2013-04-29

Adjuvant-Specific Serum Cytokine Profiles in the Context of a DNA Prime-Protein Boost HIV-1 Vaccine: A Dissertation

Rachel Buglione-Corbett
University of Massachusetts Medical School

Adjuvant-Specific Serum Cytokine Profiles in the Context of a DNA Prime-Protein Boost HIV-1 Vaccine

A Dissertation Presented

By

Rachel Mari Buglione-Corbett

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

April 29, 2013

MD/PhD Program

Adjuvant-Specific Serum Cytokine Profiles in the Context of a DNA Prime-Protein Boost HIV-1 Vaccine

A Dissertation Presented

By

Rachel Mari Buglione-Corbett

The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

_____
Shan Lu, Thesis Advisor

_____
Egil Lien, Member of Committee

_____
Michael Brehm, Member of Committee

_____
Carl Alving, Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

_____
Lawrence Stern, Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school

_____
Anthony Carruthers, Ph.D.
Dean of the Graduate School of Biomedical Sciences

MD/PhD Program

April 29, 2013

Acknowledgements

First and foremost, I would like to thank my mentor, Dr. Shan Lu, who has encouraged and supported me in my pursuit of a project that greatly interested me, and also guided me in a number of diverse collaborations and opportunities to expand my intellect and development as a scientist during the last four years. I would also like to thank all of the members of the Lu Lab for their help and support on a daily basis. Over the last four years, many of you have become valuable colleagues and friends that shaped my experience in the lab. Particularly, I would like to thank Dr. Shixia Wang for her guidance and expertise, Dr. Jill Serrano for her assistance with writing and editing, and Cindi Callaghan for her assistance and organization.

I would also like to thank Dr. Egil Lien for his guidance and collaboration on many of the projects presented in this dissertation, and members of his lab who contributed to the work described herein, including Dr. Kimberly Pouliot, Dr. Robyn Marty-Roix, and Dr. Sara Paquette. The sheer number of animal studies and the learning of new techniques would not have been possible without your valuable help, support, and intellect. I would also like to thank all members of my dissertation committee, Dr. Egil Lien, Dr. Lawrence Stern, Dr. Michael Brehm, and Dr. Carl Alving. Your input throughout my time in the lab was indispensible for shaping the direction of and completing these studies.

Finally, I would like to thank my family, and also my friends both new and old, who have stuck with me throughout my studies. Your love and support during these last several years made this accomplishment possible.

Abstract

In recent years, heterologous prime-boost vaccination constructs have emerged as a promising strategy to generate broad and protective immunity against a variety of pathogens. The utility of DNA vaccination in priming the immune system, in particular, has improved the immunogenicity of vaccines against difficult pathogens such as HIV-1. In addition, many vaccine formulations include an adjuvant to augment immune responses. However, the mechanisms and profiles of many adjuvants remain largely unknown, particularly in the context of such combination immunization approaches.

My thesis research studied the effects of several adjuvants, QS-21, aluminum hydroxide, MPL, and ISCOMATRIX™ adjuvant in the context of a previously described pentavalent HIV-1 Env DNA prime-protein boost vaccine, DP6-001. In a murine model, we quantified HIV antigen-specific humoral and T cell responses, as well as pro-inflammatory serum cytokine and chemokines, both shortly after immunization and at the termination of studies. Our data indicates that each candidate adjuvant generates a unique pattern of biomarkers as well as improved immunogenicity in the context of the DP6-001 DNA prime-protein boost vaccine.

Additionally, we examined the impact of several innate signaling pathways on the adaptive immunity raised by DP6-001 and adjuvants, as well as on the unique serum cytokine profiles. These studies provide valuable information in selection of an adjuvant for inclusion in future prime-boost strategies, with the goal of enhancing immunogenicity while minimizing reactogenicity. Furthermore, these studies provided insight about the

utility of different current adjuvants in a prime-boost formulation, and the unique immune environment induced by DNA priming.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
AIM2	Absent in melanoma 2
$ALOH_3$	Aluminum hydroxide gel
APC	Antigen-presenting cell
ASC	Apoptosis-associated speck-like protein containing a CARD
BMDC	Bone marrow-derived dendritic cells
CARD	Caspase activation and recruitment domain
CBA	Cytometric bead array
CMI	Cell-mediated immunity
CpG	Deoxycytidylate-phosphate-deoxyguanylate
CTL	Cytotoxic T Lymphocyte
DC	Dendritic cell
DLN	Draining lymph node
ELISA	Enzyme linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot assay
Env	Envelope
EP	Electroporation
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine system
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor

HBV	Hepatitis B virus
HIV-1	Human Immunodeficiency virus type 1
HPV	Human papilloma virus
ICS	Intracellular cytokine staining
ID	Intradermal
IFI16	Gamma-interferon-inducible-protein 16
IFN	Interferon
IL	Interleukin
IM	Intramuscular injection
IMX	ISCOMATRIX™ adjuvant
IP	Intraperitoneal
IRF3/7	Interferon regulatory transcription factor 3/7
mAb	Monoclonal antibody
MAVS	Mitochondrial antiviral signaling protein
MCP-1	Monocyte chemotactic protein 1
MDA5	Melanoma differentiation-associated gene 5
MIP-1α	Macrophage inflammatory protein 1 α
MIP-1β	Macrophage inflammatory protein 1 β
MPL	Monophosphoryl lipid A
MyD88	Myeloid differentiation primary response 88
NAb	Neutralizing antibody
NHP	Non-human primate

NK	Natural killer cell
NLR	Nucleotide-binding oligomerization domain (NOD) like receptors
NLRP3	NOD-like receptor 3
NZW	New Zealand White
PAMP	Pathogen-associated molecular patterns
PYHIN	Pyrin and HIN domain-containing protein family
PRR	Pattern recognition receptor
RANTES	Regulated on activation, normal T cell expressed and secreted
RIG-I	Retinoic acid-inducible gene
SHIP-1	Src homology 2 containing inositol phosphatase-1
SHIV	Simian human immunodeficiency virus
SIV	Simian immunodeficiency virus
STING	Stimulator of interferon genes
TBK1	Tank-binding kinase 1
TLR4	Toll-like receptor 4
TLR9	Toll-like receptor 9
TNF	Tumor necrosis factor
TRIF	TIR-domain-containing adaptor-inducing interferon-β

Preface

Much of the experimental work presented in this dissertation are representative of both individual and collaborative work between myself under the guidance of Dr. Shan Lu, and the Lien Lab, with the guidance of Dr. Egil Lien, and contributing efforts of Dr. Kimberly Pouliot, and Dr. Robyn Marty-Roix.

Chapters in this dissertation were taken from the following publications currently in submission or being prepared for submission:

Chapter II: Buglione-Corbett, R., Pouliot, K., Marty-Roix, R., West, K., Wang, S., Lien, E., Lu, S. Serum cytokine profiles associated with specific adjuvants used in a DNA prime-protein boost vaccination strategy. *Submitted to PLoSONE for review*.

Chapter IV: Buglione-Corbett, R., Pouliot, K., Marty-Roix, R., West, K., Wang, S., Lien, E., Lu, S. Potent adjuvant effects of ISCOMATRIX™ adjuvant as part of a DNA prime-protein boost HIV-1 vaccine formulation. *In preparation for submission.*

Chapter III in this dissertation describes work performed in collaboration with the Lien Lab, currently being prepared for publication by the Lien lab.

Other work performed during thesis studies that is not presented in this dissertation has appeared in additional publications:

Haran, J.P., Buglione-Corbett, R., Lu, S. Cytokine markers as predictors of type of respiratory infection in patients during the influenza season. (2013) Am J Emerg Med. *In press*.

Buglione-Corbett, R., Suschak, J., Wang, S., Lu, S. Chapter 18: Nucleic Acid Vaccines. In Wang, W., Singh, M. (Eds.), Biological Drug Products: Development and Strategies. *In press.*

CHAPTER I:

Introduction

Since Edward Jenner's first demonstration of effective human vaccination against smallpox in 1796 and the subsequent eradication of smallpox in the human vector, vaccine strategies and technologies have continued to develop in the face of new and challenging pathogens, as well as the urgent societal need. Two effective vaccines emerged in a rapid response to the public health crisis created by the poliovirus epidemic in the mid-20th century: Jonas Salk's inactivated poliovirus vaccine and Albert Sabin's live-attenuated poliovirus vaccine, released in 1952 and 1958, respectively (133). The emergence of new pathogens has always driven optimization and innovation in vaccine design, especially where conventional practices fail.

According to World Health Organization (WHO) reports in 2011, approximately 34 million people are currently living with human immunodeficiency virus (HIV-1) worldwide, with 2.5 million new infections and 1.7 million virus-related fatalities in 2011 (190). Since the emergence of HIV-1 and acquired immunodeficiency syndrome (AIDS) in 1981, there remains an exigent need for an effective vaccine. Conventional vaccination methods, such as live-attenuated or inactivated viral vaccines, raise considerable safety concerns. Development of an effective prophylactic vaccine remains the focus of substantial research resources, due both to the overwhelming global need as well as the intrinsic obstacles to effective immunity presented by the HIV-1 virus.

The HIV-1 virus has a high rate of mutation during replication, lacks an effective mechanism of proof-reading, and also an error-prone reverse transcriptase enzyme, which

results in the substantial diversity of viral subtypes. These characteristics also allow for a high rate of escape from virus-specific adaptive immune responses, and a high level of viral diversity within a single host (55). Subsequently, the global epidemic is characterized by a wide distribution of subtypes, or clades, of the major (M) group of HIV-1. Therefore, development of an effective vaccine must address the challenge of the large number of circulating viral subtypes even within a single region.

A major challenge of designing an HIV-1 vaccine has also been identifying viral antigens to include in the vaccine. The HIV-1 virus is comprised of an RNA genome and 9 genes encoding 15 proteins with different roles in viral function. These include the Pol enzymatic proteins [protease (PR), reverse transcriptase (RT), integrase (IN)], and several accessory proteins in the viral particle (Vif, Vpr, and Nef), gene regulatory proteins (Tat and Rev), and the viral assembly protein Vpu. Viral structural components include Gag proteins [matrix (MA), capsid (CA), nucleocapsid (NC) and p6] and two envelope (Env) subunits. The Env trimer is composed of the heavily glycosylated gp120 subunit that forms spikes expressed on the virus surface. gp120 is responsible for the binding of CD4 on the host cell surface during viral entry, and is covalently linked to the gp41 transmembrane subunit (55). Env is the primary viral antigen targeted by neutralizing antibodies (NAb) against HIV-1 (20, 113, 197).

1. HIV-1 Vaccine Trials

1.1 VaxGen Trial

Despite the overwhelming success of new subunit recombinant protein-based vaccines against hepatitis B (HBV) vaccines in the 1980s (21, 48, 116), these strategies

failed in several Phase III clinical efficacy trials conducted by VaxGen conducted between 1998 and 2003. Both the VAX004 trial, conducted in North America and the Netherlands, and the VAX003 trial, conducted in Thailand, evaluated the immunogenicity of bivalent recombinant HIV-1 envelope gp120 glycoprotein (rgp120) vaccines. The VAX004 study evaluated the immunization of at risk, HIV-1 seronegative individuals with the AIDSVAX B/B vaccine, consisting of two clade B rgp120 antigens from two different subtypes of clade B viral strains, adsorbed onto an aluminum hydroxide gel adjuvant, and administered by intramuscular (i.m.) injection at months 0, 1, 6, 12, 18, 24, and 30 (52). Despite the development of binding and neutralizing antibody responses (Nab) specific to vaccine strain rgp120, vaccination did not significantly confer protection compared to placebo. Moreover, vaccination did not positively or negatively impact disease progression in individuals that became infected during the course of the study (52). The second study, VAX003, evaluated a bivalent vaccine AIDSVAX B/E in HIV-1 negative injection drug users in Thailand, which demonstrated a similar lack of efficacy (60, 61).

These clinical results were in disappointing contrast to protection previously observed in vaccinated chimpanzees (11, 12, 46), and phase 1 and 2 clinical trials, which produced robust vaccine-induced antibody responses after vaccination with AIDSVAX (54, 63, 64, 142, 143). Furthermore, while peak antibody responses were inversely correlated with incidence of HIV-1 infection in vaccinees (61), the lack of clinical efficacy in the AIDSVAX trials suggest that recombinant protein-based vaccine induction of antibody responses may not be sufficient for protection. Indeed, definitive correlates

of protective immunity against HIV-1 remain unclear, and are not limited to the generation of humoral immunity. In light of these observations, alternative strategies focused on raising vaccine-specific T cell responses that may be sufficient for protection.

1.2 STEP Trial

In an effort to develop an HIV-1 vaccine capable of raising specific T cell responses, Merck Research Laboratories developed a recombinant adenovirus serotype 5 (rAd5) viral vector-based strategy. The trivalent rAd5 vector vaccine consisted of rAd5 encoding HIV-1 viral antigens Gag, Pol, and Nef, and was administered in a homologous prime-boost regimen at months 0, 1, and 6. Phase I clinical trials of the rAd5 vector vaccine indicated that the regimen was safe and results were encouraging, with the induction of HIV-1 Gag-specific T cell responses measured in vaccinees as measured by IFNγ ELISpot during weeks 30-78 (147). While there were concerns regarding pre-existing adenovirus-specific responses in seropositive individuals previously exposed to Ad5, clinical trials of the Merck Ad5 vaccine moved forward.

The STEP trial, a Phase IIb proof-of-concept study of the rAd5 vector vaccine, tested an identical Ad5 viral vector vaccine described above. As intended, the vaccine did induce strong T cell responses, indicated by antigen-specific production of IFNγ as measured by ELISpot in 77% of vaccinees (23, 118). Further analysis by intracellular cytokine staining (ICS) demonstrated that 41% of vaccinees produced vaccine-specific $CD4^+$ T cells with a polyfunctional profile (production of IFNγ and IL-2), while 73% of participants produced specific $CD8^+$ T cell responses (118). Unfortunately, the positive induction of cell-mediated immunity (CMI) was not correlated to protection against

infection, or mitigation of viremia in subjects that became infected during the course of the study. In fact, the STEP trial was abruptly halted in September of 2007 due to considerable safety concerns. Not only did the rAd5 vaccine fail to meet expectations of protection, but in fact the viral vector vaccine appeared to increase susceptibility to HIV-1 infection in vaccinees previously exposed to Ad5 adenovirus (95). Subsequent evaluation of samples from vaccinees indicated that $CD8^+$ T cell responses were attenuated by pre-existing NAb against Ad5 (196). Additional reports indicated that $CD4^+$ T cells specific for Ad5 vector existed but were not associated with increased susceptibility (80, 134).

In conjunction with safety concerns demonstrated with the viral vector strategy, the failure of the STEP trial raised additional questions about correlates of protective immunity. It is unclear whether the inadequate protection conferred by a vaccine designed to elicit T cell responses was due to poor induction of cell-mediated immunity (CMI), or due to the lack of robust neutralizing antibody responses. Lessons from the VaxGen and STEP trials demonstrate the need for vaccine candidates to induce both humoral and cell-mediated immune responses against HIV-1.

1.3 RV144 Trial

The Phase III RV144 trial, conducted in Thailand, volunteers were immunized with two vaccine strategies that had previously been shown to be ineffective on their own. HIV-1 negative individuals (n=16,402) received the ALVAC recombinant canarypox vector. ALVAC consists of viral vector expressing the circulating recombinant forms 01_AE gp120, subtype B transmembrane gp41, and subtype B gag

and protease, at weeks 0, 4, 12, and 24 via i.m. injection. AIDSVAX B/E recombinant gp120 boosts were formulated with aluminum hydroxide gel adjuvant and administered i.m. at weeks 12 and 24 via i.m. Vaccinees were followed for HIV infection and immune responses over three years following immunization (150).

Phase I and II clinical trials conducted on a smaller scale demonstrated that the ALVAC prime and AIDSVAX B/E boost regimen was safe and tolerable in humans. In addition, 63% of immunized participants demonstrated lymphoproliferation, and 24% of vaccinees produced $CD8^+$ T cells. Almost all vaccinees raised binding and neutralizing antibody responses against vaccine strains (132). These initial results were extremely promising, and studies were advanced to phase III trials in the RV144 study.

Vaccinees in the intent-to-treat group of RV144 demonstrated an overall 31% efficacy in protecting against HIV-1 infection in the 3.5 years of study, which was a marginal but significant and encouraging outcome (150). In fact, in a cohort of participants considered to be of a lower-risk, protection against infection was as great as 40% (145). The vaccine was shown to be safe and tolerable in immunized participants (144). Extensive follow-up studies indicated modest induction of Env-specific T cell responses in 41% of vaccinees, predominated by polyfunctional $CD4^+$ T cells producing IFNγ and IL-2 (38). Correlation was observed between subjects producing IL-2 responses and improved Env-specific antibody titers (38). Furthermore, antibody-dependent cellular toxicity was detected in 80% of vaccinees (14, 84).

Binding IgG antibody responses against the Env epitope V2 were raised in vaccinees and were correlated with protection against infection. In a comparison study

against VAX003 study samples, neutralizing antibodies against a Tier 1 panel of viral isolates were induced by the RV144 prime-boost, but to a weaker extent than observed in VAX003 (83, 125). However, persistent low levels of neutralizing antibodies generated by the RV144 vaccine are at least suggestive of the induction of long-term memory B cells (125). In fact, antibody responses in waned after the 12 months following vaccination, a factor that may have contributed to diminishing protection (145). While protection immediately following immunization was estimated at 70%, protection at the conclusion of the study (42 months) was more modest, at 31% (5).

2. Important role of antibodies in HIV-1 vaccines

2.1 Evidence from passive antibody immunization

In early preclinical studies focused on protective immunity against HIV-1, chimpanzees passively immunized with human neutralizing monoclonal antibodies (mAb) specific for HIV-1 Env gp120 epitopes were found to be protected against viral challenge by the same HIV-1 variant (49). Passive inoculation of chimpanzees with NAb against Env impacted the course and severity of disease (32). In non-human primates, the combination of HIV-1 immuno globulin and neutralizing human monoclonocal antibodies (mAbs) was either protective against subsequent challenge or resulted in a more benign disease progression (112, 114). The advantages of passive immunization and the protective importance of neutralizing antibodies were also demonstrated in models of maternal-fetal HIV-1 transmission. Passive immunization of pre-natal pregnant macaques, and post-natal immunization of their neonates, with human mAbs was observed to be either protective against infection or slowed disease progression

following simian HIV (SHIV) challenge (6, 75), even in the absence of a prenatal dose (76). These studies indicate the importance of neutralizing antibody responses in effective protection against HIV-1 infection.

The Env gp120 antigen, exposed on the surface of the virus, was found to be superior at generating a viral NAb response compared to the full-length transmembrane gp160 (12). In several studies, immunization with recombinant gp120 (rgp120) protein in non-human primates (NHP) resulted in no apparent signs of infection following challenge with homologous virus (12, 13, 62). Immunization of NHPs with rgp120-based vaccine strategies improved NAb titers against vaccine strains, with elevated specific NAb titers correlating to protection against infection during subsequent homologous viral challenge (12, 13, 62). In addition, polyvalent multiclade recombinant protein vaccine strategies generated NAb responses with improved breadth and potency, as well as improved protection and reduced viremia in immunized NHP against homologous viral challenge (30, 91).

However, despite the initial promise of protein vaccines against HIV-1 in preclinical trials, these strategies were clinically disappointing. A Phase I clinical trial of rgp120 immunization in healthy human volunteers demonstrated that vaccine-induced antibodies were capable of neutralizing laboratory-adapted viral strains, but failed to neutralize primary viral isolates (113). In comparison, HIV-infected individuals were characterized by NAb against both laboratory-adapted and primary viral strains, indicating a disparity between the vaccine-induced immune response and the immune response induced by the natural course of infection (113).

Concurrent with the study of NAb raised by protein vaccination, additional work in NHP models indicated the critical contribution of cell-mediated immunity (CMI) by both $CD8^+$ cytotoxic lymphocytes (CTLs) and $CD4^+$ helper T cells. In a series of publications, Heeney *et al.* evaluated rgp120 protein-based vaccination in rhesus macaques based not only on the induction of humoral immunity, but also vaccine antigen-specific $CD4^+$ and $CD8^+$ T cell responses (69-73, 113). Homologous viral challenge of immunized animals demonstrated sterilizing immunity. Both NAb titer and a strong, balanced Th1/Th2 helper response were identified as correlates of long-term protective immunity against homologous and heterologous viral challenge in NHP (70, 73). The production of pro-inflammatory chemokines RANTES, macrophage inflammatory protein 1α (MIP-1α) and MIP-1β produced by cytotoxic T lymphocytes (CTLs) were also correlated to protection (71). In addition, the importance of a vaccine-induced CTL response was shown to be a critical component in controlling viremia (24, 72, 164). To date, there is no human data to support this.

2.2 Evidence and new data from RV144

The modest, but promising, results from the RV144 study provide many lessons for the future direction of HIV-1 vaccine strategies, especially considering the substantial opposition faced at the inception of the trial rooted in the failure of each vaccine component individually. This study indicated that a prime-boost strategy by heterologous modalities are capable of inducing a superior immune response, and in this example, improved protection, compared to either component individually. Despite decades of research focused on the immune responses in HIV-infected individuals, and in study

participants immunized with evolving vaccine strategies, clear correlates of HIV-1 immunity towards which to strive remain elusive. It is clear from this trial, and previous failed attempts, the necessity of designing vaccines with the goal of inducing humoral responses and CMI, as well as the efficacy of prime-boost strategies in achieving this aim.

Still, questions regarding optimization remain. Was the modest efficacy and quickly waning protection observed in RV144 due to the antigens themselves, the vaccination regimen, or the vector chosen for priming? Alternatively, the choice of adjuvant may greatly impact the immunogenicity of a vaccine. Aluminum salt based adjuvants primarily promote antibody responses, and thus inclusion of a more potent and balanced immune responses may allow for substantial strides towards an effective prophylactic vaccine against HIV-1 (145, 187).

3. HIV-1 DNA vaccines

3.1 DNA vaccine history

After the initial failure of protein-based vaccines against HIV-1 based on raising NAb responses, efforts were refocused on generating a strong T cell response. DNA vaccines emerged as a novel immunization modality in the early 1990s, allowing for the uptake of antigen-encoding plasmid DNA or viral vector by antigen presenting cells (APCs), and subsequent production of vaccine antigen *in vivo* in its native conformation and with post-translational modifications. Endogenous transcription of a vaccine antigen encoded on a plasmid or viral vector makes the antigen available for processing and presentation via both major histocompatibility complex (MHC) I and II, effectively

targeting both humoral and cell-mediated immune responses to the vaccine (101, 102, 105). This approach, in theory, results in an immune response more comparable to natural infection, which is a primary objective of an HIV-1 vaccine design strategy (113), by effectively inducing the vaccine-specific CTL and helper T cell responses necessary for prophylactic immunity against HIV-1.

The utility of DNA vaccination was initially demonstrated in small animal models against a variety of pathogens. Inoculation of mice with DNA encoding influenza hemagluttinin (HA) or nucleoprotein (NP) antigen produced vaccine-specific antibody titers and CTL responses, respectively, which were protective against homologous and heterologous viral challenge (59, 155, 177). Immunogenicity of DNA vaccination was also demonstrated in small animal models of herpes simplex virus-2 (HSV-2) (117), human papillomavirus (HPV) (40), and hepatitis B virus (HBV) (36, 121). DNA vaccination has also been extensively studied in preclinical models of HIV-1. Mice immunized via i.m. inoculation with DNA plasmids encoding HIV-1 Env gp160 demonstrated potent antigen-specific NAb responses as well as lymphocyte proliferation in comparison to a recombinant protein vaccine (184). Mice immunized with Env DNA vaccine also demonstrated broad and potent NAbs concurrent with $CD4^+$ and $CD8^+$ T cell responses, with strong neutralizing activity strong even in the absence of high antibody titers (58, 104).

The efficacy and immunogenicity of HIV-1 or simian immunodeficiency virus (SIV) Env DNA vaccines was also demonstrated in NHP models, particularly when enhanced by administration routes such as electroporation (EP) or gene gun delivery

compared to i.m. injection. Gene gun or i.m. administration of a pentavalent DNA vaccine encoding SIV antigens did induce NAb titers as well as persistent CTL responses (103). While protective immunity was not achieved and $CD4^+$ T cell loss was not impacted, viremia and signs of acute infection were attenuated in immunized animals (103). Similarly, chimpanzees immunized with a polyvalent DNA vaccine encoding the HIV-1 antigens Env, Rev, and Gag/Pol also generated vaccine-specific antibody and CTL responses, correlated with partial protection against heterologous viral challenge (17).

The clinical efficacy of DNA vaccination was first demonstrated in a clinical study using a human vaccine against HBV administered using gene gun technology, which generated potent vaccine-specific $CD4^+$ and $CD8^+$ T cell responses as well as high antibody titers (158). Unfortunately, despite the promising immunogenicity of DNA vaccines in preclinical models and in the context of other pathogens, clinical trials of DNA vaccines against HIV-1 were somewhat disappointing. Immunization of asymptomatic HIV-1 seropositive individuals with DNA plasmids encoding HIV-1 antigens Env and Rev were shown to be safe in humans, but produced low and transient levels of specific humoral and cellular immune responses, with no measurable impact on viremia or disease progression (108). DNA-based immunization of HIV^+ individuals with constructs encoding the HIV-1 regulatory antigens Rev, Nef, or Tat were well tolerated and induced positive, albeit transient, vaccine-specific CTL responses; however, administration did not result in a therapeutic effect (24-26). Immunization of HIV-1 positive individuals with a DNA vaccine encoding Env and Rev was similarly tolerable, and it generated vaccine-induced lymphocyte proliferation and CTL responses (15, 16).

In addition, the vaccine induced production of IFNγ and β-chemokines MIP-1α, MIP-1β and RANTES. These markers were previously identified as correlates of protective immunity by Heeney *et al.*, and are indicative of a pro-inflammatory response (16). However, DNA vaccination alone did not appear to be sufficient for protective immunity against HIV-1 infection.

3.2 DNA Prime-protein boost vaccine

The history of HIV-1 vaccine clinical trials has shown that optimally, an HIV-1 vaccine should be capable of both producing sterilizing antibodies to prevent viral infection, and inducing diverse and specific T cell responses to assist B cell responses, as well as control and eliminate infected cells. While recombinant protein and DNA or viral vector-based immunization alone fail to activate both arms of the immune response, the combination of these two strategies has proven to be significantly more effective. A landmark 1992 study by Hu *et al.* established the utility of a prime-boost strategy (78). Macaques were primed i.m. with vaccinia vector encoding SIV_{mne} Env glycoprotein at weeks 0 and 12, followed by i.m. boosting with recombinant gp160 protein at weeks 62 and 70. While weak antibody responses were initially observed, all animals primed with vaccinia vector encoding Env antigen demonstrated notable vaccine-specific helper T cell function. Following protein boosting formulated with either an aluminum salts adjuvant or incomplete Freund's adjuvant, primed animals demonstrated a substantial increase in Env-specific antibody titers, as well as NAbs against homologous and heterologous viral antigens. Complete protective immunity was confirmed after viral challenge with SIV_{mne} in prime-boost immunized animals as compared to control animals (78).

While clinical applications of DNA vaccination alone have resulted in weak immunogenicity, it is particularly effective at priming the immune system for subsequent immunization by a different modality, such as a recombinant protein. It has been generally established that DNA vaccination promotes vaccine-specific CMI (3, 9, 156), while protein immunization promotes strong antibody responses (31, 43, 44, 65), and so the combination of these two methods in a prime-boost strategy is promising in achieving the goal of protective immunity against HIV-1.

This concept was initially demonstrated in small animal models and NHPs. Mice, guinea pigs, and rabbits immunized with a DNA vaccine encoding HIV-1 Env antigen, followed by homologous Env protein boosting, demonstrated superior CTL and NAb responses against vaccine strains compared to DNA vaccination alone (8, 153). In NHPs immunized with Env-encoding DNA plasmid and boosted with Env protein, long-lasting vaccine-specific CTLs and NAb responses were detected; in addition, animals immunized with the prime-boost were protected against homologous SHIV challenge (96).

In recent years, our lab at UMMS has extensively evaluated a pentavalent HIV-1 Env gp120 DNA prime-protein boost vaccine, called DP6-001. The DNA vaccine encodes five *env* genes from HIV-1 clades A, B, C, and E as well as a *gag* gene from a single clade. The protein boost component consists of Env gp120 proteins homologous to those encoded by the DNA vaccine, and is formulated with the saponin adjuvant QS-21.

Preclinical studies of DP6-001 and precursor DNA prime-protein boost vaccines in small animal models and NHPs established the efficacy of the combination of these two modalities. Rabbits immunized with DNA plasmids encoding gp120, and subsequently boosted with rgp120 protein, were found to raise improved NAb against primary viral isolates, while either component alone failed to produce a NAb response (185). Immunization of rhesus macaques with DP6-001 induced $CD4^+$ and $CD8^+$ T cells, characterized by the production of Th1 cytokines IFNγ and IL-2. In addition, DNA priming prior to boosting with recombinant protein augmented the vaccine induced humoral responses compared to protein immunization alone (35).

In subsequent reports, several immunized macaques rectally challenged with SHIV were protected against infection, and those that became infected demonstrated reduced viral load (137, 138). Immunized animals generated NAb against homologous and, to a lesser extent, heterologous viral isolates. Given the lessons from prior clinical observations in vaccinees and infected individuals, the DNA prime-protein boost strategy appears ideally suited for the generation of the broad and diverse immune response that is required for immunity against HIV-1. Furthermore, toxicology studies of DP6-001 in rabbits indicated minimal signs of adverse effects and ruled out DNA integration of vaccine plasmids, which supported the further evaluation of DP6-001 in humans (35).

In phase I clinical trials of DP6-001, participants were immunized either i.m. or intradermally (i.d.) with three pentavalent DNA primes at either a high (7.2 mg) or low (1.2 mg) dose, followed by two pentavalent homologous rgp120 protein boosts formulated with QS-21, as in preclinical studies. DNA priming followed by protein

immunization was shown to enhance the magnitude of cross-subtype Env-specific CMI. Robust T cell responses were detected by IFNγ ELISpot in the majority of vaccines (186). Follow-up reports identified a T cell response predominated by vaccine antigen-specific polyfunctional $CD4^+$ T cells, with low positive $CD8^+$ T cells in higher dose groups (7). In addition, vaccinees raised long-lasting antibody titers to levels comparable to those observed in infected individuals over the course of the 52-week study. Antibody responses in responders were capable of neutralizing homologous viral isolates, though neutralization against a Tier 2 panel representative of more contemporary viral strains was more modest (186).

The choice in adjuvant utilized in the DP6-001 studies is a notable difference compared to the RV144 trials. The saponin QS-21 was chosen for inclusion in DP6-001 due to its previously demonstrated ability in the clinical context of HIV antigens to induce both CMI and humoral arms of the adaptive immune response with a potency allowing for antigen dose reduction (50, 183). However, as a known hemolytic agent, QS-21 is not without adverse effects (183). In the clinical trials of DP6-001, reactogenicity predominantly comprised of self-limited injection site reactions were observed. In particular, a case of leukocytoclastic vasculitis, occurring in an individual primed with a high dose of DNA prime, was observed following protein and QS-21 boosting (7). While this case resolved within 72 hours of corticosteroid treatment, this immunization group was halted prematurely due to safety concerns. This example highlights the need to carefully consider the choice of adjuvant in an HIV-1 vaccine

strategy not only for immunogenicity, but also for safety and tolerability in a clinical setting.

4. Adjuvants

The choice of adjuvant in a vaccine formulation is a critical component of vaccine research and development. Adjuvants are a diverse group of compounds that, when formulated with vaccine antigen, improve the immunogenicity of a vaccine or modulate the immune response to immunization. Adjuvants may provide a depot effect, serve as an antigen-delivery vehicle, generally stimulate the immune system, or specifically target particular immune functions and pathways. Direct stimulation of innate immune responses has been a more recent focus of adjuvant development, with the goal of harnessing innate immunity and inflammation in order to enhance vaccine-specific adaptive immune responses.

Adjuvants must be evaluated for safety and efficacy in the context of any novel vaccine formulation, as the clinical profile and immunogenicity of adjuvants can vary in formulation with different vaccine antigens, vaccination regimens, or delivery methods. Furthermore, the choice of adjuvant depends on the type of immune response required for prophylaxis. Aluminum salts, the most clinically established and widely used adjuvant in licensed vaccines are known to primarily induce potent antibody responses as opposed to CMI. Lessons learned from clinical trials of HIV-1 vaccines indicate the need for both humoral and T cell responses, and thus, the choice of an adjuvant capable of promoting both arms of the adaptive immune system. In addition, we explored several newer adjuvant candidates better suited for inclusion in future formulations of HIV-1 vaccines.

4.1 Aluminum salt-based adjuvants

Aluminum salts, typically formulated as aluminum phosphate or aluminum hydroxide, are the most widely used adjuvants in vaccines approved for use in humans. Generically referred to as "alum," this term may include aluminum phosphate salts, and aluminum hydroxide, which is often used in a gel formulation such as Alhydrogel®. Another commonly used aluminum hydroxide-based gel is Imject® alum, a gel formulation of aluminum hydroxide and magnesium hydroxide. For the animal experiments described in this dissertation, we employed an aluminum hydroxide gel, and will herein refer to the formulation by its chemical formula, $Al(OH)_3$.

Aluminum salt-based adjuvants are typically well tolerated with minimal adverse effects, and induce potent vaccine-specific antibodies and $CD4^+$ Th2 helper responses characterized by Th2 cytokines interleukin (IL)-4 and IL-5. Despite its widespread use, alum's mechanism of action remains largely undefined. Originally, it was postulated that the depot effect was responsible for adjuvanticity. Adsorption of soluble antigen onto aluminum salts would improve uptake of the particulates by antigen-presenting cells (APCs) at the vaccination site, and also form a depot at the site for the continuous release and presentation of antigen. However, subsequent studies demonstrated that adsorption of antigen onto alum was not required for adjuvant effect (66). In fact, characterization studies of gp120 antigen formulated with a variety of aluminum hydroxide compounds showed that gp120 is rapidly desorbed from the adjuvant following immunization of guinea pigs, rabbits, and baboons (188). Furthermore, different formulations of these aluminum hydroxide adjuvants were capable of eliciting comparable binding antibody

titers specific to gp120 antigen in immunized animals (188). More recently, the activation of innate signaling pathways by either direct or indirect effects of aluminum salts have been implicated in their mechanism of action, but the dependence of adaptive immune responses on these innate signaling pathways remains controversial.

Aluminum hydroxide gel was employed as an adjuvant in the VaxGen and RV144 HIV-1 vaccine clinical trials discussed above. Each rgp120 protein immunization in the VaxGen trial was adsorbed onto 600 μg of aluminum hydroxide gel. Similarly, in the RV144 prime-boost trial, the AIDSVAX B/E boost component co-administered with the final two doses of ALVAC canarypox vector was formulated with 600 μg of aluminum hydroxide gel. While RV144 demonstrated unprecedented protection against HIV-1, antibody responses induced by the vaccine were not long lasting, and T cell responses were induced at relatively low levels. The formulation of vaccine with an aluminum salt-based adjuvant may be one reason for relatively low CMI and short-lived antibody responses (125, 145, 187). Novel vaccine adjuvants described below focus on targeting both CMI and humoral responses, and may be better candidates for future HIV-1 vaccine formulations.

4.2 Monophosphoryl Lipid A (MPLA)

Monophosphoryl lipid A (MPLA) is a low-toxicity, dephosphorylated derivative of Gram-negative bacterial lipopolysaccharide (LPS) from *Salmonella Minnesota*. The abbreviation of MPLA refers to generic monophosphoryl lipid A, so as to distinguish it from clinical grade MPL® produced by GlaxoSmithKline. While both LPS and MPLA are both agonists of Toll-like receptor 4 (TLR4), MPLA has been demonstrated to induce

0.1% of the inflammatory effects as compared to its parent molecule, LPS (2, 115). Some studies indicated that differently biased signaling pathways downstream of TLR4 are responsible for the reduced inflammation induced by MPLA, but the exact mechanism remains unclear. MPLA as an adjuvant in vaccine formulations has been clinically well tolerated with minimal adverse effects.

Due to its ability to stimulated TLR4 but with reduced inflammatory effects, MPLA was developed as a novel adjuvant system with improved Th1 responses compared to conventional aluminum salt-based adjuvants. This characteristic is advantageous in vaccines against pathogens requiring more robust Th1 immunity, such as HIV-1, malaria, and tuberculosis, which require CTL responses as well as improved T cell responses against infected cells. In addition to Th1 immune responses (characterized by IFNγ and IL-2), and the Th1-associated IgG2a isotype, MPLA induces a complex inflammatory response consisting of neutrophils, antigen-presenting cells (APCs), and natural killer (NK) cells following administration (2, 159, 162, 180). It is becoming increasingly evident that all three of these cellular responses are important in the development of HIV-1 immunity.

The extent to which MPLA induces Th1-biased immune responses depends largely on the antigen with which it is formulated, the delivery mechanism, and co-administration with another adjuvant. Indeed, MPLA is often used in combination with other adjuvants, such as in the GlaxoSmithKline (GSK) adjuvant systems. The licensed human vaccines Fendrix™ for protection against hepatitis C virus (HCV), and Cervarix™ for protection against human papilloma virus (HPV), include AS04, a

combination of aluminum hydroxide and MPL. MPLA has also been combined with QS-21 in AS01 and AS02 in anti-malarial vaccines currently being evaluated in clinical trials (39, 159).

