised (*36*). It was hypothesized that this increased risk could be due to increased frequency of virus target cells at the mucosa primed by Ad5. Thus, defining the mechanisms by which Ad5 induces gut homing potential on T cells is important for the understanding of viral vector-based HIV vaccines.

Here we investigated the mechanisms by which Ad5 can modulate the function of peripheral DCs to induce gut homing potential on CD8 T cells. Our results demonstrate that Ad5 rapidly upregulates the expression of RALDH genes in splenic conventional DCs (cDCs) that results in priming of antigen-specific CD8 T cells that co-express gut homing marker $\alpha 4\beta 7$. Impressively, this function of Ad5 is independent of signaling through Toll-like receptors (TLRs), DNA-dependent activator of IRFs (DAI; previously also known as Z-DNA binding protein 1 or ZBP-1) and some MAP kinases, but was dependant on NF-κB in DC.

3.3. Materials and Methods

3.3.1. Mice and Immunization. For immunization studies, female BALB/c mice of 6-8 weeks of age were purchased from Charles River Laboratories. Mice were immunized with 1x10^6 pfu of recombinant Ad5 expressing HIV-1 clade B Env and Gag (Ad5/Env-Gag) or non-recombinant Ad5 in sterile PBS in a final volume of 100µl with 50µl injected intramuscularly in each of the hind legs at Week 0 (prime) and Week 4 (boost). For *in vitro* co-culture assays, C57BL/6, OT-I Tg and *Nfkb1*$^{-/-}$ mice were purchased from Jackson Laboratories. The *Zbp1*$^{-/-}$ mice were provided by Dr. S.Akira (Osaka University, Japan). The *MyD88*$^{-/-}$ and *TRIF* $^{-/-}$ mice were bred onsite. All knockout mice were on a C57BL/6 genetic background. Mice were housed in specific pathogen-free conditions in the Emory Vaccine Center vivarium and were cared for under guidelines established by the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals" using protocols approved by the Emory University Institutional Animal Care and Use Committee (IACUC).

3.3.2. Isolation of LPLs. LPLs from small intestines were isolated as described previously (*215*). Briefly, small intestines were removed and Payer's patches were excised. The intestine was opened up longitudinally and cleaned of all fecal contents. Intestines were cut into small pieces and were transferred into 50ml conical tubes and shaken at 250 rpm for 20 mins. at 37°C in HBSS media (Life Technologies) supplemented with 5% FBS (Cellgro) and 2mM EDTA (Promega). After a total of two rounds of EDTA treatment, cell suspensions were passed through a strainer and the

remaining intestinal tissue was washed and minced, transferred into 50ml conical tubes and shaken for 20 mins. at 37°C in HBSS media supplemented with 5% FBS and type VIII collagenase (1.5mg/ml; Sigma). Cell suspensions were collected and passed through a strainer and were pelleted by centrifugation at 1500g. Mononuclear cells were then isolated by use of a discontinuous density gradient procedure (45% and 70%) with Percoll (GE Heathcare).

3.3.3. Tetramer and intracellular cytokine staining. Tetramer and intracellular cytokine staining was performed as described before (*216*). For tetramer analysis, cells were stained using allophycocyanin (APC)-conjugated MHC class I (H-2K^d) peptide (AMQMLKETI) tetramer (NIH tetramer core facility), CD4-FITC (clone L3T4), CD19-FITC (clone 1D3), α4β7-PE (clone DATK32) and CD8-PerCP (clone 53-6.7). CD8+ CD4- CD19- and tetramer+ cells were scored as tetramer positive cells. All antibodies were purchased from BD Pharmingen.

3.3.4. DC:OT-I co-culture. CD11c$^+$ DCs from collagenase type IV (1mg/ml; Worthington Biochemical) digested spleens were isolated using anti-CD11c-coated magnetic beads (N418 clone, Miltenyi Biotec). The resulting purity of cDCs was approximately 95%. OT-I T lymphocytes were purified from OT-I spleens using CD8 microbeads (Ly-2 clone, Miltenyi Biotec). The purity of CD8 T cells was > 95%. cDCs and OT-I T cells were co-cultured at a ratio of 1:2 for 72 hrs. To the co-culture, the $OVA_{257-264}$ SIINFEKL peptide was added (20pM). Where indicated, Retinoic Acid

(10nM; Fluka), LE540 (1μM; Wako, Japan), DEAB (10μM; Fluka), UO126 (10μM; LC Laboratories), SB203580 (20μM; LC Laboratories), SP600125 (100μM; LC Laboratories) *N*-Acetyl-L cysteine (30mM; Sigma), and Ac-YVAD-CMK (50μM; Bachem) was included during either DC infection period or the co-culture. At the end of co-culture, cell surface expression of α4β7 on total CD8 T cells were analyzed by flow cytometry. The Via-Probe dye (BD Pharmingen) was included in the staining panel to eliminate dead cells.

3.3.5. Adenoviral vectors. The E1/E3 deleted Adenovirus type 5 without a transgene (Ad5), or encoding either the HIV-1 clade B Env and Gag (Ad5/Env-Gag) or a green fluorescent protein (Ad5-GFP) were used. The viral vectors were generated by standard methods as described previously (*217*). Heat inactivation of the virus was achieved by incubation at 56°C for 1 hour. UV inactivation was achieved by exposing the virus to a 365-nm long UV lamp for 1hr.

3.3.6. Gene expression analysis

The relative quantitative real-time PCR (qRT-PCR) was performed for mRNA expression analyses as described previously (*218*). Total four individual gene expression assays were used from Applied Biosystems for Mus. musculus: aldehyde dehydrogenase family 1, subfamily A1 (*Aldh1a1*); aldehyde dehydrogenase family 1, subfamily A2 (*Aldh1a2*);

aldehyde dehydrogenase family 1, subfamily A3 (*Aldh1a3*); mouse GAPD (GAPDH) Endogenous Control (VIC/MGB Probe). Samples were tested in duplicate.

3.3.7. Statistics

Student's t-test was used to compare the differences between groups. The Bonferroni method was used to adjust the P values for multiple comparisons. Statistical analyses were performed using TIBCO Spotfire S+ 8.1. A two-sided $P < 0.05$ was considered statistically significant.

3.4. Results

3.4.1. Parenteral immunization with Ad5/Env-Gag vaccine can induce gut homing potential on antigen-specific CD8 T cells

To determine whether intramuscularly (i.m.) administered recombinant Adenovirus serotype 5 vaccine vector can induce gut homing potential on antigen-specific CD8 T cells, BALB/c mice were primed and boosted with $1x10^6$ pfu of the Ad5 expressing HIV-1 clade B Env and Gag (Ad5/Env-Gag) at weeks 0 and 4, respectively. The Gag-specific CD8 T cell responses in peripheral blood and lamina propria lymphocyte (LPL) population in the small intestines (will be referred to as 'Gut' here after) were evaluated using a H-$2K^d$ restricted Gag tetramer at various time points following the boost (Fig.3.1A, 3.1B). Expansion of gut resident Gag-specific CD8 T cells was detected as early as day 4 following the boost, peaked at day 7, contracted and

persisted as late as day 150. At the peak response, the frequency of these cells had a mean frequency of 15% of the total CD8 T cells and was 15-fold higher compared to pre-boost levels. Similar expansion/contraction kinetics was also observed in blood. However, on day 4 following boost, the mean percentage of tetramer-specific CD8 T cells in peripheral blood was 3.7-fold lower compared to the gut suggesting a preferential early homing of antigen-specific CD8 T cells to the gut upon i.m. immunization with Ad5/Env-Gag. By 2 weeks following the boost, the frequencies of Gag-specific CD8 T cells were similar in both compartments. These Gag-specific LPLs were also functional as evident from IFN-γ production (albeit at a lower level compared to tetramer+ cells) upon *ex vivo* stimulation with a Gag peptide pool that encompasses the entire Gag protein (Fig.3.1C). Gag-specific CD8 T cells were also present in the intraepithelial lymphocyte (IEL) population of the gut as early as day 7 following the boost (data not shown). However, the frequency of these cells was about 14-fold lower compared to their frequency in the lamina propria.

We next analyzed the expression of the integrin α4β7 on Gag tetramer-specific cells in blood to determine whether the early gut homing potential of these cells can be predicted from the cell surface expression of this gut homing marker (Fig.3.1D and 3.1E). The α4β7 ligand, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), is expressed by the intestinal epithelial cells and is known to mediate T cell recruitment to the gut (*219*). About 30-40% of Gag-tetramer-specific CD8 T cells in blood expressed α4β7 as early as day 4 following the boost. However, the α4β7 expression on these cells decreased rapidly by day 7, and only a small fraction (less than 10%) of tetramer positive cells retained expression on day 28. A similar pattern was also observed for the per-cell expression (as determined by the mean fluorescence intensity) of α4β7 on the tetramer

positive cells, which was highest on day 4 following the boost (Fig.3.1F). This high level of α4β7 expression on tetramer positive cells in blood on day 4 following the boost is consistent with the preferential homing of Gag-specific T cells to the gut and thus may be used as a marker to predict the gut homing potential of CD8 T cells following parenteral immunization. We also observed an increase for Gag-tetramer-specific CD8 T cells co-expressing CCR9 on day 4 following the boost but was more transient compared to α4β7 expression (data not shown).

3.4.2. Ad5 endows splenic cDC with the capacity to imprint gut homing potential on antigen-specific CD8 T cells *via* a retinoic acid dependent pathway

Upon i.m. immunization, Ad5 can either modulate peripheral DC function or target gut resident DC such that they can prime antigen-specific CD8 T cells with gut homing potential. However, it is less likely that the latter is true for this route of antigen delivery. So, we investigated whether the Ad5 can modulate peripheral DC function with regards to priming antigen-specific CD8 T cells with gut homing potential using an *in vit*ro assay system. We co-cultured the ovalbumin-specific OT-I transgenic T cells with either uninfected or Ad5 (non-recombinant) infected splenic cDCs in the presence of ovalbumin peptide (SIINFEKL) for 72 hrs and analyzed the expression of α4β7 on these T cells. To avoid any extraneous virus-induced dendritic cell death during the co-culture, Ad5 infection of cDCs was carried out for 8hrs and cells were then washed to remove cell free virus before co-culturing with the OT-I T cells. The percentage of α4β7 expressing OT-I CD8 T cells was 2.8-fold higher when primed with Ad5-infected cDCs (MOI 0.15)

in comparison to the T cells primed with uninfected cDCs (Fig.3.2A) demonstrating that Ad5 vector alone can enhance the ability of cDC to imprint gut homing potential on antigen-specific CD8 T cells. However, at higher MOI of Ad5 infection, the percentage of CD8 T cells co-expressing α4β7 was diminished (Fig.3.2A) and this could be due to death of cDC as evidenced by a decrease in T cell proliferation in vitro (data not shown). As expected, co-culture of T cells with retinoic acid (RA) induced highest levels of α4β7 on OT-I T cells.

Previous studies (*146, 213*) have shown that GALT (gut-associated lymphoid tissue) DCs, unlike peripheral DCs, have the intrinsic capacity to metabolize vitamin A (retinol) to RA that in turn imprints gut homing potential on naïve/ responding T and B cells. So, we investigated whether induction of gut homing potential on antigen-specific CD8 T cells by Ad5-infected cDC was a RA-dependent or an independent effect. Intracellularly, RA signals through either the heterodimeric (RAR/RXR) or homodimeric (RXR/RXR) retinoic acid receptors and function as ligand-induced transcription factor by interacting with retinoic acid response elements (RARE) or RX response elements (RXRE) located in the promoter regions of target genes to regulate transcription (*220, 221*). Inclusion of the retinoic acid receptor (RAR) inhibitor LE540 in the co-cultures of Ad5-infected peripheral cDC and OT-I T cells abrogated the α4β7 expression on OT-I T cells (Fig.3.2B) suggesting that Ad5 mediated induction of α4β7 on antigen-specific CD8 T cells is RA dependant. Furthermore, inclusion of LE540 during the Ad5 infection period but not in co-culture did not diminish the percent of α4β7 positive OT-I T cells suggesting that inhibition of RAR in Ad5-infected cDCs may not significantly affect their ability to induce gut homing potential on OT-I T cells. Thus, our working hypothesis is

that upon Ad5 infection, peripheral cDCs acquire the ability to synthesize RA and this RA acts on T cells to induce $\alpha4\beta7$ up regulation.

Previous studies have indicated that it is difficult to measure retinol metabolites in the supernatants of DC cultures as they are made in very small quantities(*222*). So, we resorted to measure the levels of RALDH, a key enzyme that is required for the synthesis of RA. There are three isozymes for RALDH that are encoded by *Aldh1a1*, *Aldh1a2* and *Aldh1a3* genes. We quantitated the effect of Ad5 infection on the induction of mRNA for these 3 genes in splenic cDC and found a 19-, 8- and 15-fold increase of *Aldh1a1*, *Aldh1a2* and *Aldh1a3* message, respectively at 6hrs post infection in Ad5-infected over uninfected cDCs (Fig.3.2C). In line with our hypothesis, inclusion of a RALDH inhibitor DEAB (4-diethylamino benzaldehyde), in co-cultures abrogated the ability of Ad5-infected cDC to induce gut homing potential on OT-I T cells (Fig.3.2D).

3.4.3. Inactivated Ad5 also endows splenic cDC with the capacity to imprint gut homing potential on antigen-specific CD8 T cells

To investigate whether live Ad5 is required for the ability of cDCs to induce gut homing potential on antigen-specific CD8 T cells, we infected splenic DCs either with UV inactivated or heat inactivated Ad5. UV inactivation is known to inhibit viral transcription and heat inactivation is known to inhibit viral entry (*223*). UV or heat inactivation of the virus resulted in a minimum of 5-log reduction in the viral titer (data not shown). Interestingly, neither UV nor heat inactivation diminished the ability of Ad5 to modulate cDC function to imprint gut homing potential on OT-I T cells (Fig.3.3A). In

fact, we observed a marginally higher percentage of α4β7+ CD8 T cells primed with DCs pulsed with either of the inactivated Ad5 in comparison to those primed with live Ad5-infected DCs. Consistent with the induction of gut homing potential, cDCs pulsed with UV and heat inactivated Ad5 induced a 6-11 fold upregulation of all three isoforms *Aldh1a* genes at 6 hrs following stimulation (Fig.3.3B).

Murine DCs lack the Coxsackievirus and Adenovirus Receptor (CAR) that is needed for Ad5 infectivity suggesting that Ad5 does not efficiently infect murine cDC. To confirm this, we infected splenic cDC with an Ad5 expressing GFP (Ad5-GFP) at a MOI of 2 for 24 hrs and found that less than 3% of the total cDC population was positive for GFP (Fig.3.3C). The low level of infection could be due to CAR-independent rAd5 tropism that has been reported previously (*224-226*). These results demonstrate that productive Ad5 infection per se may not be important since UV or heat inactivated Ad5 could also modulate DC function. We speculate that mere Ad5 binding to the DC is probably engaging multiple pattern recognition receptors (PRRs) and/or cell surface integrins such as α5β1, αvβ3 and αvβ5 (*227*), and that downstream signaling through these up regulates RALDH genes.

3.4.4. Signaling through multiple Pattern Recognition Receptors (PRRs) is dispensable for Ad5 infected cDCs to imprint gut homing potential on CD8 T cells

Stimulation through TLR2 on DC by zymosan can induce *Aldh1a2* (*228*) and Ad5 is known to engage multiple PRRs, including the TLRs (*229*). Thus, we investigated whether signaling through the TLRs on cDC is required for Ad5 mediated enhancement

of cDC function to prime gut homing potential on CD8 T cells. DCs from *MyD88*$^{-/-}$ and *TRIF* $^{-/-}$ mice were as efficient as DCs from wild-type B/6 mice to induce α4β7 expression on CD8 T cells following infection with Ad5 demonstrating that TLR signaling is not required for the ability of Ad5 to modulate cDC function (Fig.3.4A). In addition, we detected the induction of RALDH message by RT-PCR in cDCs from these knockout mice by Ad5 (data not shown), thus clearly ruling out any synergism between TLR signaling and RA metabolism in Ad5-infected cDCs. Interestingly, in the absence of Ad5, we noted a marked decrease in proliferation of OT-I T cells in cultures with cDC from the knockout mice (Fig. S1), however, Ad5 infection restored this defect.

Ad5 is a non-enveloped double stranded DNA virus and viral DNA recognition is known to trigger host innate immune responses. Intracellularly, viral DNA can be recognized either by endosomal TLR9 or by a cytosolic DNA sensor called the DAI (previously known as ZBP1) (*230*). To investigate the role of Ad5 DNA recognition by splenic cDC for induction of gut homing potential on CD8 T cells, we used cDCs from *Zbp1*$^{-/-}$ mice in our *in vitro* assay. Ad5-infected cDCs from *Zbp1*$^{-/-}$ mice could prime α4β7+ CD8 T cells with similar efficiency as those from wild-type B/6 mice (Fig.3.4B). Similar results were also obtained when cDCs from *TLR9*$^{-/-}$ mice were used (data not shown). These results demonstrate that Ad5-mediated modulation of cDC function to imprint gut homing potential on CD8 T cells is independent on TLR and DAI signaling.

Also, the inclusion of YVAD-cmk (*N*-Ac-Tyr-Val-Ala-Asp-chloromethyl ketone, a caspase-1 inhibitor) during Ad5 infection did not attenuate the ability of cDCs to imprint gut homing potential on OT-I T cells (Fig.3.4C). This may rule out any possible involvement of caspase-1 inflammasomes (particularly AIM2 – absent in melanoma 2, a

cytoplasmic inflammasome for dsDNA) or pro-inflammatory IL-1β (*231, 232*). However, further studies in ASC (apoptosis-related speck-like protein; an adaptor molecule that links many upstream NOD-like receptors, NLRs) knockout mice can conclusively delineate the role of inflammasomes in Ad5-mediated effect on cDCs.

DC function can be regulated by multiple MAP kinase pathways such as ERK, p38 and JNK, and Ad5 infection is known to activate these kinases (*233*). To investigate any role of MAPK activation in modulating splenic cDC function, we treated Ad5-infected cDCs with specific MAPK inhibitors UO126 (for MEK1/2), SB203580 (for p38 MAPK) and SP600125 (for JNK). Interestingly, inclusion of MAPK inhibitors during Ad5 infection did not affect the ability of cDC to imprint gut homing specificity on OT-I T cells (Fig.3.4D), thus demonstrating the lack of involvement of these MAPKs.

3.4.5. NF-κB activation in cDCs is crucial for the priming of gut homing CD8 T cells following Ad5 infection

Several studies have shown that DC activation and maturation is closely associated with NF-κB induction (*234, 235*). Ad5 infection (both in mice and humans) is known to trigger rapid production of pro-inflammatory cytokines such as TNF-α that in turn is a potent activator of NF-κB (*236, 237*). We next sought to determine if intracellular activation of NF-κB had any role in modulating Ad5-infected cDC function to prime gut homing CD8 T cells. Inclusion of a potent NF-κB inhibitor NAC (*N*-acetyl-L-cysteine) (*238*) during the Ad5 infection of cDCs (but not in co-culture) significantly reduced the percent of α4β7 positive OT-I cells (Fig.3.5A) suggesting that Ad5 mediated

enhancement of cDC function to imprint gut homing potential is dependent on activation of the NF-κB. To further confirm the role of NF-κB, we used cDCs from *Nfkb1*$^{-/-}$ mice in our *in vitro* assay. Consistent with the results obtained with the NF-κB inhibitor, Ad5 infection failed to enhance priming of α4β7+ CD8 T cells by cDCs from *Nfkb1*$^{-/-}$ mice (Fig.3.5B). Interestingly, in the absence of Ad5, we noted a marked decrease in proliferation of OT-I T cells in cultures with cDC from *Nfkb1*$^{-/-}$ mice (Fig.3.5B). However, Ad5 infection restored this defect suggesting that the failure to up regulate α4β7 by CD8 T cells in *Nfkb1*$^{-/-}$ cDC co-cultures was not due to diminished ability of these DC to get activated and present antigen.

3.5. Discussion

Tissue-specific lymphocyte trafficking is regulated by the expression of different homing receptors on these cells and the tissue resident DCs have been shown to imprint this property on them (*239*). The gut resident DC possess a unique property of metabolizing vitamin A to RA that in turn regulates expression of the gut homing receptor α4β7 on T and B cells (*146*). Our results demonstrate that the live replication deficient vector Ad5 can rapidly induce expression of vitamin A metabolizing enzymes in splenic DC *in vitro* and that correlates with the ability of this virus to rapidly prime gut homing CD8 T cells *in vivo*. These results provide an innate mechanism by which Ad5 modulates splenic DC function to prime gut homing T cells. To our knowledge, this is the first study that shows induction of vitamin A metabolizing enzymes in splenic (non-gut associated) DC by a replication-deficient viral vector. These results have important implications for developing mucosal vaccines.

Our results demonstrate that NF-κB activation in cDC is essential for Ad5 mediated induction of their ability to prime gut homing CD8 T cells. A previous study demonstrated the presence of NF-κB binding site upstream of *Aldh1a2* promoter suggesting that NF-κB can directly regulate the expression of this enzyme (*240*). Ad5 is a potent inducer of NF-κB (*241*), however the mechanism/s leading to NF-κB activation are not well understood. NF-κB can be induced by many PRRs including TLRs, retinoic acid-inducible gene I (RIG-I) and DAI. Of these, being a double stranded DNA enveloped virus, it is likely that Ad5 could target TLRs and DAI. However, our results demonstrated that signaling through TLRs and DAI in splenic cDC is not necessary for

the Ad5 mediated induction of their ability to prime gut homing CD8 T cells. It is interesting to note that the cDCs from these knockout mice were poor at stimulating OT-I T cells in comparison to cDCs from wild-type B/6 mice and Ad5 restored this defect (Fig. S1) suggesting that Ad5 can activate cDC in a TLR and DAI independent manner. A similar situation was also observed with cDC from *Nfkb1*$^{-/-}$ mice demonstrating that the failure of these DCs to prime α4β7 positive OT-I T cells was not due to lack of DC activation (Fig.3.5B). We are yet to identify the signaling pathway that leads to NF-κB dependant induction of vitamin A metabolizing enzymes in cDC by Ad5.

Vitamin A can be metabolized by any one of the three isoforms of the RALDH enzyme. Our results showed that Ad5 induces expression of all three isoforms of RALDH and it is not clear whether all three isoforms are needed for the Ad5-mediated induction of gut homing CD8 T cells by splenic DC. However, the effect of Ad5 is different from that of zymosan (a TLR2 ligand), which predominantly induced *Aldh1a2* but not *Aldh1a1*, and *Aldh1a3* (*228*). In addition, Ad5 mediated induction of RALDH enzymes was not dependant on TLR signaling. These results suggest that Ad5 is working through a pathway that is different from previously reported TLR dependant induction of RALDH enzymes in splenic cDC.

Since both DCs as well as T cells are known to have retinoic acid receptors, we investigated whether the endogenously made RA by the Ad5-infected peripheral cDCs is working in an autocrine manner (thus further conditioning these DCs somehow to prime CD8 T cells to up regulate α4β7) or in a paracrine manner on the CD8 T cells. For example, RA made by zymosan stimulated DC has been shown to act on the same DC in an autocrine manner (*228*). However in our study, the inclusion of RAR inhibitor only

during the 8 hours Ad5 infection period did not abrogate the α4β7 expression on CD8 T cells suggesting that RA made by the Ad5-stimulated DC is working mostly in a paracrine manner.

Our results demonstrate that the cytosolic DNA sensor DAI is not required for Ad5 mediated priming of gut homing CD8 T cells by splenic DC suggesting that Ad5 DNA is not required for this function. However, it is important to note that the role of DAI as a cytosolic sensor for viral DNA can be redundant as other studies in murine model have shown that DAI-deficient mice exhibit uncompromised immune responses following infection with DNA viruses (*242, 243*). A recent study using a human cell line has however identified the extra-chromosomal histone H2B as biologically crucial molecule for innate immune response to DNA viruses (*244*). Whether histone H2B plays any role as a cytosolic DNA sensor in the murine model or not needs to be further investigated.

An important implication of our findings is that they suggest an innate mechanism for the failure of recent STEP trial evaluating the efficacy of an Ad5 based HIV-1 vaccine. This trial was halted because of the presumed increased risk of HIV-1 acquisition in men that were baseline Ad5 seropositive and uncircumcised. It was hypothesized that this increased risk could be due to increased frequency of virus target cells at the mucosa primed by Ad5. Assuming that Ad5 can also imprint gut homing potential on CD4 T cells, as observed with CD8 T cells in our study and as indicated by a recent study in humans (*43*), our results suggest a mechanism by which Ad5 can induce increased frequency of HIV-1 target cells in the gut. Gaining insights into mechanisms

that regulate Ad-induced innate immune responses is important, since Ad5-based vaccines are in an advanced stage of development and are being tested in humans.

At present it is unclear which part of Ad5 (either protein or nucleic acid) is responsible for it's ability to induce vitamin A metabolizing enzymes in cDC. We speculate that this could be one of the Ad5 proteins as other studies have shown that even empty capsids derived from Ad5 can induce strong inflammatory responses (*245, 246*). Identification of this key adenoviral protein/s that can endow peripheral cDC with the ability to imprint gut homing potential on antigen-specific CD8 T cells can help in development of novel mucosal adjuvants for subunit vaccines administered *via* the parenteral route of immunization.

3.6. Legends to the Figures

Figure 3.1: Parenteral immunization with Ad5/Env-Gag vaccine can induce gut homing potential on antigen-specific CD8 T cells. BALB/c mice were primed and boosted with Ad5/Env-Gag vector at week 0 and 4 respectively. (A) Representative FACS plot showing the frequency of Gag-tetramer specific CD8 T cells in blood and in lamina propria lymphocytes (LPL) at different time points following the boost. Numbers on the FACS plots indicate Gag tetramer-specific CD8 T cells expressed as a percent of total CD8 T cells. (B) Kinetics of Gag tetramer-specific CD8 T cell response in blood and LPL following Ad5/Env-Gag boost. (C) Representative FACS plots showing Gag-specific CD8 T cell responses in spleen and LPL at one week following the Ad5/Env-Gag boost as assessed by intracellular cytokine staining. Numbers on the FACS plots indicate Gag-specific IFN-γ^+ CD8 T cells expressed as a percent of total CD8 T cells. NS, no stimulation. (D) Representative FACS plot showing $\alpha4\beta7$ expression on Gag-tetramer specific CD8 T cells in blood (gated on total CD8 lymphocytes). (E) Frequency (left panel) and mean fluorescence intensity (MFI; right panel) of $\alpha4\beta7^+$ Gag-tetramer$^+$ CD8 T cells in blood. Three to five mice were analyzed at each time point. Error bars indicate SEM. Data is representative of one of two independent experiments. * $P < 0.05$; ** $P < 0.01$. * indicate significantly higher values compared to day 0.

Figure 3.2: Ad5 endows splenic cDC with the capacity to imprint gut homing potential on antigen-specific CD8 T cells *via* a retinoic acid dependent pathway. Splenic cDCs isolated from wild-type C57BL/6 mice were either left uninfected or

infected with Ad5 (non-recombinant) for 8hrs. Following infection, cells were washed and co-cultured with OT-I T cells (CD8) in the presence of the OVA peptide for 72 hrs. (A) Representative FACS plots (left panel) showing cell surface expression of α4β7 on OT-I T cells 3 days after co-culture. Cells were gated on total CD8 T cells. Right panel, Mean frequency of $\alpha4\beta7^+$ cells (gated on total live CD8 lymphocytes) following co-culture with either uninfected cDCs or Ad5-infected cDCs at indicated MOIs. (B) Representative FACS plots (left panel) showing α4β7 expression on total live CD8 T cells following co-culture with Ad5-infected cDCs (MOI 0.15) in the absence or presence of LE540. Middle panel, frequency of $\alpha4\beta7^+$ CD8 T cells in the absence or presence of LE540 (inhibitor included in the co-culture). Data from two independent experiments (Exp.1 and Exp.2) are shown. Right panel, frequency of $\alpha4\beta7^+$ CD8 T cells with RAR inhibitor included either only during 8hrs of Ad5 infection and washed before co-culture (pre-treat) or left in the well during co-culture for 72hrs (in well). (C) Quantitative RT-PCR showing the fold change in the expression of mRNA encoding RALDH enzymes (*Aldh1a1*, *Aldh1a2* and *Aldh1a3*) in Ad5-infected cDCs over uninfected cDCs at 0, 6 and 12 hrs following infection. Results from one of the three experiments are shown. (D) Mean frequency of $\alpha4\beta7^+$ CD8 T cells following co-culture with either uninfected cDCs or Ad5-infected cDCs in the absence or presence of a RALDH inhibitor, DEAB. Error bars indicate SEM. All data are representative of at least three independent experiments. ** $P < 0.01$; *** $P < 0.001$. * indicate significantly higher value compared to the respective uninfected DCs without RA.

Figure 3.3: Inactivated Ad5 also endows splenic cDC with the capacity to imprint gut homing potential on antigen-specific CD8 T cells. Following isolation of splenic cDCs from wild-type C57BL/6 mice, they were either left uninfected or infected with Ad5 (live) or UV/ heat inactivated Ad5 (MOI 0.15) for 8hrs. After infection, cells were washed and cultured with OT-I T cells (CD8) in the presence of OVA peptide for 72 hrs. (A) Mean frequency of $\alpha4\beta7^+$ CD8 T cells following co-culture with either uninfected cDCs or live or inactivated Ad5-infected/ stimulated cDCs. (B) Quantitative RT-PCR showing the fold change in the expression of mRNA encoding RALDH enzymes (*Aldh1a1*, *Aldh1a2* and *Aldh1a3*) in Ad5-infected cDCs over uninfected cDCs at 0, 6 and 12 hrs following infection. (C) Splenic cDCs from wild-type C57BL/6 mice were infected with Ad5-GFP. After 24 hrs of infection, cells were harvested and analyzed by FACS to detect relative (to uninfected cDC) levels of expression of GFP (y-axis on flow plot). Error bars indicate SEM. Data from one of the three independent experiments is shown.

Figure 3.4: Signaling through multiple PRRs is dispensable for Ad5-infected cDCs to imprint gut homing potential on CD8 T cells. Splenic cDCs were isolated from either wild-type C57BL/6 mice or different gene knockout mice and infected with Ad5 (MOI 0.15) for 8hrs, after which, infected cells were washed and co-cultured with OT-I T cells (CD8) in the presence of the ova peptide for 72hrs. (A) Mean frequency of $\alpha4\beta7^+$ CD8 T cells following co-culture with either uninfected cDCs or Ad5-infected cDCs isolated from either wild-type C57BL/6 mice or

MyD88$^{-/-}$ or *TRIF* $^{-/-}$ mice. (B) Mean frequency of $\alpha4\beta7^+$ CD8 T cells following co-culture with either uninfected cDCs or Ad5-infected cDCs isolated from either wild-type C57BL/6 mice or *Zbp1*$^{-/-}$ mice. (C) Mean frequency of $\alpha4\beta7^+$ CD8 T cells following co-culture with Ad5-infected cDCs from wild-type C57BL/6 mice in the presence or absence of caspase-1 inhibitor YVAD-cmk. (D) Mean frequency of $\alpha4\beta7^+$ CD8 T cells following co-culture with Ad5-infected cDCs from wild-type C57BL/6 mice in the presence or absence of ERK inhibitor (UO126), p38 MAPK inhibitor (SB203580) or JNK inhibitor (SP600125). All inhibitors were included only during the 8 hrs Ad5 infection and were excluded from the 72 hrs of co-culture. Error bars indicate SEM. Data from one of the three (for Fig.4A) or one of two (for Fig.4 B-D) independent experiments is shown. ** $P < 0.01$; *** $P < 0.001$. * indicate significantly higher value compared to the respective uninfected DCs without RA.

Figure 3.5: NF-κB activation in cDCs is crucial for the priming of gut homing CD8 T cells following Ad5 infection. Splenic cDCs from either wild-type C57BL/6 mice or *Nfkb1*$^{-/-}$mice and infected with Ad5 (MOI 0.15) for 8hrs, after which, infected cells were washed and co-cultured with OT-I T cells (CD8) in the presence of the ova peptide for 72hrs. (A) Mean frequency of $\alpha4\beta7^+$ CD8 T cells following 72-hrs of co-culture with Ad5-infected cDCs (from wild-type C57BL/6 mice) in the presence or absence of NF-κB inhibitor, NAC. The inhibitor was included only during the 8 hrs of Ad5 infection and was excluded from the co-culture. (B) Mean frequency of $\alpha4\beta7^+$ cells (gated on total live CD8 lymphocytes) following 72-hrs co-culture is shown on the left and total live (Via-

Probe negative) CD8 T cell number at the end of the co-culture is sown on the right. Error bars indicate SEM. Data from one of the two independent experiments is shown. * $P < 0.05$. * indicate significantly higher value compared to the respective uninfected DCs without RA. NA, *Not available*.

Figure S1: The absolute number of OT-I T cells following Ad5-infection of cDC isolated from wild-type C57BL/6 and TLR knockout mice. Splenic cDCs were isolated from either wild-type C57BL/6 mice or *MyD88*$^{-/-}$ or *TRIF* $^{-/-}$ mice and infected with Ad5 (MOI 0.15) for 8hrs. Cells were then washed and co-cultured with OT-I T cells (CD8) in the presence of the ova peptide for 72hrs. (A) Number of $\alpha4\beta7^{+}$ CD8 T cells. (B) Number of total live (Via-Probe negative) CD8 T cells at the end of the co-culture. Data from one of the three independent experiments is shown. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. * indicate significantly higher value compared to the respective uninfected DCs without RA.

3.7. Figures

Fig3.1. Parenteral immunization with Ad5/Env-Gag vaccine can induce gut homing potential on antigen-specific CD8 T cells

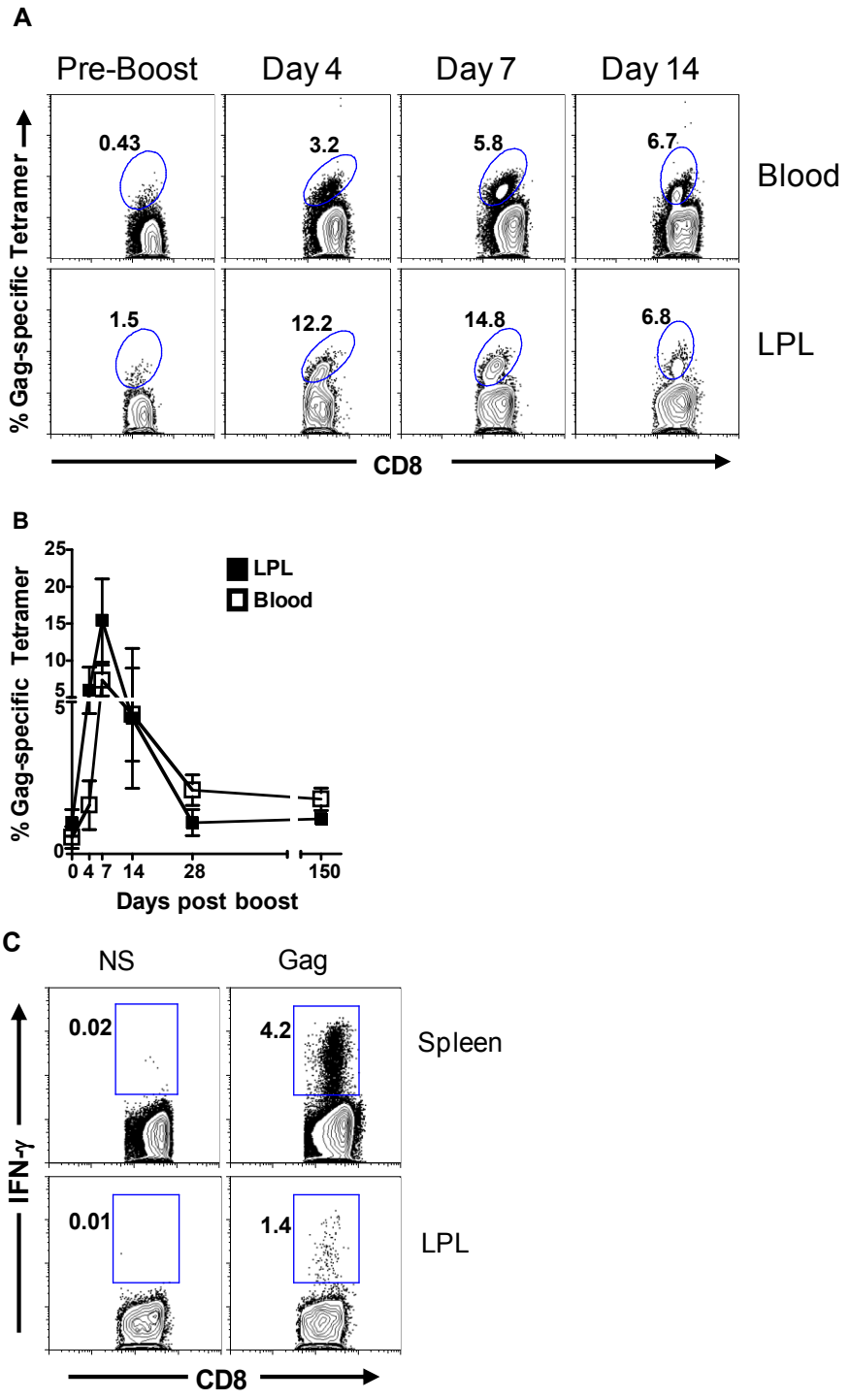


Fig 3.1. Continued

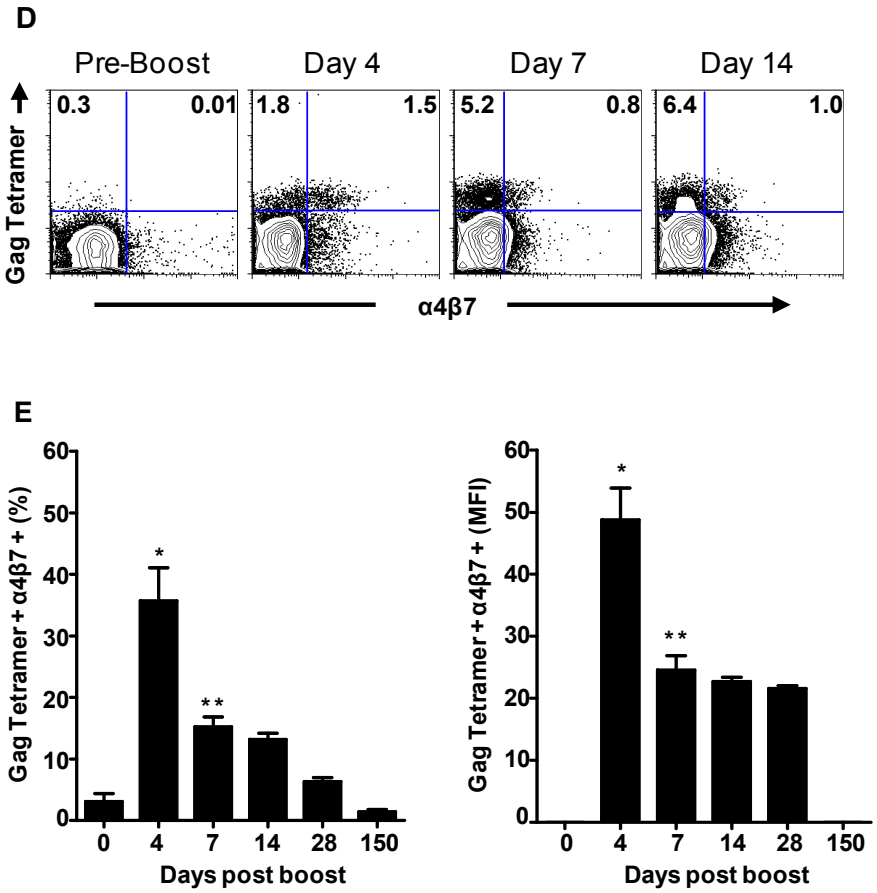


Fig.3.2. Ad5 endows splenic cDC with the capacity to imprint gut homing potential on antigen-specific CD8 T cells *via* a retinoic acid dependent pathway

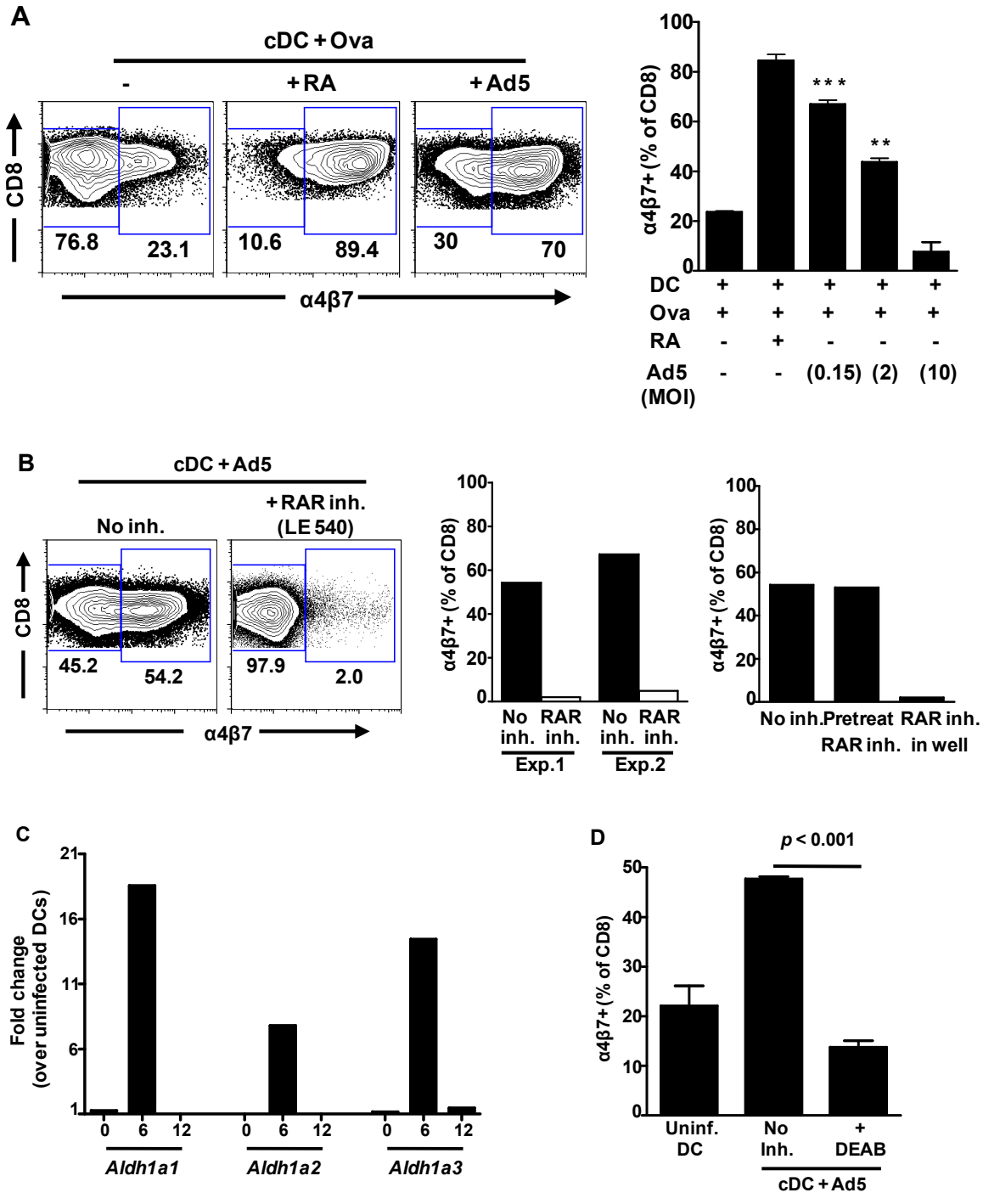


Fig.3.3. Inactivated Ad5 also endows splenic cDC with the capacity to imprint gut homing potential on antigen-specific CD8 T cells