4.3 QS-21

The QS-21 adjuvant is a purified saponin fraction derived from the *Quillaia saponaria Molina* soap bark tree. QS-21 is an acylated, water-soluble triterpene glyocoside that can be formulated with soluble antigen (87). QS-21 has been extensively evaluated in preclinical and clinical studies in formulation with HIV-1 vaccines. In the context of HIV-1 subunit protein-based vaccines, QS-21 is associated with improved Th1-biased helper T cells, antigen-specific production of IFNγ and IL-2, as well as the Th1-associated IgG isotype IgG2a (34, 87-90, 99, 107, 128, 161). QS-21 has also been shown to enhance NAb (74, 85, 100) and CTL responses (67, 129, 130, 194). The potency of this saponin adjuvant allows for reduced antigen dose in vaccine formulations (50).

However, the potency of QS-21 comes with the risk of local and systemic adverse effects. While toxicity was minimal in preclinical models and veterinary vaccines, the use of QS-21 in human vaccines is restricted due to reports of reactogenicity. A known hemolytic agent, QS-21 has been clinically associated with local adverse effects, such as mild to moderate tenderness, erythema, and pain at the injection site (7, 50, 183). Systemic symptoms after immunization, such as hypotension, fever, myalgia, and headache, have also been observed in recipients of QS-21. Our own clinical experience with QS-21 in the context of DP6-001 prime-boost immunization supported prior reports

of potent immunogenicity and necessitated the selection of a more tolerable and safe adjuvant.

QS-21 is included in several Adjuvant Systems (AS) currently being evaluated by GlaxoSmithKlein (GSK). As mentioned above, AS01 and AS02 consist of both QS-21 and MPL® in liposomes or oil-in-water emulsions, respectively, and have demonstrated improved tolerability without compromised potency in clinical trials of anti-malarial vaccine candidates (159, 180).

4.4 ISCOMATRIX™ adjuvant

ISCOMATRIX™ adjuvant, produced and trademarked by Commonwealth Serum Laboratories (CSL), Ltd. in Australia, is a novel saponin-based adjuvant system that has improved upon both the immunogenicity as well as the tolerability of QS-21. Morein *et al.* first described as 'immuno-stimulating complexes' (ISCOMs) in 1984. The optimized formulation of ISCOMATRIX™ adjuvant consists of a highly purified saponin fraction (ISCOPREP™) contained within a 40 nm diameter cage-like matrix consisting of cholesterol and a phospholipid, dipalmitoyl-sn-phosphatidylcholine (DPPC) (126). Prototypical ISCOMs required difficult and complex methods of incorporating a limited range of antigens into the structure, and employed a less highly fractionated saponin, ISCOPREP™ 703, which is comprised of a mixture of saponin fractions QH-A and QH-C (171). Current formulations of ISCOMATRIX™ adjuvant, with ISCOPREP™ saponin, are formulated with a wide variety of vaccine antigens by simple mixing, and are stable at 4^{o}C for 2-3 years (127, 141).

ISCOMATRIX™ adjuvant has been characterized as an 'integrated adjuvant system' owing to its ability to both act as an antigen delivery vehicle as well as a potent immunomodulator (127). Lymphatic duct cannulation studies performed in sheep demonstrated that antigen and ISCOMATRIX™ adjuvant traffic to draining lymph nodes (DLN) in two waves, early (2 hours) and late (24-48 hours) following injection, resulting in prolonged antigen presentation (192). At the injection site and in the DLN, antigen and ISCOMATRIX™ adjuvant are taken up by dendritic cells (DCs) and APCs. Delivery of antigen to the cytosol occurs when ISCOMATRIX™ adjuvant is internalized in an actin-dependent manner into endosomes, which are subsequently acidified, releasing vaccine antigen into the cytosolic compartment (166, 167). Antigen is made available for processing and presentation via both MHC I and II, for cross-presentation to $CD8^+$ and $CD4^+$ T cells, respectively. As a result, CTLs as well as helper T cells, aiding subsequent B cell responses, are effectively induced. As an immunomodulator, the ISCOMATRIX™ adjuvant induces a complex milieu of cytokines and chemokines, resulting in the recruitment of immune cells in the DLN while inhibiting efflux of APCs and allowing for increased and prolonged T and B cell priming, for up to 48 hours post-immunization (165, 167).

ISCOMATRIX™ adjuvant has been evaluated in 16 clinical trials to date, in the context of therapeutic vaccines against HPV (57) and HCV (41), as well as in a prophylactic vaccine against influenza. Additionally, in clinical trials ISCOMATRIX™ adjuvant has been a component of therapeutic vaccines against NY-ESO-1 positive tumors, such as in melanoma (37, 110, 131). Compiled clinical safety data recently

reported by McKenzie *et al.* indicated that local and systemic adverse effects to vaccines formulated with ISCOMATRIX™ adjuvant were mild and self-limited (120). Immunization with ISCOMATRIX™ adjuvant and vaccine antigen were demonstrated to induce strong $CD4^+$ and $CD8^+$ T cell responses in NHP and human trials of HPV, HCV, and anti-tumor vaccines. In addition, vaccine-induced CMI responses were long lasting and suggestive of a strong memory response. ISCOMATRIX™ adjuvant is also associated with the induction of balanced Th1/Th2 T cell and IgG isotype responses (109, 127, 141).

5. Innate immune signaling pathways contributing to adjuvant mechanism

Despite relatively consistent and well-defined clinical profiles, all of the adjuvant systems described herein still lack clearly elucidated mechanisms. These adjuvants all activate innate immune signaling pathways, presumably harnessing the innate immune response in order to enhance adaptive immune responses to vaccination. Here, I will expand upon the innate immune pathways demonstrated to play a role in each adjuvant mechanism, although their role in generating humoral and CMI responses remains to be characterized.

5.1 Toll-like receptor 4 (TLR4), and its adaptors MyD88 and TRIF

As described above, LPS and its derivative, MPLA, are both strong agonists of TLR4, a receptor that is present on the surface of DCs and macrophages. After engagement, two adaptor molecules of TLR4 facilitate alternate signaling pathways once the receptor has been activated. Myeloid differentiation primary response gene 88 (MyD88) is a ubiquitous adaptor molecule utilized by a number of TLR-associated and

non-TLR pathways. Briefly, activation of TLR4 recruits the MyD88 adaptor, which results in the "early" phase of activation of the transcription factor NFκB, which upon translocation to the nucleus induces pro-inflammatory cytokines. TLR4 activation may also recruit the adaptor molecule TIR-domain-containing adaptor inducing IFNβ (TRIF) in a "late" response following receptor-mediated endocytosis, which activates the transcription factor interferon regulatory factor 3 (IRF3) resulting in type I IFNs production (123).

An adoptive transfer mouse model utilizing an OVA antigen formulated with MPLA or LPS attempted to determine the differential signaling via MyD88 and TRIF (115). OVA antigen with either MPLA or LPS comparably induced OVA-specific $CD4^+$ and $CD8^+$ T cell responses. Multiplex analysis of serum cytokines induced following OVA-MPLA injection were categorized in terms of MyD88-associated (IFNγ, IL-1β, IL-6, and MIP-1α) or TRIF-associated (G-CSF, IP-10, MCP-1, and RANTES) responses. Results indicated that while both LPS and MPLA induce TRIF-associated serum cytokines, MPLA only weakly induced the MyD88-associated inflammatory responses in comparison to LPS. *In vitro* experiments with OVA protein as antigen demonstrated that signaling via MyD88 was delayed and reduced in MPLA stimulations as compared to LPS. Adoptive transfer studies in OVA-specific transgenic mice also indicated that TRIF, but not MyD88, was required for antigen-specific T cell priming after immunization with OVA protein and MPLA (115).

This bias may be responsible for the reduced toxicity of MPLA, as well as the potent Th1 response it induces, however a direct correlation to vaccine and MPLA

induced adaptive immune responses remains unclear. Subsequent comparisons of MPLA to LPS suggest that MPLA fails to induce the pro-inflammatory cytokine IL-1β; while pro-IL-1β is comparably produced, the MyD88-dependent cleavage product of the bio-active cytokine is impaired (47). Still, there is evidence that MPLA action is not devoid of MyD88 signaling, indicating that the requirement for TRIF signaling is a bias rather than exclusive selective signaling. However, this requirement may be dependent in part on antigen and immunization route. Recent work has demonstrated that a MyD88-dependent negative regulator of innate inflammatory responses, SHIP-1 (Src homology 2 containing inositol phosphatase-1), may be paradoxically responsible for mitigating the MPLA-induced TLR4 inflammatory response (28, 29).

5.2 Inflammasome

The inflammasomes are a family of large multicomponent complexes that assemble in the cytosol with caspase-1, which cleaves the inflammatory cytokines pro-IL-1β and pro-IL-18 into their matured and secreted forms. Two main subtypes of the inflammasomes are the NLRs (nucleotide-binding-and-oligomerization domain and leucine-rich-repeat-containing) or the PHYINs (pyrin domain and HIN domain-containing). While many NLRs have been characterized in humans and mice, NLRP3 is the most extensively evaluated in the context of adjuvant mechanisms of action.

Activation of the NLRP3 inflammasome occurs when a stimulant induces the assembly of NLRP3, and ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), followed by the recruitment of pro-caspase-1. Pro-caspase-1 is cleaved into its mature form, which is capable of cleaving the inactive of the

inflammatory cytokines IL-1β and IL-18 into a mature state. An initial priming signal through NFκB activation is required but not sufficient for NLRP3 activation (10). Known ligands of NLRP3 include endogenous dangers signals released during tissue damage, for example ATP and uric acid crystals, as well as several pathogens including *Klebsiella pneumonia, Listeria monocytogenes, Candida albicans*, *Staphylococcus aureus*, and influenza A. In addition, evidence indicates that potassium efflux, caused by pores formed in the cellular membrane, or the presence of reactive oxygen species, or the ingestion of certain particulates such as alum or silica, also activate the NLRP3 inflammasome (22, 149, 169).

The potential activation of the NLRP3 inflammasome by aluminum salt adjuvants remains controversial. Initial *in vitro* studies by Eisenbarth *et al.*, utilizing Imject® alum, indicated that the adjuvant indeed acted via ASC-, caspase-1-, and NLRP3-dependent pathways to produce IL-1β and IL-18. In *in vivo* studies immunizing mice with Imject® alum and model antigens, ASC-, caspase-1-, and NLRP3-deficient mice failed to mount an antigen-specific antibody response, suggesting that these pathways were also critical to adaptive immunity raised by aluminum salt adjuvants (45). Meanwhile, concurrent studies confirm that aluminum hydroxide does activate IL-1β and IL-18 via NLRP3 inflammasome pathways, but contend that this action is dispensable for its adjuvant activity (53, 119). Furthermore, it remains unclear whether the action of aluminum salts on the NLRP3 inflammasome is through direct or indirect interaction. Whereas some evidence supports a direct mechanism involving the phagocytosis of particulates and subsequent lysosomal disruption serving as an endogenous danger signal activating

NLRP3 (77), others suggest a more indirect action. Kool *et al.* speculate that administration of Imject® alum causes cell damage and cell death at the injection site, releasing the endogenous danger signal uric acid, which itself acts on the NLRP3 inflammasome (92). In a novel approach, Marichal *et al*. suggest that the cell damage induced by Imject® alum results in the release of host DNA, which, via inflammasome or nucleic-acid sensing pathways, impacts adjuvanticity (111). Therefore, while it is clear both *in vivo* and *in vitro* that aluminum salts induce the pro-inflammatory IL-1β response, the exact mechanism as well as the impact on adaptive immune response to vaccination with aluminum salt adjuvant formulations remains to be defined.

In contrast to aluminum salts, the TLR4-agonist adjuvant MPLA demonstrates impaired signaling via the NLRP3 inflammasome. The dephosphorylation of LPS to produce MPLA appears to substantially reduce the inflammation, including pro-inflammatory cytokine production, in response to TLR4 stimulation (28, 123). In fact, MPLA was shown to induce mRNA for IL-1β and the pro-form of IL-1β similarly to LPS; however, it was defective in the induction of mature IL-1β secretion and defective in the activation of caspase-1, which is required for endotoxic inflammation (136). This may or may not be attributable to TRIF-biased signaling, with weaker MyD88 induction resulting in the failure of the NLRP3 inflammasome to assemble (47).

Little is known about how saponin derivative adjuvants harness the innate immune system to enhance adaptive immunity to vaccination. Studies of ISCOMATRIX™ adjuvant thus far have not supported a mechanism via TLRs, including TLR4. However, ISCOMATRIX™ adjuvant-based vaccines exhibit variable

dependency on MyD88 activation (191). Mice immunized with a tumor protein antigen and ISCOMATRIX™ adjuvant showed that while the recruitment of immune cells and maturation of DCs in the DLN after immunization were independent of MyD88, the cross-priming by certain DC subsets to generate a $CD8^+$ T cell response, as well as natural killer (NK) cell functionality, were significantly compromised in the absence of MyD88 (191). The mechanism by which ISCOMATRIX™ adjuvant activates MyD88 signaling and other innate pathways remains unclear.

ISCOMATRIX™ adjuvant, which is by nature both a particulate and a saponin, may also function via the NLRP3 inflammasome, especially as it has been observed *in vivo* to induce an IL-1β response. Recent reports by Duewell *et al.* observed *in vitro* that the IL-1β response to ISCOMATRIX™ adjuvant occurred in a caspase-1 dependent manner, and further investigation into the role of caspase-1-dependent inflammasome pathways is currently underway (42).

Another inflammasome complex that utilizes caspase-1 and ASC is the absent in melanoma 2 (AIM2) inflammasome, which produces IL-1β and IL-18 in response to double-stranded (ds) DNA. AIM2 is unlike many other innate nucleic acid sensors, which produce type I IFNs in response to activation. The demonstration by Marichal *et al.* that host DNA released in response to OVA antigen and Imject® alum immunization was responsible for Th2-biased adaptive immune responses indicates that AIM2 (111), as well as type I IFN-producing nucleic acid sensing pathways, are potentially important in the development of future work on adjuvant mechanisms.

It is important to note that the AIM2 inflammasome is not the only nucleic acid sensing pathway. TLR9, an endosomal receptor, responds to viral and bacterial DNA including unmethylated deoxycytidylate-phosphate-deoxyguanylate (CpG) motifs, resulting in the production of type I IFN in a MyD88-dependent and interferon regulatory factor 7 (IRF7)-dependent fashion (176). Cytosolic RNA may be sensed by retinoic acid-inducible gene (RIG-I) or melanoma differentiation-associated gene 5 (MDA-5), which converge on the adaptor mitochondrial antiviral signaling protein (MAVS), and subsequently induces the production of inflammatory cytokines via NFκB or type I IFNs via Tank-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3) and IRF7 (176). Cytosolic DNA is sensed by TBK1, and requires the adaptor stimulator of interferon genes (STING), for the downstream activation of IRF3 and production of type I IFNs. dsDNA from certain viral infections can also activate gamma-interferon-inducible-protein 16 (IFI16), which associates with STING for the production of pro-inflammatory cytokines via NFκB or type I IFNs via IRF3 (176). Thus, if indeed nucleic acid sensing pathways are revealed to have a critical role in the mechanisms of studied adjuvants, there are a multitude of innate immune signaling pathways worth evaluating.

6. Research objectives

The observations from HIV-1 vaccine clinical trials have laid the groundwork for the future directions of vaccine design, and emphasize the importance of adjuvant selection. Despite poorly understood correlates of protective immunity, the polyvalent prime-protein boost regimen has emerged as the most promising avenue towards an effective vaccine, with broad and robust induction of both humoral and cell-mediated

adaptive immune responses. The original body of work described in the following chapters attempts to evaluate the unique profiles of several conventional and novel adjuvant systems in the context of a previously described HIV-1 Env DNA prime-boost vaccine strategy against HIV-1, DP6-001.

In this dissertation, I aim to compare the ability of select adjuvants to contribute to the vaccine-specific adaptive immune responses induced by DP6-001. In addition, I will utilize novel multiplex technology to characterize each adjuvant in the context of DP6-001 in terms of unique non-antigen specific serum cytokine and chemokine profiles. I expect that these profiles will indeed be unique to the DNA prime-protein boost vaccine strategy.

As a secondary objective, in the latter chapters of this dissertation, I will explore the impact of several innate immune pathways on the unique adaptive and inflammatory adjuvant profiles when formulated with DP6-002 vaccine. These studies in particular focus on two novel adjuvant systems, MPLA and ISCOMATRIX™ adjuvant, with the goal of not only elucidating their incompletely defined mechanisms, but also to aid in the selection of an adjuvant for inclusion in future optimized HIV-1 Env DNA prime-protein boost vaccines.

Preface to CHAPTER II

R. Buglione-Corbett and K. Pouliot contributed equally to animal work, including immunizations, bleeding, and termination

R. Buglione-Corbett and K. Pouliot contributed equally to Luminex experiments and data analysis, Fig. 2.2, 2.7, 2.8, 2.9

Terminal assays performed on immunized mice were performed collaboratively:

K. Pouliot and R. Marty-Roix performed ELISpot experiments and analysis, Fig 2.4

R. Buglione-Corbett performed intracellular cytokine staining, Fig. 2.5

R. Buglione-Corbett performed antibody studies and data analysis, Fig. 2.2, 2.6

R. Buglione-Corbett and S. Lu edited figures for the manuscript.

R. Buglione-Corbett, S. Lu, K. Pouliot, and E. Lien prepared the manuscript.

CHAPTER II:

Serum cytokine profiles associated with specific adjuvants used in a DNA prime-protein boost vaccination strategy

Introduction

Recently, the RV144 clinical trial using a viral vector prime-recombinant protein boost vaccine demonstrated a low level, but statistically significant, protection against HIV-1 infection in Thai volunteers (38, 125, 144, 150). The RV144 trial employed a canarypox viral vector ALVAC, encoding HIV-1 antigens env, gag, and pro, as a prime, followed by a boost with bi-clade AIDSVAX B/E recombinant gp120 protein boost adsorbed onto the aluminum hydroxide gel adjuvant. After extensive follow-up studies, new data are emerging to suggest that antibodies against certain critical areas of HIV-1 envelope proteins are responsible for protection; however, the antibody responses in the RV144 trial were not long lasting, which may have led to reduced protection during the clinical trial observation period (125, 145, 187).

Following the results of the RV144 trial, especially given the renewed interest of including recombinant Env proteins in the future HIV-1 vaccine development, the study of adjuvants is gaining more momentum as adjuvant is a critical component of most licensed recombinant protein-based human vaccines. A more immunogenic adjuvant than aluminum hydroxide gel may provide enhanced and long lasting antibody responses, which may improve the level of protection compared to that observed in the RV144 trial (145, 187).

A key consideration in vaccine development is enhancing immunogenicity while mitigating associated adverse effects. The inclusion of adjuvants in vaccine formulations has long been a method of improving vaccine efficacy, but these adjuvants are not without risk of eliciting local and systemic adverse effects. HIV-1 recombinant Env protein-based vaccines are now primarily being used as a boost component in emerging HIV-1 vaccine strategies. Thus, it is very important scientifically to examine how an adjuvant works when it is formulated with Env proteins, and in the context of hosts who have been primed by a gene-based vaccine (such as viral vector or DNA vaccine) encoding antigens similar to the boost.

Our group has demonstrated the high immunogenicity of the pentavalent DNA prime-protein boost HIV-1 vaccine formulation, DP6-001, in preclinical and phase I clinical trials (7, 186). The saponin adjuvant QS-21, derived from *Quillaia saponaria*, was included as part of the Env protein boost in the phase I clinical study of DP6-001 formulation. Previously published studies have also utilized QS-21 as part of recombinant Env protein alone vaccines in humans, demonstrating a potent immunogenicity allowing for reduced antigen dose, and characterized by improved binding and neutralizing antibody responses (50, 85). However, in these studies QS-21 has been associated with serious local reactions, including pain, induration, erythema, as well as systemic adverse effects such as hypertension, myalgia, headache, and vasovagal episodes which were rare and not proven to be caused by QS-21 directly (50, 85). We also observed in clinical trials of DP6-001, local skin reactions as well as rare skin based vasculitis associated with QS-21 adjuvanted protein boosting (7).

In the current study, the serum cytokine profiles in mice in the context of the DP6-001 DNA prime-protein boost were analyzed, in the context of several protein-adjuvant boost formulations. MPLA and ISCOMATRIX™ adjuvant were examined in the context of the DP6-001 vaccine, along with QS-21 and aluminum hydroxide gel, herein referred to by its chemical formula $Al(OH)_3$, which were tested in previous clinical studies of HIV-1 vaccines. Similar to QS-21, ISCOMATRIX™ adjuvant consists of a *Quillaia* saponin fraction, which is mixed with cholesterol and phospholipid under controlled conditions to form a cage-like structure of approximately 40 nm in diameter. The ISCOMATRIX™ adjuvant can then be formulated with virtually any antigen to make a vaccine. Complexing saponin with cholesterol and phospholipid appears to ameliorate the reactogenicity associated with using free saponin, as ISCOMATRIX™ adjuvant retains the immunogenic potency and antigen dose reduction potential of saponin but demonstrates improved tolerability (18, 120). ISCOMATRIX™ adjuvant promotes a balanced Th1/Th2 response, as well as a uniquely strong cytotoxic T cell response and long-lasting antibody responses in both animal and human models (109, 127, 141, 160). This broad, robust activation of adaptive immunity has made ISCOMATRIX™ adjuvant particularly efficacious in clinical studies of anti-tumor vaccines (37, 110, 131), as well as trials of therapeutic and protective human papilloma virus (HPV) vaccines and influenza vaccines (4, 56). Comprehensive clinical safety data has been compiled and reported for six clinical trials of ISCOMATRIX™ adjuvant with a variety of antigen formulations (120).

MPLA is low-toxicity, dephosphorylated derivative of lipopolysaccharide (LPS) (2). MPLA, like LPS, acts via Toll-like receptor (TLR) 4, but results in significantly less inflammation compared to its parent molecule (2, 115). As an adjuvant, MPLA improves vaccine-specific antibody responses, as well as induces potent Th1 responses in preclinical and clinical models, resulting in a complex inflammatory response consisting of neutrophils, antigen-presenting cells (APCs), and natural killer cells (NKs). Despite its potency, MPLA has been well tolerated in clinical trials. Currently, MPL® is a component of the licensed GlaxoSmithKline (GSK) human vaccines Fendrix® for hepatitis B in combination with aluminum phosphate and, in formulation with aluminum hydroxide in Adjuvant System AS04, in Cervarix ® for human papilloma virus (2, 115, 175). MPL® has also been clinically evaluated in GSK's AS01 adjuvant system in combination with QS-21 as a component of a malaria vaccine (159). Aluminum-based adjuvants are the most widely utilized in human vaccines due to their tolerability and consistent induction of humoral immunity. Thus, aluminum hydroxide gel [$Al(OH)_3$] has been included in the current study to provide a baseline control in the generation of an acceptable profile of tolerability (94, 119).

The relative immunogenicity of our candidate adjuvants in combination with HIV Env proteins was assessed in both C57Bl/6 and Balb/c mice, based on a comprehensive profile of vaccine-specific antibody and T cell responses, as well as non-antigen specific serum cytokines detected shortly after protein immunization. While results show that the candidate adjuvants demonstrated comparable vaccine-specific immunogenic potency by IgG ELISA and T cell ELISpot, the analysis of serum cytokines allowed us to distinguish

a profile of characteristic cytokines for each adjuvant. With this information, we will be better informed in the future for the selection of an adjuvant as part of a prime-boost HIV-1 vaccine formulation such as the polyvalent DNA prime-Env protein boost shown in the current report.

Results

Study Design and Immunization Schedule

Wild type C57Bl/6 and Balb/c mice were immunized with DP6-001 gp120 vaccines formulated with different adjuvants based on a dose and schedule from previously completed clinical studies (Fig. 1). Mice were primed i.m. three times with pentavalent gp120 DNA plasmids at weeks 0, 2, and 4 and boosted with two matched pentavalent gp120 protein boosts, formulated with either QS-21, $Al(OH)_3$, MPLA, or ISCOMATRIX™ adjuvant, at weeks 8 and 12. For an initial study examining the impact of two different DNA plasmid preparation methods, controls groups were immunized with only three pentavalent gp120 DNA immunizations, and received saline immunization in lieu of protein boosts.

Differences in serum cytokines following immunization with gp120-expressing DNA plasmids in different preparations

In previous small animal studies of the DP6-001 DNA prime-protein boost formulation, gp120 DNA plasmid components were prepared using a regular plasmid kit as described above. Prior to the analysis of serum cytokines in response to the DP6-001 and candidate adjuvants, we aimed to rule out the potential contribution of residual endotoxin content in DNA plasmid preparations to the cytokine profiles observed. DNA primes were prepared by either regular DNA plasmid kit or EndoFree DNA plasmid kit (referred to as EF DP6-001), as described above in Materials and Methods. Mice received either three DNA immunizations followed by two saline boosts, or the full course of the DP6-001 DNA prime-protein boost vaccine regimen. The protein boosts were formulated with 21, as in

our previous studies of DP6-001 vaccine (7, 35, 137, 138, 186). To characterize the systemic serum cytokines produced following adjuvanted protein immunization, in comparison to pre-immunized serum, we employed a multiplex cytokine array consisting of a panel of 12 cytokines including Th1 cytokines [Interleukin (IL)-2 and IFNγ], Th2 cytokines [IL-4], and pro-inflammatory cytokines [IL-1β, IL-6, RANTES (CCL5)]. In addition, we included cytokines and chemokines associated with activation and chemoattraction of monocytes, macrophages, NK cells, and granulocytes [MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), G-CSF], neutrophils [KC (CXCL1)] and eosinophils [Eotaxin].

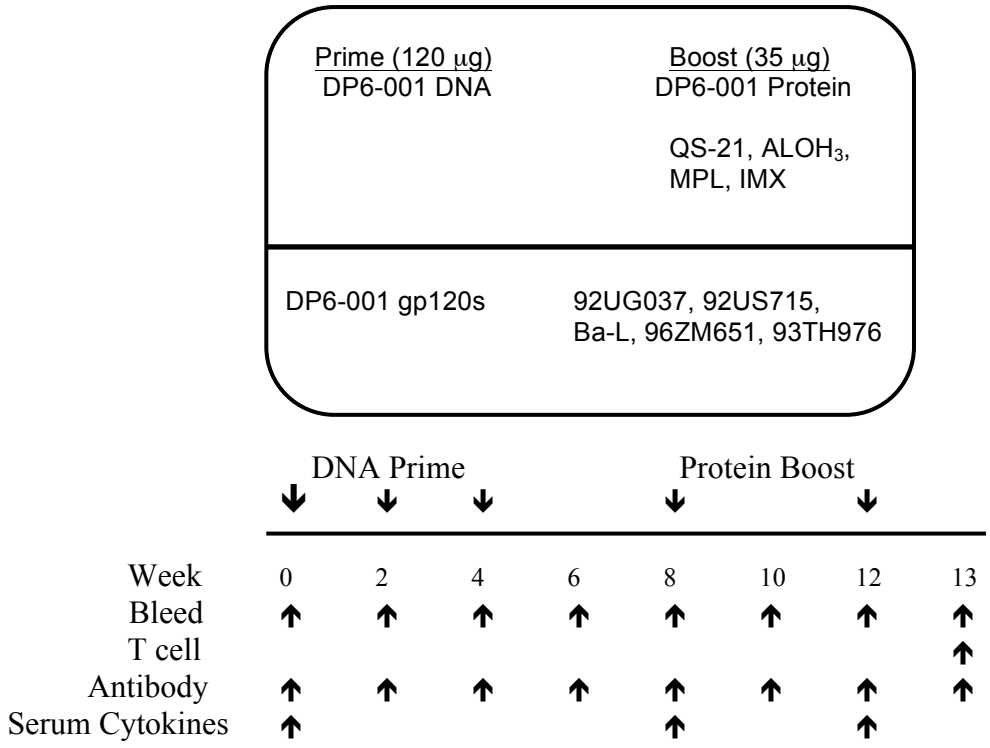


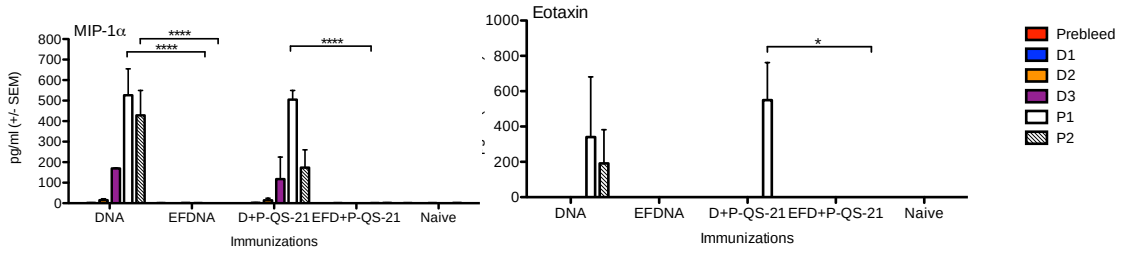
Figure 2.1. Study design and immunization schedule. Mice were immunized with three pentavalent DNA primes followed by two heterologous gp120 protein boost. The pentavalent vaccine mixture of both DNA and protein components consisted of HIV-1 Env from clades A (92UG037.8), B (92US715.6 and Bal), C (96ZM651), and E (93TH976.17). DNA and protein doses indicated are for a total of five immunogens at each immunization. Four adjuvants were tested individually as part of the protein boost. Time points of immunizations, and sample collections for different assays were indicated. (IMX = ISCOMATRIX™ adjuvant).

Overall, the different plasmid preparation methods employed did not substantially impact the levels of cytokine responses elicited at time points during the protein-adjuvant boost phase when the serum cytokine profiles will be measured. Eotaxin and MIP-1α were the only two cytokines moderately elevated in serum in mice immunized with regular DNA prep following the first protein boost as compared to EF DP6-001 but the difference dropped after the 2nd protein boost (Fig. 2.2a). Most cytokines detected in the serum clearly demonstrate the increased immune response associated with DNA prime-protein boost as compared to immunization with DNA alone. Regardless of the method of DNA vaccine preparation, the serum levels of IL-2, IL-6, MCP-1, G-CSF, and KC were significantly higher at 6 hours post-protein boost as compared to mice that received only DNA primes of either preparation (Fig. 2.2b). During DNA priming, serum levels of IL-2 and MCP-1 were low overall, regardless of DNA plasmid preparation method. A trend towards increased levels of IL-6, KC, and G-CSF during DNA priming in regular DNA preparations as compared to EF preparations, but this difference was not significant. These results suggested that our future studies of adjuvanted protein-associated serum cytokine profiles in the context of DNA prime-protein boost should focus on time points 6 hours after protein-adjuvant boosting.

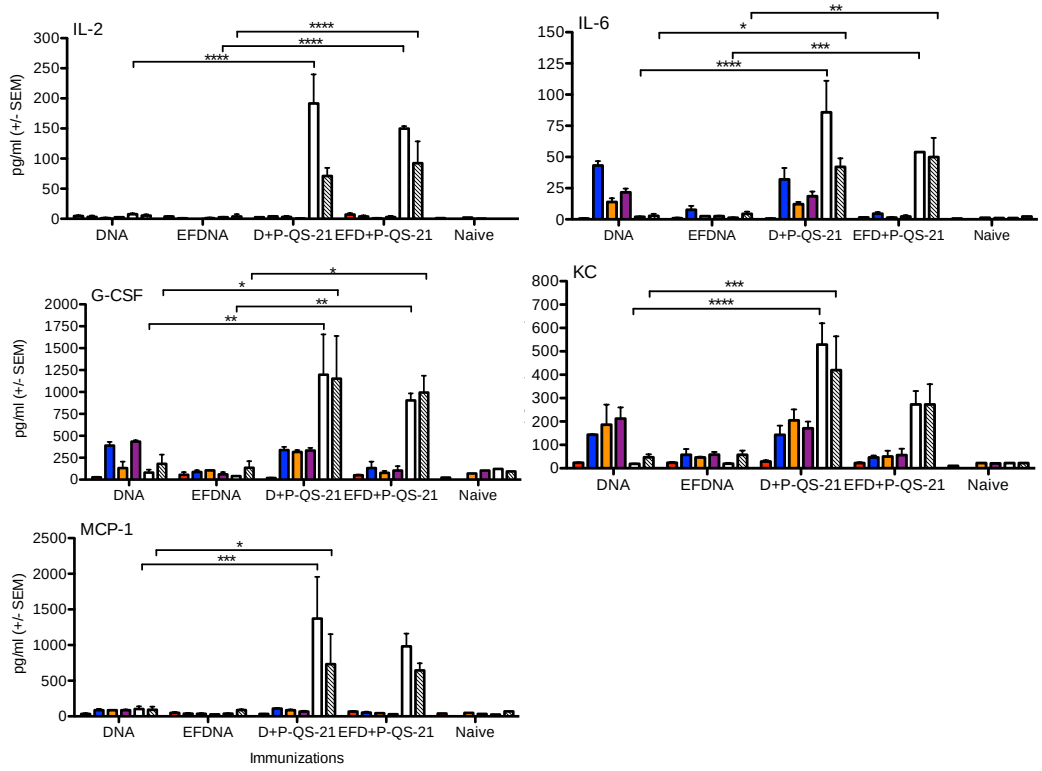
Remaining cytokines were either not above the background levels during both after DNA and protein immunizations (IFNγ and IL-4) (Fig. 2.2c), or were at low concentration only after DNA priming phase but not after protein immunization (MIP-1β and RANTES) (Fig. 2.2d).

Figure 2.2. Temporal serum cytokine levels in mice immunized with endotoxin-free (EF) DNA prime compared to regular DNA plasmid preparation. Pentavalent gp120 DNA priming components were produced with either a regular or an EF DNA plasmid preparation kit. C57Bl/6 wildtype mice were immunized with either three gp120 DNA primes and two "mock" saline boosts ('DNA' or 'EF DNA'), or with three gp120 DNA primes and two gp120 protein boosts adjuvanted by QS-21 ('D+P-QS-21' or 'EF D+P-QS-21'). Serum cytokines were quantified with a 12-plex Luminex panel in sera collected pre-immunization, and at 6 hours after each DNA prime and protein boost immunization. (a) Sera cytokines associated with endotoxin content observed after protein boosting. (b) Sera cytokines associated with the protein boost effect. (c) Sera cytokines at baseline levels. (d) Sera cytokines associated with endotoxin content during DNA immunization, but ameliorated at time of protein boosts.
Statistical analysis was performed with two-way ANOVA and Bonferroni post-test (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

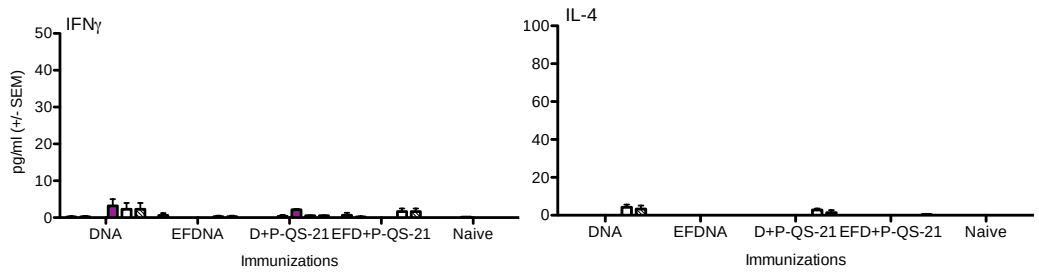
(a)


MIP-1α
Eotaxin
pg/ml (+/- SEM)
DNA
EFDNA
D+P-QS-21
EFD+P-QS-21
Naive
Immunizations
Prebleed
D1
D2
D3
P1
P2

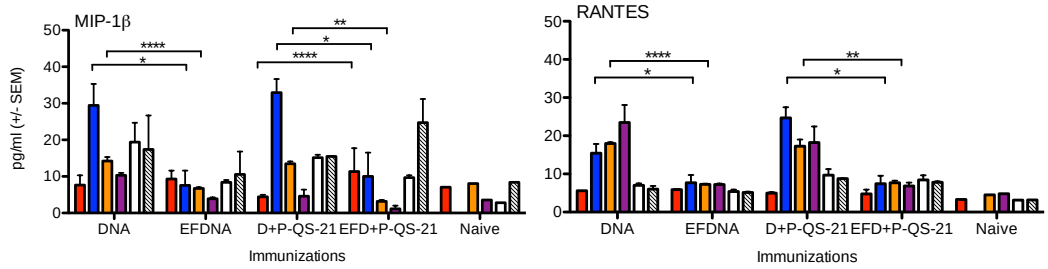
(b)


IL-2
IL-6
G-CSF
KC
MCP-1
pg/ml (+/- SEM)
DNA
EFDNA
D+P-QS-21
EFD+P-QS-21
Naive
Immunizations

(c)


IFNγ
pg/ml (+/- SEM)
50
40
30
20
10
0
DNA
EFDNA
D+P-QS-21
EFD+P-QS-21
Naive
Immunizations
IL-4
100
80
60
40
20
0
DNA
EFDNA
D+P-QS-21
EFD+P-QS-21
Naive
Immunizations

(d)


MIP-1β
pg/ml (+/- SEM)
50
40
30
20
10
0
****
*
**
*
****
DNA
EFDNA
D+P-QS-21
EFD+P-QS-21
Naive
Immunizations
RANTES
50
40
30
20
10
0
****
*
**
*
DNA
EFDNA
D+P-QS-21
EFD+P-QS-21
Naive
Immunizations

Effect of DNA prep in gp120-encoding DNA plasmid on DP6-001 vaccine-induced Env-specific IgG response

In addition to examining nonspecific serum cytokine responses, we aimed to rule out the potential impact of gp120 DNA plasmid preparations on the Env-specific IgG antibody response observed in our adjuvant studies. Sera collected from the immunized mice 7 days following the final protein boost was used to determine the Env-specific IgG endpoint titer by ELISA (Fig. 2.3). As expected, mice that received the full DP6-001 with adjuvanted protein boost demonstrated significantly higher IgG titers than mice that received DNA priming only, regardless of the method of g120 DNA plasmid preparation.