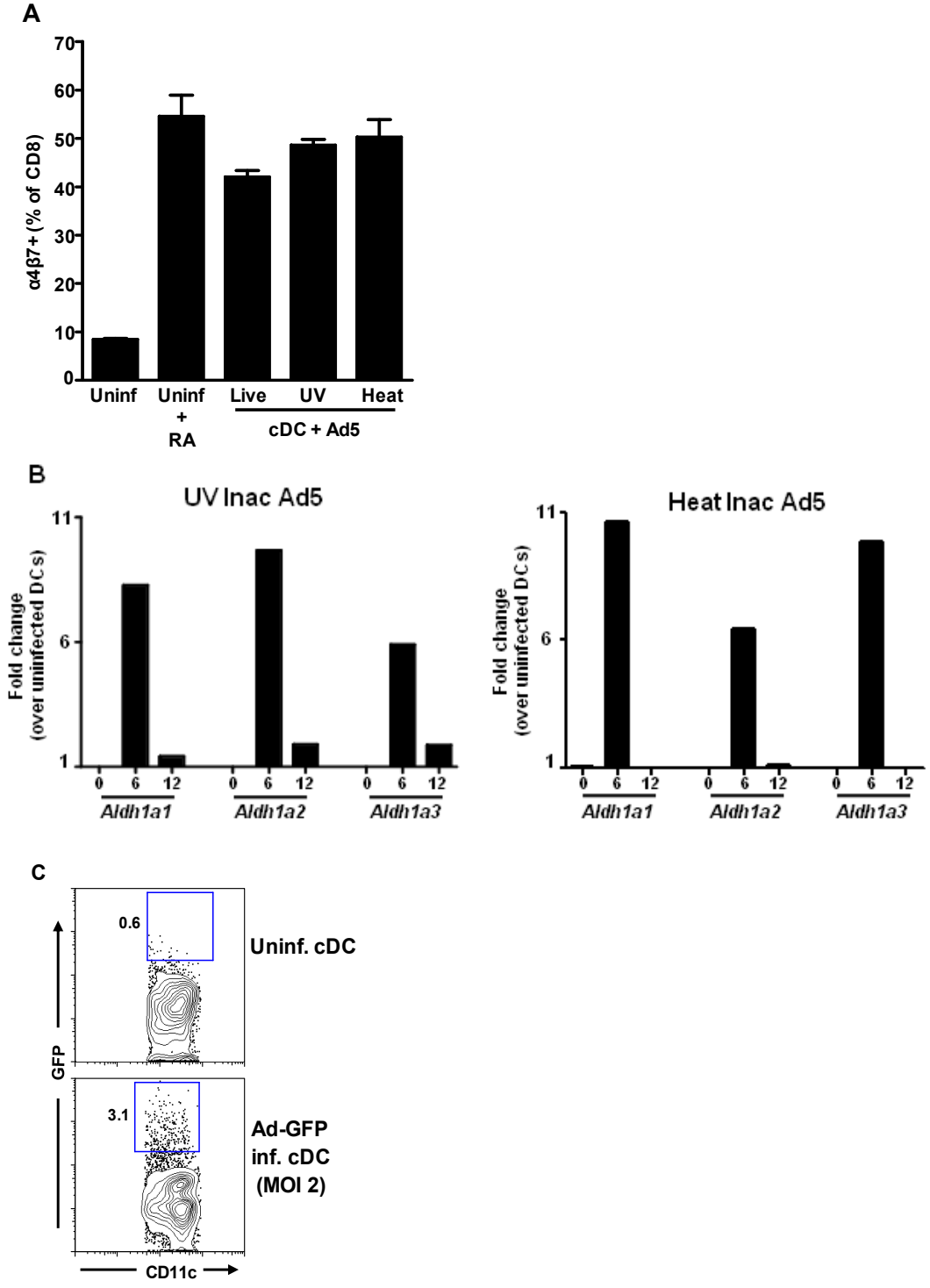


Fig.3.4. Signaling through multiple PRRs is dispensable for Ad5-infected cDCs to imprint gut homing potential on CD8 T cells

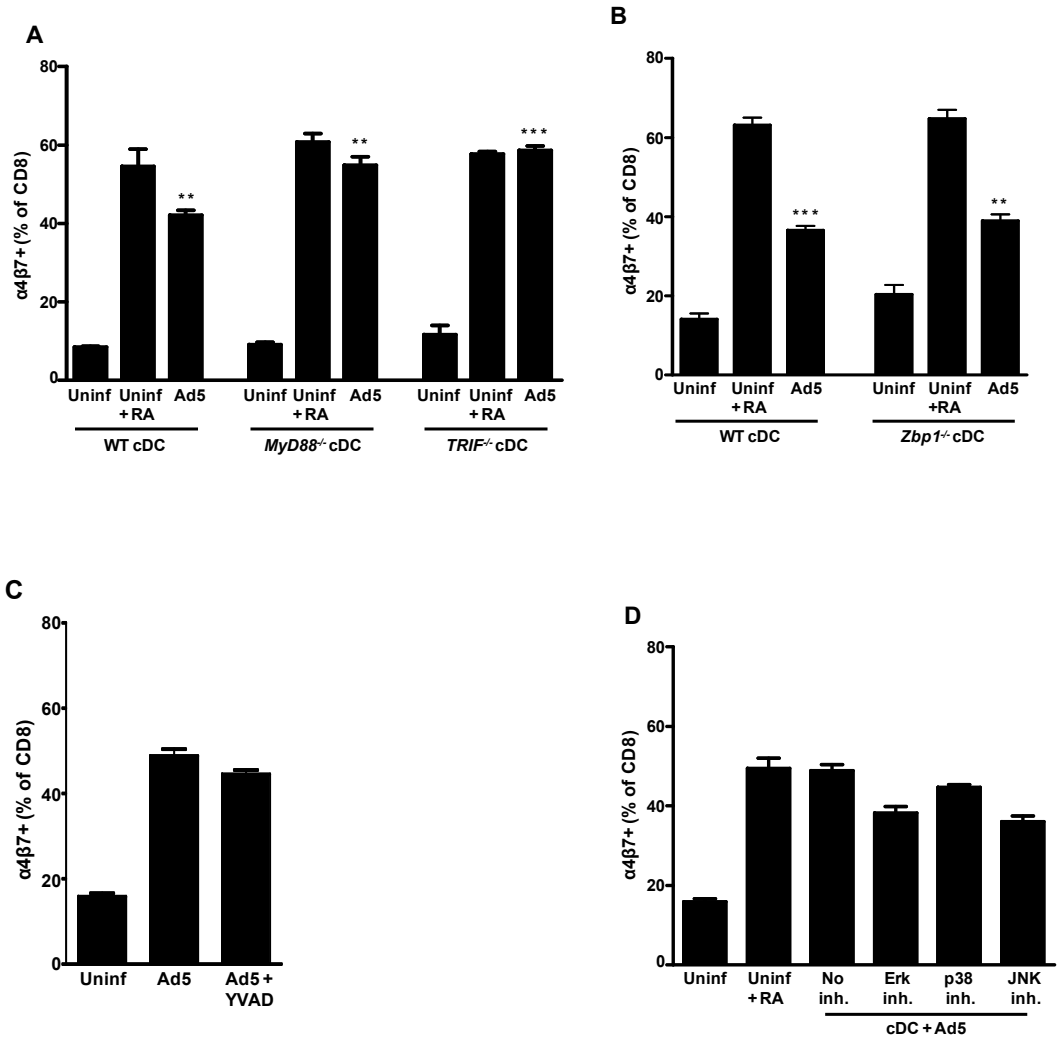


Fig.3.5. NF-κB activation in cDCs is crucial for the priming of gut homing CD8 T cells following Ad5 infection

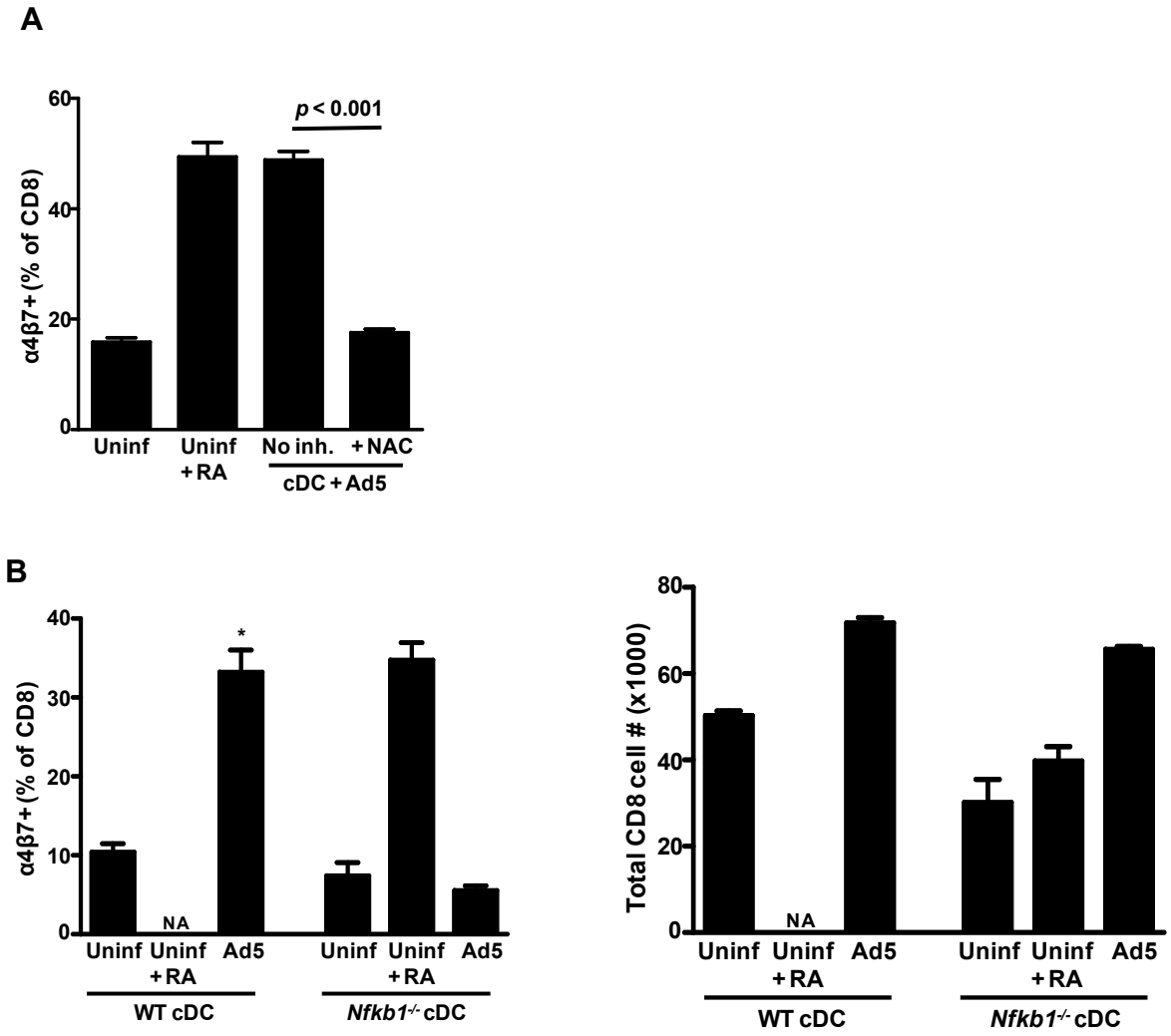
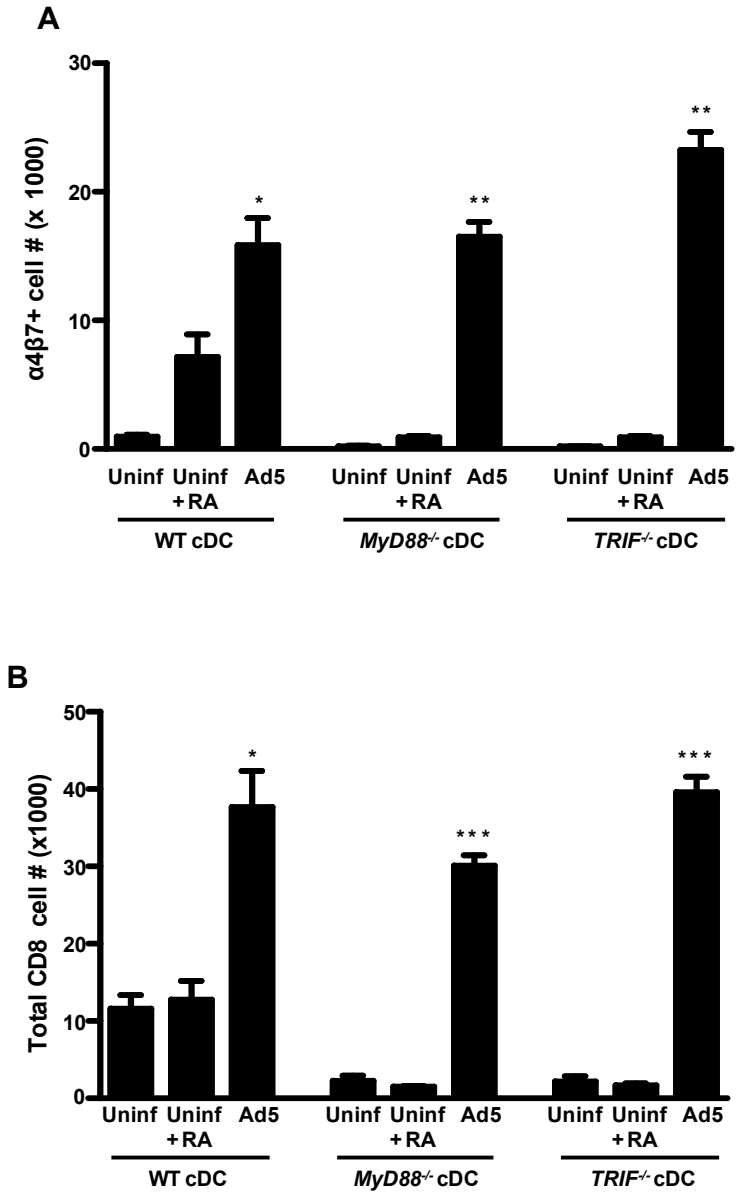


Fig. S1. The absolute number of OT-I T cells following Ad5-infection of cDC isolated from wild-type C57BL/6 and TLR knockout mice



Chapter 4

CONCLUSION

HIV and AIDS is a global pandemic and have claimed millions of lives since the disease was first diagnosed in 1981. Despite exhaustive efforts to curb the spread of infection, it is estimated that currently 33 million people are living with HIV worldwide (UNAIDS report). Over the past two to three decades, advances in antiretroviral therapies, various education/ counseling programs and barrier techniques have helped to counteract the virus transmission and mortality rates. Unfortunately antiretroviral therapies have several limitations such as long-term toxicity, emergence of drug resistant viruses and circulating recombinant forms (CRFs), poor adherence to complicated regimens, and high costs. Thus it is absolutely crucial to develop a safe and effective HIV vaccine that can save millions of lives worldwide in the future.

A recent efficacy trial in Thailand has reported for the first time a small protective effect, where the vaccine recipients had a lower rate of HIV-infection by 30% compared to the control group that received the placebo (*32*). This trial tested the prime/ boost combinations of two vaccines: priming with the ALVAC HIV vaccine which is essentially a recombinant canarypox viral vector expressing HIV-1 gp120, Gag and Pol; and boosting with the AIDSVAX recombinant gp120 protein subunit vaccine. However, some researchers have suggested that the modest success of this vaccine should be taken with a cautionary note since the volunteer cohort that was protected by this vaccine was low risk (annual incidence of infection approximately 0.3%). These efficacy results though insufficient, definitely renewed enthusiasm in the field of HIV vaccine design.

Though designing a successful HIV vaccine has been a daunting challenge so far, yet, certain key features of the virus/ infection are indicative that an efficacious vaccine can be designed. Firstly, in humans, the rate of natural transmission of the virus is low suggesting that even modest levels of protective immunity at the mucosal surface can effectively inhibit the spread of HIV. Secondly, immediately after infection, only a small population of cells gets productively infected (as evident from the viral RNA) and this founder population of infected cells must expand locally for a few days before systemic infection is established. Thirdly, both in vertical (*247*) and heterosexual transmissions (*248*), virus isolates from the newly infected individual are genetically much less diverse than viruses from the transmitter (genetic bottleneck). Fourthly, neutralizing Abs to both autologous as well as heterologous virus does eventually develop in the chronic phase of the infection (but not during acute infection).

Data from all preclinical and clinical studies thus suggests the following mandatory requirements for an ideal HIV vaccine: 1. the vaccine must generate both mucosal as well as systemic immunity. Mucosal immunity is absolutely critical to blunt the viral transmission at the portal of entry; 2. the vaccine must generate both cellular as well as humoral immunity. Both magnitude and breadth of T cell responses is important to limit viral escape. Neutralizing Abs will be critical to prevent transmission of cell-free viruses; 3. vaccine strategies must take into consideration the high genetic diversity of HIV-1. Recent studies in macaques (*249, 250*) have used the 'mosaic approach' to design vaccines to increase the breadth of immune responses to globally circulating strains of HIV-1; and 4. vaccine strategies must not result in excessive immune activation because that will only fuel viral replication/ transmission. Furthermore, to test the protective

efficacy of a candidate vaccine, the low-dose mucosal challenge model is gaining popularity since it represents a more natural/ physiological scenario of viral infection. More comprehensive understanding of HIV-1 pathogenesis and the correlates of immune protection in 'elite controllers' may provide vital clues for designing a protective HIV vaccine. In my research I have addressed two of the above points: 1. design a prototype vaccine that can induce both T and B cell responses to HIV-1 Gag (key findings are discussed in Section 4.1 below). Immune responses to conserved epitopes in Gag have been correlated to long-term control of viral load; and 2. understand the mechanism of Ad5-mediated mucosal homing of antigen-specific $CD8^+$ T cells (key findings are discussed in Section 4.2 below). Ad5-based vaccines are currently being tested in humans.

4.1. Adjuvanting HIV-1 Gag DNA vaccine with 4-1BB/ 4-1BBL

DNA vaccines have opened up a new era in the field of vaccine development, with their ease of use, safety record and broad applicability. These vaccines have been shown to be extremely efficient at priming the immune system and have been successfully used in several DNA prime/ recombinant viral vector or recombinant protein boost immunization protocols. However, DNA vaccines by themselves are relatively weak and several complementary strategies have been developed to enhance the potency of these vaccines. One such strategy is the use of co-stimulatory molecules and we have investigated the potential of co-stimulation through 4-1BB (a co-stimulatory molecule belonging to the TNFR superfamily) as an adjuvant for a HIV-1 DNA vaccine in mice

(detailed in Chapter 2). Our data suggest that for a HIV-1 vaccine where both antigen-specific cellular and humoral immunity are desirable, 4-1BBL expressed by a DNA vaccine is a superior adjuvant than anti-4-1BB agonistic Ab. Following are some of the key findings in his study:

4.1.A. Time of agonistic anti-4-1BB Ab administration may be critical in influencing Ag-specific $CD8^+$ T cell responses

Though several studies have suggested that 4-1BB is a bona-fide CD8 activating molecule, in our study the adjuvant effect was not observed for $CD8^+$ T cell responses following HIV-1 Gag DNA prime (Fig.2.1C). One caveat in our study is that we looked only in the spleen and do not know if agonistic anti-4-1BB Ab had any influence on $CD8^+$ T cell migration to other tissues (systemic and mucosal) in comparison to the non-adjuvanted controls. A suppressive effect on $CD8^+$ T cell responses was also seen when anti-4-1BB mAb was administered on day 1 post LCMV Armstrong infection (*108*). This inhibition in anti-viral $CD8^+$ T cell responses was attributed to high levels of IL-10 production, prolonged TNF-α response and upregulation of Fas.

Following MVA boost, agonistic anti-4-1BB Ab adjuvanted both $CD4^+$ and $CD8^+$ T cell responses and transient *in vivo* CD4 depletion studies suggest that the enhanced CD4 help primed in the adjuvanted group could have contributed to the enhanced $CD8^+$ T cell response. These adjuvanted T cell responses could be either due to the direct action of anti-4-1BB agonistic Ab on T cells or because of better priming by dendritic cells. A subset of murine DCs express 4-1BB, and signaling through 4-1BB has been shown to

induce increased cytokine production (IL-6 and IL-12) and also enhances the ability of DCs to stimulate T cell proliferation (*177*). Another study in murine model using 4-1BB-deficient DCs has shown that 4-1BB-mediated signaling in DCs can enhance T cell survival and also control the duration of DC-T interaction and, therefore immunogenicity (*251*).

4.1.B. Agonistic anti-4-1BB Ab and 4-1BBL DNA had differential effect on Ag-specific humoral immune responses

One key finding in this study is that the same molecular adjuvant when delivered in different forms (agonistic Ab *vs.* ligand DNA) can influence the outcome of Ag-specific humoral immune responses. Interestingly, while the agonistic Ab suppressed humoral immunity to Gag, the 4-1BBL DNA when delivered in *cis* enhanced Gag-specific IgG responses both in a heterologous as well as a homologous prime/ boost regimen (Fig.2.6). A study with β_2-microglobulin-deficient mice has shown that anti-4-1BB-mediated suppression of T-dependent humoral immunity was independent of $CD8^+$ T cells (*252*). Several other studies have shown that 4-1BB-mediated suppression of humoral immunity can be either due to CD4-dependent or CD4-independent mechanisms (Fig. 4.1). Since in our study, we saw an enhanced Ag-specific $CD4^+$ T cell responses (as evaluated by IFN-γ production) following agonistic Ab treatment, we speculate that the suppression of humoral immunity may be mediated by some CD4-independent mechanisms. However, there is evidence that a subset of helper T cells called the follicular B helper T cells (T_{FH}; distinct from Th1 and Th2 $CD4^+$ effector T cells), are

critical for providing help to B cells within the secondary lymphoid organs for Ab production (*253*). It is not known if agonistic anti-4-1BB Ab treatment can result in decreased frequencies of these T_{FH} cells and as a consequence suppress humoral immunity. As shown in Fig.4.1, upon 4-1BB receptor engagement, other cell types such as suppressor $CD8^+$ T cells and $CD4^+$ T regulatory cells can anergize $CD4^+$ T helper cells (either directly or indirectly through DCs and monocytes) and thereby result in suppression of humoral immune responses. There are other CD4-independent mechanisms that can also result in diminished B cell response. Agonistic anti-41BB treatment dramatically promotes T cell activation and these activated T cells (both CD4 and CD8) mediated down-regulation of FDCs (follicular dendritic cells) (*207*). Human monocytes express 4-1BB receptor constitutively and signaling through the receptor results in monocyte activation. These activated monocytes induce apoptosis in B cells by mechanisms that require direct cell – cell contact (*208*). We are yet to characterize these responses.

4.1.C. Unlike 4-1BBL DNA forms, agonistic anti-4-1BB Ab results in non-specific systemic immune activation

When systemic immune activation status was evaluated by CD11a expression on total lymphocytes, we found that mice vaccinated with either the membrane-bound forms (*trans* and *cis*) or the soluble form of the ligand (SPD-4-1BBL) had an identical *in vivo* activation profile as those immunized with Gag alone (Fig. 4.2). The distinct lymphocyte sub-population that had very high expression of CD11a was only restricted to the agonist

Ab treated group indicating a non-specific systemic hyper-immune activation in that group. Due to the ubiquitous expression of 4-1BB throughout the immune system on various cell types, it has been documented that even a single 200μg dose of anti-4-1BB Ab when injected in naïve mice culminated in a series of immunological abnormalities (splenomegaly, lymphadenopathy, hepatomegaly, altered trafficking of immune cells, loss of NK cells etc.) (*254*). CD11a is a cell surface molecule, expressed (on more than 90% of mature T cell, B cells, granulocytes and monocytes) as a heterodimer along with CD18 and is together known as LFA-1 (lymphocyte function-associated antigen 1). LFA-1 binds to ICAM-1 (intercellular adhesion molecule-1) and is primarily involved in intercellular adhesion, leukocyte trafficking and co-stimulation. Though CD11a is expressed on naïve cells, its expression is further up-regulated on antigen-experienced cells. From various studies it has emerged that LFA-1 (CD11a/ CD18 heterodimer) is a pivotal regulator of inflammatory processes (*255-257*). It needs to be further elucidated whether there is any link between the activation status of the immune system and the complete contradictory outcome in the humoral immune responses when 4-1BB agonistic Ab is compared to the 4-1BBL DNA. Very large amount of IFN-γ (which can be an outcome of systemic hyper-immune activation) has shown to be immunosuppressive by induction of suppressor $CD8^+$ T cells that in-turn can mediate suppression of $CD4^+$ T cells, thus hindering the induction of humoral immune response.

4.1.D. Multimeric form of the soluble 4-1BBL DNA is not immunostimulatory *in vivo*

Several studies have shown that multimerization is an important aspect for the biological activity of many members belonging to the TNF superfamily such as FasL, CD40L and GITRL (*194*). Kinetics and affinity studies has revealed that both trimeric CD40L as well as SPD-CD40L bound CD40 strongly and had similar avidity for the receptor (*195*). However, higher co-stimulatory activity of the SP-D form of the ligands has been attributed to the binding of the scaffold moiety of the collagen-like portion of SP-D to gp340 (a receptor found on macrophages and possibly DCs) (*258*), thereby resulting in receptor clustering/ generation of high-order oligomeric complexes during cell-cell interaction that ultimately culminates in more effective signaling. An interesting finding in our study is that though the soluble multimeric form (SPD-4-1BBL) showed high levels of *in vitro* activity in comparison to the membrane-bound forms of the ligand (Fig.2.3.C), yet *in vivo*, adjuvanticity was observed only with the membrane-bound form of the ligand when delivered in *cis* along with the antigen. One speculation is that maybe the soluble SPD-4-1BBL is activating the CD4 regulatory T cells (that is known to constitutively express 4-1BB receptor) and thus being less immunogenic *in vivo*. Another possibility is that soluble SPD-4-1BBL can also signal through 4-1BB on DCs and induce Stat3 (as has been shown with agonistic anti-4-1BB Ab) (*259*) and Stat3 activation has been shown to block DC maturation and induce T cell tolerance (*260*). It may be pointed out here that the VLP-incorporated 4-1BBL (generated by the *cis* form DNA plasmid; Gag-m4-1BBL) is also in a sense a soluble form of the ligand and may behave similarly as the SPD-4-1BBL *in vivo*. However we speculate that the strength of signaling

delivered by the SPD-form is several-fold greater in magnitude (since it is designed to be multimeric and can also result in receptor clustering through the scaffold moiety of the SP-D) than that delivered by few trimeric 4-1BBL molecules incorporated in the VLPs. It needs to be further explored whether such difference in strength of signaling can ultimately result in differential outcome in immune responses. Higher *in vivo* immunogenicity of the membrane-bound *cis* form of the ligand may be due to both 1) co-migration of the antigen and the adjuvant (in the form of VLPs) to the draining lymph node to prime an immune response, and 2) the ability to retain reverse signaling in the APCs upon receptor: ligand interaction. 4-1BBL-mediated reverse signaling into immature DCs results in increased levels of CD11c, 4-1BBL and IL-12, perhaps due to enhanced TNF production by 4-1BBL signaling in DCs leading to their enhanced maturation (*261*). 4-1BBL signaling-mediated upregulation of CD80, CD86 and MHCII in DCs results in an enhancement of their antigen-presenting capacity and consequently, an initiation or enhancement of immune response. More detailed studies with conditional 4-1BB or 4-1BBL knockout mice needs to be done to understand the involvement of the different cell types in generating Ag-specific immune responses following administration of the agonistic anti-4-1BB Ab or 4-1BBL DNA.

In vivo adjuvancity was however not achieved with the *trans* plasmid (m4-1BBL) that also encoded the membrane-bound trimeric form of the ligand, and immune responses were comparable to the 'no adjuvant' group. This could be due to the fact that though the antigen and the adjuvant plasmids were pre-mixed and co-injected, yet, they may not be taken up by the same APC simultaneously. The fact that supernatant from *trans* m4-1BBL plasmid transfected 293T cells could not induce enhanced proliferation

do not come as a surprise as the protein remains cell membrane-bound and there is no Gag available to induce the formation of VLPs (Fig.2.3.C).

4.1.E. Implications for HIV vaccines

Over the last couple of decades, an immense amount of concerted effort has been focused on generating B and $CD8^+$ T cell-based HIV vaccines. Though $CD8^+$ T cell based vaccines were efficacious (in terms of protection from challenges) in several preclinical studies in macaques, yet, the failure of the STEP trial (which was primarily a CTL-based approach) has clearly underscored the importance of synergizing both T and B cell immunity for conferring protection against HIV. The direct contribution of $CD4^+$ T cells in HIV immunity has remained controversial as these cells can also be targets of productive viral replication. However, it is important to maintain an optimal $CD4^+$ T cell function to be able to provide the critical "help" to antigen-specific B and $CD8^+$ T cells. A strong Ab and CTL response to the virus will result in decreased viral load and consequently render $CD4^+$ T cells less susceptible to infection. Our prototype DNA vaccine (expressing HIV-1 Gag and membrane-bound 4-1BBL) was able to generate CD4, CD8 and B cell responses in BALB/c mice. However, future studies in macaque model needs to be carried out to validate the immunogenicity data seen in mice and protection against a pathogenic SIV infection.

4.2. Understanding the mechanisms of Ad5-mediated induction of mucosal homing of antigen-specific $CD8^+$ T cells

Apart from generating both cellular and humoral immunity to the virus, successful HIV vaccines must induce both systemic as well as mucosal immunity. Several studies have shown that protective immunity at the mucosal surface is absolutely essential to prevent systemic dissemination of the virus. It may be relevant to point out here that in the recent Thai vaccine trial that reported a modest decrease in HIV acquisition (*32*), there was no effect on set-point viral load once systemic infection was established. This strongly argues for the fact that it is more crucial to prevent rather than control systemic infection and to achieve that HIV vaccines must generate protective mucosal immunity. The general consensus of the vaccine field is that only mucosal route of immunization (oral, intranasal, intrarectal or intravaginal) can generate protective, durable immunity at the mucosa. However, vaccine studies with rAd5 vector in mice and macaques, have shown that following parenteral (intramuscular) immunization, Ad5-primed antigen-specific $CD8^+$ T cells could also home to gut and confer immunity at the gut-associated mucosal tissue. Furthermore, data from the STEP trial showed a preferential expansion of gut homing $CD4^+$ T cells primed by the rAd5 vaccine vector (*43*). We thus sought to understand the mechanism underlying this phenomenon (detailed in Chapter 3). Our data suggests that Ad5 can rapidly induce the expression of retinal dehydrogenase enzymes (RALDH) in peripheral (non-gut resident) conventional DCs (cDC) and enhances their ability to prime antigen-specific $CD8^+$ T cells with gut homing potential (as indicated by the $\alpha4\beta7$ expression on $CD8^+$ T cells) (Fig. 4.3). In contrast to TLR2 ligand (zymosan) stimulated splenic cDCs (*228*) and gut-resident $CD103^+$ DCs that upregulates or

constitutively express only *Aldh1a2* respectively (*146, 262*), Ad5 stimulated splenic cDCs upregulated all three isoforms of RALDH. Interestingly, both live as well as inactivated Ad5 can modulate cDCs to upregulate all three isoforms of RALDH enzymes. This effect of Ad5 did not require signaling through Toll-like receptors (TLRs) as evident from the data with MyD88 and TRIF KO mice. Cytosolic dsDNA is either sensed/ recognized by cytosolic DNA sensor DAI (previously known as Zbp1) or AT-rich dsDNA can be converted to a 5'-triphosphate dsRNA which in-turn is sensed by retinoic acid-inducible gene I (RIG-I) (*263*). Ad5 DNA GC content is around 55% (*264*) and our data with cDCs from *Zbp1* knockout mice suggest that adenoviral DNA recognition is not critical for modulating cDC function to be able to prime gut-homing $CD8^+$ T cells. However, there may be other unidentified cytoplasmic DNA sensors that may play a pivotal role in sensing Ad5 DNA. Cytoplasmic dsDNA can also be recognized by DNA sensing inflammasomes like AIM2 (absent in melanoma 2), activation of which ultimately leads to the induction of anti-viral cytokines like IFN-β and inflammatory cytokines like IL-1β (*231*). AIM2 oligomerization upon dsDNA binding leads to caspase-1 activation and that is crucial for the proteolytic cleavage of pro-IL-1β to IL-1β. Inclusion of a specific csapase-1 inhibitor during Ad5 infection of splenic cDCs did not impede cDC function to induce upregulation of α4β7 on $CD8^+$ T cells. However, since dsDNA can also activate NALP3 inflammasomes (a caspase-1 inflammasome), further studies in ASC (apoptosis-related speck-like protein; an adaptor molecule that is downstream of several NLRs) knockout mice needs to be done to delineate the role of Ad5 DNA recognition by inflammasomes in modulating cDC function with respect to induction of RA-metabolizing enzymes. Ad5 infection is known to induce several MAPK

pathways (*233*). However, inhibiting some MAP kinase signaling cascades (ERK, JNK and p38 MAPK) using specific inhibitors during Ad5 infection, did not abrogate Ad5-infected cDC function to prime gut-homing $CD8^+$ T cells. It may be pointed out here that TLR2-mediated *Aldh1a2* upregulation in splenic DCs was ERK-dependent (*228*).

4.2.A. Ad5-induced NF-κB activation in splenic cDCs is critical for imprinting gut homing marker on $CD8^+$ T cells

Our results indicated that the key signaling/ transcription factor in splenic cDCs, the inhibition/ absence of which resulted in the abrogation of α4β7 induction on $CD8^+$ T cells, was NF-κB. Importantly, NF-κB (c-Rel) binding sites are also present upstream of murine *Aldh1a2* promoter (*240*). NF-κB can be induced by a variety of stimuli and these transcription factors are essential for DC activation and cytokine responses (*265, 266*). Ad5-infection/ stimulation is also known to activate both canonical as well as noncanonical NF-κB pathways (*233, 267, 268*). A study in murine model has shown that Ad5-infection mediated NF-κB (p65) activation can also occur independent of viral gene transcription (*241*). Ad5-infection is also known to induce Type I IFNs and pro-inflammatory cytokines which in-turn is driven by transcription factors like IRF3 and NF-κB (*269*). However, inclusion of neutralizing Abs to Type I IFNs during Ad5-infection of cDCs as well as in the DC:T cell co-culture, did not abrogate α4β7 expression on $CD8^+$ T cells. Further studies in IFNα/β receptor knockout mice need to be done to understand if Type I IFNs directly influence the upregulation of RALDH enzymes in cDCs or α4β7 on $CD8^+$ T cell surface. What is currently unknown in our

study is that which signaling cascade is being targeted by Ad5 that is ultimately leading to the induction of NF-κB. Also, which part of Ad5 (nucleic acid, capsid proteins, fiber, knob etc.) is indispensible for driving this signaling cascade? Given the rapid kinetics of induction of these enzymes in DCs following Ad5 infection/ stimulation, our results provide an innate mechanism through which Ad5 can prime antigen-specific $CD8^+$ T cells with gut homing potential.

4.2.B. RALDH enzymes in peripheral DCs can also be upregulated by a diverse array of stimulations

Other studies have also shown that RA-metabolizing enzymes in peripheral DCs can be induced under certain culture conditions or stimulations. Murine bone-marrow derived DCs (BM-DCs) when generated with GM-CSF expressed *Aldh1a2,* but Flt3 ligand generated BM-DCs did not (*270*). GM-CSF and IL-4 also synergistically induced *Aldh1a2* in splenic DCs. Another study in mouse model showed that stimulation of splenic DCs with a TLR2 ligand (zymosan) induced DCs to express *Aldh1a2* and IL-10 (*228*). It may be important to emphasize here that not all TLR stimulation can induce RALDH enzymes in splenic cDCs as stimulation with LPS (a TLR4 ligand) failed to so. A more recent study has reported that RA-producing DCs can also be found in the skin and lungs under steady-state conditions in mice (*271*). MCMV infection has also been shown to induce RALDH enzymes in splenic DCs. One striking difference between all these studies and our study with Ad5 is that, upon Ad5 stimulation/ infection, all three isoforms of *Aldh1a* was upregulated. Whether this phenomenon is unique to Ad5 or not

needs to be further understood. MVA-infected murine splenic cDCs can also prime $CD8^+$ T cells to upregulate $\alpha4\beta7$ and data with retinoic acid receptor (RAR) inhibitor and RALDH inhibitor indicate that it is also RA-mediated (unpublished observations). However, unlike Ad5, cDCs stimulated/ infected with UV-inactivated MVA showed compromised ability to prime $\alpha4\beta7$ expression on $CD8^+$ T cells in comparison to wild-type MVA. Thus for MVA, viral gene transcription may be critical to modulate cDC function with respect to inducing RA-metabolizing enzymes. It will be interesting to see if Ad5-infected human DCs could also upregulate RALDH enzymes or not and this may offer a mechanistic understanding as to how Ad5 primed gut homing $CD4^+$ T cells in the recent STEP trial.

4.2.C. Ad5 component can serve as novel mucosal adjuvant

The challenge is to identify the key Ad5 protein/s or nucleic acid that is instrumental in modulating peripheral DC function resulting in the upregulation of RALDH enzymes. It may be further extrapolated that this purified Ad5 component (which primes DCs to upregulate RALDH enzymes) can serve as a mucosal adjuvant for a subunit vaccine administered intramuscularly. One may argue that given the vast genetic diversity of HIV, subunit vaccines will not be protective. Studies in humans have shown that, though rarely generated, neutralizing Abs to conserved regions in the envelope (like 2G12 recognizing the carbohydrate epitope on HIV-1 Env (*272*); b12 recognizing the CD4 binding site (*273*); 4E10 binding to the membrane-proximal region (*274*)) do appear very late in the chronic phase of the infection but not during the acute

phase (*275, 276*). Preventative subunit vaccines thus maybe designed utilizing envelope constructs that have the conserved regions of the protein exposed so that they can now be targeted by the humoral immune system and generate a pool of neutralizing Abs that will be available at the very onset of infection. A study has shown that Retinoic Acid (RA) made by GALT DCs, along with either IL-5 or IL-6, can induce IgA production by B cells following stimulation with T-independent antigens (*213*). It will be interesting to see if the key Ad5 component that upregulates RALDH enzymes in peripheral DCs, can also serve as a B cell adjuvant that synergizes both mucosal homing and effector activity. Gaining insights into mechanisms that regulate Ad-induced innate immune responses is important, since Ad-based vaccines are in an advanced stage of development and are being tested in humans.

4.3. Legends to the Figures

Figure 4.1: Model proposing suppression of humoral immune response by agonistic anti-4-1BB Ab. Murine B cells do not express 4-1BB and effects of 4-1BB on humoral immunity has been shown to be mediated through other cell types. Suppression of humoral immunity can be either due to CD4-dependent or CD4-independent mechanisms. 4-1BB mediated co-stimulation leads to the expansion of $CD11c^+$ $CD8^+$ T cells (suppressor CD8) that produce large amounts of IFN-γ (*189*). This excessive amount of IFN-γ in turn regulates the transcription of IDO (indoleamine 2,3-dioxygenase) gene in DCs and monocytes/ macrophages (paracrine effect). Expression of IDO interferes with tryptophan metabolism which may cause selective depletion of antigen-specific $CD4^+$ T cells. 4-1BB mediated co-stimulation can also generate effector $CD8^+$ T cells that can suppress $CD4^+$ T cells via a TGF-β-dependent mechanism (*206*). $CD4^+$ $CD25^+$ T regulatory cells constitutively express 4-1BB receptor and signaling through 4-1BB can significantly augment their proliferation (*205*). These 4-1BB-expanded T_{reg} cells also retain their suppressive potential. Any one or all of these mechanisms can either deplete or anergize $CD4^+$ T cells, thus resulting in the loss of CD4 help for induction of humoral immune response. Another murine study in an autoimmune disease model (Systemic lupus erythematosus, SLE) has shown that 4-1BB-mediated signaling anergized $CD4^+$ T cells during priming at DC interface, thus resulting in a lack of $CD4^+$ T cell help and a block in the germinal center formation (*188*). There are other CD4-independent mechanisms that can also result in diminished B cell response. These include down-regulation of FDCs (follicular dendritic cells) (*207*) and B cell apoptosis induced by activated human monocytes (*208*).

Figure 4.2: Non-specific systemic immuneactivation with agonistic anti-4-1BB Ab. Mice were either vaccinated with the antigen (Gag) alone or coimmunized with the adjuvant (agonistic anti-4-1BBAb or different forms of 4-1BBL DNA). Total lymphocytes were gated on the basis of forward and side scatter. Surface expression of CD11a on lymphocyte population was analyzed by flow cytometry (using CD11a-PE) to evaluate the systemic immune activation status *in vivo*. A naïve mouse was used as a control to look at the baseline *in vivo* expression of CD11a.

Figure 4.3: A schematic representation of induction of RA-metabolizing enzymes upon Ad5-infection/ stimulation of splenic conventional DCs. Live or inactivated Ad5 infection/ stimulation can induce murine splenic cDCs to upregulate retinal dehydrogenase (RALDH) enzymes. These enzymes can metabolize retinal to retinoic acid (RA). The RA produced by the cDCs bind to the retinoic acid receptor (RAR/RXR) in $CD8^+$ T cells and upregulates the expression of $\alpha4\beta7$ on these cells. This effect of Ad5 to be able to modulate cDC function with respect to intracellular RA metabolism did not require signaling through toll-like receptors (TLRs), DNA-dependent activator of IRFs (DAI) and several MAP kinases but was dependant on NF-κB. Intracellular recognition of Ad5 DNA by inflammasomes or Type I IFNs (induced upon Ad5 infection) may not be critical in modulating splenic cDC to upregulate RALDH enzymes.