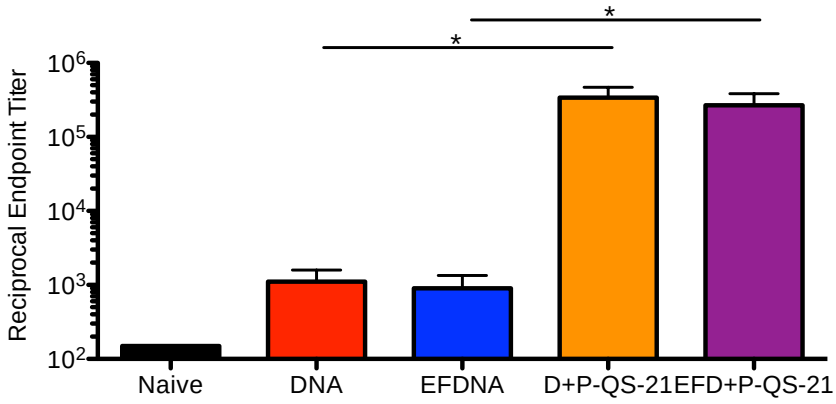


Figure 2.3. Endpoint gp120-specific IgG titer in mice immunized with endotoxin-free DNA prime versus regular DNA plasmid preparation. Total gp120-specific IgG was measured by ELISA in sera collected 7 days after the second protein boost, in week 13. Statistical analysis performed by one-way ANOVA and Tukey post-test (*: $p<0.05$).

Induction of Env-specific T cell responses by DP6-001 vaccine

Once we established the regular DNA prep was qualified for use in the current studies, T cell responses following immunization with DP6-001 with various candidate adjuvants were characterized in C57Bl/6 mouse splenocytes. ELISpot analysis was conducted to examine DP6-001 vaccine-induced production of Th1 and Th2 responses in the splenocytes of immunized mice, in response to a peptide pool (labeled 'PP') representing clade B Env consensus sequence (19). Mice that received DP6-001 formulated with QS-21 most strongly produced an Env-specific IFNγ response, with much lower levels observed in mice immunized with protein formulated with $Al(OH)_3$, MPLA, and ISCOMATRIX™ adjuvant (Fig. 2.4a). All mice receiving adjuvanted protein formulations demonstrated a positive IL-2 response to peptides (Fig. 2.4b) and minimal induction of IL-4 (Fig 2.4b) with no differences between groups. Baseline levels of Th2 cytokines IL-4 and IL-6 were notably elevated (Fig. 2.4c, d). Mice receiving protein formulated with QS-21 demonstrated a positive but not significant induction of IL-6 over background, while those receiving protein formulated with all other adjuvants showed minimal induction of IL-6 (Fig. 2.4d).

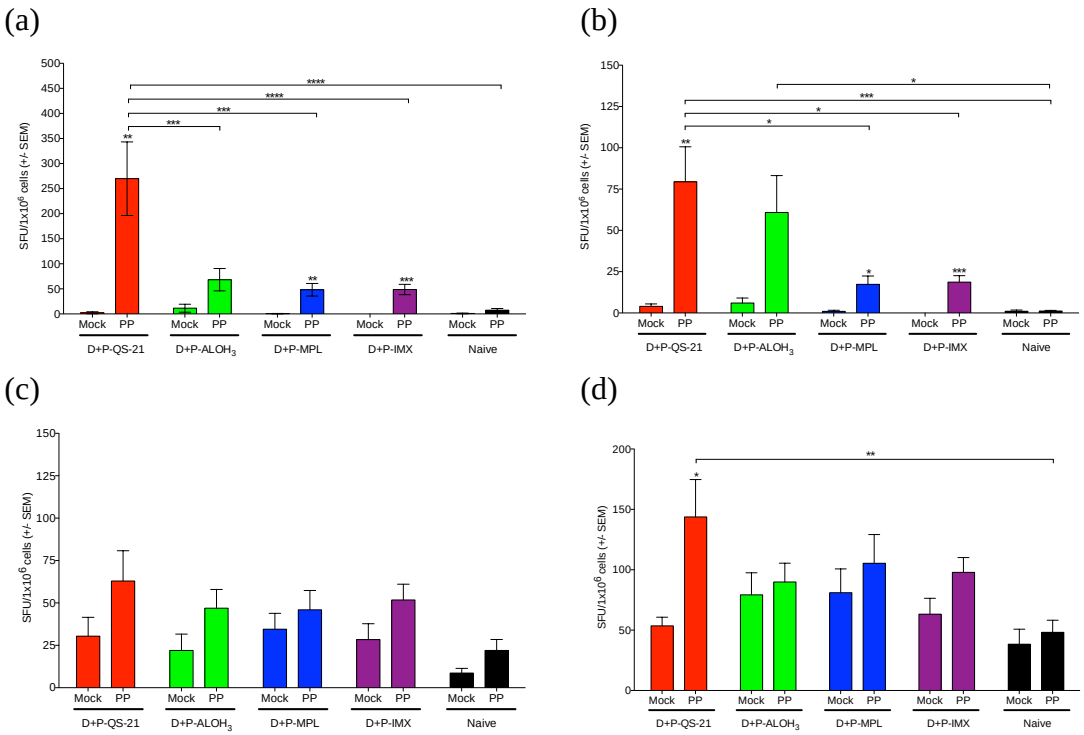


Figure 2.4. Env-specific cellular immune responses in splenocytes from mice immunized with DP6-001 and candidate adjuvants. Spleens were harvested at termination 7 days after the final protein boost. Cells were cultured for 18 hours either receiving the stimulation of a truncated HIV-1 gp120 Clade B peptide pool ('PP') or media ('mock'). Cytokine spot-forming units (SFU) per million splenocytes were visualized with a CTL Imager and analyzed with Immunospot™ software. Splenocyte production of gp120-specific Th1 cytokines (a) IFNγ and (b) IL-2, and gp120- specific Th2 cytokines (c) IL-4 and (d) IL-6 were measured. Statistical analysis of peptide stimulation over mock stimulation was calculated by Student's t-test. Significant values (*: $p < 0.05$) are represented above error bars. Statistical differences in peptide stimulation between adjuvant groups were calculated by a Two-way ANOVA and Bonferroni post-test (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). (IMX = ISCOMATRIX™ adjuvant)

Intracellular cytokine staining (ICS) was also conducted to evaluate the functionality of vaccine-specific T cells by the production of Th1 and Th2 cytokines (IFNγ, IL-2 and IL-6) in response to a pool of overlapping peptides representing the clade B consensus Env sequence. No significant difference in $CD4^+$ T cell cytokine induction was observed between adjuvanted protein groups. Similar to ELISpot results, mice immunized with protein formulated with QS-21 showed significant induction of Env-specific IFNγ (Fig. 2.5a) and IL-6 (Fig. 2.5c) by $CD4^+$ T cells, and a marginally positive IL-2 response by $CD4^+$ T cells (Fig. 2.5b). While there was a trend of positive Env-specific IFNγ responses by $CD8^+$ T cells, responses were not significantly induced above background, nor were there any significant differences between the different adjuvants (Fig. 2.5d). This result is not surprising as protein vaccines are not known for the induction of $CD8^+$ T cell responses and DP6-001 vaccine was mainly designed for the induction of antibody responses.

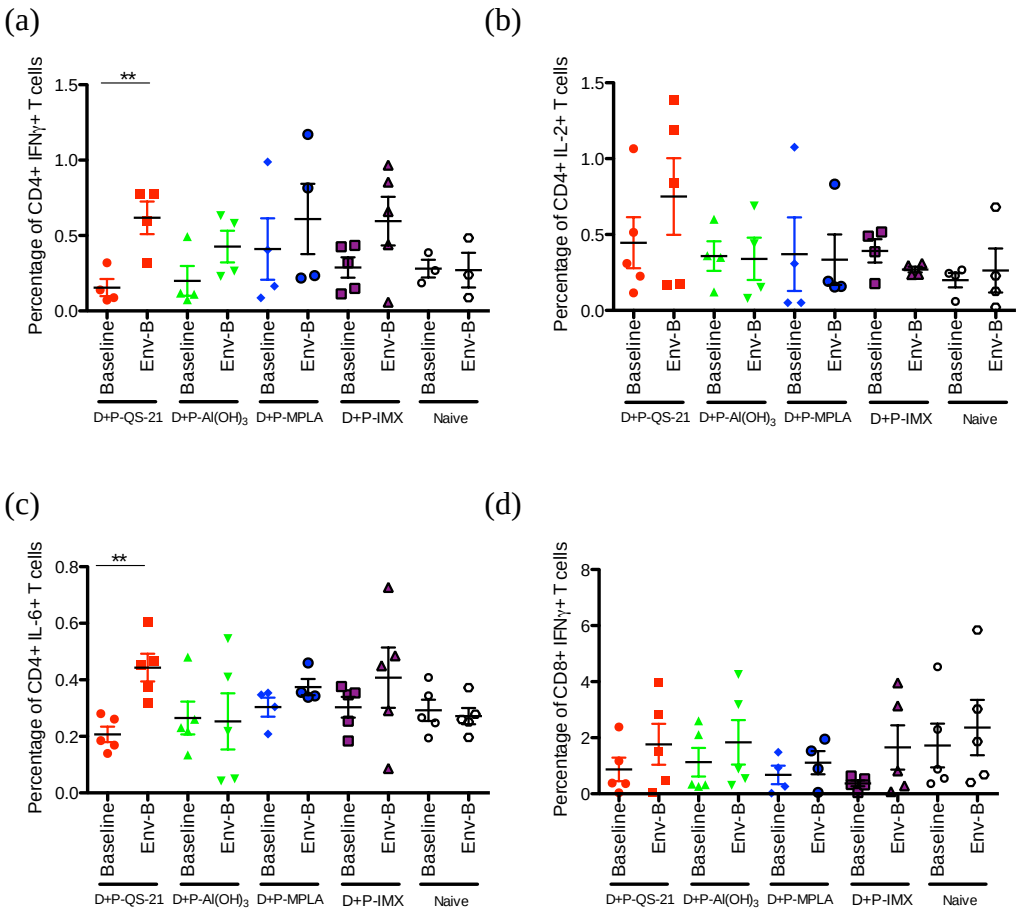


Figure 2.5 Magnitude of Env-specific CD4$^+$ and CD8$^+$ T cell responses induced by DP6-001 immunization and adjuvants. Cytokines were analyzed in murine splenocytes 7 days after final protein boost. Spleens were harvested at termination 7 days after the final protein boost. Splenocytes were cultured for 5 hours either receiving the stimulation of a consensus HIV-1 gp120 Clade B peptide pool ('Env-B') or media ('baseline'). Env-specific cytokine production by T cells was quantified by intracellular cytokine staining and samples were run on an LSR II FACS machine. Data was analyzed using FlowJo software. Shown is the production of (a) IFNγ, (b) IL-2, and (c) IL-6 (c) by CD4$^+$ T cells, and (d) IFNγ by CD8$^+$ T cells in mice vaccinated with DP6-001 and candidate adjuvants. Statistical comparisons between adjuvant groups were performed with a one-way ANOVA and Tukey post-test. Statistical significance of antigen-specific responses over background was performed with a Student's t-test.

Eliciting Env-specific IgG antibody response using DP6-001 DNA prime and protein boost formulated with candidate adjuvants

In immunogenicity studies of DP6-001 in small animals and clinical volunteers, we reported the robust induction of vaccine-specific antibody response following immunization with the polyvalent Env DNA prime-protein boost formulation DP6-001 (7, 35, 137, 138, 186). In our previous Phase I clinical study, DP6-001 protein boosts have been formulated with the saponin adjuvant, QS-21. In order to investigate the immunogenicity of alternative candidate adjuvants, particularly in comparison to QS-21, we immunized two strains of wild type mice with the pentavalent gp120 DNA prime-protein boost regimen employed in a phase I clinical trial (7, 186). Balb/c and C57Bl/6 mice were immunized with three DNA primes, followed by two protein boosts formulated with QS-21, $Al(OH)_3$, MPLA, or ISCOMATRIX™ adjuvant according to the study design outlined in Figure 2.1. Sera were collected every two weeks after DNA immunization, and six hours post-protein boosts. Sera collected from mice seven days following the second protein boost were used to ascertain the endpoint titer of Env-specific IgG responses. For antibody responses, there is limited difference between Balb/c C57Bl/6 mice, and only data from C57Bl/6 wildtype mice are presented.

Immunization with DP6-001 produced comparably robust gp120-specific IgG responses following the final protein boost, independent of the adjuvant used. Env-specific IgG levels were detectable following the third DNA immunization, and were significantly boosted by the first protein immunization. Levels of specific IgG dropped four weeks following the first protein boost, but were subsequently boosted by the second

protein immunization (Fig. 2.6a). Endpoint titer analysis showed that gp120-specific IgG levels were comparable in mice receiving formulations containing QS-21 or MPLA. IgG titers were significantly lower in mice immunized with formulations containing ISCOMATRIX™ adjuvant as compared to formulations containing MPLA and lower still in mice receiving formulations containing $Al(OH)_3$ (Fig 2.6b).

In C57Bl/6 mice, sera collected 7 days after the final protein immunization was used to characterize the IgG isotype profiles of formulations with the candidate adjuvants. HIV-1 gp120-specific IgG1 titers were comparable between mice that received formulations containing QS-21, $Al(OH)_3$, or MPLA adjuvants (Fig. 2.6c). In comparison, IgG1 titers were slightly, but not significantly lower, in mice that received formulations containing ISCOMATRIX™ adjuvant. On the other hand, gp120-specific IgG2c titers were higher in mice immunized with formulations containing QS-21 and MPLA followed by formulations containing ISCOMATRIX™ adjuvant in comparison to mice that received formulations containing $Al(OH)_3$ (Fig. 2.6d). A ratio of the endpoint titers of Env-specific IgG2c, a correlate of Th1 responses, and IgG1, a correlate of Th2 responses, were used to demonstrate the Th1 vs. Th2 responses associated with each adjuvant (Fig. 2.6d). Mice immunized with formulations containing $Al(OH)_3$ demonstrated a low IgG2c/IgG1 ratio, and therefore predominantly Th2 response. In contrast, mice immunized with formulations containing MPLA or QS-21, and to a greater extent, ISCOMATRIX™ adjuvant, demonstrated a higher IgG2c/IgG1 ratio, indicating strong Th1 responses.

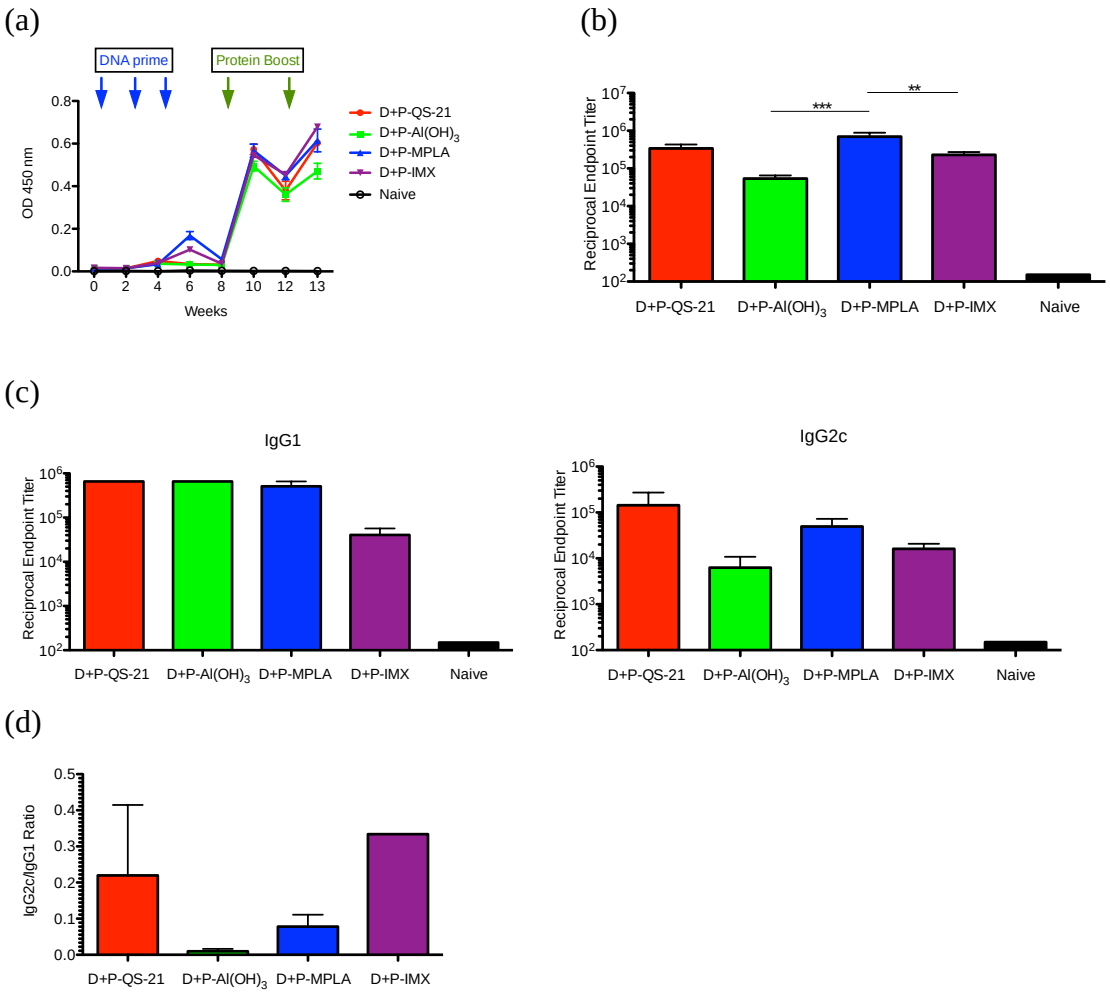


Figure 2.6. HIV-1 gp120-specific IgG response in wildtype mice immunized with DP6-001 vaccine with different adjuvants. Total gp120-specific IgG was measured by ELISA in sera collected 7 days after final protein boost, in week 13. Protein boosts were formulated with QS-21 (red), $Al(OH)_3$ (green), MPLA (blue), or ISCOMATRIX™ adjuvant (IMX) (purple). Naïve mice (black) received 'mock' saline injections in lieu of immunization. (a) Temporal gp120-specific IgG response was determined by ELISA using pooled sera samples from each group collected at two-week intervals. (b) gp120-specific endpoint IgG titer in was determined by ELISA using individual serum samples collected in week 13. (c) Endpoint IgG isotype profiles were determined by ELISA using individual serum samples collected in week 13. (d) Th1/Th2 ratio of IgG isotype responses was determined by comparing IgG2c/IgG1 ratio. Statistical significance was determined by one-way ANOVA and Tukey post-test (*: $p < .05$, **: $p < .01$, ***: $p < .001$).

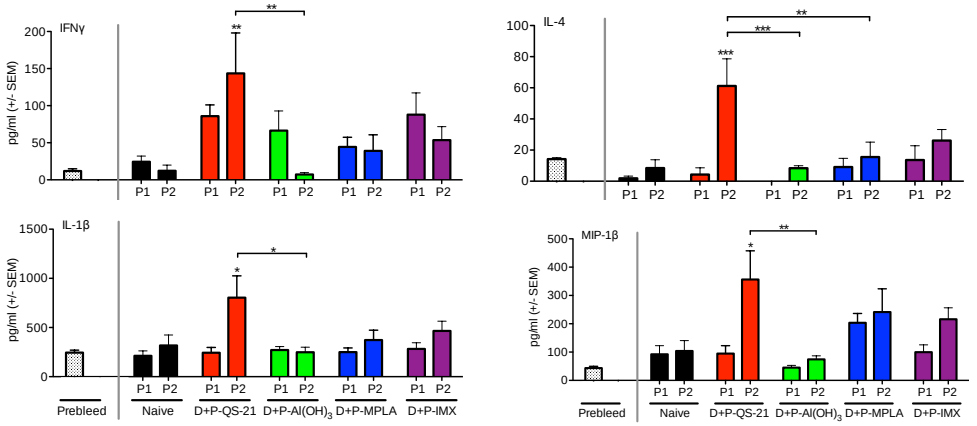
Induction of unique serum cytokine profiles following protein boost with candidate adjuvants

Throughout the DP6-001 immunization regimen in the current study, sera were collected from immunized mice at time points 6 hours following the third DNA immunization, and following both protein boosts. In order to characterize the serum cytokines produced following protein-adjuvant immunization, in comparison to pre-immunized serum, we employed a 12-plex cytokine array described above. By examining this panel of non-antigen specific, systemic cytokine responses in immunized mice 6 hours after each protein-adjuvant boost, our objective was to identify a unique profile of markers for each candidate adjuvant in the context of our prime-boost HIV vaccine. In addition, we will also identify cytokines and chemokines that are broadly induced by formulations containing the candidate adjuvants.

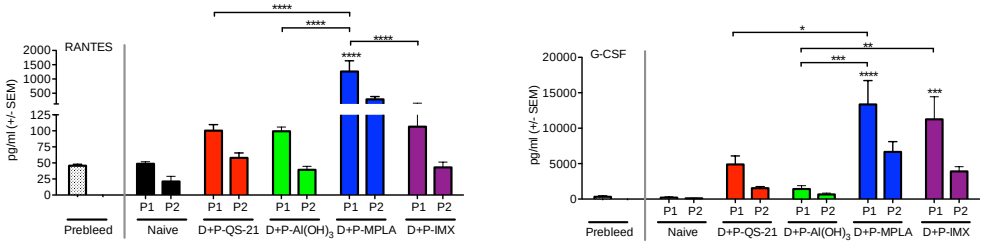
Animals immunized with DP6-001 including a boost of gp120 protein formulated with QS-21 demonstrated a unique serum cytokine profile, consisting of the Th1 cytokine IFNγ and the Th2 cytokine IL-4 (Fig. 2.7a). Immunization with formulations containing QS21 was also associated with increased levels of the pro-inflammatory marker IL-1β, and the monocyte chemoattractant MIP-1β (Fig. 2.7a). While levels of these four cytokines were relatively low in all adjuvant groups 6 hours after the first protein boost, they were clearly elevated in animals immunized with formulations containing QS21 following the second protein boost. Animals immunized with formulations containing QS-21 also demonstrated significantly higher IFNγ, IL-4, and IL-1β as compared to

Figure 2.7. Serum cytokine concentration in mice vaccinated with DP6-001 and candidate adjuvants. Mice were immunized with DP6-001 DNA prime-protein boost ('D+P'), with each protein boost formulated with QS-21 (D+P-QS-21), $Al(OH)_3$ (D+P-$Al(OH)_3$), MPLA (D+P-MPLA) or ISCOMATRIX™ adjuvant (D+P-IMX). Naïve mice received saline injections in lieu of immunization. Sera were collected pre-immunization and 6 hours following the first (P1) and second (P2) protein-adjuvant boosts. Cytokines were quantified in the serum of individual mice at a 1:4 dilution using a custom 12-plex Luminex panel. (a) QS-21 cytokine profile. (b) MPLA cytokine profile. (c) ISCOMATRIX™ adjuvant (IMX) cytokine profile. (d) Serum cytokines elevated following protein boost with formulations containing all candidate adjuvants. Significance over background is represented above error bars. Bracketed lines represent differences between adjuvant groups. Statistical significance was determined with a One-way ANOVA and Tukey post-test (*: $p < .05$, **: $p < .01$, ***: $p < .001$).

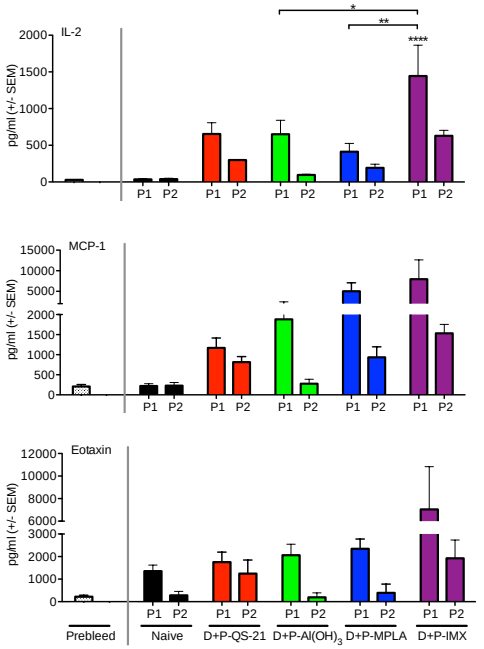
(a)


IFNγ
IL-4
IL-1β
MIP-1β
pg/ml (+/- SEM)
P1 P2
Prebleed
Naive
D+P-QS-21
D+P-Al(OH)3
D+P-MPLA
D+P-IMX

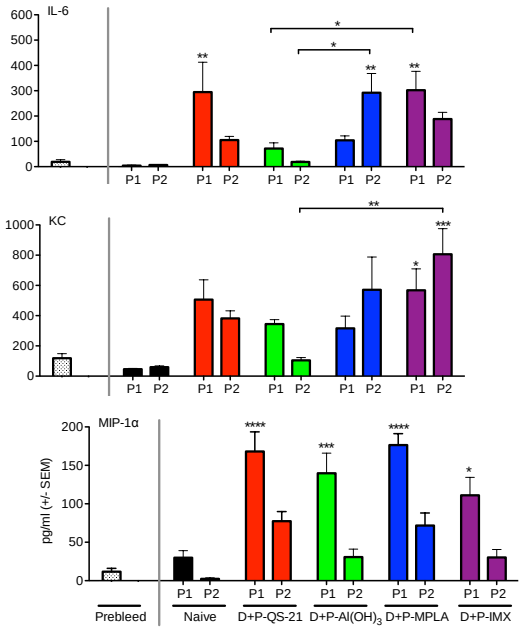
(b)


RANTES
G-CSF
pg/ml (+/- SEM)
P1 P2
Prebleed
Naive
D+P-QS-21
D+P-Al(OH)3
D+P-MPLA
D+P-IMX

(c)


IL-2
MCP-1
Eotaxin
pg/ml (+/- SEM)
P1 P2
Prebleed
Naive
D+P-QS-21
D+P-Al(OH)3
D+P-MPLA
D+P-IMX

(d)


IL-6
KC
MIP-1α
pg/ml (+/- SEM)
P1 P2
Prebleed
Naive
D+P-QS-21
D+P-Al(OH)3
D+P-MPLA
D+P-IMX

animals immunized with formulations containing $Al(OH)_3$ or MPLA. Levels of MIP-1β, a chemoattractant for natural killer (NK) cells and monocytes, were significantly higher than in $Al(OH)_3$-immunized animals. While mice immunized with formulations containing QS-21 showed significantly higher levels of IL-4 after the second protein boost in comparison to mice immunized with formulations containing ISCOMATRIX™ adjuvant, no significant differences were observed in other signature cytokines at this time point (Fig. 2.7a).

Mice immunized with DP6-001 formulated with MPLA demonstrated significantly higher levels of the multifunctional and broadly acting chemokine RANTES in serum 6 hours after the first protein boost, as compared to other protein-adjuvant formulations (Fig. 2.7b). Following the second protein-adjuvant boost, serum levels of RANTES were reduced in all groups, though in mice immunized with formulations containing MPLA the levels remained elevated compared to other formulations. In addition, levels of G-CSF were significantly increased in mice immunized with formulations containing MPLA following the first protein boost in comparison to mice immunized with formulations containing QS-21 or $Al(OH)_3$ (Fig 2.7b).

Similarly, G-CSF was significantly increased at this time point in mice immunized with formulations containing ISCOMATRIX™ adjuvant in comparison to mice immunized with formulations containing QS-21 or $Al(OH)_3$ (Fig. 2.7b). G-CSF, which induces proliferation and differentiation of granulocytes, was characteristic of formulations containing either MPLA or ISCOMATRIX™ adjuvant in the context of the DP6-001 vaccine. In addition, animals receiving DP6-001 vaccine formulated with

ISCOMATRIX™ adjuvant exhibited a unique profile of pro-inflammatory markers. The Th1 cytokine IL-2 was significantly increased following the first protein boost in mice immunized with formulations containing ISCOMATRIX™ adjuvant in comparison to formulations containing QS-21 or $Al(OH)_3$. In addition, chemoattractants for eosinophils (Eotaxin), or monocytes and DCs (MCP-1), were also characteristic of formulations containing ISCOMATRIX™ adjuvant (Fig. 2.7c). After the first protein boost, serum levels of MCP-1 in mice immunized with formulations containing QS-21 or $Al(OH)_3$ were significantly lower compared to formulations containing ISCOMATRIX™ adjuvant, while levels in mice immunized with formulations containing MPLA were comparable. However, serum levels of Eotaxin were significantly increased in mice immunized with formulations containing ISCOMATRIX™ adjuvant in comparison to mice immunized with all other adjuvanted formulations.

Several serum cytokines and chemokines in our panel were shared among different candidate adjuvants in the context of HIV-1 gp120 DNA prime-protein boost vaccine. The neutrophil chemoattractant KC was strongly elevated in serum following protein boosts in mice immunized with DP6-001 formulated with QS-21, MPLA and ISCOMATRIX™ adjuvant, as compared to naïve and mice immunized with formulations containing $Al(OH)_3$. There was no significant difference between these three adjuvanted formulations, although mice immunized with formulations containing ISCOMATRIX™ adjuvant demonstrated significantly higher levels of KC after the second protein boost as compared to formulations containing $Al(OH)_3$ (Fig. 2.7d).

IL-6, which may act as both a pro- and anti-inflammatory cytokine in response to a variety of stimuli, was strongly induced following protein boost in with formulations containing QS-21, MPLA, and ISCOMATRIX™ adjuvant, while levels in the mice immunized with formulations containing $Al(OH)_3$ were very low. After the first protein boost, levels of IL-6 in mice immunized with formulations containing QS-21 and ISCOMATRIX™ adjuvant were both elevated comparably. Interestingly, in animals receiving protein boost formulated with MPLA, after one protein boost levels of IL-6 were significantly lower than those induced by formulations containing ISCOMATRIX™ adjuvant. By the second protein boost, however, levels of IL-6 induced by the formulations containing MPLA had risen to be comparable to initial levels seen with formulations containing ISCOMATRIX™ adjuvant, while the levels of IL-6 in mice immunized with formulations containing ISCOMATRIX™ adjuvant had notably fallen (Fig. 2.7d).

Table 1 summarizes the serum cytokine profiles associated with each candidate adjuvant in the context of DP6-001, by comparing the fold increase over background for each adjuvant.

Table 2.1. Temporal serum cytokine levels in mice immunized with endotoxin-free (EF) DNA prime compared to regular DNA plasmid preparation.

	D+P+QS-21		D+P+Al(OH)$_3$		D+P+MPLA		D+P+IMX	
	P1	P2	P1	P2	P1	P2	P1	P2
IFNγ	+	++	+	-	+	+	+	+
IL-2	++	++	++	+	++	+	+++	++
IL-4	-	+	-	-	-	-	-	-
IL-6	++	+	+	-	+	++	++	+
IL-1β	-	+	-	-	-	-	-	-
Eotaxin	+	+	+	-	++	-	+++	+
KC	+	+	+	-	+	+	+	+
G-CSF	++	+	+	+	+++	++	+++	++
RANTES	+	-	+	-	++	+	+	-
MCP-1	+	+	+	-	++	++	+++	+++
MIP-1α	++	+	++	+	++	+	+	+
MIP-1β	+	+	-	-	+	+	+	+

Profiles were determined by calculating the fold increase of individual mice in each group over their respective pre-immunization cytokine levels. Data is representative of cytokine data shown in Figure 6. <2-fold increase: -, clear box. 2-10-fold increase: +, clear box. 10-30-fold increase: ++, light yellow box. >30-fold increase: +++, dark yellow box. (IMX = ISCOMATRIX™ adjuvant)

Induction of serum cytokines by a protein-adjuvant vaccine in the absence of DNA prime

In order to define the effect of DNA priming on the protein and adjuvant serum cytokine profiles, additional study was performed in C57Bl/6 wildtype mice that received only two DP6-001 protein boosts formulated with candidate adjuvants without DNA prime. Mice were immunized at weeks 0 and 4, and sera were collected for cytokine analysis at 6 hours after each protein boost. Serum cytokines were quantified in immunized mice in comparison to naïve mice using the 12-plex array described above.

Generally, serum cytokine levels associated with a protein-only vaccine were reduced (Fig. 2.8) in comparison to those observed in mice immunized with the complete DP6-001 DNA prime-protein boost regimen (Fig. 2.7). Protein-only vaccine formulated with QS-21 results in low to background levels of Th1 and Th2 cytokines, including the previously observed QS-21-associated signature cytokines, IFNγ and IL-4 (Fig. 2.8a). The inflammatory cytokine IL-1β and the chemokine MIP-1β, associated with QS-21 in the context of DP6-001 prime-boost vaccine, were substantially reduced after immunization with a protein-only vaccine. Similarly, protein vaccine formulated with QS-21 demonstrated moderate reductions in the chemokines G-CSF, MCP-1, MIP-1α, and RANTES (Fig. 2.8a). While DP6-001 prime-boost vaccine formulated with $Al(OH)_3$ produced overall low, unimpressive cytokine profiles, we observed that protein-only immunization with formulations containing $Al(OH)_3$ also demonstrated even lower or negligible levels of Th1/Th2 cytokines, inflammatory cytokines, and chemokines (Fig. 2.8b).

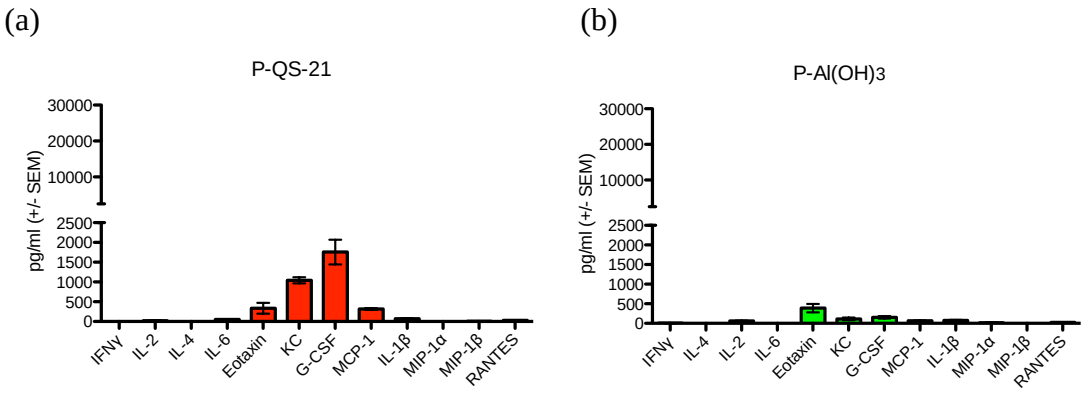


Figure 2.8. Compiled serum cytokine panels following the first protein boost in mice vaccinated with Env protein formulated with QS-21 or Al(OH)$_3$. Mice vaccinated with protein-only vaccine ('P') received immunizations at weeks 0 and 4 with the DP6-001 protein. Cytokines were quantified in serum from individual mice collected 6 hours following protein boost by a 12-plex Luminex array. Shown are serum cytokine levels 6 hours after the first protein-adjuvant boost. Mice were immunized with protein-only vaccine formulated with (a) QS-21 (red) or (b) Al(OH)$_3$ (green).

Similar studies with protein alone vaccination were conducted with formulations containing MPLA and ISCOMATRIX™ adjuvant, but also included groups that received three empty DNA vector as the prime followed with protein boost (Fig. 2.9). In the context of a prime-boost vaccine, formulations containing MPLA were primarily characterized by potent induction of G-CSF and RANTES (Fig. 2.9a). Interestingly, while Th1/Th2 cytokines and other chemokines are notably reduced following a protein-only vaccine formulated with MPLA, the MPLA formulation-associated signature chemokines G-CSF and RANTES, as well as MCP-1 and KC, are minimally changed in the absence of DNA priming (Fig. 2.9b). Vector-primed mice immunized with protein-MPLA demonstrated reduced cytokines and chemokines (Fig. 2.9c) in comparison to mice immunized with the complete DP6-001 regimen (Fig. 2.9a).

Of the signature cytokines associated with formulations containing ISCOMATRIX™ adjuvant in the context of the DP6-001 prime-boost vaccine, such as IL-2, Eotaxin, G-CSF and MCP-1 (Fig. 2.9d), we observed that IL-2, Eotaxin, and MCP-1 were substantially reduced in mice immunized with protein-only vaccine containing ISCOMATRIX™ adjuvant (Fig. 2.9e). Though still above pre-immunization levels, serum levels of G-CSF were reduced in the absence of a DNA prime (Fig. 2.9e). Control mice immunized with three empty vector DNA primes followed by DP6-001 protein boosts formulated with ISCOMATRIX™ adjuvant, however, demonstrated low or negative levels of all of the serum cytokine and chemokines analyzed (Fig. 2.9f).

In summary, from our analysis of serum cytokines associated with candidate adjuvants formulated with the DP6-001 prime-boost vaccine, several markers, IL-6, KC,

and MIP-1β, were identified that were comparably induced following protein boosting with formulations containing either QS-21, MPLA, or ISCOMATRIX™ adjuvant. Consistent with the trend of reduced cytokines associated with the absence of DNA priming, both IL-6 and MIP-1β were reduced or negative in comparison to DNA primed animals. A notable exception to this trend was the neutrophilic chemoattractant KC, which remained at comparable levels regardless of vaccine regimen (Fig. 2.8 and Fig. 2.9). The inclusion of protein-only vaccines and empty vector DNA prime controls allows us to confirm that the cytokine and chemokine profiles associated with our candidate adjuvants are unique to the context of a DNA prime-protein boost vaccine strategy, and confirmed the concept that the improved immunogenicity based on serum cytokines was due to the inclusion of DNA priming steps.

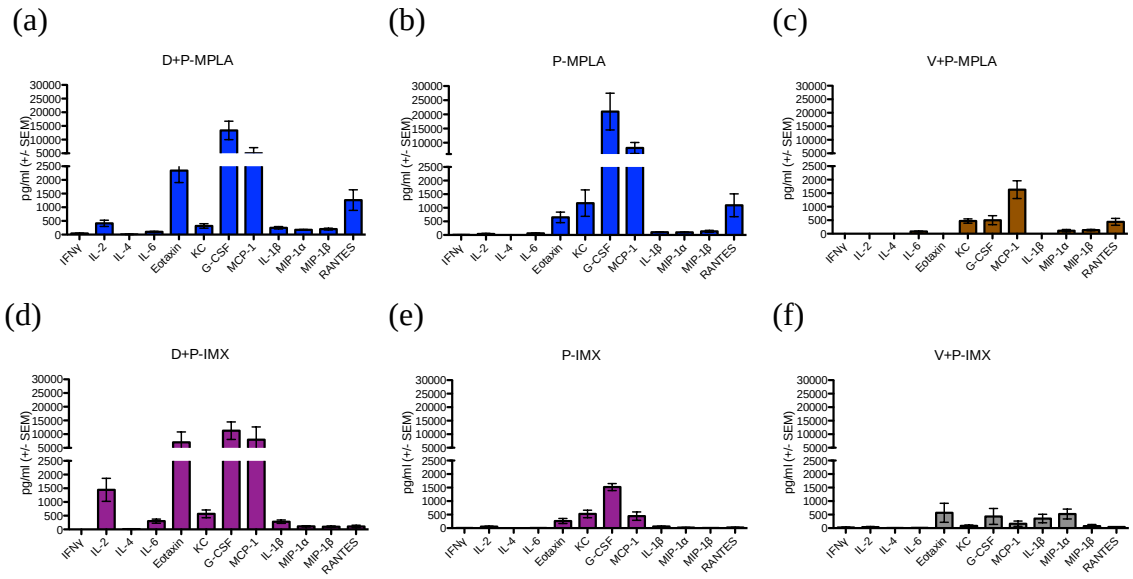


Figure 2.9. Compiled serum cytokine panels following the first protein boost in mice vaccinated with Env protein vaccine and candidate adjuvants, as compared to DNA primed-protein boosted animals. As previously described, mice were immunized with the complete DP6-001 DNA prime followed by protein boosts ('D+P') containing (a) MPLA (blue) or (d) ISCOMATRIX™ adjuvant (IMX) (purple). Mice vaccinated with protein-only vaccine ('P') received immunizations at weeks 0 and 4 with the DP6-001 protein. Protein alone vaccines were formulated with (b) MPLA (blue), or (e) ISCOMATRIX™ adjuvant (IMX) (purple). Control mice received empty vector DNA primes, followed by protein boosts ('V+P') formulated with (c) MPLA (brown) or (f) ISCOMATRIX™ adjuvant (IMX) (grey).

Discussion

In recent years, the heterologous prime-boost vaccination strategy has demonstrated considerable advantages over the classical vaccine strategies based on homologous prime-boost strategies. Results from RV144 clinical trial of viral vector prime and recombinant gp120 protein boost demonstrated unprecedented partial protection against the transmission of HIV-1 (38, 125, 144, 150). Similarly, we have previously reported that the DP6-001 vaccine formulation, consisting of a DNA prime and protein boost, was highly immunogenic in both preclinical and clinical studies (7, 35, 137, 138, 186). A Phase I clinical trial of DP6-001 vaccine showed the generation of balanced Env-specific T cell and high titer Env-specific antibody responses including broadly neutralizing antibodies in study vaccinees (186).

In both of the above studies, the Env protein boost is now recognized as a critical component. However, the roles of adjuvant in such a protein boost immunization remains unclear. Protein vaccines are formulated with adjuvants with the goal of promoting and harnessing the innate non-antigen specific, pro-inflammatory response, and ultimately enhancing the antigen-specific adaptive immune response. However, this strategy is not without the risk of adverse effects. Out of candidate adjuvants in the current study, only $Al(OH)_3$ and MPLA are components in licensed vaccines in the United States. Even well established and tolerable vaccine adjuvants such as $Al(OH)_3$ must be thoroughly evaluated for both efficacy and safety in the context of new vaccine formulations before being approved for clinical use. Vaccine antigen, dosing, route of administration, and schedule may all impact the immunogenic profile as well as the

reactogenicity of a given adjuvant. In addition, adjuvants must be selected with the pathogen-specific requirements of protection in mind. Sterilizing immunity against HIV-1 infection requires not only a strong humoral response including neutralizing antibodies, but also $CD8^+$ cytotoxic T cell responses, and $CD4^+$ T cells facilitating both the humoral and cell-mediated immunity.

In the current study, three well-studied adjuvants, QS-21, MPLA, and ISCOMATRIX™ adjuvant, in addition to the widely used $Al(OH)_3$ adjuvant as a control, all demonstrated comparable immunogenicity in the context of the DP6-001 DNA prime-protein boost in a mouse model. With the exception of $Al(OH)_3$, the other three potent adjuvants each strongly induced a vaccine-specific antibody response. While the QS-21 adjuvant was associated with the strongest induction of Env-specific $CD4^+$ T cell responses by intracellular cytokine staining and ELISpot assay, there were no significant differences in Env-specific Th1 and Th2 responses between the adjuvants evaluated as determined by ICS. Env-specific T cell ELISpot results showed that formulations containing QS-21 induced significantly greater Th1 responses as compared to other adjuvants, while formulations containing MPLA and ISCOMATRIX™ adjuvant induced relatively low positive Th1 responses. In contrast, all adjuvant formulations could elicit comparably weak Env-specific Th2 responses.

While antigen specific responses were similar for formulations containing each of the adjuvants included in the current study, the serum cytokine profiles were quite different. Multiplex arrays for nonspecific serum cytokines proved an invaluable tool in detecting differences in the non-Env specific innate responses to the DP6-001 vaccine

and candidate adjuvants. In the context of the DP6-001 prime-boost vaccine, the candidate adjuvants were easily distinguished by these cytokine profiles and chemoattractants indicative of differential immune cell recruitment. The local and systemic inflammatory environment induced by an adjuvant formulated with vaccine requires a delicate balancing act. Early resident and recruited innate immune cells release of a complex milieu of cytokines and chemoattractants that together influence subsequent waves of immune cell infiltrates, and thus enhance antigen uptake and presentation (66). Ultimately, this microenvironment shapes the antigen-specific adaptive immune response, directing a Th1 or Th2 $CD4^+$ T cell bias and enhancing B cell function and antibody production. However, overstimulation of a pro-inflammatory response may result in undesirable local and systemic symptoms, including hypersensitivity reactions, fever and myalgia.

The multifunctional cytokine IL-6 is a central player in inflammation. Along with IL-1β, IL-6 promotes the acute phase response and fever (106). IL-6 also mediates neutrophilic inflammation and the shift from innate acute inflammation to chronic inflammation and adaptive immunity (79, 81, 82, 135). In the current study, 6 hours following protein boost, we observed that IL-6 was universally induced to comparable levels by formulations containing each candidate adjuvant. This pattern was also observed for the neutrophil chemoattractant, KC. These serum cytokine levels associated with formulations containing the control adjuvant, $Al(OH)_3$, were typically low compared to all other groups. MIP-1α, which, like MIP-1β, is produced by macrophages to promote neutrophilic inflammation and immune cell recruitment (163), was produced

comparably by all adjuvanted formulations including those with $Al(OH)_3$, but at levels not significantly elevated above background. These cytokines characteristic of DP6-001 DNA prime-protein boost with all adjuvants reflects an early, acute inflammatory response to immunization, likely predominated by neutrophils.

In the current study, formulations containing QS-21 were associated with the Th1 cytokine IFNγ and the Th2 cytokine IL-4, supporting our findings of mixed Env-specific Th1/Th2 T cell responses and IgG isotyping, as well as previous reports of *Quillaia* saponin fractions (172). Elevated serum MIP-1β was characteristic of only formulations containing QS-21. IL-1β, which plays a major role in the acute phase response and fever (106), as well as neutrophilic inflammation (27, 163), was also strongly associated with formulations containing QS-21. It may be a point of interest that these characteristic cytokines were most strongly observed following the second protein boost, rather than following the first protein boost, as was the pattern for other adjuvants. This may be suggestive of a progressive inflammatory response with subsequent immunizations.

By comparison, the more clinically tolerable saponin formulation ISCOMATRIX™ adjuvant was characterized by strong IL-2, supportive of an expected Th1 and $CD8^+$ T cell response. Elevated systemic IL-6 as well as Eotaxin, a chemoattractant for eosinophils, are indicative of a Th2 response, supporting the reported mixed Th1/Th2 profile of ISCOMATRIX™ vaccines (37, 41, 109, 127, 141, 171). Granulocyte factor G-CSF and MCP-1, which recruits monocytes and macrophages subsequent to early neutrophil infiltration (195), were also associated with the protein boost containing ISCOMATRIX™ adjuvant. The distinct cytokine and chemokine

pattern observed here with DP6-001/ISCOMATRIX™ adjuvant consists of Th1 and Th2 cytokines as well as chemoattractants indicative of the recruitment of monocytes, macrophages, NK cells, and granulocytes. This profile supports a serum cytokine profile recently reported by Wilson *et al.* 6 hours following subcutaneous administration of ISCOMATRIX™ adjuvant without antigen (191). Our profile of DP6-001/ISCOMATRIX™ adjuvant is consistent with a profile of serum cytokines and immune cell infiltration at draining lymph nodes described by Duewell *et al.*, consisting of B and T cells, DCs, NK cells, and granulocytes, in mice subcutaneously immunized with OVA antigen and ISCOMATRIX™ adjuvant (42).

DP6-001 formulated with MPLA demonstrated high serum levels of granulocyte factor G-CSF, in addition to elevated serum RANTES, a T cell-produced chemoattractant for eosinophils, T cells, and NK cells. The serum cytokine profile of MPLA observed in the context of the DP6-001 Env DNA prime-protein boost may support previous evaluations of MPLA formulations in a mouse model. Mata-Haro *et al.* previously reported that in the hours following injection, MPLA formulated with OVA antigen was correlated with serum G-CSF and MCP-1, and low but positive RANTES, as compared to LPS. MyD88-associated IL-6 and MIP-1α were weakly positive, while IFNγ and IL-1β were minimal (115).

We have described here a complex picture of the differential immune responses elicited by each candidate adjuvant in the context of a novel heterologous prime-boost vaccine, with the goal of identifying correlates of immunogenicity and markers of reactogenicity that may aid in the selection of an adjuvant for future optimized vaccine

formulations. While the immunogenicity of the formulation containing our previously employed adjuvant QS-21 was comparable to formulations with our other candidate adjuvants, we may be able to correlate a unique systemic inflammatory response associated with QS-21. Formulations with all three potent adjuvants, with the exception of $Al(OH)_3$, demonstrated comparable serum levels of IL-6 and KC, and low but positive levels of MIP-1α. Beyond this, the additional pro-inflammatory environment of serum IL-1β and MIP-1β associated specifically with QS-21 may contribute to reported adverse events. The predominance of markers for acute inflammation and fever, as well as products and mediators of neutrophilic inflammation, is of particular interest given the nature of a previously reported vasculitis associated with DP6-001 and QS-21 (7). Leukocytoclastic vasculitis is due to the toxic effect of neutrophilic degranulation products on the endothelial cells of small vasculature (174).

In contrast to QS-21, formulations containing MPLA and ISCOMATRIX™ adjuvant likely demonstrate potentially milder reactogenic profiles. Both MPLA and ISCOMATRIX™ adjuvant are largely characterized by broadly acting chemoattractants recruiting a varied population of immune cells, including granulocytes, NK cells, monocytes, DCs, and macrophages. However, formulations containing either of these adjuvants demonstrate notably lower levels of additional inflammatory cytokines and chemokines such as IL-1β and MIP-1β in comparison to QS-21.

In the process of evaluating different adjuvants with the DP6-001 formulation, we observed that serum cytokine responses demonstrate unique kinetics in a prime-boost vaccine regimen. The optimal time to detect high level cytokine responses was at 6 hours

after the protein boost, while early studies demonstrated low to negative responses at the end of DNA priming immunization. Repeated boost with protein-adjuvant vaccines actually led to a reduced cytokine response following subsequent protein boosts, except with the use of QS-21.

In the current study, we performed several control immunizations to confirm the correlation of our unique adjuvant serum cytokine profiles to their use in the context of a unique DNA prime-protein boost vaccine strategy. In animals immunized with a protein-only vaccine formulated with QS-21, $Al(OH)_3$, MPLA, or ISCOMATRIX™ adjuvant, serum cytokine and chemokine panels were largely reduced in comparison to those in DNA-primed animals. A similar trend was observed in animals that received an empty vector DNA prime and protein boost formulated with our candidate adjuvants. These serum cytokine profiles from protein-only or empty vector primed vaccine formulations suggest that the serum cytokine and chemokine profiles we have defined for QS-21, MPLA, and ISCOMATRIX™ adjuvant are unique to the context of a DNA prime-protein boost vaccine strategy.

A few notable exceptions to this trend were observed. In mice immunized with protein formulated with MPLA, the markers G-CSF and RANTES, associated with MPLA in the context of the full DP6-001 regimen, were apparently uncompromised in the absence of DNA priming. In addition, the neutrophil chemoattractant KC remained at relatively unchanged levels in the context of formulations containing QS-21, MPLA, or ISCOMATRIX™ adjuvant, regardless of vaccination strategy. This observation suggests that an early immune response to vaccination characterized by strong neutrophilia was

common to all tested vaccine strategies and adjuvants, and was minimally impacted by the presence or absence of DNA priming.

The current study also suggested that while trace amount of endotoxin contaminants will lead to elevated levels of only a few cytokines, this has a minimal effect on the overall levels of Env-specific antibody responses. These findings ruled out the potential contribution of endotoxin contamination to vaccine-induced immunogenicity and the observed profiles of non-antigen specific serum cytokines. This analysis confirmed that the adjuvant effects we observed with our various DP6-001 formulations were related to the adjuvant used, and not potentiated by method of DNA preparation.

In summary, we have reported that two potent candidate adjuvants MPLA and ISCOMATRIX™ adjuvant demonstrate comparable immunogenicity to our previously employed adjuvant QS-21, in the context of a heterologous, multiclade HIV-1 Env DNA prime-protein boost regimen. However, these adjuvants differ considerably in terms of induction of pro-inflammatory cytokines and chemokines responsible for local and systemic immune cell recruitment. This study provides critical insight about these adjuvants in formulation with Env antigen, as well as in the context of a DNA-primed immune system. In addition, the distinct cytokine and chemokine profiles defined for each adjuvant shed light on useful correlates of vaccine immunogenicity as well as pro-inflammatory markers of reactogenicity for guidance in adjuvant selection for optimized Env prime-boost formulations.

Preface to CHAPTER III

R. Buglione-Corbett and K. Pouliot contributed equally to animal work, including immunizations, bleeding, and termination

R. Buglione-Corbett and K. Pouliot contributed equally to Luminex experiments and data analysis, Fig. 3.5, 3.9

Terminal assays performed on immunized mice were performed collaboratively:

K. Pouliot and R. Marty-Roix performed ELISpot experiments and analysis, Fig 3.4, 3.8

R. Buglione-Corbett performed antibody studies and data analysis, Fig. 3.2, 3.3, 3.6, 3.7

K. Pouliot, R. Marty-Roix, and E. Lien edited figures for the manuscript.

K. Pouliot, R. Marty-Roix, E. Lien, **R. Buglione-Corbett** and S. Lu prepared the manuscript.

CHAPTER III:

Impact of innate signaling pathways on the adaptive and innate profiles of Monophosphoryl Lipid A and other adjuvants in the context of a DNA prime-protein boost vaccine

Introduction

Many vaccines are formulated with an adjuvant component that is capable of eliciting an immunomodulatory or immunostimulatory effect with the purpose of enhancing the adaptive immune response to the vaccine antigen, and thus improved efficacy. Classically, compounds such as aluminum salts have been utilized in licensed human vaccines, including vaccines against diphtheria/tetanus/pertussis (DTaP) and hepatitis B virus (HBV), which deliver protein antigen to the immune system in order to raise protective immunity. Aluminum salts have been used in humans for the last 70 years in part due to their ability to raise a vaccine-specific antibody response, and also because of their clinical tolerability (66, 119). More recently, novel adjuvant systems that specifically activate innate immune signaling pathways to ultimately enhance adaptive immunity have emerged as candidates for inclusion in human vaccines. Interactions with receptors for a variety of innate immune pathways impact vaccine-specific immune responses, but also induce the secretion of pro-inflammatory cytokines and chemokines, and recruitment of a diverse population of immune cells that shape and direct the humoral and cell-mediated immune response. Work to clearly understand the mechanism of adjuvants described herein is still underway.

Every vaccine and its respective adjuvant must be evaluated for efficacy and safety in terms of specific antigen, dosage, and delivery method. In this study, we report on the evaluation of a previously described pentavalent HIV-1 Env DNA prime-protein boost vaccine DP6-001 (7, 137, 138, 186) in the context of three candidate adjuvants, with the goal of better understanding the innate pathways uniquely harnessed by each adjuvant. Protein components of the DP6-001 vaccine were formulated with QS-21 as previously evaluated in preclinical and clinical studies or with the well-established aluminum salt-based adjuvant, aluminum hydroxide gel, referred to by its chemical formula $Al(OH)_3$, as a control. Alternatively, DP6-001 was formulated with MPLA, an adjuvant that has recently been approved for use in licensed human vaccines. MPLA may be a clinically advantageous approach particularly in vaccines against HIV-1, as it is capable of inducing a strong antibody response as well as Th1-biased $CD4^+$ T cells (2, 115, 162, 175, 181, 182, 189). MPL® is currently approved in formulation with aluminum hydroxide in the adjuvant system AS04 produced by GlaxoSmithKline (GSK) for the Fendrix® vaccine against HBV and the Cervarix® vaccine for human papillomavirus (HPV). Additionally, AS01 and AS02, formulations of MPL® with QS-21, are currently undergoing clinical trials for anti-malarial vaccines (159, 180).

MPLA is a derivative of LPS from Gram negative bacteria that is characterized by reduced toxicity in comparison to its parent molecule while still serving as an immunomodulatory compound (2). LPS is highly toxic and produces a systemic inflammatory response via signaling through the pattern recognition receptor (PRR) Toll-like receptor 4 (TLR4). Through the process of acid-base hydrolysis, LPS is

dephosphorylated and a hydroxytetradecanoyl group is removed, resulting in the less toxic MPLA derivative (2, 151, 152). Like LPS, MPLA is an agonist of TLR4 and requires signaling via TLR4 to mount an adaptive immune response. However, the dependency of MPLA's adjuvanticity on the activation of downstream signaling pathways remains less clear. In particular, controversy remains regarding the relative bias of signaling via MyD88 or TRIF downstream of TLR4 activation.

In a report by Mata-Haro *et al.* comparing the innate and adaptive responses to OVA protein antigen formulated with LPS or MPLA, it was suggested that TRIF-biased signaling with a concurrent reduction of activity via MyD88 adaptor and MyD88-dependent inflammatory responses, was responsible for the reduced toxicity observed clinically with MPLA (115). More recent studies have investigated whether this bias may be due to either a passive signaling bias or active inhibition of MyD88-dependent responses (29). Our immunization studies of DP6-001 and adjuvants included genetically modified mice that are deficient in the required MPLA receptor, TLR4, or the adaptor molecule MyD88. This allowed us to evaluate the contribution of MPLA activation of TLR4 signaling to the adaptive immune response in the context of the DP6-001 vaccine, and also determine the dependence of vaccine-induced humoral and CMI on downstream signaling via MyD88. In addition, we observed the dependence of the non-antigen specific serum pro-inflammatory cytokine and chemokine profiles established in Chapter II to be associated with DP6-001/MPLA immunization on intact signaling via either TLR4 or MyD88.

Unlike MPLA, the mechanisms of both alum adjuvants and QS-21 via innate immune signaling pathways remain largely unknown, and neither appear to activate any of the known TLRs. Evidence indicates that aluminum salts interact directly with DC membranes in order to facilitate and maintain processing and presentation of antigen, and also inhibits release of IL-12, consistent with its Th2-biased response (51). Aluminum salts have been extensively evaluated *in vitro* and *in vivo* for activation of the NLRP3 inflammasome complex in DCs and other APCs, which results in the production of pro-inflammatory cytokines IL-1β and IL-18. As described in Chapter I, aluminum salts, along with other particulates such as asbestos or silica, may act directly on the NLRP3 inflammasome, inducing assembly of inflammasome components and secretion of cytokines. Conversely, administration of aluminum salts may cause tissue damage and cell death resulting in the release of 'danger signals,' including host DNA, which themselves induce the NLRP3 or other inflammasome complexes (22, 45, 53, 92, 97, 119, 149). Maturation and secretion of pro-inflammatory cytokines via the NLRP3 inflammasome requires a first signal via MyD88 for the transcription of pro-cytokines (10). Thus, while it is unlikely that the adjuvant effect of aluminum-based adjuvants, like $Al(OH)_3$, is impacted by deficient TLR4 signaling, the MyD88 adaptor molecule is an important checkpoint for a variety of innate pathways that may be involved in the adjuvant effect of aluminum salts. By similar logic, the saponin adjuvant QS-21, which is a known hemolytic agent associated with inflammation and reactogenicity, may well activate innate signaling pathways by producing cell damage and release of danger signals at the injection site.

The exact nature of the linkage between this innate inflammatory response and the generation of adaptive immunity to adjuvanted vaccine remains unclear. The studies discussed in this chapter aim to determine the dependence of the antigen-specific responses (defined by antibody and T cell response) and non-antigen specific serum cytokine profiles associated with DP6-001 DNA prime-protein boost vaccine and candidate adjuvants, on intact signaling via TLR4 or MyD88, with a particular focus on the novel adjuvant system MPLA.

Results

Study Design and Immunization Schedule

For these studies, C57Bl/6 wildtype mice or mice deficient in TLR4 or in MyD88 were immunized with the DP6-001 vaccine as previously described in Chapter II. Knockout mice were generated as previously described (1). Mice were immunized i.m. with the pentavalent DNA prime-protein regimen weeks 0, 2, and 4 and then boosted with DP6-001 pentavalent protein and one of three adjuvants (QS-21, $Al(OH)_3$, or MPLA) in weeks 8 and 12 according the schedule in Figure 3.1. As a control, some mice were primed with an empty vector DNA plasmid pSW3891, and then boosted with DP6-001 protein and MPLA. Throughout the immunization schedule, sera were collected from each mouse biweekly, and 6 hours following the final DNA prime and each protein-adjuvant boost. At termination 7 days after the final protein boost, sera and spleens were harvested from immunized mice for downstream application.

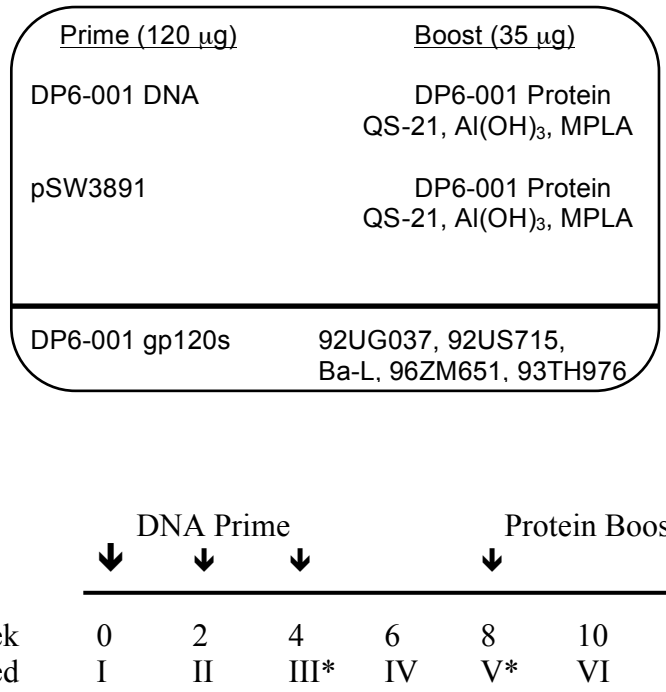


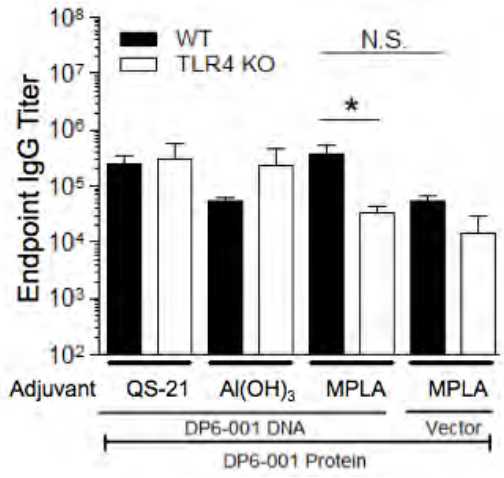
Figure 3.1. Study design and immunization schedule. Mice were immunized i.m. with three DNA primes containing either DP6-001 or pSW3891 DNA two weeks apart. Mice were immunized i.m. twice with DP6-001 protein formulated with either QS-21, $Al(OH)_3$ or MPLA. Blood was harvested every two weeks. (* Mice bled 6 hours after immunization)

TLR4 is required for antibody responses in DP6-001/MPLA immunized mice

In sera collected from immunized mice at termination, 7 days after the final protein-adjuvant boost, total Env-specific IgG antibody in serum was measured by ELISA. All immunized mice produced a positive, vaccine antigen-specific IgG antibody in comparison to naïve mice (Fig. 3.2a). As observed in previous studies of wildtype mice, there was minimal difference between Env-specific endpoint titers in response to DP6-001 when formulated with different adjuvants. While not significant, animals immunized with an empty vector prime followed by boosting with DP6-001 protein and MPLA produced more modest Env-specific IgG endpoint titers compared to DP6-001 DNA primed animals (Fig. 3.2a). Notably, animals deficient in TLR4 demonstrated positive but significantly reduced endpoint IgG titers in comparison to wildtype mice, following immunization with DP6-001/MPLA (Fig. 3.2a). This dependency on TLR4 was not observed in mice immunized with DP6-001 and either QS-21 or $Al(OH)_3$.

Temporal production of Env-specific IgG was quantified in sera collected biweekly throughout the immunization schedule by ELISA. While animals immunized with vector prime did not demonstrate a detectable vaccine-specific IgG response in serum until after protein-MPLA boosting, there was no significant difference in the endpoint IgG titers between DP6-001 or vector primed wildtype animals (Fig. 3.2b). A slight reduction was observed in the temporal induction of IgG during protein-MPLA boost time points in TLR deficient mice immunized with either DP6-001 or vector prime and MPLA, as compared to wildtype mice (Fig. 3.2b).

(a)



(b)

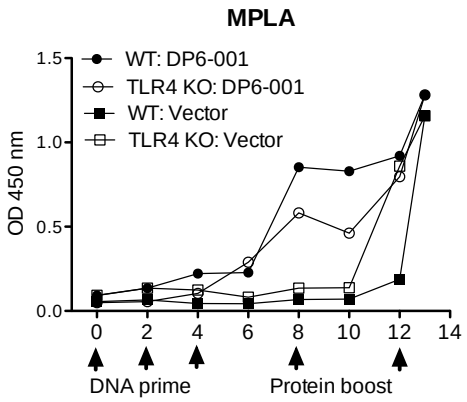
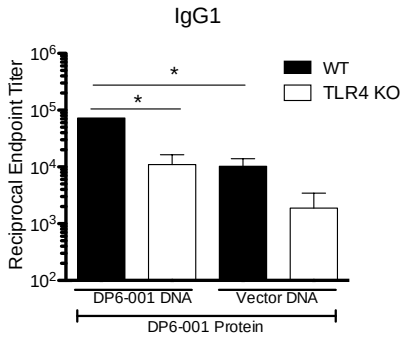


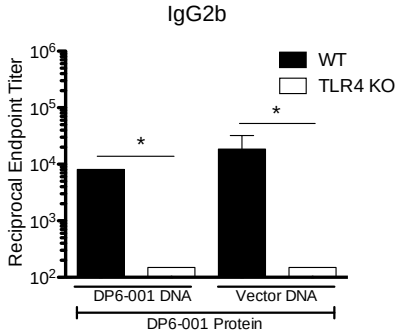
Figure 3.2. **Optimal Env-specific IgG antibody responses in mice immunized with MPLA/DP6-001 are dependent upon TLR4.** (a) Env-specific endpoint IgG titer in wildtype and TLR4-deficient mice immunized with DP6-001 or pSW3891 vector prime followed by DP6-001 protein adjuvanted with QS-21, $Al(OH)_3$ or MPLA. (b) Temporal gp120-specific IgG response was determined by ELISA using pooled sera samples from each group collected at two-week intervals. Statistical significance was determined by Student's t test (* $p < 0.05$).

Characterization of IgG isotypes indicative of a Th1- or Th2-biased response were quantified in serum collected at termination by ELISA (Fig. 3.3). Production of the Th2-associated isotype IgG1 was significantly greater in wild type mice immunized with DP6-001/MPLA compared to mice primed with empty vector (Fig. 3.3a). Furthermore, a moderate but significant reduction in Env-specific IgG1 was observed in TLR4 deficient mice compared to wild type (Fig. 3.3a). In contrast, Th1-associated isotypes IgG2b and IgG2c were comparable between wild type mice immunized with either DP6-001 or vector prime and protein-MPLA boost (Fig. 3.3b,c). However, endpoint titers of both IgG2b and IgG2c were significantly reduced nearly to background levels in both groups of immunized TLR4 deficient mice, indicating a dependency of the Th1 response to DP6-001/MPLA on intact TLR4 signaling (Fig. 3.3b,c).

(a)



(b)



(c)

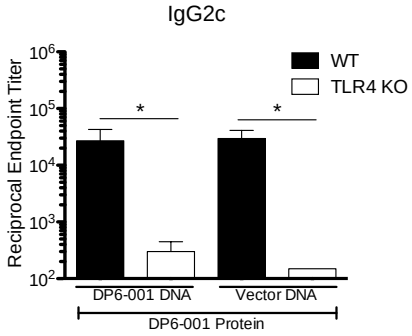


Figure 3.3. Optimal Env-specific IgG isotype responses in MPLA/DP6-001 immunized mice are dependent upon TLR4. Endpoint IgG isotype titers were determined in terminal sera from wildtype and TLR4-deficient mice by ELISA. (a) IgG1, (b) IgG2b, and (c) IgG2c. Statistical significance was determined by Student's t test (* $p < 0.05$).

TLR4 is required for T-cell mediated IFNγ responses in DP6-001/MPLA immunized mice

Upon termination of study, 7 days following final protein boost, splenocytes were harvested from immunized mice in order to quantify the vaccine-induced antigen-specific T cell responses. Splenocytes were incubated with abbreviated peptide pools of overlapping 15mers representing a small region from a clade B consensus sequence covering the length of gp120. Peptide pools stimulated production of Th1 (IFNγ, IL-2) and Th2 (IL-4, IL-6) cytokines by vaccine-specific T cells as measured by ELISpot. As negative and positive controls, splenocytes were alternatively incubated with either RPMI media or PMA and ionomycin, respectively. With a particular focus on the T cell responses associated with MPL, we observed that Env-specific production of IFNγ was greater in DP6-001 prime-boost immunized mice compared to vector primed mice (Fig. 3.4). In both immunization groups adjuvanted by MPLA, deficiency in TLR4 signaling resulted in significantly reduced Env-specific IFNγ by ELISpot (Fig. 3.4). These results indicate that the T cell response induced by the DP6-001 vaccine is enhanced by Env-encoding DNA plasmid priming, and that the adjuvant effect of MPLA required intact TLR4 signaling.

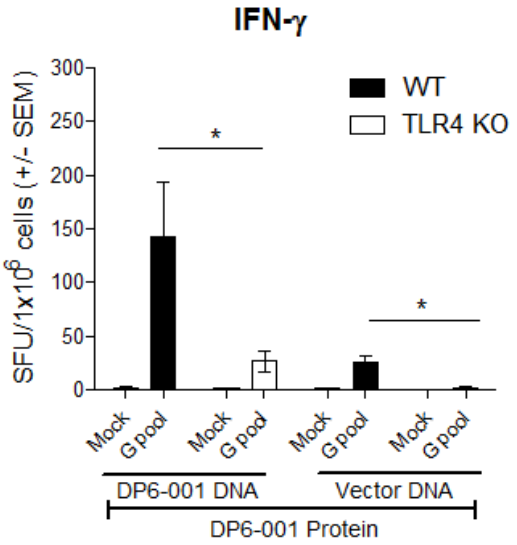


Figure 3.4. Env-specific T-cell mediated IFNγ response in DP6-001/MPLA or pSW3891/MPLA-immunized mice are dependent upon TLR4. After immunization with DP6-001/MPLA or pSW3891 vector prime and DP6-001 protein boost with MPL, spleens were harvested at termination 7 days after the final protein boost. Single cell suspensions of splenocytes were seeded into 96-well multiscreen filter plates and stimulated for 18 hours with Env-specific peptides ("G pool") or media alone ("mock"). IFNγ release was measured by ELISpot assay and spots quantified using CTL software. Statistical analysis of peptide stimulation over mock stimulation was calculated by Student's t-test (*: $p < 0.05$).

TLR4 is required for pro-inflammatory responses in DP6-001/MPLA immunized mice following protein boosting

Initial screening of a large panel of non-antigen specific inflammatory cytokines and chemokines in immunized wildtype mice identified a panel of analytes that were significantly induced above background by immunization with DP6-001 and candidate adjuvants. Levels of cytokines and chemokines in the sera of immunized mice were highly elevated at time points 6 hours after each protein-adjuvant boost. We have previously identified non-antigen specific serum cytokines and chemokines characteristic of the DP6-001 vaccine and candidate adjuvants at time points 6 hours after protein-adjuvant boosting (Buglione-Corbett, *et al*., unpublished data). In wildtype mice immunized with DP6-001 DNA prime and protein boost, we have observed that formulation of protein boost with MPLA resulted in a unique profile of elevated serum RANTES and G-CSF. We also noted several analytes that were commonly induced following protein boosts formulated with all candidate adjuvants, including IL-6. Overall, of the 12-plex panel of serum cytokines and chemokines assessed in immunized mice, minimal differences were observed between wildtype mice immunized with DP6-001 and either QS-21 or $Al(OH)_3$. However, serum levels of MPLA-associated G-CSF and RANTES, as well as IL-6, were significantly reduced in TLR4 deficient mice immunized with DP6-001/MPLA compared to wildtype mice (Fig. 3.5). These results suggest that the pro-inflammatory environment uniquely induced by MPLA in association with DP6-001 vaccine is dependent on its action via TLR4.