4.4. Figures

Fig.4.1. Model proposing suppression of humoral immune response by agonistic anti-4-1BB Ab

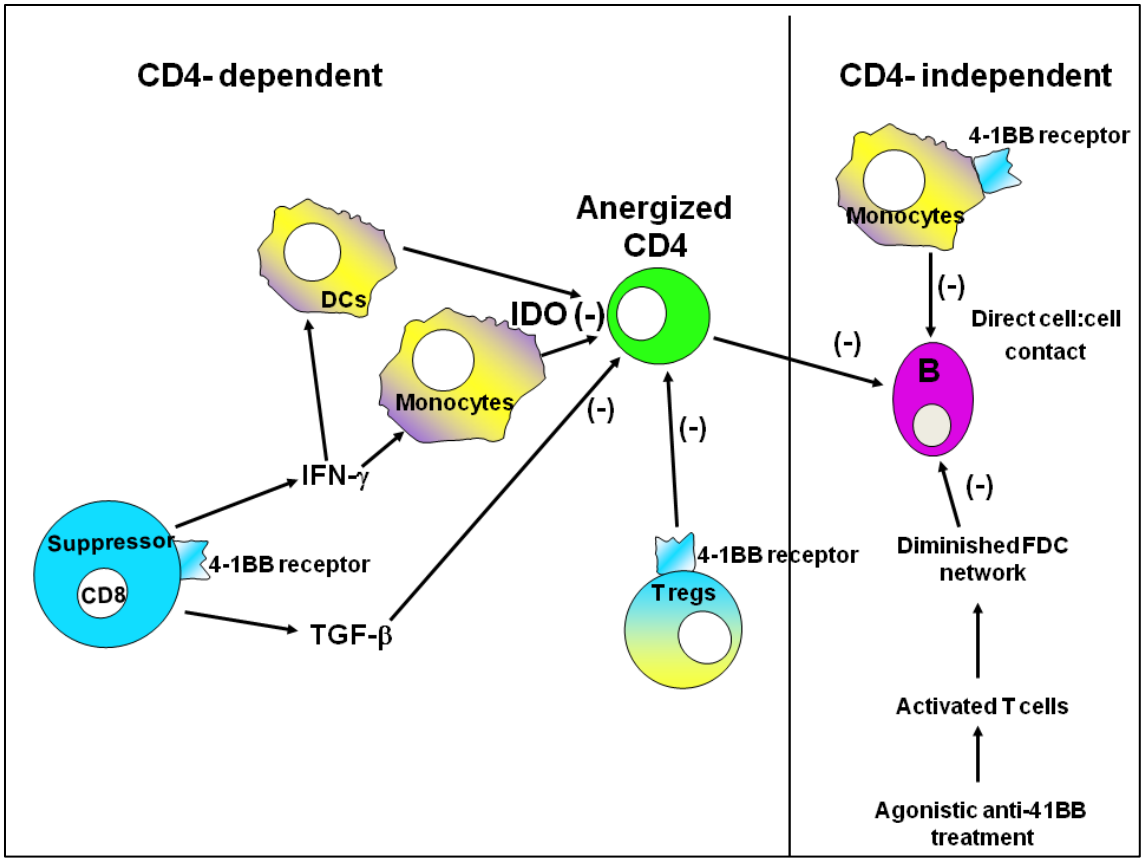


Fig.4.2. Non-specific systemic immuneactivation with agonistic anti-4-1BB Ab

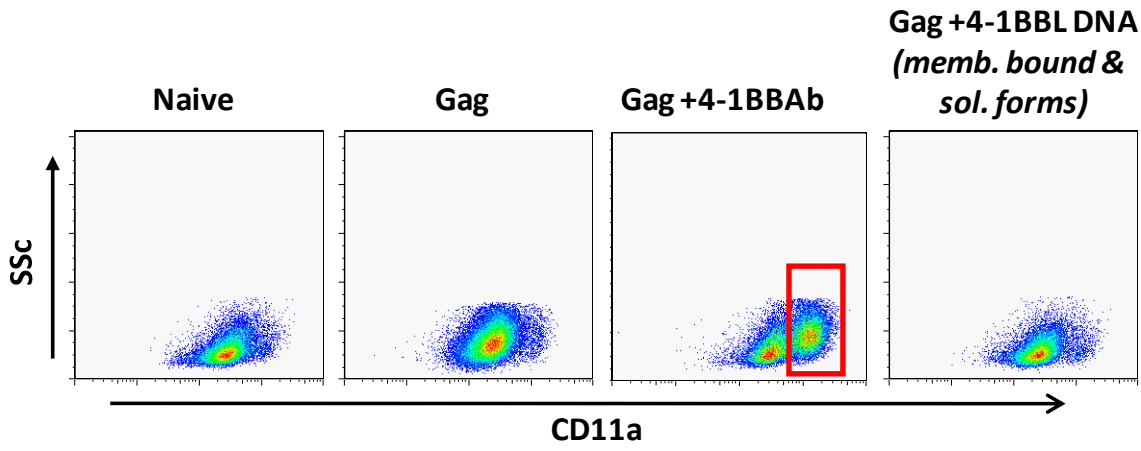
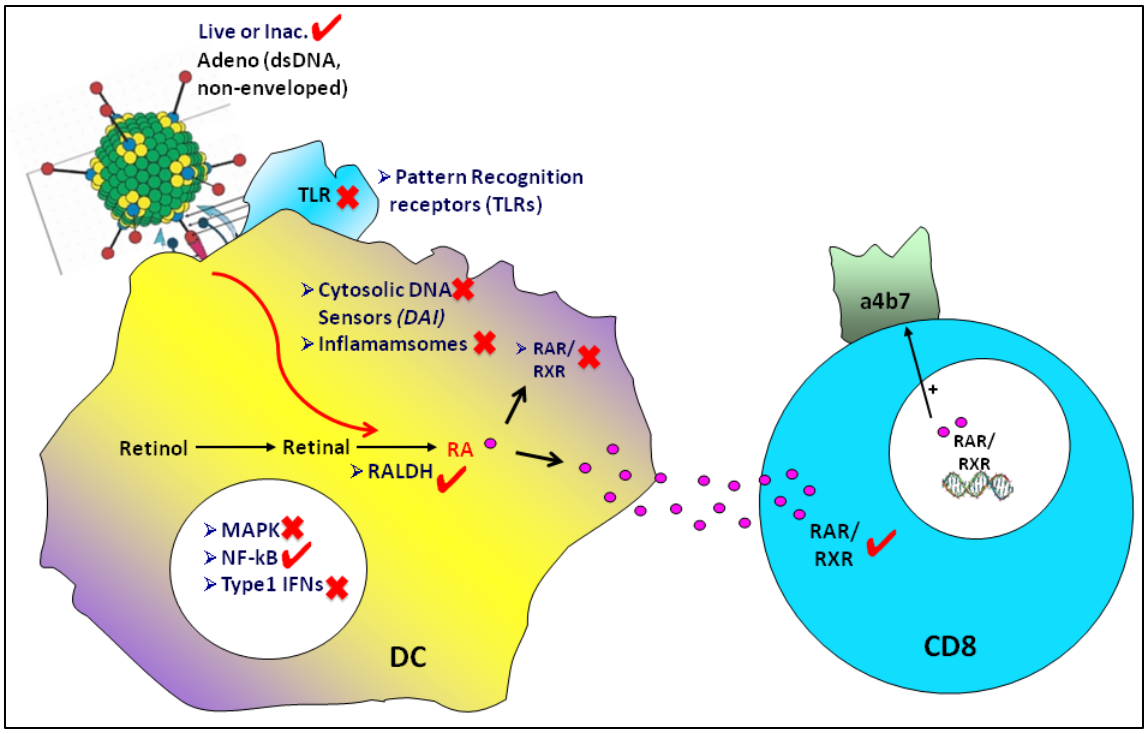


Fig.4.3. A schematic representation of induction of RA-metabolizing enzymes upon Ad5-infection/ stimulation of splenic conventional DCs



References

1. Veronese, F. D., DeVico, A. L., Copeland, T. D., Oroszlan, S., Gallo, R. C., and Sarngadharan, M. G. (1985) Characterization of gp41 as the transmembrane protein coded by the HTLV-III/LAV envelope gene, *Science 229*, 1402-1405.
2. Robey, W. G., Safai, B., Oroszlan, S., Arthur, L. O., Gonda, M. A., Gallo, R. C., and Fischinger, P. J. (1985) Characterization of envelope and core structural gene products of HTLV-III with sera from AIDS patients, *Science 228*, 593-595.
3. Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A. (1996) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor, *Science 272*, 872-877.
4. Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., and Sodroski, J. (1996) The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates, *Cell 85*, 1135-1148.
5. Chan, D. C., Fass, D., Berger, J. M., and Kim, P. S. (1997) Core structure of gp41 from the HIV envelope glycoprotein, *Cell 89*, 263-273.
6. Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J., and Wiley, D. C. (1997) Atomic structure of the ectodomain from HIV-1 gp41, *Nature 387*, 426-430.
7. 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. (1999) Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells, *Science 286*, 1353-1357.

8. 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. (1999) Rapid infection of oral mucosal-associated lymphoid tissue with simian immunodeficiency virus, *Science 285*, 1261-1265.
9. 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. (2002) HIV preferentially infects HIV-specific CD4+ T cells, *Nature 417*, 95-98.
10. Steffens, C. M., Managlia, E. Z., Landay, A., and Al-Harthi, L. (2002) Interleukin-7-treated naive T cells can be productively infected by T-cell-adapted and primary isolates of human immunodeficiency virus 1, *Blood 99*, 3310-3318.
11. Eckstein, D. A., Penn, M. L., Korin, Y. D., Scripture-Adams, D. D., Zack, J. A., Kreisberg, J. F., Roederer, M., Sherman, M. P., Chin, P. S., and Goldsmith, M. A. (2001) HIV-1 actively replicates in naive CD4(+) T cells residing within human lymphoid tissues, *Immunity 15*, 671-682.
12. Geijtenbeek, T. B. H., Kwon, D. S., Torensma, R., Van Vliet, S. J., Van Duijnhoven, G. C. F., Middel, J., Cornelissen, I. L. M. H. A., Nottet, H. S. L. M., KewalRamani, V. N., Littman, D. R., Figdor, C. G., and Van Kooyk, Y. (2000) DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells, *Cell 100*, 587-597.

13. 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. (1998) Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection, *Science 280*, 427-431.

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

15. McCune, J. M. (2001) The dynamics of CD4+ T-cell depletion in HIV disease, *Nature 410*, 974-979.

16. Dion, M.-L., Poulin, J.-F., Bordi, R., Sylvestre, M., Corsini, R., Kettaf, N., Dalloul, A., Boulassel, M.-R., Debre, P., Routy, J.-P., Grossman, Z., Sekaly, R.-P., and Cheynier, R. (2004) HIV infection rapidly induces and maintains a substantial suppression of thymocyte proliferation, *Immunity 21*, 757-768.

17. Bentwich, Z., Kalinkovich, A., Weisman, Z., and Grossman, Z. (1998) Immune activation in the context of HIV infection, *Clin Exp Immunol 111*, 1-2.

18. Ascher, M. S., and Sheppard, H. W. (1990) AIDS as immune system activation. II. The panergic imnesia hypothesis, *J Acquir Immune Defic Syndr 3*, 177-191.

19. 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. (2006) Microbial translocation is a cause of systemic immune activation in chronic HIV infection, *Nature Medicine 12*, 1365-1371.

20. Moss, P. A., Rowland-Jones, S. L., Frodsham, P. M., McAdam, S., Giangrande, P., McMichael, A. J., and Bell, J. I. (1995) Persistent high frequency of human immunodeficiency virus-specific cytotoxic T cells in peripheral blood of infected donors, *Proc Natl Acad Sci U S A 92*, 5773-5777.

21. Moore, J. P., Kitchen, S. G., Pugach, P., and Zack, J. A. (2004) The CCR5 and CXCR4 Coreceptors-Central to Understanding the Transmission and Pathogenesis of Human Immunodeficiency Virus Type 1 Infection, *AIDS Research and Human Retroviruses 20*, 111-126.

22. Gulick, R. M., Mellors, J. W., Havlir, D., Eron, J. J., Gonzalez, C., McMahon, D., Richman, D. D., Valentine, F. T., Jonas, L., Meibohm, A., Emini, E. A., and Chodakewitz, J. A. (1997) Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy, *N Engl J Med 337*, 734-739.

23. Emini, E. A., Schleif, W. A., Nunberg, J. H., Conley, A. J., Eda, Y., Tokiyoshi, S., Putney, S. D., Matsushita, S., Cobb, K. E., and Jett, C. M. (1992) Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody, *Nature 355*, 728-730.

24. 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. (1999) Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes, *Science 283*, 857-860.

25. 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. (1999) Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques, *J Exp Med 189*, 991-998.

26. Parren, P. W., Moore, J. P., Burton, D. R., and Sattentau, Q. J. (1999) The neutralizing antibody response to HIV-1: viral evasion and escape from humoral immunity, *AIDS 13 Suppl A*, S137-162.

27. Chackerian, B., Rudensey, L. M., and Overbaugh, J. (1997) Specific N-linked and O-linked glycosylation modifications in the envelope V1 domain of simian immunodeficiency virus variants that evolve in the host alter recognition by neutralizing antibodies, *Journal of Virology 71*, 7719-7727.

28. Koenig, S., Conley, A. J., Brewah, Y. A., Jones, G. M., Leath, S., Boots, L. J., Davey, V., Pantaleo, G., Demarest, J. F., and Carter, C. (1995) 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 1*, 330-336.

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

30. Amara, R. R., Villinger, F., Altman, J. D., Lydy, S. L., O'Neil, S. P., Staprans, S. I., Montefiori, D. C., Xu, Y., Hemdon, 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. (2001) Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine, *Science 292*, 69-74.

31. 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. (2000) Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination, *Science 290*, 486-492.
32. Rerks-Ngarm, S., Pitisuttithum, P., Nitayaphan, S., Kaewkungwal, J., Chiu, J., Paris, R., Premsri, N., Namwat, C., de Souza, M., Adams, E., Benenson, M., Gurunathan, S., Tartaglia, J., McNeil, J. G., Francis, D. P., Stablein, D., Birx, D. L., Chunsuttiwat, S., Khamboonruang, C., Thongcharoen, P., Robb, M. L., Michael, N. L., Kunasol, P., and Kim, J. H. (2009) Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand, *N Engl J Med 361*, 2209-2220.
33. Cohen, J. (2003) Public health: AIDS vaccine trial produces disappointment and confusion, *Science 299*, 1290-1291.
34. Gilbert, P. B., Peterson, M. L., Follmann, D., Hudgens, M. G., Francis, D. P., Gurwith, M., Heyward, W. L., Jobes, D. V., Popovic, V., Self, S. G., Sinangil, F., Burke, D., and Berman, P. W. (2005) Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a

phase 3 HIV-1 preventive vaccine trial, *Journal of Infectious Diseases 191*, 666-677.

35. Graham Barney, S., and Mascola John, R. (2005) Lessons from failure--preparing for future HIV-1 vaccine efficacy trials, *J Infect Dis 191*, 647-649.
36. 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. (2008) Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial, *Lancet 372*, 1881-1893.
37. Desrosiers, R. C. (2004) Prospects for an AIDS vaccine, *Nature Medicine 10*, 221-223.
38. Baba, T. W., Jeong, Y. S., Pennick, D., Bronson, R., Greene, M. F., and Ruprecht, R. M. (1995) Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques, *Science 267*, 1820-1825.
39. Shiver John, W., Fu, T.-M., Chen, L., Casimiro Danilo, R., Davies, M.-E., Evans Robert, K., Zhang, Z.-Q., Simon Adam, J., Trigona Wendy, L., Dubey Sheri, A., Huang, L., Harris Virginia, A., Long Romnie, S., Liang, X., Handt, L., Schleif William, A., Zhu, L., Freed Daniel, C., Persaud Natasha, V., Guan, L., Punt Kara, S., Tang, A., Chen, M., Wilson Keith, A., Collins Kelly, B., Heidecker Gwendolyn, J., Fernandez, V. R., Perry Helen, C., Joyce Joseph, G., Grimm Karen, M., Cook James, C., Keller Paul, M., Kresock Denise, S., Mach, H., Troutman Robert, D., Isopi Lynne, A., Williams Donna, M., Xu, Z., Bohannon Kathryn, E., Volkin

David, B., Montefiori David, C., Miura, A., Krivulka Georgia, R., Lifton Michelle, A., Kuroda Marcelo, J., Schmitz Jorn, E., Letvin Norman, L., Caulfield Michael, J., Bett Andrew, J., Youil, R., Kaslow David, C., and Emini Emilio, A. (2002) Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity, *Nature 415*, 331-335.

40. Farina, S. F., Gao, G.-P., Xiang, Z. Q., Rux, J. J., Burnett, R. M., Alvira, M. R., Marsh, J., Ertl, H. C. J., and Wilson, J. M. (2001) Replication-defective vector based on a chimpanzee adenovirus, *Journal of Virology 75*, 11603-11613.
41. 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. (2003) Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: Efficient human cell infection and bypass of preexisting adenovirus immunity, *Journal of Virology 77*, 8263-8271.
42. Shiver, J. W., and Emini, E. A. (2004) Recent advances in the development of HIV-1 vaccines using replication-incompetent adenovirus vectors, *Annual Review of Medicine 55*, 355-372.
43. Benlahrech, A., Harris, J., Meiser, A., Papagatsias, T., Hornig, J., Hayes, P., Lieber, A., Athanasopoulos, T., Bachy, V., Csomor, E., Daniels, R., Fisher, K., Gotch, F., Seymour, L., Logan, K., Barbagallo, R., Klavinskis, L., Dickson, G., and Patterson, S. (2009) Adenovirus vector vaccination induces expansion of memory CD4 T cells

with a mucosal homing phenotype that are readily susceptible to HIV-1, *Proc Natl Acad Sci U S A 106*, 19940-19945.

44. Meyer, H., Sutter, G., and Mayr, A. (1991) Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence, *Journal of General Virology 72*, 1031-1038.

45. Sutter, G., and Moss, B. (1992) Nonreplicating vaccinia vector efficiently expresses recombinant genes, *Proc Natl Acad Sci U S A 89*, 10847-10851.

46. Schneider, J., Gilbert, S. C., Blanchard, T. J., Hanke, T., Robson, K. J., Hannan, C. M., Becker, M., Sinden, R., Smith, G. L., and Hill, A. V. (1998) Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara, *Nat Med 4*, 397-402.

47. McShane, H., Brookes, R., Gilbert, S. C., and Hill, A. V. (2001) Enhanced immunogenicity of CD4(+) t-cell responses and protective efficacy of a DNA-modified vaccinia virus Ankara prime-boost vaccination regimen for murine tuberculosis, *Infect Immun 69*, 681-686.

48. Stittelaar, K. J., Kuiken, T., de Swart, R. L., van Amerongen, G., Vos, H. W., Niesters, H. G. M., van Schalkwijk, P., van der Kwast, T., Wyatt, L. S., Moss, B., and Osterhaus, A. D. M. E. (2001) Safety of modified vaccinia virus Ankara (MVA) in immune-suppressed macaques, *Vaccine 19*, 3700-3709.

49. Moorthy, V. S., McConkey, S., Roberts, M., Gothard, P., Arulanantham, N., Degano, P., Schneider, J., Hannan, C., Roy, M., Gilbert, S. C., Peto, T. E. A., and Hill, A. V. S. (2003) Safety of DNA and modified vaccinia virus Ankara vaccines

against liver-stage P. falciparum malaria in non-immune volunteers, *Vaccine 21*, 1995-2002.

50. Dorrell, L., Williams, P., Suttill, A., Brown, D., Roberts, J., Conlon, C., Hanke, T., and McMichael, A. (2007) Safety and tolerability of recombinant modified vaccinia virus Ankara expressing an HIV-1 gag/multiepitope immunogen (MVA.HIVA) in HIV-1-infected persons receiving combination antiretroviral therapy, *Vaccine 25*, 3277-3283.

51. Irvine, K. R., Chamberlain, R. S., Shulman, E. P., Surman, D. R., Rosenberg, S. A., and Restifo, N. P. (1997) Enhancing efficacy of recombinant anticancer vaccines with prime/boost regimens that use two different vectors, *J Natl Cancer Inst 89*, 1595-1601.

52. 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. (2001) Reduction of simian-human immunodeficiency virus 89.6P viremia in rhesus monkeys by recombinant modified vaccinia virus Ankara vaccination, *Journal of Virology 75*, 5151-5158.

53. Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L. (1990) Direct gene transfer into mouse muscle in vivo, *Science 247*, 1465-1468.

54. Babiuk, L. A., Pontarollo, R., Babiuk, S., Loehr, B., and van Drunen Littel-van den Hurk, S. (2003) Induction of immune responses by DNA vaccines in large animals, *Vaccine 21*, 649-658.

55. Manam, S., Ledwith, B. J., Barnum, A. B., Troilo, P. J., Pauley, C. J., Harper, L. B., Griffiths, T. G., 2nd, Niu, Z., Denisova, L., Follmer, T. T., Pacchione, S. J., Wang, Z., Beare, C. M., Bagdon, W. J., and Nichols, W. W. (2000) Plasmid DNA vaccines: tissue distribution and effects of DNA sequence, adjuvants and delivery method on integration into host DNA, *Intervirology 43*, 273-281.

56. Pal, R., Yu, Q., Wang, S., Kalyanaraman, V. S., Nair, B. C., Hudacik, L., Whitney, S., Keen, T., Hung, C. L., Hocker, L., Kennedy, J. S., Markham, P., and Lu, S. (2006) Definitive toxicology and biodistribution study of a polyvalent DNA prime/protein boost human immunodeficiency virus type 1 (HIV-1) vaccine in rabbits, *Vaccine 24*, 1225-1234.

57. MacGregor, R. R., Boyer, J. D., Ciccarelli, R. B., Ginsberg, R. S., and Weiner, D. B. (2000) Safety and immune responses to a DNA-based human immunodeficiency virus (HIV) type I env/rev vaccine in HIV-infected recipients: follow-up data, *J Infect Dis 181*, 406.

58. Calarota, S., Bratt, G., Nordlund, S., Hinkula, J., Leandersson, A. C., Sandstrom, E., and Wahren, B. (1998) Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients, *Lancet 351*, 1320-1325.

59. Andre, S., Seed, B., Eberle, J., Schraut, W., Bultmann, A., and Haas, J. (1998) Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage, *Journal of Virology 72*, 1497-1503.

60. Bojak, A., Wild, J., Deml, L., and Wagner, R. (2002) Impact of codon usage modification on T cell immunogenicity and longevity of HIV-1 gag-specific DNA vaccines, *Intervirology 45*, 275-286.

61. Barouch, D. H., Santra, S., Tenner-Racz, K., Racz, P., Kuroda, M. J., Schmitz, J. E., Jackson, S. S., Lifton, M. A., Freed, D. C., Perry, H. C., Davies, M.-E., Shiver, J. W., and Letvin, N. L. (2002) Potent CD4+ T cell responses elicited by a bicistronic HIV-1 DNA vaccine expressing gp120 and GM-CSF, *Journal of Immunology 168*, 562-568.

62. Svanholm, C., Lowenadler, B., and Wigzell, H. (1997) Amplification of T-cell and antibody responses in DNA-based immunization with HIV-1 Nef by co-injection with a GM-CSF expression vector, *Scandinavian Journal of Immunology 46*, 298-303.

63. Robinson, H. L., Montefiori, D. C., Villinger, F., Robinson, J. E., Sharma, S., Wyatt, L. S., Earl, P. L., McClure, H. M., Moss, B., and Amara, R. R. (2006) Studies on GM-CSF DNA as an adjuvant for neutralizing Ab elicited by a DNA/MVA immunodeficiency virus vaccine, *Virology 352*, 285-294.

64. Lai, L., Vodros, D., Kozlowski, P. A., Montefiori, D. C., Wilson, R. L., Akerstrom, V. L., Chennareddi, L., Yu, T., Kannanganat, S., Ofielu, L., Villinger, F., Wyatt, L. S., Moss, B., Amara, R. R., and Robinson, H. L. (2007) GM-CSF DNA: an adjuvant for higher avidity IgG, rectal IgA, and increased protection against the acute phase of a SHIV-89.6P challenge by a DNA/MVA immunodeficiency virus vaccine, *Virology 369*, 153-167.

65. Kim, J. J., Yang, J. S., VanCott, T. C., Lee, D. J., Manson, K. H., Wyand, M. S., Boyer, J. D., Ugen, K. E., and Weiner, D. B. (2000) Modulation of antigen-specific humoral responses in rhesus macaques by using cytokine cDNAs as DNA vaccine adjuvants, *J Virol 74*, 3427-3429.

66. Kim, J. J., Yang, J. S., Manson, K. H., and Weiner, D. B. (2001) Modulation of antigen-specific cellular immune responses to DNA vaccination in rhesus macaques through the use of IL-2, IFN-g, or IL-4 gene adjuvants, *Vaccine 19*, 2496-2505.

67. Kim, J. J., Ayyavoo, V., Bagarazzi, M. L., Chattergoon, M. A., Dang, K., Wang, B., Boyer, J. D., and Weiner, D. B. (1997) In vivo engineering of a cellular immune response by coadministration of IL-12 expression vector with a DNA immunogen, *J Immunol 158*, 816-826.

68. Tsuji, T., Hamajima, K., Fukushima, J., Xin, K. Q., Ishii, N., Aoki, I., Ishigatsubo, Y., Tani, K., Kawamoto, S., Nitta, Y., Miyazaki, J., Koff, W. C., Okubo, T., and Okuda, K. (1997) Enhancement of cell-mediated immunity against HIV-1 induced by coinnoculation of plasmid-encoded HIV-1 antigen with plasmid expressing IL-12, *J Immunol 158*, 4008-4013.

69. Boyer, J. D., Kim, J., Ugen, K., Cohen, A. D., Ahn, L., Schumann, K., Lacy, K., Bagarazzi, M. L., Javadian, A., Ciccarelli, R. B., Ginsberg, R. S., MacGregor, R. R., and Weiner, D. B. (1999) HIV-1 DNA vaccines and chemokines, *Vaccine 17 Suppl 2*, S53-64.

70. Kim, J. J., Yang, J. S., Dentchev, T., Dang, K., and Weiner, D. B. (2000) Chemokine gene adjuvants can modulate immune responses induced by DNA vaccines, *J Interferon Cytokine Res 20*, 487-498.

71. Sasaki, S., Amara, R. R., Oran, A. E., Smith, J. M., and Robinson, H. L. (2001) Apoptosis-mediated enhancement of DNA-raised immune responses by mutant caspases, *Nat Biotechnol 19*, 543-547.

72. Sasaki, S., Amara, R. R., Yeow, W. S., Pitha, P. M., and Robinson, H. L. (2002) Regulation of DNA-raised immune responses by cotransfected interferon regulatory factors, *J Virol 76*, 6652-6659.

73. Kim, J. J., Bagarazzi, M. L., Trivedi, N., Hu, Y., Kazahaya, K., Wilson, D. M., Ciccarelli, R., Chattergoon, M. A., Dang, K., Mahalingam, S., Chalian, A. A., Agadjanyan, M. G., Boyer, J. D., Wang, B., and Weiner, D. B. (1997) Engineering of in vivo immune responses to DNA immunization via codelivery of costimulatory molecule genes, *Nat Biotechnol 15*, 641-646.

74. Goebels, N., Michaelis, D., Wekerle, H., and Hohlfeld, R. (1992) Human myoblasts as antigen-presenting cells, *J Immunol 149*, 661-667.

75. Kim, J. J., Nottingham, L. K., Wilson, D. M., Bagarazzi, M. L., Tsai, A., Morrison, L. D., Javadian, A., Chalian, A. A., Agadjanyan, M. G., and Weiner, D. B. (1998) Engineering DNA vaccines via co-delivery of co-stimulatory molecule genes, *Vaccine 16*, 1828-1835.

76. Kim, J. J., Tsai, A., Nottingham, L. K., Morrison, L., Cunning, D. M., Oh, J., Lee, D. J., Dang, K., Dentchev, T., Chalian, A. A., Agadjanyan, M. G., and Weiner, D. B. (1999) Intracellular adhesion molecule-1 modulates beta-chemokines and directly costimulates T cells in vivo, *J Clin Invest 103*, 869-877.

77. Stone Geoffrey, W., Barzee, S., Snarsky, V., Kee, K., Spina Celsa, A., Yu, X.-F., and Kornbluth Richard, S. (2006) Multimeric soluble CD40 ligand and GITR ligand as adjuvants for human immunodeficiency virus DNA vaccines, *J Virol 80*, 1762-1772.

78. Miyahira, Y., Akiba, H., Katae, M., Kubota, K., Kobayashi, S., Takeuchi, T., Garcia-Sastre, A., Fukuchi, Y., Okumura, K., Yagita, H., and Aoki, T. (2003) A Potent Adjuvant Effect of Ligand to Receptor Activator of NF-kB Gene for Inducing Antigen-Specific CD8+ T Cell Response by DNA and Viral Vector Vaccination, *Journal of Immunology 171*, 6344-6348.

79. Kwon, B., Lee, H. W., and Kwon, B. S. (2002) New insights into the role of 4-1BB in immune responses: beyond CD8+ T cells, *Trends in Immunology 23*, 378-380.

80. Nishimoto, H., Lee, S.-W., Hong, H., Potter, K. G., Maeda-Yamamoto, M., Kinoshita, T., Kawakami, Y., Mittler, R. S., Kwon, B. S., Ware, C. F., Croft, M., and Kawakami, T. (2005) Costimulation of mast cells by 4-1BB, a member of the tumor necrosis factor receptor superfamily, with the high-affinity IgE receptor, *Blood 106*, 4241-4248.

81. Heinisch, I. V., Bizer, C., Volgger, W., and Simon, H. U. (2001) Functional CD137 receptors are expressed by eosinophils from patients with IgE-mediated allergic responses but not by eosinophils from patients with non-IgE-mediated eosinophilic disorders, *J Allergy Clin Immunol 108*, 21-28.

82. Wilcox, R. A., Chapoval, A. I., Gorski, K. S., Otsuji, M., Shin, T., Flies, D. B., Tamada, K., Mittler, R. S., Tsuchiya, H., Pardoll, D. M., and Chen, L. (2002) Cutting edge: expression of functional CD137 receptor by dendritic cells, *Journal of Immunology 168*, 4262-4267.

83. Goodwin, R. G., Din, W. S., Davis-Smith, T., Anderson, D. M., Gimpel, S. D., Sato, T. A., Maliszewski, C. R., Brannan, C. I., Copeland, N. G., and Jenkins, N. A. (1993) Molecular cloning of a ligand for the inducible T cell gene 4-1BB: a

member of an emerging family of cytokines with homology to tumor necrosis factor, *Eur J Immunol 23*, 2631-2641.

84. Locksley, R. M., Killeen, N., and Lenardo, M. J. (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology, *Cell 104*, 487-501.

85. Cannons, J. L., Lau, P., Ghumman, B., DeBenedette, M. A., Yagita, H., Okumura, K., and Watts, T. H. (2001) 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy, *Journal of Immunology 167*, 1313-1324.

86. Vinay, D. S., and Kwon, B. S. (1998) Role of 4-1BB in immune responses, *Seminars in Immunology 10*, 481-489.

87. Saoulli, K., Lee, S. Y., Cannons, J. L., Yeh, W. C., Santana, A., Goldstein, M. D., Bangia, N., DeBenedette, M. A., Mak, T. W., Choi, Y., and Watts, T. H. (1998) CD28-independent, TRAF2-dependent costimulation of resting T cells by 4-1BB ligand, *J Exp Med 187*, 1849-1862.

88. Hsu, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. (1996) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways, *Cell 84*, 299-308.

89. Nam, K.-O., Kang, H., Shin, S.-M., Cho, K.-H., Kwon, B., Kwon Byoung, S., Kim, S.-J., and Lee, H.-W. (2005) Cross-linking of 4-1BB activates TCR-signaling pathways in CD8+ T lymphocytes, *J Immunol 174*, 1898-1905.

90. Kienzle, G., and Von Kempis, J. (2000) CD137 (ILA/4-1BB), expressed by primary human monocytes, induces monocyte activation and apoptosis of B lymphocytes, *International Immunology 12*, 73-82.

91. Cannons, J. L., Lau, P., Ghumman, B., DeBenedette, M. A., Yagita, H., Okumura, K., and Watts, T. H. (2001) 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy, *J Immunol 167*, 1313-1324.

92. Bukczynski, J., Wen, T., and Watts, T. H. (2003) Costimulation of human CD28- T cells by 4-1BB ligand, *European Journal of Immunology 33*, 446-454.

93. Lee, H.-W., Park, S.-J., Choi Beom, K., Kim Hyun, H., Nam, K.-O., and Kwon Byoung, S. (2002) 4-1BB promotes the survival of CD8+ T lymphocytes by increasing expression of Bcl-xL and Bfl-1, *J Immunol 169*, 4882-4888.

94. Watts, T. H. (2005) TNF/TNFR family members in costimulation of T cell responses, *Annual Review of Immunology 23*, 23-68.

95. Staerck, L., Scholz, C., Doerken, B., and Daniel, P. T. (2005) Costimulation by CD137/4-1BB inhibits T cell apoptosis and induces Bcl-xL and c-FLIPshort via phosphatidylinositol 3-kinase and AKT/protein kinase B, *European Journal of Immunology 35*, 1257-1266.

96. Shuford, W. W., Klussman, K., Tritchler, D. D., Loo, D. T., Chalupny, J., Siadak, A. W., Brown, T. J., Emswiler, J., Raecho, H., Larsen, C. P., Pearson, T. C., Ledbetter, J. A., Aruffo, A., and Mittler, R. S. (1997) 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses, *Journal of Experimental Medicine 186*, 47-55.

97. Tan, J. T., Whitmire, J. K., Murali-Krishna, K., Ahmed, R., Altman, J. D., Mittler, R. S., Sette, A., Pearson, T. C., and Larsen, C. P. (2000) 4-1BB costimulation is

required for protective anti-viral immunity after peptide vaccination, *J Immunol 164*, 2320-2325.

98. Wiethe, C., Dittmar, K., Doan, T., Lindenmaier, W., and Tindle, R. (2003) Provision of 4-1BB Ligand Enhances Effector and Memory CTL Responses Generated by Immunization with Dendritic Cells Expressing a Human Tumor-Associated Antigen, *Journal of Immunology 170*, 2912-2922.
99. Myers, L., Lee, S. W., Rossi, R. J., Lefrancois, L., Kwon, B. S., Mittler, R. S., Croft, M., and Vella, A. T. (2006) Combined CD137 (4-1BB) and adjuvant therapy generates a developing pool of peptide-specific CD8 memory T cells, *International Immunology 18*, 325-333.
100. Bukczynski, J., Wen, T., Ellefsen, K., Gauldie, J., and Watts, T. H. (2004) Costimulatory ligand 4-1BBL (CD137L) as an efficient adjuvant for human antiviral cytotoxic T cell responses, *Proceedings of the National Academy of Sciences of the United States of America 101*, 1291-1296.
101. Gramaglia, I., Cooper, D., Miner, K. T., Kwon, B. S., and Croft, M. (2000) Co-stimulation of antigen-specific CD4 T cells by 4-1BB ligand, *Eur J Immunol 30*, 392-402.
102. Melero, I., Shuford, W. W., Neby, S. A., Aruffo, A., Ledbetter, J. A., Hellstrom, K. E., Mittler, R. S., and Chen, L. (1997) Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors, *Nature Medicine 3*, 682-685.
103. Miller, R. E., Jones, J., Le, T., Whitmore, J., Boiani, N., Gliniak, B., and Lynch, D. H. (2002) 4-1BB-specific monoclonal antibody promotes the generation of tumor-

specific immune responses by direct activation of CD8 T cells in a CD40-dependent manner, *Journal of Immunology 169*, 1792-1800.

104. Ito, F., Li, Q., Shreiner, A. B., Okuyama, R., Jure-Kunkel, M. N., Teitz-Tennenbaum, S., and Chang, A. E. (2004) Anti-CD137 Monoclonal Antibody Administration Augments the Antitumor Efficacy of Dendritic Cell-Based Vaccines, *Cancer Research 64*, 8411-8419.
105. Cuadros, C., Dominguez, A. L., Lollini, P.-L., Croft, M., Mittler, R. S., Borgstroem, P., and Lustgarten, J. (2005) Vaccination with dendritic cells pulsed with apoptotic tumors in combination with Anti-OX40 and Anti-4-1BB monoclonal antibodies induces T cell-mediated protective immunity in Her-2/neu transgenic mice, *International Journal of Cancer 116*, 934-943.
106. Halstead, E. S., Mueller Yvonne, M., Altman John, D., and Katsikis Peter, D. (2002) In vivo stimulation of CD137 broadens primary antiviral CD8+ T cell responses, *Nat Immunol 3*, 536-541.
107. Bertram, E. M., Dawicki, W., Sedgmen, B., Bramson, J. L., Lynch, D. H., and Watts, T. H. (2004) A Switch in Costimulation from CD28 to 4-1BB during Primary versus Secondary CD8 T Cell Response to Influenza In Vivo, *Journal of Immunology 172*, 981-988.
108. Zhang, B., Maris, C. H., Foell, J., Whitmire, J., Niu, L., Song, J., Kwon, B. S., Vella, A. T., Ahmed, R., Jacob, J., and Mittler, R. S. (2007) Immune suppression or enhancement by CD137 T cell costimulation during acute viral infection is time dependent, *J Clin Invest 117*, 3029-3041.

109. Wang, S., Kim, Y. J., Bick, C., Kim, S. H., and Kwon, B. S. (1998) The potential roles of 4-1BB costimulation in HIV type 1 infection, *AIDS Res Hum Retroviruses 14*, 223-231.

110. Serghides, L., Bukczynski, J., Wen, T., Wang, C., Routy, J.-P., Boulassel, M.-R., Sekaly, R.-P., Ostrowski, M., Bernard, N. F., and Watts, T. H. (2005) Evaluation of OX40 Ligand as a Costimulator of Human Antiviral Memory CD8 T Cell Responses: Comparison with B7.1 and 4-1BBL, *Journal of Immunology 175*, 6368-6377.

111. Munks Michael, W., Mourich Dan, V., Mittler Robert, S., Weinberg Andrew, D., and Hill Ann, B. (2004) 4-1BB and OX40 stimulation enhance CD8 and CD4 T-cell responses to a DNA prime, poxvirus boost vaccine, *Immunology 112*, 559-566.

112. Harrison, J. M., Bertram, E. M., Boyle, D. B., Coupar, B. E. H., Ranasinghe, C., and Ramshaw, I. A. (2006) 4-1BBL coexpression enhances HIV-specific CD8 T cell memory in a poxvirus prime-boost vaccine, *Vaccine 24*, 6867-6874.

113. Myers, L. M., and Vella, A. T. (2005) Interfacing T-cell effector and regulatory function through CD137 (4-1BB) co-stimulation, *Trends in Immunology 26*, 440-446.

114. Sun, Y., Lin, X., Chen, H. M., Wu, Q., Subudhi, S. K., Chen, L., and Fu, Y.-X. (2002) Administration of agonistic anti-4-1BB monoclonal antibody leads to the amelioration of experimental autoimmune encephalomyelitis, *Journal of Immunology 168*, 1457-1465.

115. Foell, J., Strahotin, S., O'Neil, S. P., McCausland, M. M., Suwyn, C., Haber, M., Chander, P. N., Bapat, A. S., Yan, X.-J., Chiorazzi, N., Hoffmann, M. K., and

Mittler, R. S. (2003) CD137 costimulatory T cell receptor engagement reverses acute disease in lupus-prone NZB * NZW F1 mice, *Journal of Clinical Investigation 111*, 1505-1518.

116. Seo, S. K., Choi, J. H., Kim, Y. H., Kang, W. J., Park, H. Y., Suh, J. H., Choi, B. K., Vinay, D. S., and Kwon, B. S. (2004) 4-1BB-mediated immunotherapy of rheumatoid arthritis, *Nature Medicine 10*, 1088-1094.
117. 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. (2004) CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract, *J Exp Med 200*, 749-759.
118. Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., Figdor, C. G., and van Kooyk, Y. (2000) DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells, *Cell 100*, 587-597.
119. Zhang, L., Lewin, S. R., Markowitz, M., Lin, H. H., Skulsky, E., Karanicolas, R., He, Y., Jin, X., Tuttleton, S., Vesanen, M., Spiegel, H., Kost, R., van Lunzen, J., Stellbrink, H. J., Wolinsky, S., Borkowsky, W., Palumbo, P., Kostrikis, L. G., and Ho, D. D. (1999) Measuring recent thymic emigrants in blood of normal and HIV-1-infected individuals before and after effective therapy, *J Exp Med 190*, 725-732.
120. Mehandru, S., Poles, M. A., Tenner-Racz, K., Horowitz, A., Hurley, A., Hogan, C., Boden, D., Racz, P., and Markowitz, M. (2004) Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract, *J Exp Med 200*, 761-770.

121. Levesque, M. C., Moody, M. A., Hwang, K. K., Marshall, D. J., Whitesides, J. F., Amos, J. D., Gurley, T. C., Allgood, S., Haynes, B. B., Vandergrift, N. A., Plonk, S., Parker, D. C., Cohen, M. S., Tomaras, G. D., Goepfert, P. A., Shaw, G. M., Schmitz, J. E., Eron, J. J., Shaheen, N. J., Hicks, C. B., Liao, H. X., Markowitz, M., Kelsoe, G., Margolis, D. M., and Haynes, B. F. (2009) Polyclonal B cell differentiation and loss of gastrointestinal tract germinal centers in the earliest stages of HIV-1 infection, *PLoS Med 6*, e1000107.

122. 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. (2000) Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection, *Nat Med 6*, 200-206.

123. 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. (2000) Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies, *Nat Med 6*, 207-210.

124. 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. (1999) Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes, *Science 283*, 857-860.

125. 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. (1999) Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques, *J Exp Med 189*, 991-998.

126. Beyrer, C., Artenstein, A. W., Rugpao, S., Stephens, H., VanCott, T. C., Robb, M. L., Rinkaew, M., Birx, D. L., Khamboonruang, C., Zimmerman, P. A., Nelson, K. E., and Natpratan, C. (1999) Epidemiologic and biologic characterization of a cohort of human immunodeficiency virus type 1 highly exposed, persistently seronegative female sex workers in northern Thailand. Chiang Mai HEPS Working Group, *J Infect Dis 179*, 59-67.

127. Mazzoli, S., Trabattoni, D., Lo Caputo, S., Piconi, S., Ble, C., Meacci, F., Ruzzante, S., Salvi, A., Semplici, F., Longhi, R., Fusi, M. L., Tofani, N., Biasin, M., Villa, M. L., Mazzotta, F., and Clerici, M. (1997) HIV-specific mucosal and cellular immunity in HIV-seronegative partners of HIV-seropositive individuals, *Nat Med 3*, 1250-1257.