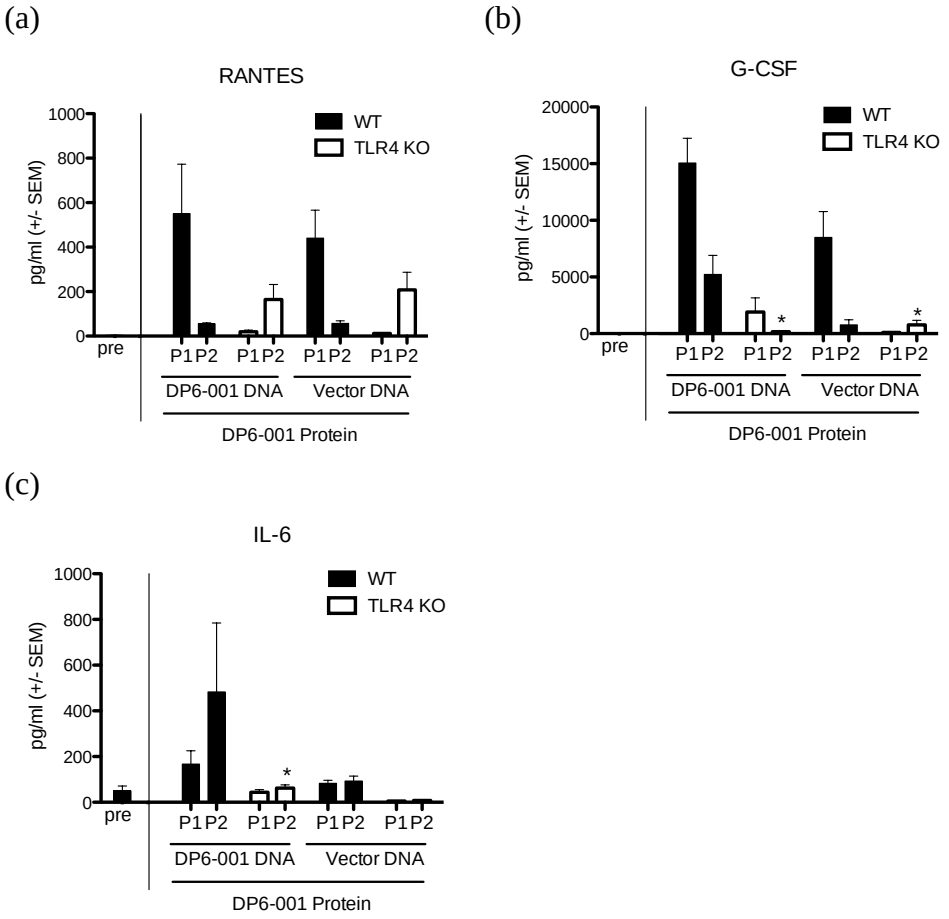


Figure 3.5. Optimal induction of pro-inflammatory cytokines requires TLR4 in DP6/MPL-immunized mice. Sera were collected pre-immunization and 6 hours following protein-adjuvant boosts. Cytokines were quantified in the serum of individual mice at a 1:4 dilution using a custom 12-plex Luminex panel. Shown are significant decreases in chemoattractants and proliferative factors after protein boosting in TLR4 deficient mice immunized with DP6-001 adjuvanted with MPLA or other adjuvants: (a) RANTES, (b) G-CSF, and (c) IL-6. Statistical significance was determined by Student's t test (* $p < 0.05$).

MyD88 is required for antibody responses in DP6-001/MPLA immunized mice

Endpoint titers of Env-specific IgG was determined by ELISA in sera collected upon termination 7 days after the final protein-adjuvant boost. While MyD88 deficiency did not substantially impact the antibody response induced by DP6-001 formulated with either QS-21 or $Al(OH)_3$, MyD88 deficiency had a partial but significant negative impact on the Env-specific IgG response to DP6-001/MPLA (Fig. 3.6a,b). The vaccine-induced antibody responses were further characterized by IgG isotyping as previously described. As was observed in TLR4 mice immunized with DP6-001 and MPLA adjuvant, there was a moderate but significant reduction in the induction of Th2-associated IgG1 endpoint titers in MyD88 deficient mice as compared to wild type mice (Fig. 3.7a). Furthermore, a more substantial impact on Th1-associated isotypes IgG2b and IgG2c was observed in MyD88 deficient mice immunized with either DP6-001 DNA primed mice or vector primed mice and MPLA (Fig. 3.7b,c). Therefore, as observed in immunization studies with TLR4 deficient mice, it appears that a vaccine-induced Th1-biased adaptive immune response requires intact MyD88 signaling downstream of TLR4 signaling.

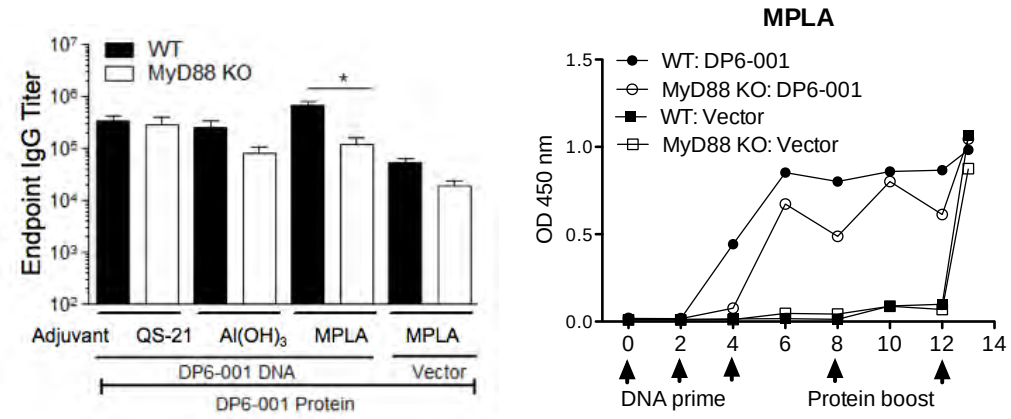
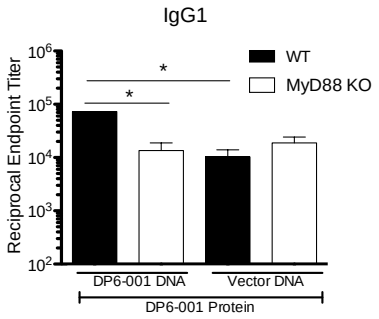
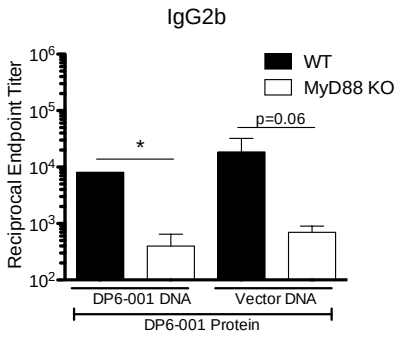


Figure 3.6. Optimal Env-specific IgG antibody responses in MPLA/DP6-001 immunized mice are dependent upon MyD88. Env-specific endpoint IgG titer in wild type (WT) and MyD88-deficient mice immunized with DP6-001 or vector pSW3891 prime followed by DP6-001 protein adjuvanted with QS-21, $Al(OH)_3$ or MPLA. Statistical analysis of peptide stimulation over mock stimulation was calculated by Student's t-test (*: $p < 0.05$).

(a)



(b)



(c)

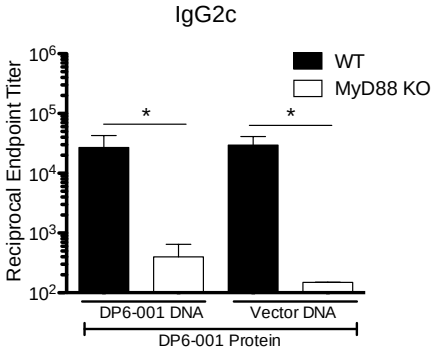


Figure 3.7. **Optimal Env-specific IgG isotype responses in MPL/DP6-001 immunized mice are dependent upon MyD88.** Endpoint IgG isotype titers were determined in terminal sera from wildtype and MyD88-deficient mice by ELISA. (a) IgG1, (b) IgG2b, and (c) IgG2c. Statistical significance was determined by Student's t test (* $p < 0.05$).

MyD88 is required for T-cell mediated IFNγ responses in DP6-001/MPLA immunized mice

As previously described, splenocytes harvested from immunized mice 7 days after the final protein boost were assessed for their ability to generate vaccine-specific T cell responses by ELISpot. Generally, mice immunized with DP6-001 and candidate adjuvants demonstrated modest but positive induction of Env-specific Th1 (IFNγ, IL-2) and Th2 (IL-4, IL-6) cytokines in response to stimulation with overlapping peptide pools representative of gp120. In MyD88 deficient mice, a significant reduction in the Env-specific IFNγ response was observed after immunization with DP6-001 formulated with or MPLA (Fig. 3.8). Control mice designed to focus on MPLA were immunized with empty vector prime and DP6-001 protein-MPLA boosts. The specific induction of IFNγ was much more modest in wild type mice in comparison to DP6-001 DNA primed mice (Fig. 3.8). Furthermore, while it was previously observed that the IFNγ was significantly reduced in TLR4 deficient control mice, there was no change in IFNγ response between MyD88 deficient mice immunized with vector control compared to wild type (Fig. 3.8).

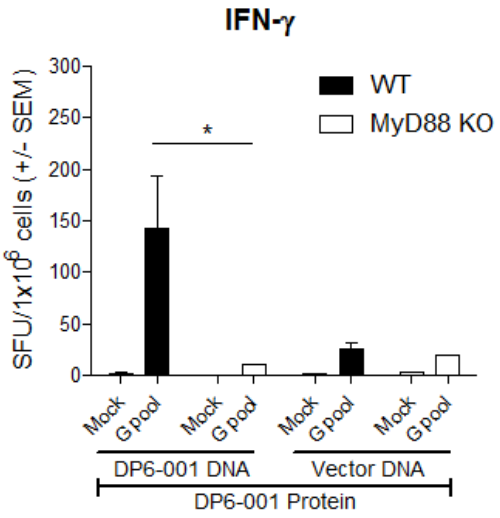


Figure 3.8. T-cell mediated IFNγ response in DP6-001/MPLA immunized mice are dependent upon MyD88. After immunization with DP6-001/MPLA or pSW3891 vector prime and DP6-001 protein boost with MPLA, spleens were harvested at termination 7 days after the final protein boost. Single cell suspensions of splenocytes were seeded into 96-well multiscreen filter plates and stimulated for 18 hours with Env-specific peptides ("G pool") or media alone ("mock"). IFNγ release was measured by ELISpot assay and spots quantified using CTL software. Statistical analysis of peptide stimulation over mock stimulation was calculated by Student's t-test (*: $p < 0.05$).

MyD88 is required for pro-inflammatory responses in DP6-001/MPLA immunized mice following protein boosting

Serum cytokines and chemokines were quantified by multiplex in sera collected from immunized mice 6 hours after each protein-adjuvant group, with a particular focus on analytes associated uniquely with DP6-001/MPLA. As was observed in TLR deficient mice, the characteristic induction of G-CSF by DP6-001/MPLA was reduced after protein-MPLA boosting in immunized MyD88 deficient mice, and this reduction was significant after the second protein boost (Fig. 3.9b). Additionally, serum levels of the inflammatory cytokine IL-6, which was associated with protein boosting in all adjuvant groups, was reduced by MyD88 deficiency (Fig. 3.9c). While a mild reduction was observed in DP6-001 groups adjuvanted by QS-21, this reduction was significant in mice immunized with DP6-001 protein boosts formulated with MPLA.

In contrast to these observations, the chemokine RANTES, which has previously been shown to be associated with DP6-001/MPLA vaccination and to also require intact TLR4 signaling for robust serum levels, was unchanged in MyD88 deficient mice in comparison to wild type mice (Fig. 3.9a). Similarly, while TLR4 deficiency had a significant negative impact on the pro-inflammatory chemokines MIP-1α, MIP-1β, and MCP-1 associated with DP6-001/MPLA immunization, the macrophage inflammatory proteins MIP-1α and MIP-1β were unchanged in MyD88 deficient mice compared to wild type (data not shown). With a focus on the DP6-001/MPLA, specifically, we identified a dependency of the adjuvant profile on TLR4, but only a partial dependency on intact signaling downstream of TLR4 via MyD88.

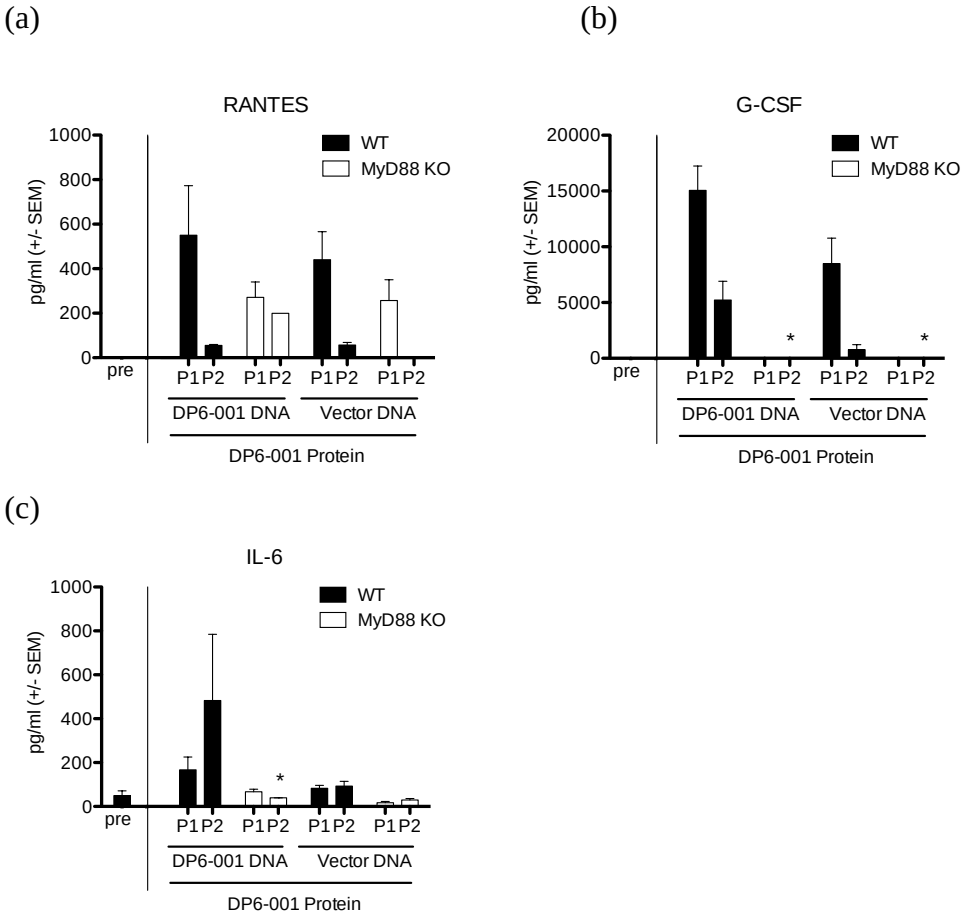


Figure 3.9. Optimal induction of pro-inflammatory cytokines requires MyD88 in DP6/MPLA-immunized mice. Sera were collected pre-immunization and 6 hours following protein-adjuvant boosts. Cytokines were quantified in the serum of individual mice at a 1:4 dilution using a custom 12-plex Luminex panel. Shown are significant decreases in chemoattractants and proliferative factors after protein boosting in MyD88 deficient mice immunized with DP6-001 adjuvanted with MPLA: (a) RANTES, (b) G-CSF, and (c) IL-6. Statistical significance was determined by Student's t test (* $p < 0.05$).

Discussion

In the current chapter, we describe the dependence of adaptive and innate immune responses to vaccination with DP6-001 and candidate adjuvants on intact signaling via TLR4 or the adaptor MyD88. Specifically, we focused on the recently approved MPLA adjuvant, in the unique context of DP6-001 prime-boost vaccine. It is well established that MPLA depends on TLR4 signaling for its adjuvanticity, like its parent molecule LPS. However, it remains unclear the dependency of both induction of pro-inflammatory serum cytokines as well as adaptive immune responses on multiple signaling pathways downstream of TLR4 activation. Furthermore, DNA priming provides a unique environment for subsequent immunizations with antigen and adjuvant. The studies described herein aim to highlight the impact of DNA priming on the innate signaling pathways utilized by MPLA and its immunogenicity in the context of DP6-001.

As previously described, while MPLA and LPS both signal via TLR4 and both potently induce immune responses, MPLA is less inflammatory and demonstrates reduced pro-inflammatory cytokines in comparison to LPS (28, 29, 115, 123, 136, 175). Reports based on a model of OVA protein vaccine formulated with MPLA attribute the reduced inflammatory response of MPLA to biased signaling downstream of TLR4. Mata-Haro *et al.* indicate that MPLA activates TRIF-biased signaling, resulting in the production of TRIF-associated serum cytokines after immunization, including G-CSF, RANTES and MCP-1. Conversely, immunization with LPS results in increased production of MyD88-associated IFNγ, IL-1β, IL-6, and MIP-1α (115). Making a clinically relevant link between innate and adaptive immune responses to vaccination,

they utilized transgenic OVA-specific mice in order to demonstrate that OVA-specific T cell priming required intact TRIF signaling but not MyD88. These reports suggest that MPLA only weakly activates MyD88-dependent pathways downstream of TLR4 (115). This would appear to be supported by reports that MPLA results in decreased production of pro-inflammatory cytokines indicative of MyD88 signaling, such as IL-1β (29, 123, 136). It was also demonstrated that while MPLA and LPS comparably induce the transcription of pro-IL-1β and pro-IL-18, MPLA fails to induce sufficient MyD88 signaling for expression and assembly of the NLRP3 inflammasome and therefore, secretion of mature pro-inflammatory cytokines (123). Some reports have suggested that this less inflammatory bias of MPLA signaling via TLR4 is due to active inhibition of MyD88 rather than a passive bias of "weak" MyD88 activation (29). Though the mechanism is unclear, the ability of MPLA to harness TLR4 signaling and immunostimulation without the toxic inflammation that characterizes LPS makes it clinically appealing.

In Chapter II, we defined a profile of adaptive and innate immune responses to MPLA formulated with DP6-001 prime-boost vaccine, in terms of antigen-specific antibody and T cell responses as well as non-antigen specific serum cytokines and chemokines induced by protein-MPLA boosting. In the current chapter, we further explored how signaling via TLR4 and the MyD88 adaptor impacts these immune responses to DP6-001 and select adjuvants, with a particular focus on MPLA. We also confirmed that while QS-21 and $Al(OH)_3$ do not appear to activate TLR4, they might to some extent activate MyD88-dependent pathways.

As we expected, total Env-specific IgG antibody responses to either DP6-001 or empty vector primed immunization formulated with MPLA were significantly decreased in TLR4 deficient animals; however, this reduction was only partial. More specifically, analysis of IgG isotypes induced by vaccination with MPLA showed that Th1-associated responses were completely abrogated by TLR4 deficiency, while Th2-associated responses were only partially reduced. Perhaps surprisingly, MyD88 deficiency had a similar impact on Env-specific antibody responses. These results indicate that while intact TLR4 signaling is required for an improved humoral response to DP6-001/MPLA, MyD88 signaling also contributes to the adjuvant effect of MPLA. The overall reduced antibody response observed in the vector prime control with MPLA demonstrates the contribution of both DNA priming and MPLA adjuvant to improved humoral immunity. In contrast to MPLA, we did not observe any impact of either TLR4 or MyD88 deficiency on the humoral response associated with DP6-001 and QS-21 or $Al(OH)_3$.

In parallel with these results, we also noticed a significant contribution of both TLR4 and MyD88 signaling to Env-specific Th1 responses to DP6-001/MPLA as measured by IFNγ ELISpot. In addition, Env-specific IFNγ in splenocytes from mice immunized with DP6-001/QS-21 was significantly reduced in MyD88 deficient mice, suggesting a possible role for MyD88 adaptor in an undefined mechanism of QS-21. Meanwhile, Th2 responses as measured by IL-4 or IL-6 ELISpot were similar between all adjuvant groups, and were not impacted by TLR4 or MyD88 deficiency.

In Chapter II, we established that there were unique profiles of serum cytokine and chemokines induced by each adjuvant and the DP6-001 vaccine. In our studies,

MPLA was characterized by potent induction of G-CSF and RANTES at time points 6 hours after protein-adjuvant boosting. This profile is supported by reports profiling MPLA formulated with OVA antigen (115, 182). We also identified that serum IL-6 was induced following protein immunization with all of our candidate adjuvants, including MPLA. These serum cytokines were more strongly induced by DP6-001/MPLA in comparison to mice primed with empty vector; however, in both cases, serum levels of G-CSF and RANTES were significantly reduced by TLR4 deficiency. Serum levels of IL-6 were also reduced by TLR4 deficiency. In contrast, MyD88 had a less obvious impact on serum cytokine profiles. In mice immunized with DP6-001/MPLA, both G-CSF and IL-6 were significantly reduced in the absence of MyD88; however, RANTES was unchanged between wild type and MyD88 deficient mice. Notably, MyD88 deficiency did not have a significant impact on the sera cytokine profiles of DP6-001 vaccine formulated with either QS-21 or $Al(OH)_3$.

Altogether, our results are supportive of the dependence of MPLA adjuvanticity on intact TLR4 signaling. Furthermore, these results demonstrate that both the vaccine-specific adaptive immunity and early pro-inflammatory cytokine profiles associated with MPLA are not independent of MyD88 signaling. Finally, induction of chemokines G-CSF and RANTES, and the pro-inflammatory cytokine IL-6, by MPLA were enhanced by a DNA primed vaccine strategy. G-CSF is involved in the production of granulocytes, as well as the survival, proliferation, and function of neutrophils. RANTES serves as a chemoattractant recruiting leukocytes, eosinophils and basophils to sites of inflammation and also contributing to NK cell activation. The induction of these two broadly acting

chemokines shortly after protein-MPLA boosting suggests an inflammatory response characterized by recruitment of a diverse population of immune cells. Evaluation of cellular infiltrates by histology and flow cytometry associated with subcutaneous immunization with MPLA indicated a rapid infiltration of neutrophils within 12 hours (182). In addition, initial reduction in circulating lymphocytes and subsequent recruitment to draining lymph nodes were also observed after MPLA administration (182). Gene expression studies indicate that MPLA, given intraperitoneally, supports a diverse microenvironment characterized by Th1 and pro-inflammatory cytokines, as well as chemoattractants for granulocytes and monocytes, and immune cell infiltrate predominated by APCs, neutrophils, and NK cells (93). The consistent trend of the induction of G-CSF, RANTES, and IL-6 shortly after MPLA administration formulated with a variety of antigens is supportive of the rapid and diverse recruitment of immune cells, which serve to support and enhance adaptive immune responses (115, 182).

The activation of pattern-recognition receptors such as TLR4 on DCs and other APCs at the injection site contributes to the adjuvant effect of MPLA, by increasing local inflammation. This response influences the recruitment of lymphocytes and other inflammatory cells that are responsible for the uptake and presentation of antigen for the generation of adaptive immunity. In this chapter, we have demonstrated the induction of broadly acting cytokines and chemokines (IL-6, RANTES, and G-CSF) associated with DP6-001/MPLA, consistent with other reports. The induction of this non-antigen specific inflammatory response is enhanced in subsequent immunizations by a DNA primed environment. This inflammatory response to MPLA is dependent on intact

signaling via TLR4 as well as the adaptor MyD88. Therefore, not only the adaptive T cell and antibody responses, but also the innate inflammation induced by MPLA adjuvant, involves TLR4 and MyD88 signaling.

Preface to CHAPTER IV

R. Buglione-Corbett and K. Pouliot contributed equally to animal work, including immunizations, bleeding, and termination

R. Buglione-Corbett and K. Pouliot contributed equally to Luminex experiments and data analysis, Fig. 4.8

Terminal assays performed on immunized mice were performed collaboratively:

K. Pouliot performed early CBA experiments and data analysis, Fig. 4.2, 4.4, 4.6

R. Buglione-Corbett performed antibody studies and data analysis, Fig. 4.3 4.5, 4.7

R. Buglione-Corbett performed rabbit immunizations, bleeding, and antibody studies with the assistance of technicians in the Lu laboratory, Fig. 4.9, 4.10

R. Buglione-Corbett and S. Lu edited figures for the manuscript.

R. Buglione-Corbett, S. Lu, K. Pouliot, and E. Lien prepared the manuscript.

CHAPTER IV:

Potent adjuvant effects of ISCOMATRIX™ adjuvant as part of a DNA prime-protein boost HIV-1 vaccine formulation

Introduction

Effective prophylactic vaccination against Human Immunodeficiency Virus (HIV-1) requires induction of a diverse and specific immune response, consisting of neutralizing antibodies specific to viral epitopes, as well as effector $CD4^+$ and $CD8^+$ T cells. Development of a vaccine with the ability to stimulate both the humoral and cellular arms of the adaptive immune system to produce sterilizing immunity remains elusive. In approaching the complex problem of HIV-1 vaccination, the choice of an adjuvant capable of stimulating both humoral and cellular immune responses is a critical component of a protective vaccine.

'Immunostimulatory complexes' (ISCOMs) were first described as an adjuvant and antigen delivery system by Morein *et al*. in 1984 (126). The ISCOMATRIX™ adjuvant formulation is a particulate adjuvant that consists of cholesterol and dipalmitoyl-sn-phosphatidylcholine (DPCC) in a cage-like matrix, and purified ISCOPREP™ saponin Fraction C derived from *Quillaja saponaria*, with which protein antigen can be formulated. ISCOMATRIX™ adjuvant is easily formulated with a range of antigens by simple mixing. ISCOMATRIX™ adjuvant demonstrates a promising alternative to other saponin adjuvants such as QS-21, which, while potently immunogenic, are associated with hemolytic activity and a range of adverse effects in preclinical and clinical models (85, 99, 129, 130, 183). The formulation of the saponin fraction within this cholesterol-

phospholipid matrix results offers the potential of improved tolerability without sacrificing potency (170, 171). In addition, ISCOMATRIX™ adjuvant is both an effective delivery vehicle for antigen, as well as a strong inducer of an immunomodulatory response. As a vaccine component, ISCOMATRIX™ adjuvant is not only formulated with antigen by simple mixing, but is also stable for several years when stored at 2-8^{o}C, an advantage for use in large scale production and wide distribution of vaccines (141).

The precise mechanisms of many saponin-based adjuvants remain to be elucidated. ISCOMATRIX™ adjuvant effectively facilitates antigen-adjuvant complex uptake by and activation of dendritic cells (DCs) locally and in draining lymph nodes, resulting in cross-presentation to $CD8^{+}$ T cells via MHC I, as well as presentation to $CD4^{+}$ T cells, indirectly contributing to specific B cell function, via MHC II (157, 166, 167). ISCOMATRIX™ adjuvant also stimulates local and systemic induction of pro-inflammatory cytokines and chemokines that play an important role in the recruitment and activation of immune cells such as DCs, B cells, T cells, and NK cells in the DLN (42, 191). Lymphatic cannulation studies have demonstrated that ISCOMATRIX™ adjuvant also aids in the trafficking of antigen-adjuvant complexes to DLNs, temporarily reducing LN cellular output, effectively prolonging presentation of antigen to immune cells (192, 193). ISCOMATRIX™ adjuvant is particularly effective in facilitating the cross-presentation of antigen via MHC I, thus promoting a potent CTL response, a desired quality in the vaccine design against a number of pathogens as well as anti-tumor vaccines. Wilson *et al.* demonstrated that the action of ISCOMATRIX™ adjuvant on

DCs and cross-presentation to $CD8^+$ T cells, as well as vaccine-specific adaptive immune responses, are to some extent dependent on the adaptor protein MyD88 (191).

The integrated delivery and immunomodulatory functions of ISCOMATRIX™ adjuvant result in strong adaptive immune responses. In guinea pigs, using an HIV-1 gp120 antigen, ISCOMATRIX™ adjuvant was demonstrated to allow for protein antigen dose reduction up to 100-fold while enhancing neutralizing antibody responses in comparison to an aluminum-based adjuvant (18). Formulation of protein antigens including HPV16 E6 and E7, and H5N1 influenza hemaglutinin (HA), with ISCOMATRIX™ adjuvant have also demonstrated in small animal models durable specific antibody responses with reduced antigen dose, as compared to formulations with aluminum-based adjuvants (18, 122, 173). The induced antibody response is long lasting, as demonstrated in nonhuman primates receiving hepatitis C virus core protein with ISCOMATRIX™ adjuvant (146).

The broad, multifunctional induction of vaccine-specific immunity associated with ISCOMATRIX™ adjuvant makes it an ideal adjuvant for applications ranging from prophylactic vaccines against viral and intracellular pathogens, to therapeutic vaccines against cancer. Clinically, up to 1600 individuals participating in 16 Phase I clinical trials have received doses of ISCOMATRIX™ adjuvant ranging from 22.5-90 ISCO™ Units, including vaccines for hepatitis C virus (HCV), human papilloma virus (HPV), and influenza, as well as therapeutic cancer vaccines. Rimmelzwaan *et al.* demonstrated the ISCOMATRIX™ adjuvant formulated vaccines produced more rapid immunity to influenza as compared to traditional vaccines (154). A substantial body of work has

utilized the highly immunogenic NY-ESO-1 cancer testis tumor antigen with ISCOMATRIX™ adjuvant in humans, resulting in the induction of broad tumor-specific antibodies, as well as $CD4^+$ and $CD8^+$ T cell responses, which could be correlated to improved clinical outcome (37) and long-term persistent immunity (131). ISCOMATRIX™ adjuvant has also demonstrated therapeutic applications in vaccines for HPV16 in HIV-infected individuals (4), in HPV-associated cervical neoplasia (56), and in individuals chronically infected with HCV (41). ISCOMATRIX™ adjuvant has been well-tolerated in clinical trials, with adverse effects being mild and typically self-limited local reactions (120).

In clinical trials, ISCOMATRIX™ adjuvant has typically been formulated with recombinant protein. The current study focuses on the adjuvant effect of ISCOMATRIX™ adjuvant in the unique context of a previously described polyvalent HIV-1 gp120 DNA prime-protein boost construct, DP6-001. We have previously demonstrated the immunogenicity of DP6-001 in human volunteers, with the induction of Env-specific antibody responses with neutralizing ability, as well as induction of vaccine-specific T cell responses (7, 186). The protein boost component of DP6-001 was formulated with the saponin adjuvant QS-21 in preclinical and clinical evaluations. As a saponin-based complex with reduced reactogenicity but uncompromised potency, ISCOMATRIX™ adjuvant is a promising alternative to QS-21. In addition, protective immunity against HIV-1 infection requires the action of both the humoral and cellular arms of the adaptive immune response, and so a broadly acting integrated adjuvant

system like ISCOMATRIX™ adjuvant is an obvious choice for inclusion in future Env DNA prime-protein boost vaccine formulations.

In the current study, we have demonstrated the efficacy of a the DP6-001 gp120 DNA prime-protein boost formulated with ISCOMATRIX™ adjuvant in generating a robust antibody response in both Balb/c and C57Bl/6 mice, and New Zealand White rabbits, in comparison to DP6-001 formulated with aluminum hydroxide gel adjuvant, $Al(OH)_3$. Improved strength and breadth of neutralizing antibodies was demonstrated in rabbits receiving ISCOMATRIX™ adjuvant. Serum cytokines following each protein-adjuvant immunization were quantified in mice, demonstrating potent induction of Th1 and Th2 cytokines by DP6-001 formulated with ISCOMATRIX™ adjuvant. Immunization of MyD88 deficient mice with DP6-001 DNA prime and protein-ISCOMATRIX™ adjuvant boost demonstrated a partial albeit incomplete reduction in humoral and serum cytokine responses, consistent with a role for MyD88 in the adjuvant activity of ISCOMATRIX™ adjuvant. Our data also suggests that DNA priming itself may impact the dependence of an ISCOMATRIX™ adjuvant-based vaccine on MyD88 signaling. To our knowledge, this is the first study of the adjuvanticity of ISCOMATRIX™ adjuvant in the context of a DNA prime-protein boost vaccine construct utilizing an HIV-1 Env gp120 antigen.

Results

Vaccine-induced temporal serum cytokine profiles following protein-adjuvant boost in Balb/c wildtype mice

Wildtype Balb/c mice received three DP6-001 DNA followed by DP6-001 protein boosts adjuvanted by either ISCOMATRIX™ adjuvant or our control adjuvant $Al(OH)_3$. Control animals received an empty vector DNA prime followed by a protein boost with ISCOMATRIX™ adjuvant. Naïve animals received a saline injection. Sera were collected at two-week intervals and following each immunization, as well as one week following final protein boost, according to Figure 4.1. Serum cytokines were quantified by Cytometric Bead Array (CBA) in samples collected pre-immunization, at 6 hours following each protein boost, and upon termination at 7 days after the final protein boost. All immunized and naïve animals demonstrated background levels of the measured serum cytokines prior to immunization and upon termination at week 13, with the strongest levels of serum cytokine production measured following each protein boost.

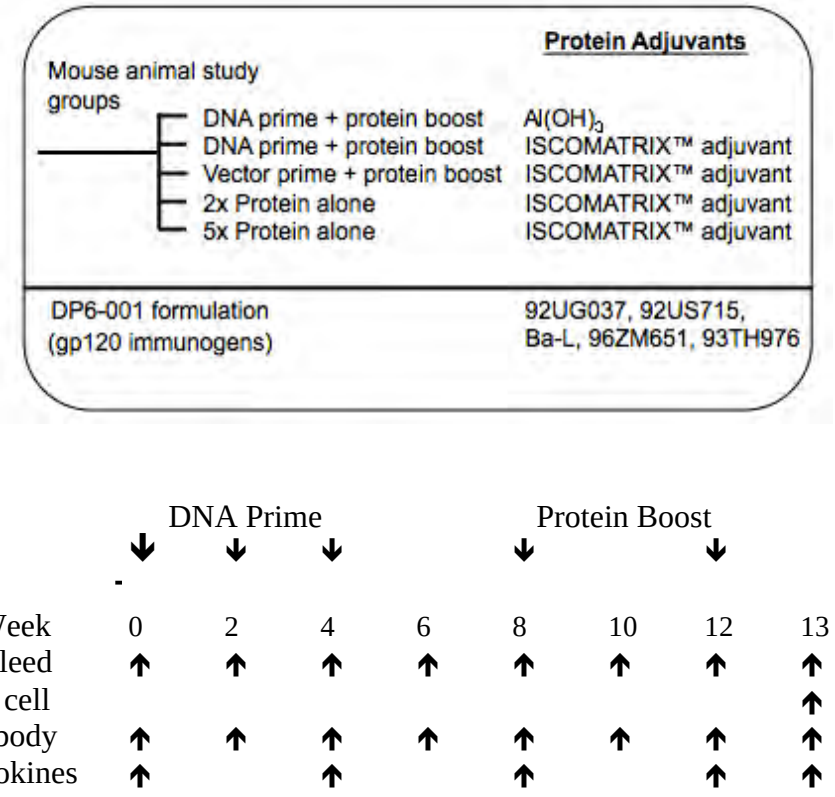


Figure 4.1. Study design and immunization schedule. Mice were immunized with three pentavalent DNA primes followed by two heterologous gp120 protein boosts. The pentavalent vaccine mixture of both DNA and protein components consisted of HIV-1 Env from clades A (92UG037.8), B (92US715.6 and Bal), C (96ZM651), and E (93TH976.17). As a control, mice were immunized with empty pSW3891 vector primes followed by DP6-001 protein and adjuvant boosts. DNA and protein doses indicated are for a total of five immunogens at each immunization. Two adjuvants, $Al(OH)_3$ or ISCOMATRIX™ adjuvant were tested individually as part of the protein boost. Time points of immunizations, and sample collections for different assays were indicated. (IMX = ISCOMATRIX™ adjuvant)

Mice immunized with DP6-001/ISCOMATRIX™ adjuvant produced a more diverse and Th1/Th2 balanced serum cytokine profile following each protein boosting as compared to animals immunized with D6-001/$Al(OH)_3$, or with vector prime and DP6-001 protein boost formulated with ISCOMATRIX™ adjuvant (Fig. 4.2). Immunization with DP6-001/ISCOMATRIX™ adjuvant induced significantly elevated serum levels of Th1 cytokines IFNγ (Fig. 4.2a) and IL-2 (Fig. 4.2b), as well as the Th2-associated cytokine IL-6 (Fig. 4.2c). Notably, all three serum cytokines were more prominent at 6 hours following the first protein boost versus the second boost. Conversely, DP6-001/$Al(OH)_3$ resulted in low positive serum cytokine responses. Priming with empty vector as opposed to DP6-001 DNA resulted in background levels of Th1 serum cytokines. Serum levels of IL-6 were also reduced in vector-primed animals boosted with DP6-001 protein formulated with ISCOMATRIX™ adjuvant, but remained moderately positive compared to background (Fig. 4.2c).

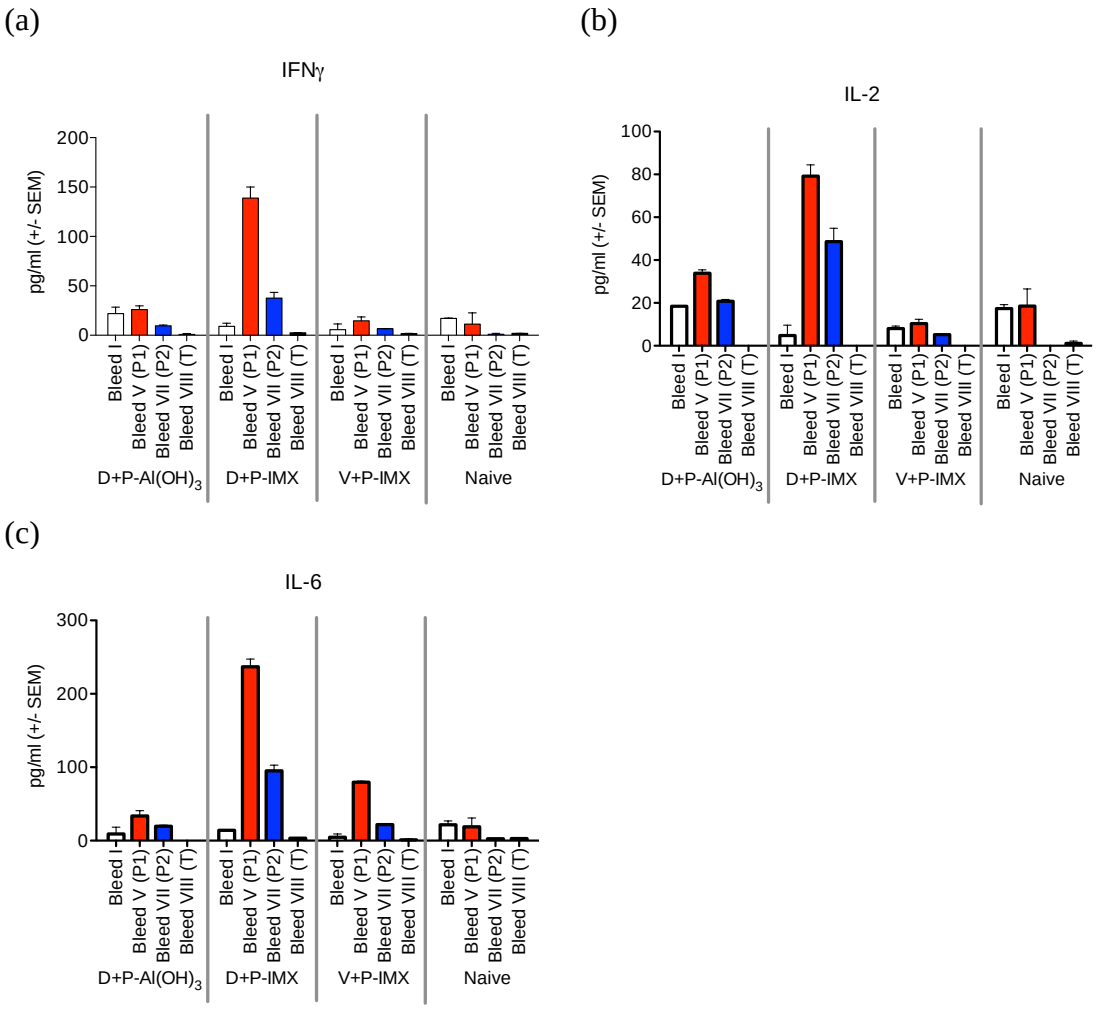
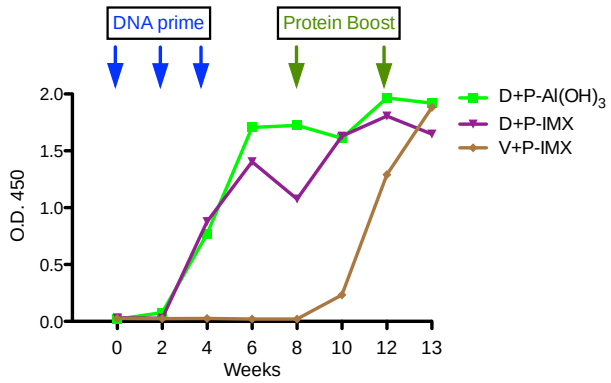


Figure 4.2. Temporal induction of Th1 and Th2 serum cytokines in wildtype Balb/c mice. Wildtype Balb/c mice were immunized with DP6-001 and $Al(OH)_3$ (D+P-$Al(OH)_3$) or ISCOMATRIX™ adjuvant (D+P-IMX), or with an empty vector DNA prime followed by protein boost formulated with ISCOMATRIX™ adjuvant (V+P-IMX). Naïve mice received saline injections. Sera were collected pre-immunization (Bleed I), 6 hours following the first (Bleed V 'P1') and second (Bleed VII 'P2') protein-adjuvant boost, and at termination 7 days after the final protein boost (Bleed VIII 'T'). Levels of serum cytokines in individual mice were measured by CBA. Sera were evaluated for levels of Th1 cytokines (a) IFNγ, (b) IL-2, or (c) the Th2-associated cytokine IL-6. (IMX = ISCOMATRIX™ adjuvant)

Induction of Env-specific IgG antibody responses in Balb/c wildtype mice

We have previously reported robust antibody responses to immunization with DP6-001 formulated with QS-21 in small animals, non-human primates, and clinical settings (139, 186). In wildtype Balb/c mice, we did not observe a significant difference in the end-point titer of Env-specific IgG responses at one week following the final protein boost in mice immunized with DP6-001 and either $Al(OH)_3$ or ISCOMATRIX™ adjuvant (Fig. 4.3b). In addition, the temporal course of antibody induction was similar in immunized mice regardless of adjuvant (Fig. 4.3a). Mice immunized with empty vector prime and protein-ISCOMATRIX™ adjuvant boost also demonstrated comparable Env-specific endpoint IgG to mice receiving the full DNA prime. While Env-specific IgG levels in vector-primed mice remained low compared to DP6-001 DNA-primed mice during initial immunizations, antibody levels rose sharply following the first and second protein boosts to reach levels comparable to those seen in animals immunized with the complete DP6-001 prime-boost vaccine.

(a)



(b)

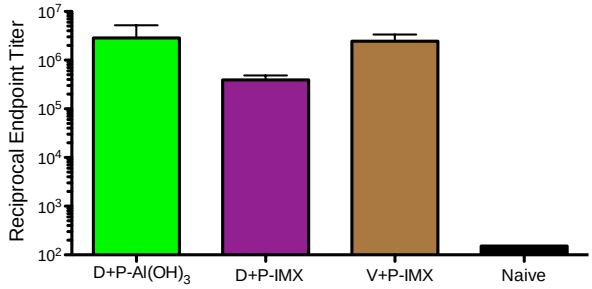


Figure 4.3. Env-specific IgG response in wildtype Balb/c mice immunized with DP6-001 vaccine with different adjuvants. Total gp120-specific IgG was measured by ELISA in sera collected from immunized Balb/c mice 7 days after final protein boost, in week 13. Protein boosts were adjuvanted by $Al(OH)_3$ (green), or ISCOMATRIX™ adjuvant (purple). Control mice were immunized with empty vector prime followed by DP6-001 protein boost formulated with ISCOMATRIX™ adjuvant (brown). Naïve mice (black) received "mock" saline injections in lieu of immunization. (a) Temporal gp120-specific IgG response was determined by ELISA using pooled sera samples from each group collected at two-week intervals. (b) Env-specific endpoint IgG titer in was determined by ELISA using individual serum samples collected in week 13.

Vaccine-induced serum cytokines following protein-adjuvant boost in C57Bl/6 wildtype mice

C57Bl/6 wildtype mice were immunized according to Figure 1, and sera was collected prior to immunization, 6 hours after each protein boost, and 7 days after final protein boost at termination. Serum cytokine levels were quantified by CBA. As observed in Balb/c wildtype mice, the highest cytokine levels were measured at 6 hours following the first protein boost, with reduced levels following the second protein boost (Fig. 4.4). At termination, serum cytokine levels were at background levels. Animals immunized with DP6-001 and either $Al(OH)_3$ or ISCOMATRIX™ adjuvant demonstrated comparable levels of Th1 cytokines IFNγ (Fig. 4.4a) and IL-2 (Fig. 4.4b) following the first protein boost. IFNγ levels dropped in both groups to background levels by the second protein boost. Animals immunized with DP6-001/ISCOMATRIX™ adjuvant demonstrated a more durable IL-2 response through the second protein boost as compared to animals receiving DP6-001/$Al(OH)_3$. Serum IL-6 was more prominently induced following each protein boost in animals immunized with DP6-001/ISCOMATRIX™ adjuvant as compared to the low positive response observed in mice receiving DP6-001/$Al(OH)_3$ (Fig. 4.4c). Generally, serum cytokine responses in mice immunized with vector prime and protein-ISCOMATRIX™ adjuvant boost were low or at background levels compared to animals immunized with the complete prime-boost regimen. Serum IL-6 was moderately elevated in vector-primed animals only after the second protein boost before falling again to baseline levels (Fig. 4.4c).

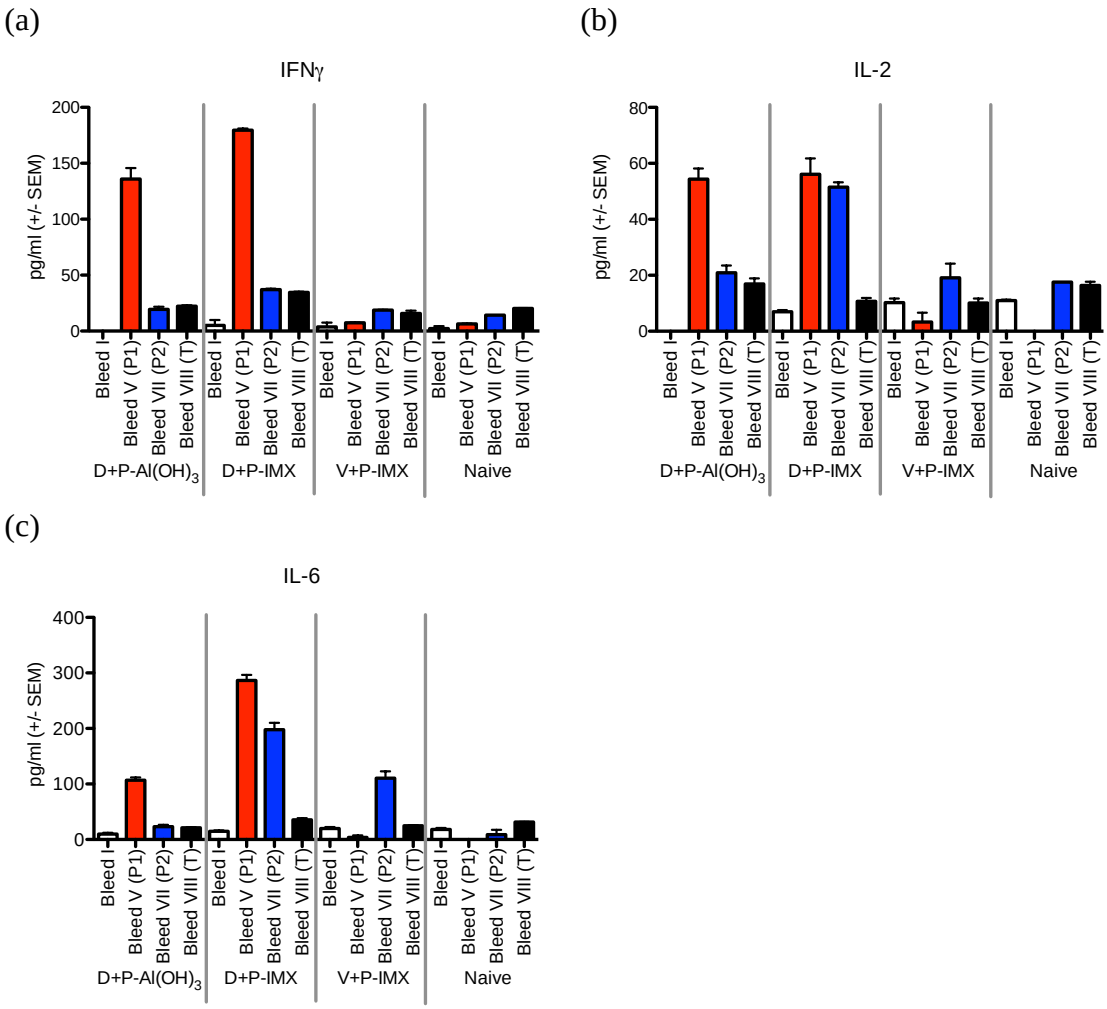


Figure 4.4. Temporal induction of Th1 and Th2 serum cytokines in wildtype C57Bl/6 mice. Wildtype C57Bl/6 mice were immunized with DP6-001 and $Al(OH)_3$ (D+P-$Al(OH)_3$) or ISCOMATRIX™ adjuvant (D+P-IMX), or with an empty vector DNA prime followed by protein boost formulated with ISCOMATRIX™ adjuvant (V+P-IMX). Naïve mice received saline injections. Sera were collected pre-immunization (Bleed I), 6 hours following the first (Bleed V 'P1') and second (Bleed VII 'P2') protein-adjuvant boost, and at termination 7 days after the final protein boost (Bleed VIII 'T'). Levels of serum cytokines in individual mice were measured by CBA. Sera were evaluated for levels of Th1 cytokines (a) IFNγ, (b) IL-2, or (c) the Th2-associated cytokine IL-6. (IMX = ISCOMATRIX™ adjuvant)

Induction of Env-specific IgG antibody responses in C57Bl/6 wildtype and MyD88 deficient mice

As we observed in Balb/c mice, there was no significant difference between immunization groups in the Env-specific IgG endpoint titers (Fig. 4.5b). In addition, the temporal course of specific IgG induction was relatively similar between mice immunized with DP6-001 and either $Al(OH)_3$ or ISCOMATRIX™ adjuvant (Fig. 4.5a). In mice immunized with vector prime and protein-ISCOMATRIX™ adjuvant boost, Env-specific IgG titers were low during priming immunizations, and following protein boosts rose to levels comparable to mice immunized with the full DP6-001 vaccine regimen (Fig. 4.5a). In MyD88 deficient mice immunized with DP6-001/$Al(OH)_3$, the final titer of Env-specific IgG was significantly decreased as compared to wildtype (Fig. 4.5d). While slight reductions in IgG titers were observed in MyD88 deficient mice immunized with either DP6-001/ISCOMATRIX™ adjuvant or vector prime and protein-ISCOMATRIX™ adjuvant boost, this difference was not significant.

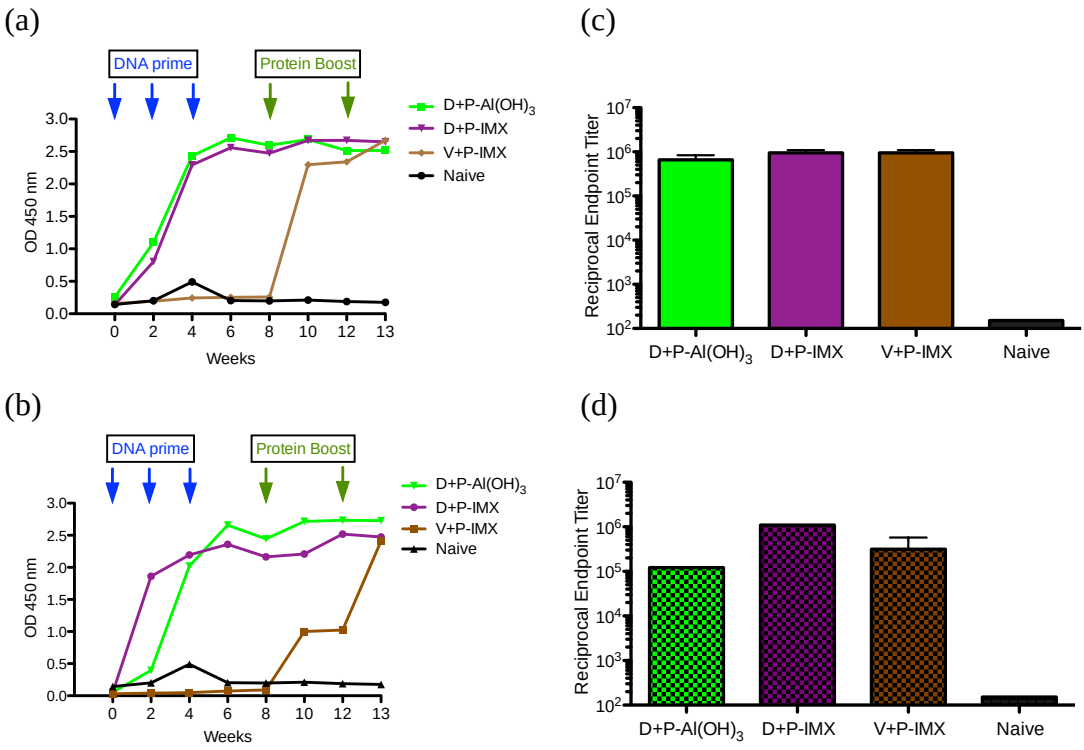


Figure 4.5. Env-specific IgG response in wildtype C57Bl/6 mice immunized with DP6-001 vaccine with different adjuvants. Total Env-specific IgG was measured by ELISA in sera collected from immunized C57Bl/6 mice 7 days after final protein boost, in week 13. Protein boosts were adjuvanted by $Al(OH)_3$ (green), or ISCOMATRIX™ adjuvant (purple). Control mice were immunized with empty vector prime followed by DP6-001 protein boost formulated with ISCOMATRIX™ adjuvant (brown). Naïve mice (black) received "mock" saline injections in lieu of immunization. Temporal Env-specific IgG response was determined by ELISA using pooled sera samples from each group collected at two-week intervals from immunized (a) wildtype or (b) MyD88 deficient mice. Env-specific endpoint IgG titer was determined by ELISA using individual serum samples collected in week 13 from immunized (c) wildtype or (d) MyD88 deficient mice. (IMX = ISCOMATRIX™ adjuvant)

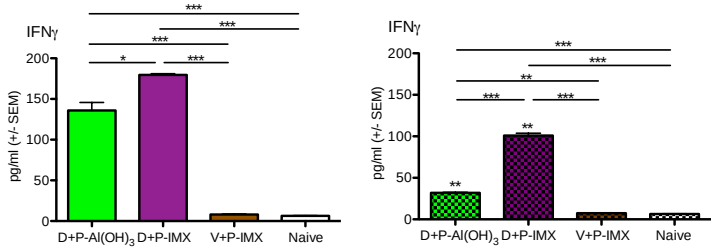
Serum cytokine profiles of DP6-001 and adjuvants in C57Bl/6 wildtype and MyD88 deficient mice by CBA

Pro-inflammatory serum cytokines were quantified by CBA in wildtype C57Bl/6 and MyD88 deficient mice at 6 hours following the first protein boost (Fig. 4.6). The first protein boost time point was identified in earlier assays to demonstrate the highest levels of serum cytokines. Animals immunized with DP6-001/ISCOMATRIX™ adjuvant demonstrated significantly elevated IFNγ (Fig. 4.6a), TNFα (Fig. 4.6c), and IL-6 (Fig. 4.6d) as compared to animals immunized with DP6-001/$Al(OH)_3$ or vector prime and protein-ISCOMATRIX™ adjuvant boost. Notably, in MyD88 deficient mice immunized with DP6-001/ISCOMATRIX™ adjuvant, serum IFNγ (Fig. 4.6a) and IL-6 (Fig. 4.6d) are significantly reduced as compared to wildtype. In MyD88 deficient mice immunized with DP6-001/$Al(OH)_3$, serum levels of IFNγ (Fig. 4.6a), TNFα (Fig. 4.6c), and IL-6 (Fig. 4.6d) are significantly reduced compared to wildtype. Serum cytokines induced in mice immunized with vector prime and protein-ISCOMATRIX™ adjuvant boost were typically low or at background levels in comparison to DP6-001/ISCOMATRIX™ adjuvant. However, serum TNFα associated with vector prime and protein-ISCOMATRIX™ adjuvant boost was significantly elevated above background, though at significantly lower levels than those observed in DP6-001/ISCOMATRIX™ adjuvant groups (Fig. 4.6c). In vector primed mice receiving a protein-ISCOMATRIX™ adjuvant boost, serum TNFα was significantly reduced in MyD88 deficient mice (Fig. 4.6c).

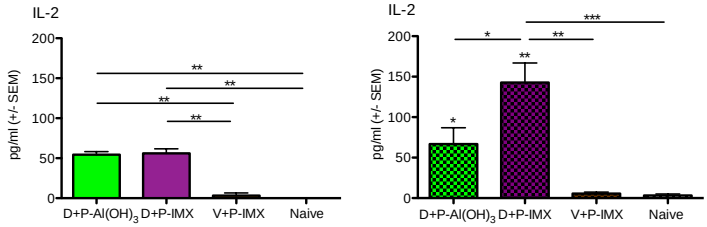
The Th1-associated cytokine IL-2 was comparably elevated in mice immunized with DP6-001/$Al(OH)_3$ or DP6-001/ISCOMATRIX™ adjuvant, but not in mice immunized with vector prime and protein-ISCOMATRIX™ adjuvant boost (Fig. 4.6b). Interestingly, serum IL-2 was significantly greater in MyD88 deficient mice immunized with DP6-001 and either $Al(OH)_3$ or ISCOMATRIX™ adjuvant (Fig. 4.6b). Similar results were observed for the anti-inflammatory cytokine IL-10, which was strongly elevated in MyD88 deficient mice immunized with DP6-001 and either $Al(OH)_3$ or ISCOMATRIX™ adjuvant, as compared to the baseline levels observed in all wildtype mice (Fig. 4.6e). This phenomenon was not observed to such a great extent in mice immunized with a vector prime and DP6-001 protein boost formulated with ISCOMATRIX™ adjuvant.

Figure 4.6. Differential induction of Th1 and Th2 serum cytokines in C57Bl/6 wildtype and MyD88 deficient mice after first protein-adjuvant boost. Wildtype C57Bl/6 mice were immunized with DP6-001 and $Al(OH)_3$ (green) or ISCOMATRIX™ adjuvant (purple), or with an empty vector DNA prime followed by protein boost formulated with ISCOMATRIX™ adjuvant (brown). Naïve mice received saline injections (white). Sera were collected pre-immunization, 6 hours following each protein-adjuvant group, and at termination 7 days after the final protein boost. Shown are levels of serum cytokines in individual mice measured by CBA after first protein-adjuvant boost in wildtype (solid) or MyD88 deficient (checkered) mice. Sera were evaluated for (a) IFNγ, (b) IL-2, (c) TNF, (d) IL-6, or (e) IL-10. Statistical significance was determined by a one-way ANOVA and a Tukey post-test (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). (IMX = ISCOMATRIX™ adjuvant)

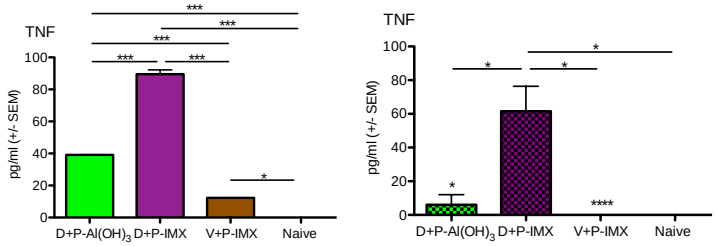
(a)


IFNγ
pg/ml (+/- SEM)
D+P-Al(OH)3
D+P-IMX
V+P-IMX
Naive
IFNγ
pg/ml (+/- SEM)
D+P-Al(OH)3
D+P-IMX
V+P-IMX
Naive

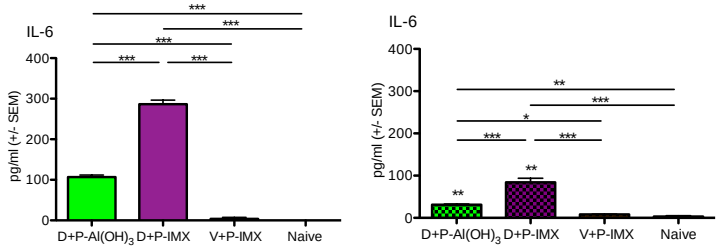
(b)


IL-2
pg/ml (+/- SEM)
D+P-Al(OH)3
D+P-IMX
V+P-IMX
Naive
IL-2
pg/ml (+/- SEM)
D+P-Al(OH)3
D+P-IMX
V+P-IMX
Naive

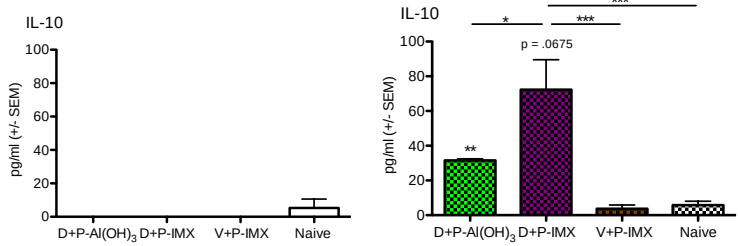
(c)


TNF
pg/ml (+/- SEM)
D+P-Al(OH)3
D+P-IMX
V+P-IMX
Naive
TNF
pg/ml (+/- SEM)
D+P-Al(OH)3
D+P-IMX
V+P-IMX
Naive

(d)


IL-6
pg/ml (+/- SEM)
D+P-Al(OH)3
D+P-IMX
V+P-IMX
Naive
IL-6
pg/ml (+/- SEM)
D+P-Al(OH)3
D+P-IMX
V+P-IMX
Naive

(e)


IL-10
pg/ml (+/- SEM)
D+P-Al(OH)3
D+P-IMX
V+P-IMX
Naive
IL-10
p = .0675
pg/ml (+/- SEM)
D+P-Al(OH)3
D+P-IMX
V+P-IMX
Naive

Impact of DNA priming on antibody and sera cytokine responses in MyD88 deficient mice

In a subsequent study, mice were immunized with two DP6-001 protein boosts and ISCOMATRIX™ adjuvant in the absence of DNA priming. As previously shown in Figure 4.6, we did not observe a significant impact of MyD88 deficient on the Env-specific IgG responses produced in mice immunized with DP6-001/ISCOMATRIX™ adjuvant (Fig. 4.7a, c). These results suggest that MyD88 is minimally involved in the specific humoral response induced by ISCOMATRIX™ adjuvant in the context of a DNA prime-protein boost vaccine. In the context of immunization with DP6-001 protein formulated with ISCOMATRIX™ adjuvant in the absence of DNA priming, a significant decrease in Env-specific IgG titers was observed in MyD88 deficient mice as compared with wildtype (Fig. 4.7b, d). This suggests either the impact of the extended (five) immunizations included in the full DP6-001 regimen, or the impact of DNA priming on the utilization of and dependence on the MyD88 adaptor molecule, and subsequent generation of humoral immunity.

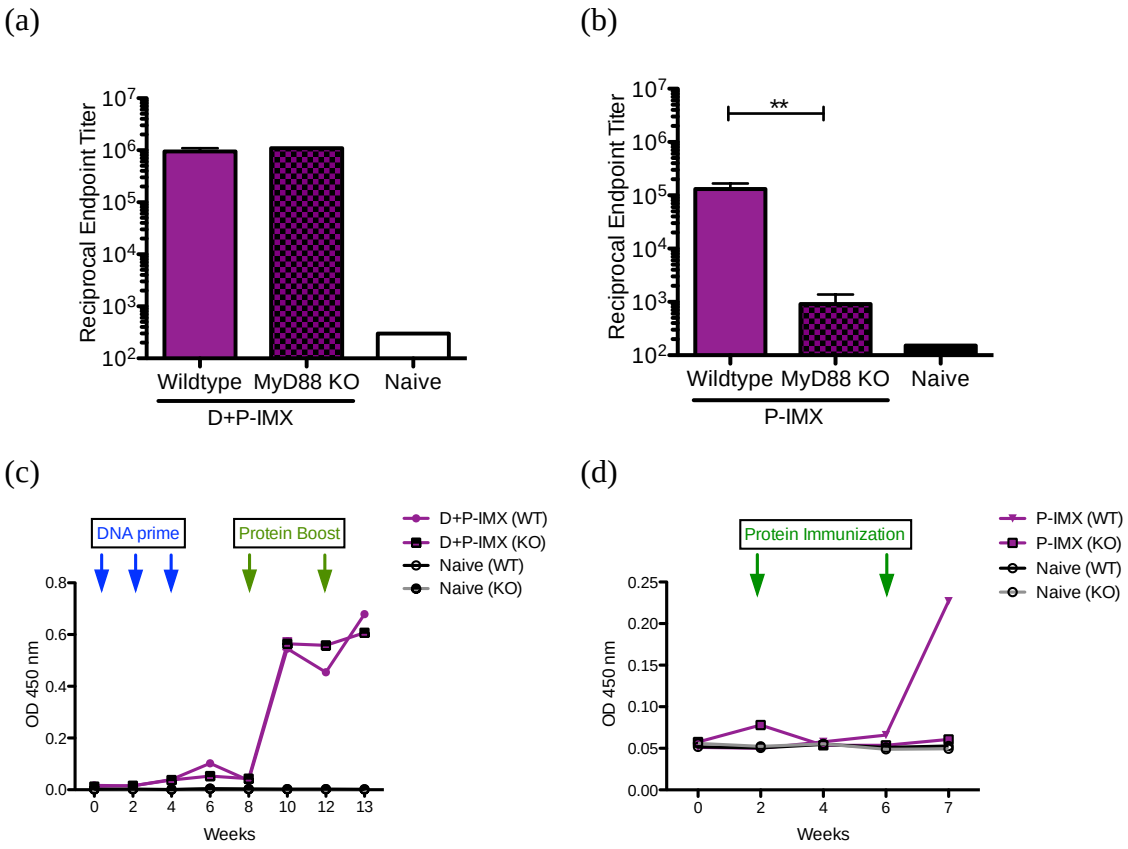


Figure 4.7. DNA priming improves Env-specific IgG response to DP6-001 and ISCOMATRIX™ adjuvant and alters dependency on MyD88. Total Env-specific IgG was measured by ELISA in sera collected from immunized C57Bl/6 mice 7 days after final protein boost, in week 13. DNA-primed wildtype (solid, 'WT') and MyD88 deficient (checkered, 'KO') mice were immunized with DP6-001 DNA prime-protein boost and ISCOMATRIX™ adjuvant (a-b). In the absence of DNA priming, wildtype and MyD88 deficient mice were immunized with two DP6-001 protein boosts and ISCOMATRIX™ adjuvant (c-d). Naïve mice were immunized with saline injections. Env-specific endpoint IgG titer was determined by ELISA on individual serum samples collected 7 days after final protein boost from immunized (a) wildtype or (b) MyD88 deficient mice. Temporal Env-specific IgG response was determined by ELISA using pooled sera samples from each group collected at two-week intervals from immunized (c) wildtype or (d) MyD88 deficient mice. Statistical significance was determined by a student's t test (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). (IMX = ISCOMATRIX™ adjuvant)

Serum cytokines and chemokines were quantified by Luminex array in mice immunized with DP6-001 protein-ISCOMATRIX™ adjuvant in the absence of DNA priming. As we have previously noted, serum cytokines levels were typically most strongly induced in the 6 hours following the first protein immunization. In comparison to mice receiving the complete DP6-001/ISCOMATRIX™ adjuvant vaccine, overall serum cytokines were notably lower in mice following only two protein-ISCOMATRIX™ adjuvant immunizations (Fig. 4.8a, c). Serum KC, G-CSF, and MCP-1, were significantly reduced in MyD88 deficient mice immunized with DP6-001 protein formulated with ISCOMATRIX™ adjuvant as compared to wildtype mice immunize with protein-ISCOMATRIX™ adjuvant vaccine (Fig. 4.8c-d). In comparison, these characteristic cytokines were unchanged in wildtype and MyD88 deficient mice immunized with DP6-001/ISCOMATRIX™ adjuvant (Fig. 4.8a-b). Moderate reductions in serum IL-2, Eotaxin, IL-1β were observed in MyD88 deficient mice only in the absence of any DNA prime (Fig. 4.8c-d).

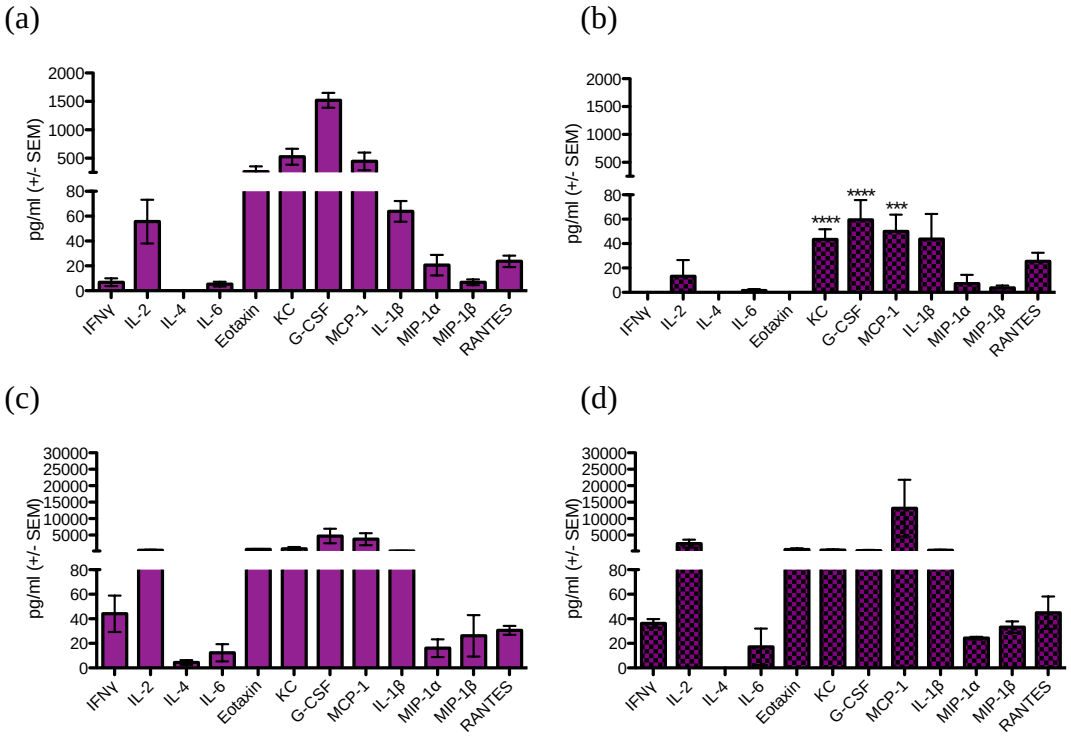


Figure 4.8. Serum cytokine levels after first protein boost in wildtype or MyD88 deficient mice vaccinated with DP6-001 and ISCOMATRIX™ adjuvant in the presence or absence of DNA priming. In the absence of DNA priming, wildtype (solid) and MyD88 deficient (checkered) mice were immunized with two DP6-001 protein boosts and ISCOMATRIX™ adjuvant (a-b). DNA primed wildtype and MyD88 deficient mice were immunized with DP6-001 DNA prime-protein boost and ISCOMATRIX™ adjuvant (c-d). Sera were collected pre-immunization and 6 hours following both protein-adjuvant boosts. Cytokines were quantified in the serum of individual mice at a 1:4 dilution using a custom 12-plex Luminex panel. Shown are serum cytokine panels in (a) wildtype and (b) MyD88 deficient mice immunized with DP6-001 protein formulated with ISCOMATRIX™ adjuvant, or (c) wildtype and (d) MyD88 deficient mice immunized with DP6-001 DNA prime-protein boost formulated with ISCOMATRIX™ adjuvant. Statistical significance was determined with a One-way ANOVA and Tukey post-test (*: $p < .05$, **: $p < .01$, ***: $p < .001$).

DP6-001 formulated with ISCOMATRIX™ adjuvant produced Env-specific IgG and robust neutralizing antibody responses in rabbit

In a study scaled up from mouse immunization studies, White New Zealand rabbits received the DP6-001 DNA prime-protein boost vaccine adjuvanted by either $Al(OH)_3$ or ISCOMATRIX™ adjuvant. Controls rabbits received an empty vector DNA prime followed by a DP6-001 protein boost formulated with ISCOMATRIX™ adjuvant. Naïve animals received saline injections. Sera were collected at biweekly and monthly intervals throughout the immunization schedule. Env-specific endpoint IgG titer was measured in sera collected 7 days following the final protein boost. Rabbits immunized with DP6-001/$Al(OH)_3$ and DP6-001/ISCOMATRIX™ adjuvant demonstrated comparable Env-specific IgG induction by temporal OD, with serum IgG levels increasing slightly during DNA priming (Fig. 4.9a). Both groups demonstrated a notable rise in specific antibody response following first protein boost, and these levels were maintained by subsequent protein boosting. In rabbits immunized with vector prime and protein-ISCOMATRIX™ adjuvant boost, Env-specific IgG in the sera was not detectable until the first protein boost; however, Env-specific IgG was substantially boosted by the second protein-ISCOMATRIX™ adjuvant immunization, rising to levels comparable to rabbits immunized with the complete DP6-001 regimen. End-point titers of Env-specific IgG were not statistically different between immunization groups (Fig. 4.9b).

Previous formulations of the polyvalent Env DP6-001 vaccine in small animal and clinical models were capable of eliciting broadly neutralizing antibody responses. NAb titer, defined as the serum dilution at which it is possible to neutralize 50% of virus

infection, was performed as previously described (178, 179). Immunized rabbit sera were assessed for neutralizing activity against sensitive viral isolates SF162 and ss1196 (Fig. 4.10). No significant difference was observed between immunization groups in neutralizing antibody titers against either sensitive viral isolate. Rabbits receiving DP6-001 and either $Al(OH)_3$ or ISCOMATRIX™ adjuvant, as well as those receiving vector prime followed by DP6-001 protein boost formulated with ISCOMATRIX™ adjuvant, were similarly able to neutralize SF162 (Fig. 4.10a) and ss1196 (Fig. 4.10b).