128. Kaul, R., Trabattoni, D., Bwayo, J. J., Arienti, D., Zagliani, A., Mwangi, F. M., Kariuki, C., Ngugi, E. N., MacDonald, K. S., Ball, T. B., Clerici, M., and Plummer, F. A. (1999) HIV-1-specific mucosal IgA in a cohort of HIV-1-resistant Kenyan sex workers, *AIDS 13*, 23-29.

129. Kaul, R., Plummer, F. A., Kimani, J., Dong, T., Kiama, P., Rostron, T., Njagi, E., MacDonald, K. S., Bwayo, J. J., McMichael, A. J., and Rowland-Jones, S. L.

(2000) HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi, *J Immunol 164*, 1602-1611.

130. Sundling, C., Schon, K., Morner, A., Forsell, M. N., Wyatt, R. T., Thorstensson, R., Karlsson Hedestam, G. B., and Lycke, N. Y. (2008) CTA1-DD adjuvant promotes strong immunity against human immunodeficiency virus type 1 envelope glycoproteins following mucosal immunization, *J Gen Virol 89*, 2954-2964.
131. Lehner, T., Mitchell, E., Bergmeier, L., Singh, M., Spallek, R., Cranage, M., Hall, G., Dennis, M., Villinger, F., and Wang, Y. (2000) The role of gammadelta T cells in generating antiviral factors and beta-chemokines in protection against mucosal simian immunodeficiency virus infection, *Eur J Immunol 30*, 2245-2256.
132. Kang, S. M., and Compans, R. W. (2003) Enhancement of mucosal immunization with virus-like particles of simian immunodeficiency virus, *J Virol 77*, 3615-3623.
133. Jiang, J. Q., Patrick, A., Moss, R. B., and Rosenthal, K. L. (2005) CD8+ T-cell-mediated cross-clade protection in the genital tract following intranasal immunization with inactivated human immunodeficiency virus antigen plus CpG oligodeoxynucleotides, *J Virol 79*, 393-400.
134. Wang, Y., Abel, K., Lantz, K., Krieg, A. M., McChesney, M. B., and Miller, C. J. (2005) The Toll-like receptor 7 (TLR7) agonist, imiquimod, and the TLR9 agonist, CpG ODN, induce antiviral cytokines and chemokines but do not prevent vaginal transmission of simian immunodeficiency virus when applied intravaginally to rhesus macaques, *J Virol 79*, 14355-14370.

135. Belyakov, I. M., Ahlers, J. D., Clements, J. D., Strober, W., and Berzofsky, J. A. (2000) Interplay of cytokines and adjuvants in the regulation of mucosal and systemic HIV-specific CTL, *J Immunol 165*, 6454-6462.

136. Van Damme, L., Ramjee, G., Alary, M., Vuylsteke, B., Chandeying, V., Rees, H., Sirivongrangson, P., Mukenge-Tshibaka, L., Ettiegne-Traore, V., Uaheowitchai, C., Karim, S. S., Masse, B., Perriens, J., and Laga, M. (2002) Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial, *Lancet 360*, 971-977.

137. Honey, K. (2007) Microbicide trial screeches to a halt, *J Clin Invest 117*, 1116.

138. Veazey, R. S., Klasse, P. J., Schader, S. M., Hu, Q., Ketas, T. J., Lu, M., Marx, P. A., Dufour, J., Colonno, R. J., Shattock, R. J., Springer, M. S., and Moore, J. P. (2005) Protection of macaques from vaginal SHIV challenge by vaginally delivered inhibitors of virus-cell fusion, *Nature 438*, 99-102.

139. 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. (2003) Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120, *Nat Med 9*, 343-346.

140. Li, Q., Estes, J. D., Schlievert, P. M., Duan, L., Brosnahan, A. J., Southern, P. J., Reilly, C. S., Peterson, M. L., Schultz-Darken, N., Brunner, K. G., Nephew, K. R., Pambuccian, S., Lifson, J. D., Carlis, J. V., and Haase, A. T. (2009) Glycerol monolaurate prevents mucosal SIV transmission, *Nature 458*, 1034-1038.

141. Chang, T. L., Chang, C. H., Simpson, D. A., Xu, Q., Martin, P. K., Lagenaur, L. A., Schoolnik, G. K., Ho, D. D., Hillier, S. L., Holodniy, M., Lewicki, J. A., and Lee, P.

P. (2003) Inhibition of HIV infectivity by a natural human isolate of Lactobacillus jensenii engineered to express functional two-domain CD4, *Proc Natl Acad Sci U S A 100*, 11672-11677.

142. Liu, X., Lagenaur, L. A., Simpson, D. A., Essenmacher, K. P., Frazier-Parker, C. L., Liu, Y., Tsai, D., Rao, S. S., Hamer, D. H., Parks, T. P., Lee, P. P., and Xu, Q. (2006) Engineered vaginal lactobacillus strain for mucosal delivery of the human immunodeficiency virus inhibitor cyanovirin-N, *Antimicrob Agents Chemother 50*, 3250-3259.
143. Nochi, T., Takagi, H., Yuki, Y., Yang, L., Masumura, T., Mejima, M., Nakanishi, U., Matsumura, A., Uozumi, A., Hiroi, T., Morita, S., Tanaka, K., Takaiwa, F., and Kiyono, H. (2007) Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination, *Proc Natl Acad Sci U S A 104*, 10986-10991.
144. Nochi, T., Yuki, Y., Matsumura, A., Mejima, M., Terahara, K., Kim, D. Y., Fukuyama, S., Iwatsuki-Horimoto, K., Kawaoka, Y., Kohda, T., Kozaki, S., Igarashi, O., and Kiyono, H. (2007) A novel M cell-specific carbohydrate-targeted mucosal vaccine effectively induces antigen-specific immune responses, *J Exp Med 204*, 2789-2796.
145. Bjersing, J. L., Telemo, E., Dahlgren, U., and Hanson, L. A. (2002) Loss of ileal IgA+ plasma cells and of CD4+ lymphocytes in ileal Peyer's patches of vitamin A deficient rats, *Clin Exp Immunol 130*, 404-408.
146. Iwata, M., Hirakiyama, A., Eshima, Y., Kagechika, H., Kato, C., and Song, S. Y. (2004) Retinoic acid imprints gut-homing specificity on T cells, *Immunity 21*, 527-538.

147. Berlin, C., Berg, E. L., Briskin, M. J., Andrew, D. P., Kilshaw, P. J., Holzmann, B., Weissman, I. L., Hamann, A., and Butcher, E. C. (1993) Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1, *Cell 74*, 185-195.

148. Briskin, M., Winsor-Hines, D., Shyjan, A., Cochran, N., Bloom, S., Wilson, J., McEvoy, L. M., Butcher, E. C., Kassam, N., Mackay, C. R., Newman, W., and Ringler, D. J. (1997) Human mucosal addressin cell adhesion molecule-1 is preferentially expressed in intestinal tract and associated lymphoid tissue, *Am J Pathol 151*, 97-110.

149. Kunkel, E. J., Campbell, J. J., Haraldsen, G., Pan, J., Boisvert, J., Roberts, A. I., Ebert, E. C., Vierra, M. A., Goodman, S. B., Genovese, M. C., Wardlaw, A. J., Greenberg, H. B., Parker, C. M., Butcher, E. C., Andrew, D. P., and Agace, W. W. (2000) Lymphocyte CC chemokine receptor 9 and epithelial thymus-expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: Epithelial expression of tissue-specific chemokines as an organizing principle in regional immunity, *J Exp Med 192*, 761-768.

150. Svensson, M., Marsal, J., Ericsson, A., Carramolino, L., Broden, T., Marquez, G., and Agace, W. W. (2002) CCL25 mediates the localization of recently activated CD8alphabeta(+) lymphocytes to the small-intestinal mucosa, *J Clin Invest 110*, 1113-1121.

151. Arthos, J., Cicala, C., Martinelli, E., Macleod, K., Van Ryk, D., Wei, D., Xiao, Z., Veenstra, T. D., Conrad, T. P., Lempicki, R. A., McLaughlin, S., Pascuccio, M., Gopaul, R., McNally, J., Cruz, C. C., Censoplano, N., Chung, E., Reitano, K. N.,

Kottilil, S., Goode, D. J., and Fauci, A. S. (2008) HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells, *Nat Immunol 9*, 301-309.

152. Johansson-Lindbom, B., Svensson, M., Wurbel, M. A., Malissen, B., Marquez, G., and Agace, W. (2003) Selective generation of gut tropic T cells in gut-associated lymphoid tissue (GALT): requirement for GALT dendritic cells and adjuvant, *J Exp Med 198*, 963-969.
153. Belyakov, I. M., Isakov, D., Zhu, Q., Dzutsev, A., and Berzofsky, J. A. (2007) A novel functional CTL avidity/activity compartmentalization to the site of mucosal immunization contributes to protection of macaques against simian/human immunodeficiency viral depletion of mucosal CD4+ T cells, *J Immunol 178*, 7211-7221.
154. Belyakov, I. M., Derby, M. A., Ahlers, J. D., Kelsall, B. L., Earl, P., Moss, B., Strober, W., and Berzofsky, J. A. (1998) Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge, *Proc Natl Acad Sci U S A 95*, 1709-1714.
155. Belyakov, I. M., Ahlers, J. D., Brandwein, B. Y., Earl, P., Kelsall, B. L., Moss, B., Strober, W., and Berzofsky, J. A. (1998) The importance of local mucosal HIV-specific CD8(+) cytotoxic T lymphocytes for resistance to mucosal viral transmission in mice and enhancement of resistance by local administration of IL-12, *J Clin Invest 102*, 2072-2081.

156. Wang, J., Thorson, L., Stokes, R. W., Santosuosso, M., Huygen, K., Zganiacz, A., Hitt, M., and Xing, Z. (2004) Single mucosal, but not parenteral, immunization with recombinant adenoviral-based vaccine provides potent protection from pulmonary tuberculosis, *J Immunol 173*, 6357-6365.

157. Staats, H. F., Montgomery, S. P., and Palker, T. J. (1997) Intranasal immunization is superior to vaginal, gastric, or rectal immunization for the induction of systemic and mucosal anti-HIV antibody responses, *AIDS Res Hum Retroviruses 13*, 945-952.

158. Imaoka, K., Miller, C. J., Kubota, M., McChesney, M. B., Lohman, B., Yamamoto, M., Fujihashi, K., Someya, K., Honda, M., McGhee, J. R., and Kiyono, H. (1998) Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus-specific immune responses in reproductive tissues, *J Immunol 161*, 5952-5958.

159. Rudin, A., Riise, G. C., and Holmgren, J. (1999) Antibody responses in the lower respiratory tract and male urogenital tract in humans after nasal and oral vaccination with cholera toxin B subunit, *Infect Immun 67*, 2884-2890.

160. Sasaki, S., Hamajima, K., Fukushima, J., Ihata, A., Ishii, N., Gorai, I., Hirahara, F., Mohri, H., and Okuda, K. (1998) Comparison of intranasal and intramuscular immunization against human immunodeficiency virus type 1 with a DNA-monophosphoryl lipid A adjuvant vaccine, *Infect Immun 66*, 823-826.

161. Barackman, J. D., Ott, G., and O'Hagan, D. T. (1999) Intranasal immunization of mice with influenza vaccine in combination with the adjuvant LT-R72 induces

potent mucosal and serum immunity which is stronger than that with traditional intramuscular immunization, *Infect Immun 67*, 4276-4279.

162. Bergquist, C., Lagergard, T., Lindblad, M., and Holmgren, J. (1995) Local and systemic antibody responses to dextran-cholera toxin B subunit conjugates, *Infect Immun 63*, 2021-2025.
163. Enose, Y., Ui, M., Miyake, A., Suzuki, H., Uesaka, H., Kuwata, T., Kunisawa, J., Kiyono, H., Takahashi, H., Miura, T., and Hayami, M. (2002) Protection by intranasal immunization of a nef-deleted, nonpathogenic SHIV against intravaginal challenge with a heterologous pathogenic SHIV, *Virology 298*, 306-316.
164. Crotty, S., Miller, C. J., Lohman, B. L., Neagu, M. R., Compton, L., Lu, D., Lu, F. X., Fritts, L., Lifson, J. D., and Andino, R. (2001) Protection against simian immunodeficiency virus vaginal challenge by using Sabin poliovirus vectors, *J Virol 75*, 7435-7452.
165. Quiding-Jarbrink, M., Nordstrom, I., Granstrom, G., Kilander, A., Jertborn, M., Butcher, E. C., Lazarovits, A. I., Holmgren, J., and Czerkinsky, C. (1997) Differential expression of tissue-specific adhesion molecules on human circulating antibody-forming cells after systemic, enteric, and nasal immunizations. A molecular basis for the compartmentalization of effector B cell responses, *J Clin Invest 99*, 1281-1286.
166. Eriksson, K., Quiding-Jarbrink, M., Osek, J., Moller, A., Bjork, S., Holmgren, J., and Czerkinsky, C. (1998) Specific-antibody-secreting cells in the rectums and genital tracts of nonhuman primates following vaccination, *Infect Immun 66*, 5889-5896.

167. Belyakov, I. M., Hel, Z., Kelsall, B., Kuznetsov, V. A., Ahlers, J. D., Nacsa, J., Watkins, D. I., Allen, T. M., Sette, A., Altman, J., Woodward, R., Markham, P. D., Clements, J. D., Franchini, G., Strober, W., and Berzofsky, J. A. (2001) Mucosal AIDS vaccine reduces disease and viral load in gut reservoir and blood after mucosal infection of macaques, *Nat Med 7*, 1320-1326.

168. Mayer, L., and Shao, L. (2004) Therapeutic potential of oral tolerance, *Nat Rev Immunol 4*, 407-419.

169. Tatsis, N., Lin, S. W., Harris-McCoy, K., Garber, D. A., Feinberg, M. B., and Ertl, H. C. (2007) Multiple immunizations with adenovirus and MVA vectors improve CD8+ T cell functionality and mucosal homing, *Virology 367*, 156-167.

170. Lin, S. W., Cun, A. S., Harris-McCoy, K., and Ertl, H. C. (2007) Intramuscular rather than oral administration of replication-defective adenoviral vaccine vector induces specific CD8+ T cell responses in the gut, *Vaccine 25*, 2187-2193.

171. Kaufman, D. R., Liu, J., Carville, A., Mansfield, K. G., Havenga, M. J., Goudsmit, J., and Barouch, D. H. (2008) Trafficking of antigen-specific CD8+ T lymphocytes to mucosal surfaces following intramuscular vaccination, *J Immunol 181*, 4188-4198.

172. Pal, R., Venzon, D., Santra, S., Kalyanaraman, V. S., Montefiori, D. C., Hocker, L., Hudacik, L., Rose, N., Nacsa, J., Edghill-Smith, Y., Moniuszko, M., Hel, Z., Belyakov, I. M., Berzofsky, J. A., Parks, R. W., Markham, P. D., Letvin, N. L., Tartaglia, J., and Franchini, G. (2006) Systemic immunization with an ALVAC-HIV-1/protein boost vaccine strategy protects rhesus macaques from CD4+ T-cell

loss and reduces both systemic and mucosal simian-human immunodeficiency virus SHIVKU2 RNA levels, *J Virol 80*, 3732-3742.

173. Santosuosso, M., McCormick, S., Roediger, E., Zhang, X., Zganiacz, A., Lichty, B. D., and Xing, Z. (2007) Mucosal luminal manipulation of T cell geography switches on protective efficacy by otherwise ineffective parenteral genetic immunization, *J Immunol 178*, 2387-2395.
174. Ranasinghe, C., Turner, S. J., McArthur, C., Sutherland, D. B., Kim, J. H., Doherty, P. C., and Ramshaw, I. A. (2007) Mucosal HIV-1 pox virus prime-boost immunization induces high-avidity CD8+ T cells with regime-dependent cytokine/granzyme B profiles, *J Immunol 178*, 2370-2379.
175. Vinay, D. S., and Kwon, B. S. (1998) Role of 4-1BB in immune responses, *Seminars in immunology 10*, 481-489.
176. Watts, T. H. (2005) TNF/TNFR family members in costimulation of T cell responses, *Annual review of immunology 23*, 23-68.
177. Wilcox, R. A., Chapoval, A. I., Gorski, K. S., Otsuji, M., Shin, T., Flies, D. B., Tamada, K., Mittler, R. S., Tsuchiya, H., Pardoll, D. M., and Chen, L. (2002) Cutting edge: Expression of functional CD137 receptor by dendritic cells, *J Immunol 168*, 4262-4267.
178. Cannons, J. L., Lau, P., Ghumman, B., DeBenedette, M. A., Yagita, H., Okumura, K., and Watts, T. H. (2001) 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T cells with similar efficacy, *J Immunol 167*, 1313-1324.

179. Shuford, W. W., Klussman, K., Tritchler, D. D., Loo, D. T., Chalupny, J., Siadak, A. W., Brown, T. J., Emswiler, J., Raecho, H., Larsen, C. P., Pearson, T. C., Ledbetter, J. A., Aruffo, A., and Mittler, R. S. (1997) 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses, *The Journal of experimental medicine 186*, 47-55.

180. Tan, J. T., Whitmire, J. K., Murali-Krishna, K., Ahmed, R., Altman, J. D., Mittler, R. S., Sette, A., Pearson, T. C., and Larsen, C. P. (2000) 4-1BB costimulation is required for protective anti-viral immunity after peptide vaccination, *J Immunol 164*, 2320-2325.

181. Wiethe, C., Dittmar, K., Doan, T., Lindenmaier, W., and Tindle, R. (2003) Provision of 4-1BB ligand enhances effector and memory CTL responses generated by immunization with dendritic cells expressing a human tumor-associated antigen, *J Immunol 170*, 2912-2922.

182. Myers, L., Lee, S. W., Rossi, R. J., Lefrancois, L., Kwon, B. S., Mittler, R. S., Croft, M., and Vella, A. T. (2006) Combined CD137 (4-1BB) and adjuvant therapy generates a developing pool of peptide-specific CD8 memory T cells, *International immunology 18*, 325-333.

183. Melero, I., Shuford, W. W., Newby, S. A., Aruffo, A., Ledbetter, J. A., Hellstrom, K. E., Mittler, R. S., and Chen, L. (1997) Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors, *Nature medicine 3*, 682-685.

184. Miller, R. E., Jones, J., Le, T., Whitmore, J., Boiani, N., Gliniak, B., and Lynch, D. H. (2002) 4-1BB-specific monoclonal antibody promotes the generation of tumor-

specific immune responses by direct activation of CD8 T cells in a CD40-dependent manner, *J Immunol 169*, 1792-1800.

185. Halstead, E. S., Mueller, Y. M., Altman, J. D., and Katsikis, P. D. (2002) In vivo stimulation of CD137 broadens primary antiviral CD8+ T cell responses, *Nature immunology 3*, 536-541.
186. Bukczynski, J., Wen, T., Ellefsen, K., Gauldie, J., and Watts, T. H. (2004) Costimulatory ligand 4-1BBL (CD137L) as an efficient adjuvant for human antiviral cytotoxic T cell responses, *Proceedings of the National Academy of Sciences of the United States of America 101*, 1291-1296.
187. Sun, Y., Lin, X., Chen, H. M., Wu, Q., Subudhi, S. K., Chen, L., and Fu, Y. X. (2002) Administration of agonistic anti-4-1BB monoclonal antibody leads to the amelioration of experimental autoimmune encephalomyelitis, *J Immunol 168*, 1457-1465.
188. Foell, J., Strahotin, S., O'Neil, S. P., McCausland, M. M., Suwyn, C., Haber, M., Chander, P. N., Bapat, A. S., Yan, X. J., Chiorazzi, N., Hoffmann, M. K., and Mittler, R. S. (2003) CD137 costimulatory T cell receptor engagement reverses acute disease in lupus-prone NZB x NZW F1 mice, *The Journal of clinical investigation 111*, 1505-1518.
189. Seo, S. K., Choi, J. H., Kim, Y. H., Kang, W. J., Park, H. Y., Suh, J. H., Choi, B. K., Vinay, D. S., and Kwon, B. S. (2004) 4-1BB-mediated immunotherapy of rheumatoid arthritis, *Nature medicine 10*, 1088-1094.
190. Smith, J. M., Amara, R. R., Campbell, D., Xu, Y., Patel, M., Sharma, S., Butera, S. T., Ellenberger, D. L., Yi, H., Chennareddi, L., Herndon, J. G., Wyatt, L. S.,

Montefiori, D., Moss, B., McClure, H. M., and Robinson, H. L. (2004) DNA/MVA vaccine for HIV type 1: effects of codon-optimization and the expression of aggregates or virus-like particles on the immunogenicity of the DNA prime, *AIDS research and human retroviruses 20*, 1335-1347.

191. Robinson, H. L., Sharma, S., Zhao, J., Kannanganat, S., Lai, L., Chennareddi, L., Yu, T., Montefiori, D. C., Amara, R. R., Wyatt, L. S., and Moss, B. (2007) Immunogenicity in macaques of the clinical product for a clade B DNA/MVA HIV vaccine: elicitation of IFN-gamma, IL-2, and TNF-alpha coproducing CD4 and CD8 T cells, *AIDS Res Hum Retroviruses 23*, 1555-1562.
192. Stone, G. W., Barzee, S., Snarsky, V., Kee, K., Spina, C. A., Yu, X. F., and Kornbluth, R. S. (2006) Multimeric soluble CD40 ligand and GITR ligand as adjuvants for human immunodeficiency virus DNA vaccines, *Journal of virology 80*, 1762-1772.
193. Liu, J., Wyatt, L. S., Amara, R. R., Moss, B., and Robinson, H. L. (2006) Studies on in vitro expression and in vivo immunogenicity of a recombinant MVA HIV vaccine, *Vaccine 24*, 3332-3339.
194. Locksley, R. M., Killeen, N., and Lenardo, M. J. (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology, *Cell 104*, 487-501.
195. Haswell, L. E., Glennie, M. J., and Al-Shamkhani, A. (2001) Analysis of the oligomeric requirement for signaling by CD40 using soluble multimeric forms of its ligand, CD154, *Eur J Immunol 31*, 3094-3100.
196. Schneider, P., Holler, N., Bodmer, J. L., Hahne, M., Frei, K., Fontana, A., and Tschopp, J. (1998) Conversion of membrane-bound Fas(CD95) ligand to its soluble

form is associated with downregulation of its proapoptotic activity and loss of liver toxicity, *The Journal of experimental medicine 187*, 1205-1213.