Notable differences emerged upon further dissecting the quality of vaccine-induced antibody responses. Immunized sera were examined for neutralizing ability against a previously described NIH tier 2 clade B standardized panel of 12 primary viral isolates (98) (Fig. 4.10c). Sera from rabbits immunized with either DP6-001/$Al(OH)_3$ or vector prime and protein-ISCOMATRIX™ adjuvant boost failed to elicit greater than 5% of positive neutralizing events against the less sensitive Tier 2 panel. Rabbits immunized with DP6-001/ISCOMATRIX™ adjuvant demonstrated a much broader neutralizing response, averaging 20% positive neutralizing events against Tier 2 viral isolates (Fig. 4.10c). These results identify the importance of both DNA priming as well as choice of adjuvant in inducing an effective, broad and potent neutralizing antibody response against HIV-1.

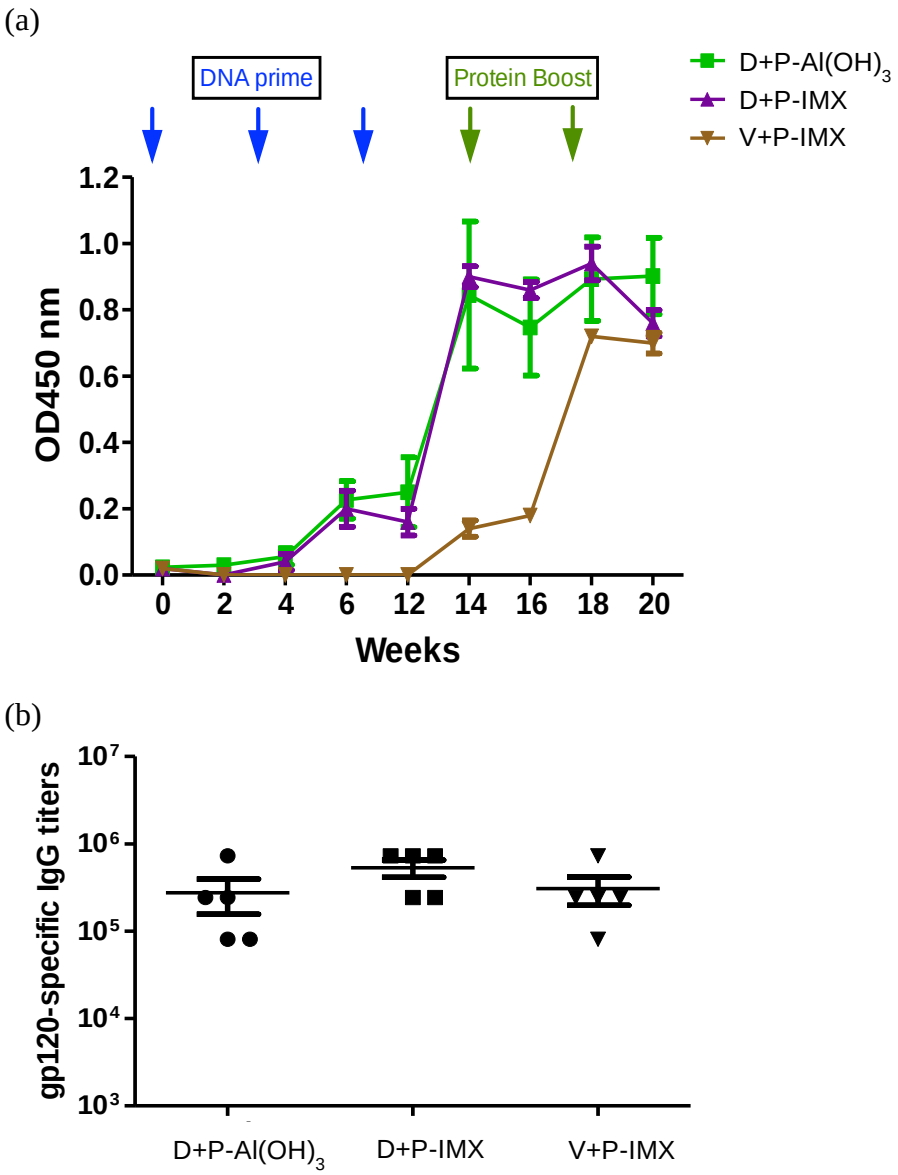


Figure 4.9. HIV-1 Env-specific induction of total IgG in rabbits immunized with DP6-001 and ISCOMATRIX™ adjuvant. Rabbits were immunized with DP6-001 DNA prime-protein boost and either $Al(OH)_3$ (D+P-$Al(OH)_3$) or ISCOMATRIX™ adjuvant (D+P-IMX). As a control, rabbits were immunized with empty vector DNA prime followed by DP6-001 protein boosts formulated with ISCOMATRIX™ adjuvant (V+P-IMX). Naïve animals received saline injection in lieu of immunization (black). (a) Temporal Env-specific IgG response was determined by ELISA using pooled sera samples from each group collected at two-week intervals from immunized rabbits. (b) Env-specific endpoint IgG titer was determined by ELISA using individual serum samples collected in week 20 after the final protein-adjuvant boost. (IMX = ISCOMATRIX™ adjuvant)

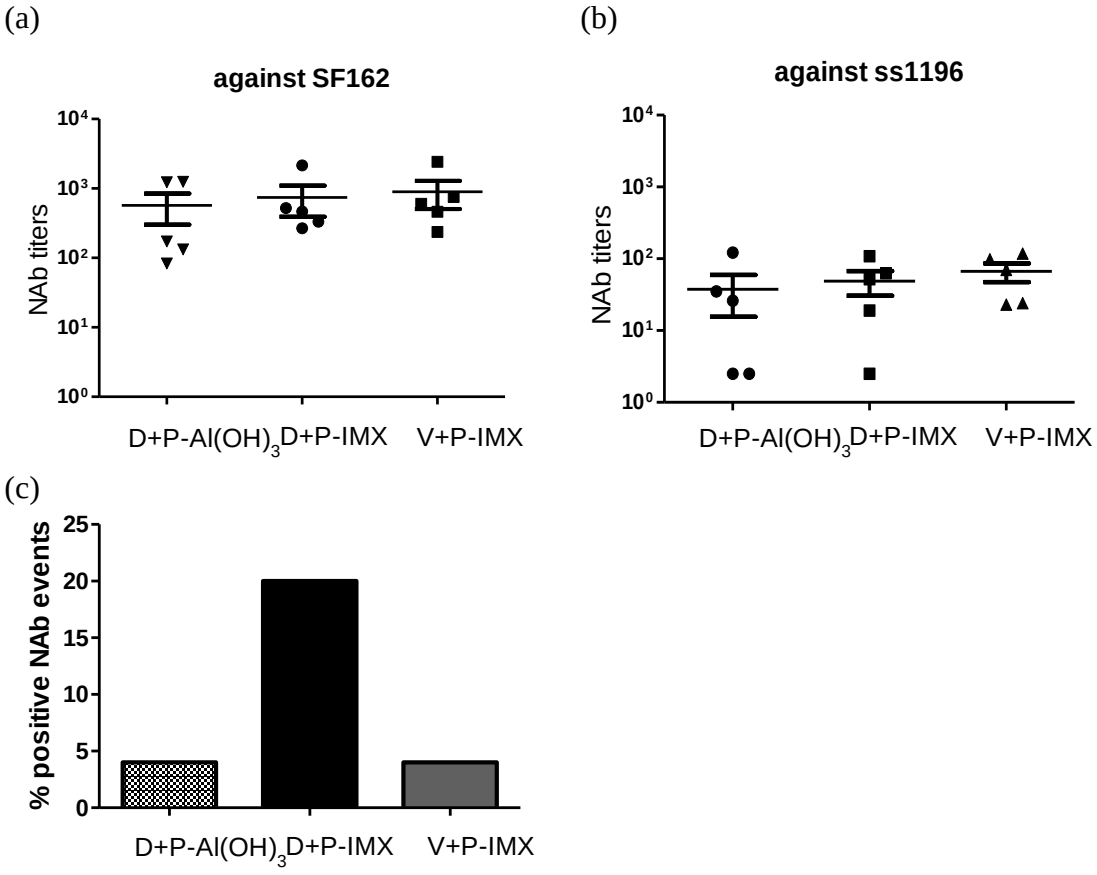


Figure 4.10. ISCOMATRIX™ adjuvant improves breadth of neutralizing antibody response in rabbits immunized with DP6-001. Rabbits were immunized with DP6-001 DNA prime-protein boost and either $Al(OH)_3$ (D+P-$Al(OH)_3$) or ISCOMATRIX™ adjuvant (D+P-IMX). Control rabbits were immunized with empty vector DNA prime followed by DP6-001 protein boosts formulated with ISCOMATRIX™ adjuvant (V+P-IMX). NAb titer is defined as the serum dilution at which it is possible to inhibit 50% of viral infection. Neutralizing antibody titers were determined against two Clade B viral isolates sensitive to neutralization: (a) SF162 and (b) ss1196. (c) Neutralization of a previously described Tier 2B panel of 12 primary viruses by serum from rabbits immunized with DP6-001 and different adjuvants. (IMX = ISCOMATRIX™ adjuvant)

Discussion

Protective immunity against complex pathogens such as HIV-1 requires a vaccine that is capable of inducing a diverse and potent immune response consisting of both humoral and cell-mediated immunity. The importance of polyfunctional $CD4^+$ helper T cells, $CD8^+$ T cells, and broad neutralizing antibodies have been clearly defined in the effective prophylaxis against HIV-1 challenge. In several clinical trials, including the widely publicized Thailand RV144 trial and our lab's own trial of the DP6-001 vaccine, a DNA or viral vector prime followed by a protein boost has been employed as an effective and potent strategy to strongly activate both arms of the immune response and confer some level of protective immunity. The choice of adjuvant to be paired with the protein boost component critically impacts the immunogenicity an efficacy of a vaccine.

In recent years, the integrated adjuvant system ISCOMATRIX™ adjuvant has emerged as an ideal candidate for inclusion in future formulations of HIV-1 vaccines, due to its ability to serve as a delivery system for vaccine adjuvant, and also have a substantial and diverse immunomodulatory effect on innate immune cell recruitment and function, and subsequent cross-presentation by dendritic cells and a potent $CD8^+$ T cell response. ISCOMATRIX™ adjuvant-based vaccines have been demonstrated to induce potent humoral and cell-mediated immunity while remaining well tolerated and safe in 16 Phase I trials of prophylactic and therapeutic vaccines.

While ISCOMATRIX™ adjuvant has been clinically evaluated with recombinant protein-based vaccines, and pre-clinically in the context of recombinant protein-based vaccines against HIV-1, the current study is the first report of ISCOMATRIX™ adjuvant

in the context of an HIV-1 Env DNA prime-protein boost vaccine strategy. The primary objectives of this study were to confirm the immunogenicity of ISCOMATRIX™ adjuvant formulated with the previously described DP6-001 in preclinical models, and also identify the impact of DNA priming on an ISCOMATRIX™ adjuvant-based protein boost.

In the current study, we demonstrated in both mouse and rabbit models, the ability of an Env DNA prime-protein boost vaccine formulated with ISCOMATRIX™ to generate strong Env-specific IgG responses comparable to levels associated with DP6-001/$Al(OH)_3$ immunization or vector prime and protein-ISCOMATRIX™ adjuvant boost. In rabbits, vaccine-induced antibodies generated with DP6-001/$Al(OH)_3$, DP6-001/ISCOMATRIX™ adjuvant, or vector prime and protein-ISCOMATRIX™ adjuvant were similarly able to neutralize sensitive HIV-1 viral isolates SF162 and ss1196. The superior immunogenicity of the DP6-001/ISCOMATRIX™ adjuvant vaccine formulation was evident upon more in-depth evaluation of the neutralizing antibody response generated in immunized rabbits. Animals immunized with DP6-001/ISCOMATRIX™ adjuvant were able to generate broadly neutralizing antibodies against 20% of a clade B Tier 2 panel of more resistant viral isolates, while those immunized with DP6-001/$Al(OH)_3$, or with vector prime and protein-ISCOMATRIX™ adjuvant boost in the absence of DNA priming, performed poorly. These data highlight not only the potent immunogenicity of the ISCOMATRIX™ adjuvant, but also the substantial impact of a multiclade DNA prime encoding Env antigen on the breadth and potency of vaccine-induced humoral immunity.

We also evaluated the ability of DP6-001/ISCOMATRIX™ adjuvant vaccine to generate characteristic serum cytokines in comparison to DP6-001/$Al(OH)_3$ vaccine or an empty vector DNA prime. In the current study we identified that 6 hours following the first protein boost yielded the most robust serum cytokine response. In a recent study by Wilson *et al.*, a serum cytokine profile was developed for ISCOMATRIX™ adjuvant 6 hours after subcutaneous administration, with the Th1 and Th2 cytokines, as well as the chemokines G-CSF, KC, and MIP-1α, standing out over background levels (191). Wilson *et al.* correlated this rapid but transient induction of a unique serum cytokine signature with activation of DCs and NKs in the draining lymph node, suggesting that the generation of a potent and unique systemic cytokine response facilitates the innate and adaptive immune response to vaccination with ISCOMATRIX™ adjuvant (191). In concurrent work by our lab, we have defined a profile of serum markers associated with DP6-001/ISCOMATRIX™ adjuvant immunization (Buglione-Corbett *et al.*, unpublished data), including IL-2, MCP-1, Eotaxin, and G-CSF, as well as markers commonly induced by all of our vaccine formulations, IL-6, KC, and MIP-1α. In the current study, we demonstrated DP6-001/$Al(OH)_3$ and, to a greater extent, DP6-001/ISCOMATRIX™ adjuvant, were able to induce strong non-antigen specific Th1 and Th2 serum cytokines following the first protein boost. In these groups, serum cytokine levels typically fell following the second protein-adjuvant boost, and returned to background levels by termination 7 days after the second protein-adjuvant boost.

In contrast, wildtype animals primed with empty vector DNA generated low to background levels of IFNγ, IL-2, and IL-6 following either of two DP6-001 protein-

ISCOMATRIX™ adjuvant boosts. This trend was also observed for TNF and IL-10 in a broader CBA panel focusing on serum cytokine levels 6 hours after the first protein-adjuvant boost only. Similarly, non-antigen specific serum cytokines and chemokines analyzed by multiplex array following only two immunizations with DP6-001 protein formulated with ISCOMATRIX™ adjuvant in absence of DNA priming were reduced across the board in comparison to DNA-primed animals. These results indicate the critical impact of Env DNA priming in the generation of an ISCOMATRIX™ adjuvant-associated potent and diverse inflammatory cytokine profile.

In this study, we also examined the dependence of the DP6-001/ISCOMATRIX™ adjuvant vaccine on the broadly utilized innate immune signaling adaptor, MyD88. In a recent study by Wilson *et al.* utilizing a recombinant protein tumor antigen vaccine formulated with ISCOMATRIX™ adjuvant, MyD88 was found to be essential for adaptive immune responses such as $CD8^+$ T cell responses, NK cell function, and vaccine-specific antibody responses, while other TLR deficiencies did not compromise adaptive immunity. However, MyD88 appeared to be dispensable for activation of dendritic cells and cross-presentation to $CD8^+$ T cells (191). Wilson *et al.* suggest that ISCOMATRIX™ adjuvant produces an indirect effect on innate signaling pathways via the generation of a pro-inflammatory cytokine and chemokine environment, and propose the coexistence of both MyD88-dependent and independent mechanisms of action for the ISCOMATRIX™ adjuvant (191). Our current study of the role of MyD88 in ISCOMATRIX™ adjuvant focused on its impact on adaptive immunity and sera cytokine

profiles, as well as its changing function in the context of a DNA prime-protein boost vaccine strategy.

Env-specific IgG endpoint titers associated with DP6-001/$Al(OH)_3$, DP6-001/ISCOMATRIX™ adjuvant, or vector prime and DP6-001 protein boost formulated with ISCOMATRIX™ adjuvant, were minimally impacted in MyD88 deficient mice. Initial serum cytokine analysis by CBA indicated some reduction in Th1 and Th2 responses associated with DP6-001/ISCOMATRIX™ adjuvant in MyD88 deficient mice, while serum cytokines induced by vector prime and protein-ISCOMATRIX™ adjuvant boost were overall low. An expanded multiplex panel of serum cytokines and chemokines indicated that mice immunized with only DP6-001 protein formulated with ISCOMATRIX™ adjuvant also generated a reduced serum cytokine response in comparison to DP6-001/ISCOMATRIX™ adjuvant immunizations.

Follow-up studies employing immunization with two DP6-001 protein boosts formulated with ISCOMATRIX™ adjuvant, however, indicated a dependence of vaccine-induced humoral responses on MyD88 in the absence of DNA priming. In addition, by a multiplex array, MyD88 deficiency had a much more significant impact on sera cytokine and chemokine profiles in protein-ISCOMATRIX™ adjuvant immunized mice that was observed in DP6-001/ISCOMATRIX™ adjuvant groups. These data highlight the significant impact of a DNA priming component on the innate and adaptive immune responses, showing that DNA primed vaccines generate not only improved humoral immunity and pro-inflammatory response. However, we also demonstrate that the use of a DNA prime reduces the dependence of these responses on MyD88 signaling

in comparison to our protein-only vaccine, or the recombinant protein-based vaccine with ISCOMATRIX™ adjuvant employed by Wilson *et al.* (191).

While an evident but poorly understood role for MyD88 has been identified in the adjuvanticity of ISCOMATRIX™ adjuvant, the mechanism of this integrated adjuvant system remains unclear. Studies of other particulate adjuvants have implicated inflammasome pathways such as NLRP3, either directly or indirectly, in their mechanism of action (169). In addition, recent work by Duewell *et al.* introduced the secretion of mature IL-1β by DCs *in vitro* in response to stimulation with ISCOMATRIX™ adjuvant in a caspase-1-dependent manner, indicating stimulation of any number of caspase-1-dependent inflammasome pathways (42). The further elucidation of the signaling pathways involved in ISCOMATRIX™ adjuvant-induced adaptive immunity, with a particular focus on the unique impact of DNA priming on these mechanisms, are a component of ongoing research by this lab.

In conclusion, the ISCOMATRIX™ adjuvant system is a promising prospect for inclusion in future formulations of HIV-1 Env vaccines. In the context of a DNA prime-protein boost strategy, ISCOMATRIX™ adjuvant was associated with improved neutralizing antibody responses, as well as a potent pro-inflammatory cytokine and chemokine environment that contributes to the NK cell and DC activation, cross-presentation, and potent $CD8^+$ T cell response characteristic of ISCOMATRIX™ adjuvant. The data presented in the current study contributes to our selection of an adjuvant for future optimized HIV-1 Env DNA prime-protein boosts, and also expands

our understanding of both the impact of a DNA prime component and also the underlying mechanisms of ISCOMATRIX™ adjuvant in the context of a novel vaccine strategy.

CHAPTER V:

Materials and Methods

HIV-1 gp120 DNA vaccine

The gp120-expressing DNA vaccine component of the DP6-001 formulation was composed of equal amounts of five plasmids encoding codon-optimized gp120 genes from primary HIV-1 isolates: A (92UG037.8), B (92US715.6), Ba-L, Czm (96ZM651), and E (93TH976.17) in the common vector pSW3891 as previously described (138). DNA vaccine plasmids were grown up in HB101 strain of *E. coli*, and prepared using a Plasmid Giga Kit (Qiagen, Valencia, CA). Endotoxin-free DNA plasmids were prepared using EndoFree Plasmid Mega Kit (Qiagen). A colorimetric test of the endotoxin levels in individual DNA preparations confirmed that EndoFree plasmids were associated with an average of 12.1 EU/ml, while standard DNA plasmid preparations were associated with 1000-fold greater EU/ml (ranging from 1460 EU/ml to greater than 25,000 EU/ml). DNA plasmid expression was confirmed by transient expression in 293T cells and Western blot.

HIV-1 gp120 Protein Production

The protein component of DP6-001 was composed of equal parts of five recombinant gp120 proteins homologous to DNA vaccine components. These gp120 proteins were produced in CHO cell lines by Advanced Bioscience Laboratory, Inc. (ABL) as previously described (186). Final protein product consisted of 7 μg/ per gp120 protein at each immunization, in Dulbecco's phosphate buffered saline (DPBS) (Gibco, Invitrogen, Grand Islands, NY).

Formulation of DP6-001 gp120 Protein With Adjuvants

Protein mixes were formulated prior to immunization with the candidate adjuvants immediately prior to immunization. 35 μg total gp120 protein was simply mixed with 5 μg QS-21 in DPBS. 35 μg total gp120 protein was adsorbed onto 175 μg aluminum hydroxide gel [$Al(OH)_3$ gel] (Sigma Aldrich Corp., St. Louis, MO) by mixing, followed by a 20 minute incubation at room temperature. Protein and $Al(OH)_3$ mixtures were then spun down at 12,000 rpm for 10 minutes, the supernatant was removed, and the pellet was resuspended in the final volume of DPBS for immunization. Stock synthetic MPLA (Avanti Polar Lipids, Inc., Alabaster, AL) was dissolved in 1 ml DMSO, followed by 9 ml dH_2O, and opsonized for complete dissolution. 35 μg total gp120 protein was simply mixed with 25 μg MPLA. 35 μg total gp120 protein was simply mixed in DBPS with 1.5 ISCO™ Units of ISCOMATRIX™ adjuvant (CSL Limited, Parkville, Victoria, Australia). 1.5 ISCO™ Units is equivalent to 1.5 μg of ISCOPREP™ saponin and to allow easier comparison with other adjuvants the μg measurement is used in this dissertation.

Mouse Immunizations

BALB/c (6-8 weeks old, mixed sex) and C57Bl/6 (6-8 weeks old, mixed sex) mice were obtained from Taconic Farms and maintained in Department of Animal Medicine animal facility at University of Massachusetts Medical School, according to an IACUC-approved protocol. Mice received i.m. DNA immunizations of total 120 μg gp120 DNA plasmid, divided between each quadriceps at weeks 0, 2, and 4. Sera were collected at each immunization time point. After the third DNA immunization, sera were collected 6

hours following immunization. Mice were bled at week 6. Mice received protein boosts of 7 μg/mouse per gp120 protein for a total protein immunization of 35 μg at each immunization. Protein boosts were formulated with candidate adjuvants as described above, and administered via two i.m. injections divided between each quadriceps at weeks 8 and 12. Mice were bled multiple times for serum antibody and cytokine analysis (Fig. 1). Mice were terminated 7 days after the final protein boost according to an IACUC approved procedure, at which time sera and spleens were collected.

Rabbit Immunizations

New Zealand White (NZW) rabbits (6-8 weeks) were purchased from Millbrook Farm (Amherst, MA). Rabbits were house in the Department of Animal Medicine animal facility at University of Massachusetts Medical School, according to an IACUC-approved protocol. Rabbits received i.m. DNA immunizations of total 400 μg gp120 DNA plasmid in each lumbar muscle at weeks 0, 2, and 4. Rabbits received i.m. protein boost for of total 100 μg gp120 protein formulated with candidate adjuvants at weeks 8 and 12. Rabbit protein immunizations consisted of 500 μg/rabbit of $Al(OH_3)$ gel (Sigma) or 50 μg/rabbit ISCOMATRIX™ adjuvant (CSL Ltd.). Protein and adjuvant formulations were prepared as described above. 1.5 ISCO™ Units is equivalent to 1.5 μg of ISCOPREP™ saponin and to allow easier comparison with other adjuvants the μg measurement is used in this article. Sera were collected biweekly throughout the immunization schedule.

Enzyme Linked Immunosorbent Assay (ELISA)

gp120-specific antibody responses were assessed by ELISA, performed as previously described with some modifications (179). Briefly, 96-well EIA/RIA microtiter plates (Costar, Corning, NY) were coated with 5 μg/well ConA diluted in PBS for 1 hr. Between each step, plates were washed with PBS and 0.1% Triton X-100 five times using AquaMax2000 automatic plate washer (Molecular Devices, Sunnyvale, CA). Plates were coated with 1 μg/ml of the five recombinant gp120 protein mix used in immunizations. Wells were blocked overnight (4% whey by weight whey dilution buffer and 5% powdered milk) at 4^{o}C. Plates were incubated with 100 μl of serially diluted mouse sera in duplicate for 1 hr. Biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) was added at 1.5 μg/ml for 1 hr. Horseradish-peroxidase (HRP)-conjugated streptavidin (Vector Laboratories) at 0.5 μg/ml was added and incubated for 1 hr. Plates were developed with TMB substrate (Sigma-Aldrich, St. Louis, MO) for 5 minutes, followed by the addition of 2 N H_2SO_4. Optical density (OD) of 450 nm (OD_{450}) minus the background of plate absorbance at 630 nm, was read on a Multiskan FC (Thermo Fischer Scientific, Waltham, MA). The endpoint titer was determined as the highest dilution at which the OD_{450} equaled twice the OD_{450} of negative control wells. Statistically significant differences between titers were analyzed using Student's *t* test.

For temporal antibody responses, pooled mouse sera dilutions of 1:250 or 1:500 were prepared for each collection time point. For mouse IgG isotyping ELISA, standard curve wells were coated with 0.5 μg/ml IgG2c or IgG1 coating antibody (Southern Biotech, Birmingham, AL) at 1:3 serial dilutions from a starting dilution of 1:1000.

Biotin-conjugated IgG2c or IgG1 detection antibody (Southern Biotech) was applied at 0.5 μg/ml. Plates were washed, developed, and endpoint titers were determined as described above. Env-specific antibody ELISAs were performed on rabbit sera as described above.

HIV-1 Neutralization Assay

Neutralization assays on rabbit sera were performed as previously described (124, 178, 179). Briefly, HIV-1 pseudovirus was produced in HEK293T cells by cotransfection with $pSG3^{\Delta env}$ backbone (NIH AIDS Research References and Reagent Program) and a plasmid encoding gp160. Pseudovirus was titered out using TZM-bl cell line. Rabbit sera were incubated for 1 hr at 37°C with 200 $TCID_{50}$ of pseudovirus. This mixture was added to $1x10^5$ TZM-bl cells in 20 μg/ml DEAE Dextran, for 48 hr at 37°C. Plates were developed with luciferase reagent (Promega, Madison, WI). Percent change in luciferase activity in the presence of pre-immune sera compared to in the presence of sera from immunized mice was used to calculate percent neutralization.

Splenocyte Preparation

Spleens were harvested 7 days following the second protein boost. Spleens were homogenized in complete RPMI media, with 10% heat-inactivated FBS (HyClone, Logan, UT), and 1% Penicillin-Streptomycin. Single-cell suspensions were made by pressing each spleen through a screen, and washing with media. Red blood cells were lysed with Red Blood Cell Lysis Buffer (Sigma). Cells were washed, counted, and diluted to a final concentration of $1x10^7$ cells/ml.

Intracellular Cytokine Staining

All fluorophore-conjugated antibodies, unless otherwise noted, were obtained from BD Pharmingen (San Diego, CA). Splenocytes were cultured in 96-well cell culture round-bottom plates (Costar) at $1x10^6$ cells per well. Splenocytes were co-incubated with 2 μg/ml human IL-2, GolgiPlug (BD Biosciences, San Diego, CA), and peptide. Positive controls were stimulated with BD Leukocyte Activating Cocktail (BD Pharmingen, San Diego, CA). Antigen-specific stimulation consisted peptides 8771-8886 from a consensus clade B peptide pool (Cat. No. 9480, NIH AIDS Research & Reference Program, Germantown, MD) covering the region of gp120, composed of 115 15mer peptides overlapping by 11 amino acids each, at an individual peptide concentration of 2 μg/ml. Mock-stimulated splenocytes were treated with media, hIL-2, and GolgiPlug alone. Splenocytes were incubated at 37°C for 5 hr, after which cells were washed in 2% FBS/PBS staining buffer. Non-specific binding was blocked by incubating with 5 μg/ml α-Fcγ R III/II (2.4 G2) antibody (BD Pharmingen) at 4°C for 10 minutes. Cells were washed in staining buffer, and then incubated with LIVE/DEAD Fixable Blue (Invitrogen, Carlsbad, CA). Cells were washed in staining buffer, and then incubated with anti-CD4-Alexa700 and anti-CD8-PerCPCy5.5 at 0.4 μg/ml for 20 minutes at 4°C. Cells were washed in staining buffer, and were then fixed and permeabilized in Cytofix/Cytoperm (BD Biosciences) in the dark at 4°C for 20 minutes. Cells were washed in 1X PermWash (BD Biosciences). Cells were stained with anti-IFNγ-FITC, anti-IL-2-PECy7, anti-IL-4-APC, anti-IL-5-APC, anti-IL-6-PE, and anti-IL-17-Pacific Blue, diluted in 1X Permwash, for 30 minutes at 4°C. Cells were washed in Permwash,

and resuspended in staining buffer. Stained splenocytes were analyzed on an LSRII FACS machine (BD Biosciences, San Jose, CA), and data was analyzed using FlowJo software (Treestar, Ashland, OR).

T Cell ELISpot

ELISpot reagents (IL-2, IL-4 IFNγ) were obtained from Mabtech (Mariemont, OH) or from BD Biosciences (IL-6) (San Diego, CA). ELISpots were performed according to manufacturer's instructions. Pre-coated MSIP PVDF-plates (Millipore, Billerica, MA) were seeded with splenocytes from immunized mice (prepared as above) at a $2.5x10^5$ cells/well. Positive controls were stimulated with 20 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) and 500 ng/ml ionomycin (Sigma-Aldrich). Antigen-specific stimulation was performed with truncated peptide pools derived from clade B consensus Env including the V2/V3 pool (8836-8844) (19) (provided by NIH AIDS Reagent Repository), at an individual peptide concentration of 2 μg/ml. . Mock-stimulated wells received media only. Plates were incubated 18-20 hr at 37^oC in 5% CO_2. Positive spots were visualized on a CTL Imager and counting was performed with Immunospot™ software (Cellular Technology Ltd., Shaker Heights, OH)

Cytometric Bead Array (CBA)

Cytokines were quantified in sera collected before immunization, and 6 hr following each protein boost, using a Th1/Th2/Th17 CBA kit from BD Biosciences (San Diego, CA). The panel included: IFNγ, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17, TNFα. Prior to assay, sera were diluted 1:6 in sample diluent. Samples were read using an LSRII FACS

machine (BD Biosciences), and the data was analyzed using FCAP Array Software v3.0 (BD Bioscience).

Luminex

Cytokines and chemokines were quantified in serum collected before immunization, and 6 hr following each protein boost, using a custom Bio-Plex cytokine assay (Bio-Rad, Hercules, CA) according to manufacturer's instructions. The panel of cytokines and chemokines included: IFNγ, IL-1β, IL-2, IL-4, IL-6, Eotaxin, G-CSF, KC, MIP-1α, MIP-1β, MCP-1, and RANTES. Prior to assay, serum samples were diluted 1:4 in sample diluent. Samples were read on a Bio-Plex 200 system with Bio-Plex Manager software (Bio-Rad).

BMDC Preparation

Bl6.129 wildtype or AIM2 deficient mice were used to prepare BMDCs, as previously described (148, 191). Briefly bone marrow cells were plated at $1x10^6$ cells per 10 cm plate in 10 ml, and grown for 10-12 days in 10% GM-CSF (Invitrogen) in complete RPMI (Gibco) containing 10% FBS and 1% penicillin and streptomycin at 37^oC. At days 3, 6, and 8, cells were fed at with 5 ml/plate fresh complete media. At day 10-12, cells were harvested, counted, and replated in 96-well plates (Corning) at $2x10^5$ cells/well in complete media. Cells were rested overnight.

BMDC Adjuvant Stimulation

Cells were primed for 2 hours with 1 μg/ml LPS prior to adjuvant stimulation as previously described (191). After priming, supernatant and stimulation were removed and replaced with adjuvant stimulations. Cells were stimulated with adjuvants in the

following concentrations: 10 μg/ml QS-21, 100 μg/ml $Al(OH)_3$, 20 μg/ml MPLA, or 10 μg/ml ISCOMATRIX™ adjuvant. As a positive control, 1.25 mM ATP was added to LPS-primed wells in the last hour of the overnight adjuvant stimulation. Select samples were co-incubated with 2 mM Z-Vad-fmk pan-caspase inhibitor. After 18-hour incubation at 37^{o}C, supernatants were harvested and stored at -20^{o}C for downstream application.

IL-1β ELISA

Stimulated cell supernatant were assayed for production of IL-1β by ELISA (BD Biosciences) according to manufacturer's instructions.

Statistical Analysis

All data is presented as the mean of individual mice +/- standard error of the mean (SEM). Statistical analysis was performed using a Student's *t* test, a one-way ANOVA followed by a Tukey post-test, or a two-way ANOVA followed by a Bonferroni post-test.

CHAPTER VI:

Final Comments and Conclusions

For the last three decades since the emergence of HIV-1 onto the global stage, a primary focus of HIV-1 research has been the design of an effective vaccine that is capable of eliciting sufficient virus-specific immune responses to confer protection against viral infection. Until very recently, clinical attempts have not been successful, but have contributed valuable insight into immune correlates of protective immunity. Based on the failures of the VaxGen trials, which targeted on the induction of humoral immunity, and the Merck STEP trial, which instead focused on eliciting T cell responses, we have learned the importance of vaccine induction of both humoral and cell-mediated immune responses. The unprecedented, albeit modest, protection elicited in the RV144 trial demonstrated the improved efficacy of a prime-boost strategy in both vaccine-specific antibody and T cell responses. Still, while 31% protection is a promising advancement, substantial effort is still necessary to develop a vaccine capable of protecting the entire population against HIV-1 infection.

In addition to lessons about immune correlates of protective immunity, clinical trials of novel HIV-1 vaccine strategies have also emphasized the importance of adjuvant selection in vaccine design. The use of a conventional, antibody-biased adjuvant like aluminum hydroxide gel in the RV144 trial, may well have contributed to the limitations to vaccine-induced immunogenicity. In contrast, our own clinical studies of an HIV-1 DNA prime-protein boost vaccine, DP6-001, utilized an adjuvant that potently stimulates

vaccine-specific antibodies, helper T cells, and cytotoxic T cells, but at the cost of clinical tolerability.

In the work presented in this dissertation, I attempted to characterize several conventional and novel adjuvant systems in the unique context of the DP6-001 DNA prime-protein boost vaccine. Specifically, I aimed to define the adaptive immune responses associated with mice immunized with DP6-001 and adjuvants in terms of vaccine induction of Env-specific T cells and IgG antibody responses. In these studies I also established distinct profiles of pro-inflammatory serum cytokines and chemokines associated with each adjuvant. Additionally, I confirmed that these were unique to the DNA primed DP6-001 vaccine strategy, implicating a role for DNA priming in the inflammatory response to subsequent immunization with protein and adjuvants.

In terms of adaptive immunity, the select adjuvants $Al(OH)_3$, QS-21, MPLA, and ISCOMATRIX™ adjuvant all comparably induced humoral responses in immunized mice, and while QS-21 most potently induced Env-specific Th1 cytokine production, both MPLA and ISCOMATRIX™ adjuvant also induced positive responses. However, distinct patterns associated with each adjuvant emerged upon evaluation of a panel of non-antigen specific pro-inflammatory serum cytokines and chemokines at time points 6 hours after protein-adjuvant immunization. These studies identified several biomarkers induced shortly after protein-adjuvant induction in all immunization groups, including IL-6, KC, and MIP-1α. DP6-001/$Al(OH)_3$ demonstrated an overall low serum cytokine and chemokine profile. DP6-001/QS-21 was characterized by Th1 cytokine IFNγ, Th2 cytokine IL-4, as well as pro-inflammatory factors IL-1β and MIP-1β. DP6-001/MPLA

was uniquely characterized by the broadly acting chemokines G-CSF and RANTES. DP6-001/ISCOMATRIX™ adjuvant was associated with potent induction of IL-2, Eotaxin, and MCP-1. These distinct profiles are not only characteristic of each adjuvant, but are also unique to the context of the DP6-001 DNA prime-protein boost vaccine strategy itself. The adjuvant profiles described in this dissertation provide insight into

A secondary aim of the research presented in this dissertation was to further explore the innate immune mechanisms utilized by the examined adjuvants, particularly given that the mechanisms of even widely employed adjuvants like $Al(OH)_3$ remain undefined. Adjuvants must be evaluated in the context of each new vaccine formulation, and the DNA prime-protein boost may provide a unique environment for the action of adjuvants on innate immune pathways. With plans in mind to move forward with an optimized HIV-1 prime boost vaccine formulated with an improved adjuvant, these mechanistic studies focused on MPLA and ISCOMATRIX™ adjuvant specifically.

In the context of DP6-001, I confirmed a critical role for TLR4 signaling in the antibody and T cell responses enhanced by MPLA. In addition, intact TLR4 activation was required to generate serum cytokines and chemokines associated with DP6-001/MPLA, chiefly RANTES and G-CSF, as well as IL-6, which was associated with all adjuvants. More interestingly, in light of literature reports suggesting that MPLA induces weak MyD88 signaling and TRIF-biased activation downstream of TLR4, I also identified a critical role for MyD88 in the adaptive immune response to DP6-001/MPLA. Serum cytokine profiles were more variably affected by MyD88 deficiency. I also reported reduced adaptive and innate inflammatory responses to immunization with

empty vector prime and DP6-001 protein-MPLA boost, highlighting the improved immunogenicity and inflammatory response provided by the DNA prime component.