197. Gramaglia, I., Cooper, D., Miner, K. T., Kwon, B. S., and Croft, M. (2000) Co-stimulation of antigen-specific CD4 T cells by 4-1BB ligand, *European journal of immunology 30*, 392-402.
198. Cooper, D., Bansal-Pakala, P., and Croft, M. (2002) 4-1BB (CD137) controls the clonal expansion and survival of CD8 T cells in vivo but does not contribute to the development of cytotoxicity, *European journal of immunology 32*, 521-529.
199. Tan, J. T., Whitmire, J. K., Ahmed, R., Pearson, T. C., and Larsen, C. P. (1999) 4-1BB ligand, a member of the TNF family, is important for the generation of antiviral CD8 T cell responses, *J Immunol 163*, 4859-4868.
200. Bertram, E. M., Lau, P., and Watts, T. H. (2002) Temporal segregation of 4-1BB versus CD28-mediated costimulation: 4-1BB ligand influences T cell numbers late in the primary response and regulates the size of the T cell memory response following influenza infection, *J Immunol 168*, 3777-3785.
201. Harrison, J. M., Bertram, E. M., Boyle, D. B., Coupar, B. E., Ranasinghe, C., and Ramshaw, I. A. (2006) 4-1BBL coexpression enhances HIV-specific CD8 T cell memory in a poxvirus prime-boost vaccine, *Vaccine 24*, 6867-6874.
202. Munks, M. W., Mourich, D. V., Mittler, R. S., Weinberg, A. D., and Hill, A. B. (2004) 4-1BB and OX40 stimulation enhance CD8 and CD4 T-cell responses to a DNA prime, poxvirus boost vaccine, *Immunology 112*, 559-566.

203. Calarota, S. A., Hokey, D. A., Dai, A., Jure-Kunkel, M. N., Balimane, P., and Weiner, D. B. (2008) Augmentation of SIV DNA vaccine-induced cellular immunity by targeting the 4-1BB costimulatory molecule, *Vaccine 26*, 3121-3134.

204. Sun, Y., Chen, H. M., Subudhi, S. K., Chen, J., Koka, R., Chen, L., and Fu, Y. X. (2002) Costimulatory molecule-targeted antibody therapy of a spontaneous autoimmune disease, *Nature medicine 8*, 1405-1413.

205. Zheng, G., Wang, B., and Chen, A. (2004) The 4-1BB costimulation augments the proliferation of CD4+CD25+ regulatory T cells, *J Immunol 173*, 2428-2434.

206. Myers, L., Takahashi, C., Mittler, R. S., Rossi, R. J., and Vella, A. T. (2003) Effector CD8 T cells possess suppressor function after 4-1BB and Toll-like receptor triggering, *Proc Natl Acad Sci U S A 100*, 5348-5353.

207. Sun, Y., Blink, S. E., Chen, J. H., and Fu, Y. X. (2005) Regulation of follicular dendritic cell networks by activated T cells: the role of CD137 signaling, *J Immunol 175*, 884-890.

208. Kienzle, G., and von Kempis, J. (2000) CD137 (ILA/4-1BB), expressed by primary human monocytes, induces monocyte activation and apoptosis of B lymphocytes, *Int Immunol 12*, 73-82.

209. Wyatt, L. S., Shors, S. T., Murphy, B. R., and Moss, B. (1996) Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model, *Vaccine 14*, 1451-1458.

210. Rosel, J. L., Earl, P. L., Weir, J. P., and Moss, B. (1986) Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late

genes deduced by structural and functional analysis of the HindIII H genome fragment, *J Virol 60*, 436-449.

211. Neutra, M. R., and Kozlowski, P. A. (2006) Mucosal vaccines: the promise and the challenge, *Nat Rev Immunol 6*, 148-158.
212. Belyakov, I. M., and Berzofsky, J. A. (2004) Immunobiology of mucosal HIV infection and the basis for development of a new generation of mucosal AIDS vaccines, *Immunity 20*, 247-253.
213. Mora, J. R., Iwata, M., Eksteen, B., Song, S. Y., Junt, T., Senman, B., Otipoby, K. L., Yokota, A., Takeuchi, H., Ricciardi-Castagnoli, P., Rajewsky, K., Adams, D. H., and von Andrian, U. H. (2006) Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells, *Science 314*, 1157-1160.
214. Masopust, D., Choo, D., Vezys, V., Wherry, E. J., Duraiswamy, J., Akondy, R., Wang, J., Casey, K. A., Barber, D. L., Kawamura, K. S., Fraser, K. A., Webby, R. J., Brinkmann, V., Butcher, E. C., Newell, K. A., and Ahmed, R. Dynamic T cell migration program provides resident memory within intestinal epithelium, *J Exp Med 207*, 553-564.
215. Denning, T. L., Wang, Y. C., Patel, S. R., Williams, I. R., and Pulendran, B. (2007) Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses, *Nat Immunol 8*, 1086-1094.
216. Ganguly, S., Liu, J., Pillai, V. B., Mittler, R. S., and Amara, R. R. Adjuvantive effects of anti-4-1BB agonist Ab and 4-1BBL DNA for a HIV-1 Gag DNA vaccine: different effects on cellular and humoral immunity, *Vaccine 28*, 1300-1309.

217. Kawakami, Y., Li, H., Lam, J. T., Krasnykh, V., Curiel, D. T., and Blackwell, J. L. (2003) Substitution of the adenovirus serotype 5 knob with a serotype 3 knob enhances multiple steps in virus replication, *Cancer Res 63*, 1262-1269.

218. Mandl, J. N., Barry, A. P., Vanderford, T. H., Kozyr, N., Chavan, R., Klucking, S., Barrat, F. J., Coffman, R. L., Staprans, S. I., and Feinberg, M. B. (2008) Divergent TLR7 and TLR9 signaling and type I interferon production distinguish pathogenic and nonpathogenic AIDS virus infections, *Nat Med 14*, 1077-1087.

219. Butcher, E. C., and Picker, L. J. (1996) Lymphocyte homing and homeostasis, *Science 272*, 60-66.

220. Balmer, J. E., and Blomhoff, R. (2002) Gene expression regulation by retinoic acid, *J Lipid Res 43*, 1773-1808.

221. Bastien, J., and Rochette-Egly, C. (2004) Nuclear retinoid receptors and the transcription of retinoid-target genes, *Gene 328*, 1-16.

222. Svensson, M., Johansson-Lindbom, B., Zapata, F., Jaensson, E., Austenaa, L. M., Blomhoff, R., and Agace, W. W. (2008) Retinoic acid receptor signaling levels and antigen dose regulate gut homing receptor expression on CD8+ T cells, *Mucosal Immunol 1*, 38-48.

223. Russell, W. C., Valentine, R. C., and Pereira, H. G. (1967) The effect of heat on the anatomy of the adenovirus, *J Gen Virol 1*, 509-522.

224. Cheng, C., Gall, J. G., Kong, W. P., Sheets, R. L., Gomez, P. L., King, C. R., and Nabel, G. J. (2007) Mechanism of ad5 vaccine immunity and toxicity: fiber shaft targeting of dendritic cells, *PLoS Pathog 3*, e25.

225. Wickham, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. (1993) Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment, *Cell 73*, 309-319.

226. Nemerow, G. R. (2000) Cell receptors involved in adenovirus entry, *Virology 274*, 1-4.

227. Jooss, K., and Chirmule, N. (2003) Immunity to adenovirus and adeno-associated viral vectors: implications for gene therapy, *Gene Ther 10*, 955-963.

228. Manicassamy, S., Ravindran, R., Deng, J., Oluoch, H., Denning, T. L., Kasturi, S. P., Rosenthal, K. M., Evavold, B. D., and Pulendran, B. (2009) Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity, *Nat Med 15*, 401-409.

229. Hartman, Z. C., Kiang, A., Everett, R. S., Serra, D., Yang, X. Y., Clay, T. M., and Amalfitano, A. (2007) Adenovirus infection triggers a rapid, MyD88-regulated transcriptome response critical to acute-phase and adaptive immune responses in vivo, *J Virol 81*, 1796-1812.

230. Takaoka, A., Wang, Z., Choi, M. K., Yanai, H., Negishi, H., Ban, T., Lu, Y., Miyagishi, M., Kodama, T., Honda, K., Ohba, Y., and Taniguchi, T. (2007) DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response, *Nature 448*, 501-505.

231. Hornung, V., Ablasser, A., Charrel-Dennis, M., Bauernfeind, F., Horvath, G., Caffrey, D. R., Latz, E., and Fitzgerald, K. A. (2009) AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC, *Nature 458*, 514-518.

232. Fernandes-Alnemri, T., Yu, J. W., Datta, P., Wu, J., and Alnemri, E. S. (2009) AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA, *Nature 458*, 509-513.

233. Tamanini, A., Nicolis, E., Bonizzato, A., Bezzerri, V., Melotti, P., Assael, B. M., and Cabrini, G. (2006) Interaction of adenovirus type 5 fiber with the coxsackievirus and adenovirus receptor activates inflammatory response in human respiratory cells, *J Virol 80*, 11241-11254.

234. Neumann, M., Fries, H., Scheicher, C., Keikavoussi, P., Kolb-Maurer, A., Brocker, E., Serfling, E., and Kampgen, E. (2000) Differential expression of Rel/NF-kappaB and octamer factors is a hallmark of the generation and maturation of dendritic cells, *Blood 95*, 277-285.

235. Rescigno, M., Martino, M., Sutherland, C. L., Gold, M. R., and Ricciardi-Castagnoli, P. (1998) Dendritic cell survival and maturation are regulated by different signaling pathways, *J Exp Med 188*, 2175-2180.

236. Ginsberg, H. S., Moldawer, L. L., Sehgal, P. B., Redington, M., Kilian, P. L., Chanock, R. M., and Prince, G. A. (1991) A mouse model for investigating the molecular pathogenesis of adenovirus pneumonia, *Proc Natl Acad Sci U S A 88*, 1651-1655.

237. Beg, A. A., Finco, T. S., Nantermet, P. V., and Baldwin, A. S., Jr. (1993) Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation, *Mol Cell Biol 13*, 3301-3310.

238. Schreck, R., Albermann, K., and Baeuerle, P. A. (1992) Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review), *Free Radic Res Commun 17*, 221-237.

239. Villablanca, E. J., Russo, V., and Mora, J. R. (2008) Dendritic cell migration and lymphocyte homing imprinting, *Histol Histopathol 23*, 897-910.

240. Wang, X., Sperkova, Z., and Napoli, J. L. (2001) Analysis of mouse retinal dehydrogenase type 2 promoter and expression, *Genomics 74*, 245-250.

241. Borgland, S. L., Bowen, G. P., Wong, N. C., Libermann, T. A., and Muruve, D. A. (2000) Adenovirus vector-induced expression of the C-X-C chemokine IP-10 is mediated through capsid-dependent activation of NF-kappaB, *J Virol 74*, 3941-3947.

242. Ishii, K. J., Kawagoe, T., Koyama, S., Matsui, K., Kumar, H., Kawai, T., Uematsu, S., Takeuchi, O., Takeshita, F., Coban, C., and Akira, S. (2008) TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines, *Nature 451*, 725-729.

243. Wang, Z., Choi, M. K., Ban, T., Yanai, H., Negishi, H., Lu, Y., Tamura, T., Takaoka, A., Nishikura, K., and Taniguchi, T. (2008) Regulation of innate immune responses by DAI (DLM-1/ZBP1) and other DNA-sensing molecules, *Proc Natl Acad Sci U S A 105*, 5477-5482.

244. Kobiyama, K., Takeshita, F., Jounai, N., Sakaue-Sawano, A., Miyawaki, A., Ishii, K. J., Kawai, T., Sasaki, S., Hirano, H., Ishii, N., Okuda, K., and Suzuki, K. (2010) Extrachromosomal histone H2B mediates innate antiviral immune responses induced by intracellular double-stranded DNA, *J Virol 84*, 822-832.

245. Kafri, T., Morgan, D., Krahl, T., Sarvetnick, N., Sherman, L., and Verma, I. (1998) Cellular immune response to adenoviral vector infected cells does not require de novo viral gene expression: implications for gene therapy, *Proc Natl Acad Sci U S A 95*, 11377-11382.

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

247. Wolinsky, S. M., Wike, C. M., Korber, B. T., Hutto, C., Parks, W. P., Rosenblum, L. L., Kunstman, K. J., Furtado, M. R., and Munoz, J. L. (1992) Selective transmission of human immunodeficiency virus type-1 variants from mothers to infants, *Science 255*, 1134-1137.

248. 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. (2004) Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission, *Science 303*, 2019-2022.

249. Barouch, D. H., O'Brien, K. L., Simmons, N. L., King, S. L., Abbink, P., Maxfield, L. F., Sun, Y. H., La Porte, A., Riggs, A. M., Lynch, D. M., Clark, S. L., Backus, K., Perry, J. R., Seaman, M. S., Carville, A., Mansfield, K. G., Szinger, J. J., Fischer, W., Muldoon, M., and Korber, B. (2010) Mosaic HIV-1 vaccines expand the breadth and depth of cellular immune responses in rhesus monkeys, *Nat Med 16*, 319-323.

250. Santra, S., Liao, H. X., Zhang, R., Muldoon, M., Watson, S., Fischer, W., Theiler, J., Szinger, J., Balachandran, H., Buzby, A., Quinn, D., Parks, R. J., Tsao, C. Y., Carville, A., Mansfield, K. G., Pavlakis, G. N., Felber, B. K., Haynes, B. F., Korber, B. T., and Letvin, N. L. (2010) Mosaic vaccines elicit CD8+ T lymphocyte responses that confer enhanced immune coverage of diverse HIV strains in monkeys, *Nat Med 16*, 324-328.

251. Choi, B. K., Kim, Y. H., Kwon, P. M., Lee, S. C., Kang, S. W., Kim, M. S., Lee, M. J., and Kwon, B. S. (2009) 4-1BB functions as a survival factor in dendritic cells, *J Immunol 182*, 4107-4115.

252. Mittler, R. S., Bailey, T. S., Klussman, K., Trailsmith, M. D., and Hoffmann, M. K. (1999) Anti-4-1BB monoclonal antibodies abrogate T cell-dependent humoral immune responses in vivo through the induction of helper T cell anergy, *J Exp Med 190*, 1535-1540.

253. Chtanova, T., Tangye, S. G., Newton, R., Frank, N., Hodge, M. R., Rolph, M. S., and Mackay, C. R. (2004) T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells, *J Immunol 173*, 68-78.

254. Niu, L., Strahotin, S., Hewes, B., Zhang, B., Zhang, Y., Archer, D., Spencer, T., Dillehay, D., Kwon, B., Chen, L., Vella, A. T., and Mittler, R. S. (2007) Cytokine-mediated disruption of lymphocyte trafficking, hemopoiesis, and induction of lymphopenia, anemia, and thrombocytopenia in anti-CD137-treated mice, *J Immunol 178*, 4194-4213.

255. Watts, G. M., Beurskens, F. J., Martin-Padura, I., Ballantyne, C. M., Klickstein, L. B., Brenner, M. B., and Lee, D. M. (2005) Manifestations of inflammatory arthritis are critically dependent on LFA-1, *J Immunol 174*, 3668-3675.

256. Gordon, E. J., Myers, K. J., Dougherty, J. P., Rosen, H., and Ron, Y. (1995) Both anti-CD11a (LFA-1) and anti-CD11b (MAC-1) therapy delay the onset and diminish the severity of experimental autoimmune encephalomyelitis, *J Neuroimmunol 62*, 153-160.

257. Kakimoto, K., Nakamura, T., Ishii, K., Takashi, T., Iigou, H., Yagita, H., Okumura, K., and Onoue, K. (1992) The effect of anti-adhesion molecule antibody on the development of collagen-induced arthritis, *Cell Immunol 142*, 326-337.

258. Holmskov, U., Mollenhauer, J., Madsen, J., Vitved, L., Gronlund, J., Tornoe, I., Kliem, A., Reid, K. B., Poustka, A., and Skjodt, K. (1999) Cloning of gp-340, a putative opsonin receptor for lung surfactant protein D, *Proc Natl Acad Sci U S A 96*, 10794-10799.

259. Zhang, B., Zhang, Y., Niu, L., Vella, A. T., and Mittler, R. S. (2010) Dendritic cells and Stat3 are essential for CD137-induced CD8 T cell activation-induced cell death, *J Immunol 184*, 4770-4778.

260. Cheng, F., Wang, H. W., Cuenca, A., Huang, M., Ghansah, T., Brayer, J., Kerr, W. G., Takeda, K., Akira, S., Schoenberger, S. P., Yu, H., Jove, R., and Sotomayor, E. M. (2003) A critical role for Stat3 signaling in immune tolerance, *Immunity 19*, 425-436.

261. Schwarz, H. (2005) Biological activities of reverse signal transduction through CD137 ligand, *J Leukoc Biol 77*, 281-286.

262. Johansson-Lindbom, B., Svensson, M., Pabst, O., Palmqvist, C., Marquez, G., Forster, R., and Agace, W. W. (2005) Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing, *J Exp Med 202*, 1063-1073.

263. Hornung, V., and Latz, E. (2010) Intracellular DNA recognition, *Nat Rev Immunol 10*, 123-130.

264. Mei, Y. F., Skog, J., Lindman, K., and Wadell, G. (2003) 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 84*, 2061-2071.

265. Ouaaz, F., Arron, J., Zheng, Y., Choi, Y., and Beg, A. A. (2002) Dendritic cell development and survival require distinct NF-kappaB subunits, *Immunity 16*, 257-270.

266. Yoshimura, S., Bondeson, J., Foxwell, B. M., Brennan, F. M., and Feldmann, M. (2001) Effective antigen presentation by dendritic cells is NF-kappaB dependent: coordinate regulation of MHC, co-stimulatory molecules and cytokines, *Int Immunol 13*, 675-683.

267. Lyakh, L. A., Koski, G. K., Young, H. A., Spence, S. E., Cohen, P. A., and Rice, N. R. (2002) Adenovirus type 5 vectors induce dendritic cell differentiation in human CD14(+) monocytes cultured under serum-free conditions, *Blood 99*, 600-608.

268. Tamanini, A., Rolfini, R., Nicolis, E., Melotti, P., and Cabrini, G. (2003) MAP kinases and NF-kappaB collaborate to induce ICAM-1 gene expression in the early phase of adenovirus infection, *Virology 307*, 228-242.

269. Nociari, M., Ocheretina, O., Schoggins, J. W., and Falck-Pedersen, E. (2007) Sensing infection by adenovirus: Toll-like receptor-independent viral DNA recognition signals activation of the interferon regulatory factor 3 master regulator, *J Virol 81*, 4145-4157.

270. Yokota, A., Takeuchi, H., Maeda, N., Ohoka, Y., Kato, C., Song, S. Y., and Iwata, M. (2009) GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity, *Int Immunol 21*, 361-377.

271. Guilliams, M., Crozat, K., Henri, S., Tamoutounour, S., Grenot, P., Devilard, E., de Bovis, B., Alexopoulou, L., Dalod, M., and Malissen, B. (2010) Skin-draining lymph nodes contain dermis-derived CD103(-) dendritic cells that constitutively produce retinoic acid and induce Foxp3(+) regulatory T cells, *Blood 115*, 1958-1968.

272. Calarese, D. A., Scanlan, C. N., Zwick, M. B., Deechongkit, S., Mimura, Y., Kunert, R., Zhu, P., Wormald, M. R., Stanfield, R. L., Roux, K. H., Kelly, J. W., Rudd, P. M., Dwek, R. A., Katinger, H., Burton, D. R., and Wilson, I. A. (2003) Antibody domain exchange is an immunological solution to carbohydrate cluster recognition, *Science 300*, 2065-2071.

273. Kwong, P. D., Doyle, M. L., Casper, D. J., Cicala, C., Leavitt, S. A., Majeed, S., Steenbeke, T. D., Venturi, M., Chaiken, I., Fung, M., Katinger, H., Parren, P. W., Robinson, J., Van Ryk, D., Wang, L., Burton, D. R., Freire, E., Wyatt, R., Sodroski, J., Hendrickson, W. A., and Arthos, J. (2002) HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites, *Nature 420*, 678-682.

274. Cardoso, R. M., Brunel, F. M., Ferguson, S., Zwick, M., Burton, D. R., Dawson, P. E., and Wilson, I. A. (2007) Structural basis of enhanced binding of extended and helically constrained peptide epitopes of the broadly neutralizing HIV-1 antibody 4E10, *J Mol Biol 365*, 1533-1544.

275. Stamatatos, L., Morris, L., Burton, D. R., and Mascola, J. R. (2009) Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine?, *Nat Med 15*, 866-870.

276. Gray, E. S., Madiga, M. C., Moore, P. L., Mlisana, K., Abdool Karim, S. S., Binley, J. M., Shaw, G. M., Mascola, J. R., and Morris, L. (2009) Broad neutralization of human immunodeficiency virus type 1 mediated by plasma antibodies against the gp41 membrane proximal external region, *J Virol 83*, 11265-11274.