The studies presented in these chapters also evaluated the novel adjuvant system ISCOMATRIX™ adjuvant formulated with DP6-001 vaccine in both mice and rabbit models. In both animal models, the induction of vaccine-specific serum IgG antibody responses were similar between adjuvant groups, the superiority of ISCOMATRIX™ adjuvant emerged in the rabbit model upon evaluation of the breadth and strength of neutralizing ability against a panel of Tier 2 viruses. I also observed enhanced induction of serum cytokines and chemokines associated with DP6-001/ISCOMATRIX™ adjuvant, which was reduced in the absence of DNA priming. Of particular note, while I did not observe a critical role for MyD88 signaling in the adaptive or inflammatory immune responses to DP6-001/ISCOMATRIX™ adjuvant, MyD88 deficiency had a significant negative impact on the adaptive and inflammatory profiles to immunization with protein and ISCOMATRIX™ adjuvant in the absence of DNA priming. These results not only demonstrate the strong immune response induced by DP6-001/ISCOMATRIX™ adjuvant, but also strongly indicate a role for DNA priming in shaping the inflammatory response to subsequent immunizations with antigen and adjuvant.

As a final note, I began preliminary *in vitro* work drawing on recent trends in the literature exploring roles for innate inflammasome signaling in the mechanism of all of the adjuvants described in this dissertation. While this work was performed *in vitro* and only in the absence of antigen, I confirmed the ability of $Al(OH)_3$ and ISCOMATRIX™

adjuvant to induce the production of IL-1β in LPS-primed BMDCs, and also indicated that QS-21 induces a similar response and may thus owe its adjuvanticity to a similar mechanism. Furthermore, this IL-1β response was completely and significantly diminished with the inhibition of capase-1, and modestly but significantly reduced in cells generated from AIM2 deficient mice. While these experiments were preliminary in nature, they lay the groundwork for the future directions for our lab, exploring more deeply in the innate mechanisms for vaccine adjuvants. Our *in vivo* experiments clearly suggest a role for DNA priming not only in the enhancement of vaccine-specific immunogenicity, but also in providing a unique setting for the innate immune pathways harnessed by subsequent vaccine and adjuvant immunizations.

In summary, in this dissertation I have established a method by which unique adjuvant profiles in the context of our vaccine can be defined, based on adaptive immune responses and serum cytokine and chemokine induction. Furthermore, these profiles are unique to the setting of a DNA prime-protein boost immunization regimen utilizing HIV-1 Env antigens, rather than being characteristic of a protein-adjuvant vaccine in the absence of DNA priming. Finally, we have identified roles for both TLR4 and MyD88 signaling in the generation of adaptive and pro-inflammatory immune responses to DP6-001 and MPLA adjuvant; meanwhile, any role for MyD88 signaling in the immune responses to other adjuvants appears to vary based on the vaccine regimen. This body of work clearly warrants further investigation of the impact of DNA priming on subsequent immunization and adjuvant use, sheds additional light on the involvement of our selected adjuvants in innate inflammasome pathways that are increasingly the focus of adjuvant

research, and provides an opportunity to make a critical clinically relevant link between innate immune responses to adjuvants and the adaptive immune responses to which they contribute.

The original impetus for our study of adjuvants was a clinical case of leukocytoclastic vasculitis, and other, milder adverse effects associated with our DP6-001 vaccine and the QS-21 adjuvant (86). We aimed to explore the causes of inflammation and adverse effects of vaccine adjuvants, and also identify markers indicative of inflammation and of immunogenicity, which might better inform us in our selection of an ideal adjuvant from a panel of candidates. However, this question is not so clear cut, as samples from human subjects immunized with DP6-001 and QS-21 were not assessed for correlates of reactogenicity. Furthermore, there is mounting evidence that the reliable animal models upon which so much preclinical work is based may not be truly predictive of clinical outcomes. Indeed, preclinical toxicology and immunogenicity studies of DP6-001 and QS-21 in rabbits did not reveal local or systemic reactogenicity associated with the vaccine, encouraging the move towards a clinical application (140).

This issue may particularly evident in the case of inbred mice used for models of inflammation and immunological responses. A recent article by Seok *et al.* summarizes results of published models of acute inflammatory stresses in murine and human models, with a focus on the gene response patterns in mice and compared to humans in the context of varied sources of acute inflammation, including endotoxemia, trauma, and burns (168). Given the dominance of the mouse model in preclinical research, it was surprisingly to find that at the transcriptional level, the response in mice to endotoxemia

and acute injury and inflammation varied so greatly from the human response. It has been established that mice tolerate a substantially higher challenge of endotoxin in comparison to humans, although the kinetics of cytokine induction in response to endotoxin are not wholly disparate between the two models (33). Why then, if such a basic immunological response is so different at the genetic level, do we rely so heavily on the mouse model in the study of human immunological responses? Clearly new approaches sounds be explored to improve the quality of animal models, perhaps moving towards humanized mouse models, or to develop alternative ways to simulate the human model (68).

Still, the preclinical adjuvant research presented in this dissertation is not without significance. We clearly identified superior immunogenicity of novel adjuvant systems compared to classical adjuvants, and also established unique profiles of inflammatory markers consistently associated with each candidate adjuvant. In addition, we have demonstrated the substantial impact of a DNA priming component on the immunogenicity and inflammatory cytokine profiles elicited upon subsequent immunization. Certainly we may observe the impact of different adjuvants on the immunogenicity of the DP6-001 vaccine in a preclinical model in a clinically relevant way, particularly in terms of the improved neutralizing antibodies elicited by MPLA and ISCOMATRIX™ adjuvant compared to classical adjuvants.

In the absence of clear human correlates of reactogenicity, it is difficult to characterize the nature of adverse effects that might be predicted by these inflammatory markers, or indeed to define the specific qualities of an ideal adjuvant. Given what we

know about the balanced humoral and cell-mediated immunity (Th1/Th2 responses as well as CTLs) necessitated by a complex pathogen such as HIV-1, and evidence from clinical studies of each adjuvant, we can speculate that the novel adjuvants MPLA and ISCOMATRIX™ are better candidates for inclusion in future HIV-1 vaccine formulations that aluminum hydroxide adjuvants or QS-21.

Our preclinical work with multiplex analysis of serum cytokines suggests that strong and balanced induction of Th1 and Th2 cytokines may be favorable and associated with improved immunogenicity; potent pro-inflammatory cytokines such as the elevated IL-1β associated with QS-21, may be of predictive value for reactogenicity. We might also speculate that cytokine and chemokine profiles suggestive of a diverse and rapid recruitment of innate immune cells, resulting in improved antigen trafficking and presentation. This would be best further studied by studying the temporal immune cell recruitment at the sites of immunization and at draining lymph nodes, alongside serum cytokines and chemoattractants in both preclinical and clinical models. Further still, the mechanisms of these adjuvants on the innate and adaptive immune systems remain poorly defined. More thorough elucidation of the signaling pathways activated by these candidate adjuvants would help to define the relationship of these serum marker profiles to the innate and adaptive immune responses shaped by each adjuvant.

Definition of mechanisms of action of these adjuvants as well as the mechanism of DNA priming and its impact on subsequent immunizations, will likely be the focus of future preclinical investigation by this lab, based on the data described herein. In addition, optimized vaccine formulations including ISCOMATRIX™ adjuvant are

moving towards clinical trials. The preclinical work in this dissertation has laid the groundwork for future development of the DNA prime-protein boost HIV-1 vaccine.

Preface to Appendix I

R. Buglione-Corbett designed, performed, and analyzed the preliminary *in vitro* experiments presented in this chapter, Fig. AI.1.

Appendix I:

Preliminary *in vitro* studies exploring innate pathways to guide future adjuvant studies

Introduction

In light of our *in vivo* findings regarding the impact of DNA priming on the MyD88 dependency of adaptive immune responses and serum cytokine profiles to ISCOMATRIX™ adjuvant, and considering the current direction of research into adjuvant mechanisms, we decided to further explore our candidate adjuvants *in vitro*. Bone marrow-derived dendritic cells (BMDCs) derived from wildtype mice or mice deficient in immune signaling pathways were stimulated with candidate adjuvants, and IL-1β secretion was quantified as a determination of inflammasome activation. We examined adjuvant activity in the presence or absence of a caspase-1 inhibitor molecule, in order to generally evaluate the dependence of adjuvant-induced IL-1β secretion on caspase-1-dependent inflammasome complexes such as NLRs and AIM2. The work described in this Appendix highlights simple pilot *in vitro* studies, which serve mainly to confirm a future direction for the adjuvant work to be performed by our lab.

As described in previous chapters, the NLRP3 inflammasome has been the most widely investigated inflammasome protein complex in the context of adjuvants, especially aluminum salts and to a lesser extent, ISCOMATRIX™ adjuvant. In both cases, it remains unclear whether the particulate adjuvants themselves stimulate assembly of the inflammasome complex and subsequent secretion of pro-inflammatory cytokines, or if administration of adjuvant results in tissue and cellular damage, the products of

which are inflammasome ligands. It is the latter hypothesis that suggests the potential importance of nucleic acid sensing pathways in adjuvant mechanisms. As described in Chapter I, while many nucleic acid sensing pathways result in the production of type I IFNs, the AIM2 inflammasome, like NLRP3, recruits caspase-1 and results in the secretion of IL-1β and IL-18 in response to dsDNA. Recent studies by Marichal *et al.* suggest that aluminum salts induce cell death, resulting in the release of host cell DNA. The host DNA activates innate nucleic acid sensors, and the subsequent secretion of pro-inflammatory cytokines helps shape the adaptive immune response to immunization with aluminum salt-based adjuvants (111).

A novel role for cell damage products in the adjuvanticity of alumimun salts suggest that these pathways may also be relevant for our other adjuvants of interest, in particular the saponin adjuvants QS-21 and ISCOMATRIX™ adjuvant. QS-21 is a known hemolytic agent with reactogenic characteristics and strong induction of inflammatory cytokines including IL-1β, but very little is known about its mechanism. While our *in vivo* data does not suggest a dependence on TLR4 or MyD88 signaling for the generation of adaptive immunity or serum cytokine profiles, at least in the context of DP6-001, QS-21 may directly or indirectly act on inflammasome pathways. While ISCOMATRIX™ adjuvant is considerably more tolerable than QS-21, it also contains a saponin fraction and is also particulate in nature, characteristics that may cause cell damage upon administration. Therefore, with the recent work on $Al(OH)_3$ in mind, we aimed to take an exploratory approach and investigate the activation of the AIM2 inflammasome by our candidate adjuvant *in vitro* in the absence of antigen.

Results

Generation of BMDCs and Adjuvant Stimulations

In these pilot experiments, bone marrow-derived dendritic cells (BMDCs) were generated from naïve wildtype or mice deficinent in innate immune signaling pathways as previously described (10, 149). Bone marrow was harvested from individual mice, and the cells were grown in complete RPMI with 10% FBS and 1% penicillin and streptomycin for 12 days in the presence of 10% GM-CSF for the selective growth of BMDCs. After maturation, cells were counted and seeded into 96-well plates for stimulation the next day. Cells were washed with complete media and BMDCs were incubated for 18 hours, with either QS-21, $Al(OH)_3$, MPLA, or ISCOMATRIX™ adjuvant in the absence of antigen.

Adjuvant induction of IL-1β production in wildtype BMDCs

BMDCs generated from Bl6.129 wildtype mice were primed with LPS for 2 hours, before being stimulated with adjuvant overnight (42, 149). Based on previous methods described in the literature, BMDCs were stimulated with 10 μg/ml of QS-21 or ISCOMATRIX™ adjuvant, 20 μg/ml of MPLA, or 100 μg/ml of $Al(OH)_3$. As a read-out of inflammasome activation, IL-1β was measured in the supernatant by ELISA. LPS-primed BMDCs stimulated with either QS-21, $Al(OH)_3$, or ISCOMATRIX™ adjuvant produced comparably robust IL-1β. As previously described in Chapter I, a first signal via MyD88 and NFκB activation is required for production of pro-cytokines and assembly of inflammasome components (10). Thus, as we would expect, adjuvant stimulations in the absence of LPS priming resulted in poor IL-1β production.

Furthermore, LPS priming alone was insufficient for induction of IL-1β. As it has been previously established that the LPS derivative, MPLA, has impaired activation of the NLRP3 inflammasome, MPLA alone also did not induce IL-1β (Fig AI.1).

Adjuvant-induced IL-1β production in wildtype BMDCs requires caspase-1

The small molecule z-VAD-fmk (z-VAD) binds to the catalytic site of caspases and inhibits their enzymatic function. While z-VAD inhibits several caspases, it very strongly inhibits caspase-1 and is frequently used in studies of the NLRP3 inflammasome. Wildtype BMDCs were primed with LPS for 2 hours and then stimulated with adjuvant in the presence and absence of z-VAD. Addition of the caspase-1 inhibitor completely abrograted the IL-1β induction by QS-21, $Al(OH)_3$ and ISCOMATRIX™ adjuvant (Fig AI.1).

Reduction of IL-1β in AIM2 deficient BMDCs stimulated by adjuvant

In order to explore a potential role for the AIM2 inflammasome in the mechanism of each candidate adjuvant, the *in vitro* stimulations described above were performed in parallel in BMDCs generated from AIM2 deficient mice. Interestingly, AIM2 deficiency significantly reduced IL-1β production in response to $Al(OH)_3$ or ISCOMATRIX™ adjuvant, but this negative impact was only partial. In AIM2 deficient BMDCs stimulated with QS-21, there was a modest reduction in IL-1β induction compared to wildtype. However, the addition of z-VAD to adjuvant stimulations in AIM2 deficient mice completely eliminated the IL-1β response to QS-21, $Al(OH)_3$, and ISCOMATRIX™ adjuvant, similar to the effect observed in wildtype BMDCs (Fig AI.1).

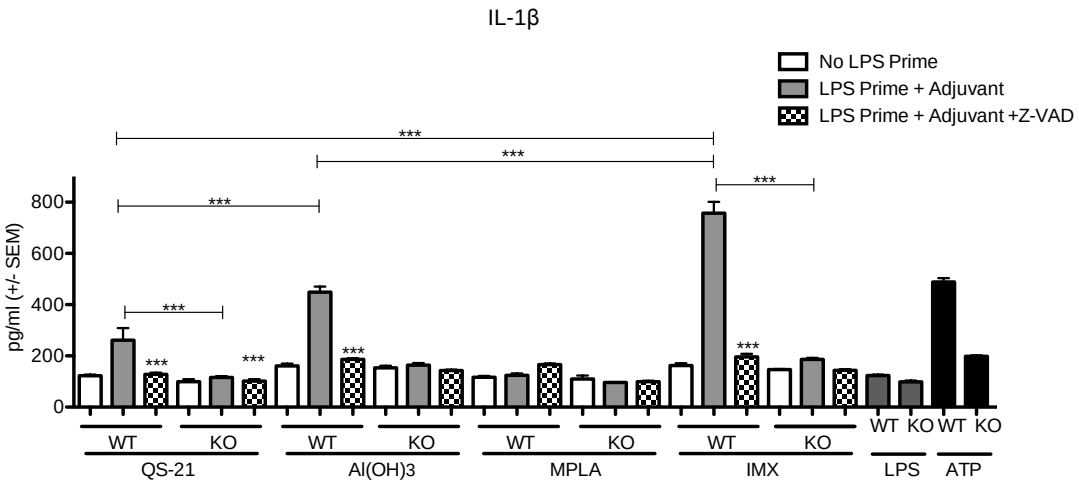


Figure AI.1. Adjuvants induced IL-1β production in LPS-primed BMDCs from wildtype and AIM2 deficient mice in the absence of antigen. BMDCs were generated from the bone marrow of Bl6.129 wildtype (WT) or AIM2 deficient (KO) mice. After maturation, BMDCs were plated at $2x10^5$ cells/well, and primed with 1 μg/ml LPS for 2 hours. After priming, BMDCs were incubated with adjuvants for 18 hours overnight at the following concentrations (grey): 10 μg/ml QS-21, 100 μg/ml $Al(OH)_3$, 20 μg/ml MPLA, or 10 μg/ml ISCOMATRIX™ adjuvant. As a control, BMDCs were incubated with adjuvants in the absence of LPS priming (white). Select adjuvant stimulations of BMDCs were co-incubated with the pan-caspase-1 inhibitor Z-vad (checkered). Statistical differences were determined by a two-way ANOVA and a Bonferroni post-test. (*: $p < .05$, **: $p < .01$, ***: $p < .001$). (IMX = ISCOMATRIX™ adjuvant)

Discussion

Innate signaling pathways, and inflammasomes in particular, are the primary focus in current adjuvant research. Though the goal of adjuvants in vaccine design is to harness the innate immune response and promote inflammatory responses in order to promote antigen presentation and generation of adaptive immunity, the mechanism by which these adjuvants accomplish these immunomodulatory effects remains unclear. Aluminum salts in particular have been extensively evaluated for activation of the NLRP3 inflammasome, and while it is clear that alum-based adjuvants induce pro-inflammatory cytokines in an NLRP3-dependent manner, a clear link between this response and adaptive immunity subsequent to vaccination with antigen *in vivo* remains tenuous (45, 53, 77, 92, 97, 119, 169).

Our *in vivo* experience with select adjuvants in the context of DP6-001 DNA prime-protein boost, in comparison to empty vector primed immunizations or protein-only immunizations, demonstrate that the DNA priming component itself may uniquely affect the immunogenicity and inflammatory profiles of adjuvants in subsequent protein immunizations. The influence of DNA priming may even be to the extent of reduced dependence on innate pathways involving MyD88, for example, for the generation of adaptive immunity.

Consistent with previous reports, LPS-primed BMDCs stimulated with aluminum hydroxide gel or ISCOMATRIX™ adjuvant induced IL-1β in a caspase-1-dependent manner (42). QS-21 also induced IL-1β in a caspase-1-dependent manner. While this has not to our knowledge been previously demonstrated in this model, it is not

unexpected for several reasons: serum IL-1β associated with QS-21 in the context of DP6-001, the similarities between QS-21 and ISCOMATRIX™ adjuvant, and the likely cell damage caused by QS-21 administration. However, the complete inhibition of IL-1β in the presence of a caspase-1 inhibitor suggests that, like $Al(OH)_3$ and ISCOMATRIX™ adjuvant, QS-21 may be acting via a caspase-1-dependent inflammasome complex.

Based on recent reports that aluminum hydroxide induces cell death and subsequent release of host DNA, which is responsible for shaping the adaptive immune response to alum-adjuvanted vaccination (111), we chose to evaluate a potential role for the caspase-1-dependent dsDNA sensor AIM2 in the IL-1β response to adjuvant stimulations. We hypothesized that, similarly to aluminum hydroxide adjuvants, the saponins QS-21 and ISCOMATRIX™ adjuvant may also cause cell damage and subsequently activate nucleic acid sensing pathways. While we did observe a reduction in the IL-1β response in AIM2 deficient BMDCs with all candidate adjuvants, the effect was only partial. Adjuvant-induced IL-1β was completely absent in AIM2 deficient BMDCs with the addition of z-VAD. These results suggest that while AIM2 signaling may contribute to the IL-1β response to stimulation with QS-21, ISCOMATRIX™ adjuvant or $Al(OH)_3$, it is not required. However, intact caspase functionality is required for the induction of IL-1β for each of these adjuvants, indicating the possible involvement of multiple inflammasome pathways.

In the preliminary experiments described in this chapter, we sought to define potential future directions for our group's continued adjuvant research. As we move forward with optimized HIV-1 DNA prime-protein boost vaccines, and with the

ISCOMATRIX™ adjuvant selected for use in future vaccine formulations, we aim to better understand both the mechanism of ISCOMATRIX™ adjuvant itself, and also how its mechanism may be impacted by the unique context of DNA priming. In addition, inclusion of QS-21 in these experiments may provide greater insight into the mechanism of action of saponins in general, and also a potential mechanism for the reactogenicity associated with QS-21 in comparison to more tolerable saponins-related systems like ISCOMATRIX™ adjuvant.

CHAPTER VII:

Bibliography

1. **Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity 9:143-150.**
2. **Alving, C. R., and M. Rao. 2008. Lipid A and liposomes containing lipid A as antigens and adjuvants. Vaccine 26:3036-3045.**
3. **Amara, R. R., F. Villinger, J. D. Altman, S. L. Lydy, S. P. O'Neil, S. I. Staprans, D. C. Montefiori, Y. Xu, J. G. Herndon, L. S. Wyatt, M. A. Candido, N. L. Kozyr, P. L. Earl, J. M. Smith, H. L. Ma, B. D. Grimm, M. L. Hulsey, J. Miller, H. M. McClure, J. M. McNicholl, B. Moss, and H. L. Robinson. 2001. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science 292:69-74.**
4. **Anderson, J. S., J. Hoy, R. Hillman, M. Barnden, B. Eu, A. McKenzie, and C. Gittleson. 2009. A randomized, placebo-controlled, dose-escalation study to determine the safety, tolerability, and immunogenicity of an HPV-16 therapeutic vaccine in HIV-positive participants with oncogenic HPV infection of the anus. J Acquir Immun Defic Syndr 52:371-381.**
5. **Andersson, K. M., A. D. Paltiel, and D. K. Owens. 2011. The potential impact of an HIV vaccine with rapidly waning protection on the epidemic in Southern Africa: examining the RV144 trial results. Vaccine 29:6107-6112.**
6. **Baba, T. W., V. Liska, R. Hofmann-Lehmann, J. Vlasak, W. Xu, S. Ayehunie, L. A. Cavacini, M. R. Posner, H. Katinger, G. Stiegler, B. J. Bernacky, T. A. Rizvi, R. Schmidt, L. R. Hill, M. E. Keeling, Y. Lu, J. E. Wright, T. C. Chou, and R. M. Ruprecht. 2000. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. Nature medicine 6:200-206.**
7. **Bansal, A., B. Jackson, K. West, S. Wang, S. Lu, J. S. Kennedy, and P. A. Goepfert. 2008. Multifunctional T-Cell Characteristics Induced by a Polyvalent DNA Prime/Protein Boost Human Immunodeficiency Virus Type 1 Vaccine Regimen Given to Healthy Adults Are Dependent on the Route and Dose of Administration. J Virol 82:6458-6469.**
8. **Barnett, S. W., S. Rajasekar, H. Legg, B. Doe, D. H. Fuller, J. R. Haynes, C. M. Walker, and K. S. Steimer. 1997. Vaccination with HIV-1 gp120 DNA induces immune responses that are boosted by a recombinant gp120 protein subunit. Vaccine 15:869-873.**
9. **Barouch, D. H., S. Santra, J. E. Schmitz, M. J. Kuroda, T. M. Fu, W. Wagner, M. Bilska, A. Craiu, X. X. Zheng, G. R. Krivulka, K. Beaudry, M. A. Lifton, C. E. Nickerson, W. L. Trigona, K. Punt, D. C. Freed, L. Guan, S. Dubey, D. Casimiro, A. Simon, M. E. Davies, M. Chastain, T. B. Strom, R. S. Gelman, D. C. Montefiori, M. G. Lewis, E. A. Emini, J. W. Shiver, and N. L.**

Letvin. 2000. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. Science 290:486-492.

10. **Bauernfeind, F. G., G. Horvath, A. Stutz, E. S. Alnemri, K. MacDonald, D. Speert, T. Fernandes-Alnemri, J. Wu, B. G. Monks, K. A. Fitzgerald, V. Hornung, and E. Latz. 2009. Cutting Edge: NF- B Activating Pattern Recognition and Cytokine Receptors License NLRP3 Inflammasome Activation by Regulating NLRP3 Expression. J immunol 183:787-791.**
11. **Berman, P. W. 1998. Development of bivalent rgp120 vaccines to prevent HIV type 1 infection. AIDS research and human retroviruses 14 Suppl 3:S277-289.**
12. **Berman, P. W., T. J. Gregory, L. Riddle, G. R. Nakamura, M. A. Champe, J. P. Porter, F. M. Wurm, R. D. Hershberg, E. K. Cobb, and J. W. Eichberg. 1990. Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. Nature 345:622-625.**
13. **Berman, P. W., K. K. Murthy, T. Wrin, J. C. Vennari, E. K. Cobb, D. J. Eastman, M. Champe, G. R. Nakamura, D. Davison, M. F. Powell, J. Bussiere, D. P. Francis, T. Matthews, T. J. Gregory, and J. F. Obijeski. 1996. Protection of MN-rgp120-immunized chimpanzees from heterologous infection with a primary isolate of human immunodeficiency virus type 1. The Journal of infectious diseases 173:52-59.**
14. **Bonsignori, M., J. Pollara, M. A. Moody, M. D. Alpert, X. Chen, K. K. Hwang, P. B. Gilbert, Y. Huang, T. C. Gurley, D. M. Kozink, D. J. Marshall, J. F. Whitesides, C. Y. Tsao, J. Kaewkungwal, S. Nitayaphan, P. Pitisuttithum, S. Rerks-Ngarm, J. H. Kim, N. L. Michael, G. D. Tomaras, D. C. Montefiori, G. K. Lewis, A. DeVico, D. T. Evans, G. Ferrari, H. X. Liao, and B. F. Haynes. 2012. Antibody-Dependent Cellular Cytotoxicity-Mediating Antibodies from an HIV-1 Vaccine Efficacy Trial Target Multiple Epitopes and Preferentially Use the VH1 Gene Family. J Virol 86:11521-11532.**
15. **Boyer, J. D., M. A. Chattergoon, K. E. Ugen, A. Shah, M. Bennett, A. Cohen, S. Nyland, K. E. Lacy, M. L. Bagarazzi, and T. J. Higgins. 1999. Enhancement of cellular immune response in HIV-1 seropositive individuals: a DNA-based trial. Clin Immunol 90:100-107.**
16. **Boyer, J. D., A. D. Cohen, S. Vogt, K. Schumann, B. Nath, L. Ahn, K. Lacy, M. L. Bagarazzi, T. J. Higgins, and Y. Baine. 2000. Vaccination of Seronegative Volunteers with a Human Immunodeficiency Virus Type 1 envlrev DNA Vaccine Induces Antigen-Specific Proliferation and Lymphocyte Production of β-Chemokines. The Journal of infectious diseases 181:476.**
17. **Boyer, J. D., K. E. Ugen, B. Wang, M. Agadjanyan, L. Gilbert, M. L. Bagarazzi, M. Chattergoon, P. Frost, A. Javadian, and W. V. Williams. 1997. Protection of chimpanzees from high-dose heterologous HIV-1 challenge by DNA vaccination. Nature medicine 3:526-532.**

18. Boyle, J., D. Eastman, C. Millar, S. Camuglia, J. Cox, M. Pearse, J. Good, and D. Drane. 2007. The utility of ISCOMATRIX adjuvant for dose reduction of antigen for vaccines requiring antibody responses. Vaccine 25:2541-2544.

19. Brown, S. A., T. D. Lockey, C. Slaughter, K. S. Slobod, S. Surman, A. Zirkel, A. Mishra, V. R. Pagala, C. Coleclough, P. C. Doherty, and J. L. Hurwitz. 2005. T cell epitope "hotspots" on the HIV Type 1 gp120 envelope protein overlap with tryptic fragments displayed by mass spectrometry. AIDS research and human retroviruses 21:165-170.

20. Brown, S. A., S. L. Surman, R. Sealy, B. G. Jones, K. S. Slobod, K. Branum, T. D. Lockey, N. Howlett, P. Freiden, P. Flynn, and J. L. Hurwitz. 2010. Heterologous Prime-Boost HIV-1 Vaccination Regimens in Pre-Clinical and Clinical Trials. Viruses 2:435-467.

21. Brown, S. E., C. Stanley, C. R. Howard, A. J. Zuckerman, and M. W. Steward. 1986. Antibody responses to recombinant and plasma derived hepatitis B vaccines. British medical journal 292:159-161.

22. Bryant, C., and K. A. Fitzgerald. 2009. Molecular mechanisms involved in inflammasome activation. Trends Cell Biol 19:455-464.

23. Buchbinder, S. P., D. V. Mehrotra, A. Duerr, D. W. Fitzgerald, R. Mogg, D. Li, P. B. Gilbert, J. R. Lama, M. Marmor, C. Del Rio, M. J. McElrath, D. R. Casimiro, K. M. Gottesdiener, J. A. Chodakewitz, L. Corey, M. N. Robertson, and T. Step Study Protocol. 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.

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

25. Calarota, S. A., A. Kjerrström, K. B. Islam, and B. Wahren. 2001. Gene combination raises broad human immunodeficiency virus-specific cytotoxicity. Hum Gen Ther 12:1623-1637.

26. Calarota, S. A., A. C. Leandersson, G. Bratt, J. Hinkula, D. M. Klinman, K. J. Weinhold, E. Sandström, and B. Wahren. 1999. Immune responses in asymptomatic HIV-1-infected patients after HIV-DNA immunization followed by highly active antiretroviral treatment. J Immunol 163:2330-2338.

27. Cassatella, M. A. 1995. The production of cytokines by polymorphonuclear neutrophils. Immunol Today 16:21-26.

28. Cekic, C., C. R. Casella, C. A. Eaves, A. Matsuzawa, H. Ichijo, and T. C. Mitchell. 2009. Selective Activation of the p38 MAPK Pathway by Synthetic Monophosphoryl Lipid A. J Biol Chem 284:31982-31991.

29. Cekic, C., C. R. Casella, D. Sag, F. Antignano, J. Kolb, J. Suttles, M. R. Hughes, G. Krystal, and T. C. Mitchell. 2011. MyD88-Dependent SHIP1 Regulates Proinflammatory Signaling Pathways in Dendritic Cells after Monophosphoryl Lipid A Stimulation of TLR4. J Immunol 186:3858-3865.

30. Cho, M. W., Y. B. Kim, M. K. Lee, K. C. Gupta, W. Ross, R. Plishka, A. Buckler-White, T. Igarashi, T. Theodore, R. Byrum, C. Kemp, D. C. Montefiori, and M. A. Martin. 2001. Polyvalent envelope glycoprotein vaccine elicits a broader neutralizing antibody response but is unable to provide sterilizing protection against heterologous Simian/human immunodeficiency virus infection in pigtailed macaques. J Virol 75:2224-2234.

31. Clements-Mann, M. L., K. Weinhold, T. J. Matthews, B. S. Graham, G. J. Gorse, M. C. Keefer, M. J. McElrath, R. H. Hsieh, J. Mestecky, S. Zolla-Pazner, J. Mascola, D. Schwartz, R. Siliciano, L. Corey, P. F. Wright, R. Belshe, R. Dolin, S. Jackson, S. Xu, P. Fast, M. C. Walker, D. Stablein, J. L. Excler, J. Tartaglia, E. Paoletti, and et al. 1998. Immune responses to human immunodeficiency virus (HIV) type 1 induced by canarypox expressing HIV-1MN gp120, HIV-1SF2 recombinant gp120, or both vaccines in seronegative adults. NIAID AIDS Vaccine Evaluation Group. The Journal of infectious diseases 177:1230-1246.

32. Conley, A. J., J. A. Kessler, 2nd, L. J. Boots, P. M. McKenna, W. A. Schleif, E. A. Emini, G. E. Mark, 3rd, H. Katinger, E. K. Cobb, S. M. Lunceford, S. R. Rouse, and K. K. Murthy. 1996. The consequence of passive administration of an anti-human immunodeficiency virus type 1 neutralizing monoclonal antibody before challenge of chimpanzees with a primary virus isolate. J Virol 70:6751-6758.

33. Copeland, S., H. S. Warren, S. F. Lowry, S. E. Calvano, D. Remick, I. and, and t. H. R. t. I. Investigators. 2005. Acute inflammatory response to endotoxin in mice and humans. Clinical and diagnostic laboratory immunology 12:60-67.

34. Coughlin, R. T., A. Fattom, C. Chu, A. C. White, and S. Winston. 1995. Adjuvant activity of QS-21 for experimental E. coli 018 polysaccharide vaccines. Vaccine 13:17-21.

35. Cristillo, A. D., S. Wang, M. S. Caskey, T. Unangst, L. Hocker, L. He, L. Hudacik, S. Whitney, T. Keen, T.-H. W. Chou, S. Shen, S. Joshi, V. S. Kalyanaraman, B. Nair, P. Markham, S. Lu, and R. Pal. 2006. Preclinical evaluation of cellular immune responses elicited by a polyvalent DNA prime/protein boost HIV-1 vaccine. Virology 346:151-168.

36. Davis, H. L., M. J. McCluskie, J. L. Gerin, and R. H. Purcell. 1996. DNA vaccine for hepatitis B: evidence for immunogenicity in chimpanzees and comparison with other vaccines. Proceedings of the National Academy of Sciences of the United States of America 93:7213-7218.

37. Davis, I. D., W. Chen, H. Jackson, P. Parente, M. Shackleton, W. Hopkins, Q. Chen, N. Dimopoulos, T. Luke, and R. Murphy. 2004. Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4+ and CD8+ T cell responses in humans. Proceedings of the National Academy of Sciences of the United States of America 101:10697.

38. de Souza, M. S., S. Ratto-Kim, W. Chuenarom, A. Schuetz, S. Chantakulkij, B. Nuntapinit, A. Valencia-Micolta, D. Thelian, S. Nitayaphan, P. Pitisuttithum, R. M. Paris, J. Kaewkungwal, N. L. Michael, S. Rerks-Ngarm, B. Mathieson, M. Marovich, J. R. Currier, J. H. Kim, and t. M. o. P. H.-T. A. V. E. G. Collaborators. 2012. The Thai Phase III Trial (RV144) Vaccine Regimen Induces T Cell Responses That Preferentially Target Epitopes within the V2 Region of HIV-1 Envelope. J Immunol 188:5166-5176.

39. Didierlaurent, A. M., S. Morel, L. Lockman, S. L. Giannini, M. Bisteau, H. Carlsen, A. Kielland, O. Vosters, N. Vanderheyde, F. Schiavetti, D. Larocque, M. Van Mechelen, and N. Garcon. 2009. AS04, an Aluminum Salt- and TLR4 Agonist-Based Adjuvant System, Induces a Transient Localized Innate Immune Response Leading to Enhanced Adaptive Immunity. J Immunol 183:6186-6197.

40. Donnelly, J. J., D. Martinez, K. U. Jansen, R. W. Ellis, D. L. Montgomery, and M. A. Liu. 1996. Protection against papillomavirus with a polynucleotide vaccine. The Journal of infectious diseases 173:314-320.

41. Drane, D., E. Maraskovsky, R. Gibson, S. Mitchell, M. Barnden, A. Moskwa, D. Shaw, B. Gervase, S. Coates, and M. Houghton. 2009. Priming of CD4+ and CD8+ T cell responses using a HCV core ISCOMATRIX™ vaccine. Hum Vaccin 5:151-157.

42. Duewell, P., U. Kisser, K. Heckelsmiller, S. Hoves, P. Stoitzner, S. Koernig, A. B. Morelli, B. E. Clausen, M. Dauer, A. Eigler, D. Anz, C. Bourquin, E. Maraskovsky, S. Endres, and M. Schnurr. 2011. ISCOMATRIX adjuvant combines immune activation with antigen delivery to dendritic cells in vivo leading to effective cross-priming of CD8+ T cells. J Immunol 187:55-63.

43. Earl, P. L., W. Sugiura, D. C. Montefiori, C. C. Broder, S. A. Lee, C. Wild, J. Lifson, and B. Moss. 2001. Immunogenicity and protective efficacy of oligomeric human immunodeficiency virus type 1 gp140. J Virol 75:645-653.

44. Earl, P. L., L. S. Wyatt, D. C. Montefiori, M. Bilska, R. Woodward, P. D. Markham, J. D. Malley, T. U. Vogel, T. M. Allen, D. I. Watkins, N. Miller, and B. Moss. 2002. Comparison of vaccine strategies using recombinant env-gag-pol MVA with or without an oligomeric Env protein boost in the SHIV rhesus macaque model. Virology 294:270-281.

45. Eisenbarth, S. C., O. R. Colegio, W. O'Connor, F. S. Sutterwala, and R. A. Flavell. 2008. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature 453:1122-1126.

46. el-Amad, Z., K. K. Murthy, K. Higgins, E. K. Cobb, N. L. Haigwood, J. A. Levy, and K. S. Steimer. 1995. Resistance of chimpanzees immunized with recombinant gp120SF2 to challenge by HIV-1SF2. Aids 9:1313-1322.

47. Embry, C. A., L. Franchi, G. Nunez, and T. C. Mitchell. 2011. Mechanism of Impaired NLRP3 Inflammasome Priming by Monophosphoryl Lipid A. Sci Signal 4:ra28-ra28.

48. Emini, E. A., R. W. Ellis, W. J. Miller, W. J. McAleer, E. M. Scolnick, and R. J. Gerety. 1986. Production and immunological analysis of recombinant hepatitis B vaccine. The Journal of infection 13 Suppl A:3-9.

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

50. Evans, T. G., M. J. McElrath, T. Matthews, D. Montefiori, K. Weinhold, M. Wolff, M. C. Keefer, E. G. Kallas, L. Corey, G. J. Gorse, R. Belshe, B. S. Graham, P. W. Spearman, D. Schwartz, M. J. Mulligan, P. Goepfert, P. Fast, P. Berman, M. Powell, D. Francis, and N. A. V. E. Group. 2001. QS-21 promotes an adjuvant effect allowing for reduced antigen dose during HIV-1 envelope subunit immunization in humans. Vaccine 19:2080-2091.

51. Flach, T. L., G. Ng, A. Hari, M. D. Desrosiers, P. Zhang, S. M. Ward, M. E. Seamone, A. Vilaysane, A. D. Mucsi, Y. Fong, E. Prenner, C. C. Ling, J. Tschopp, D. A. Muruve, M. W. Amrein, and Y. Shi. 2012. Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. Nature medicine:1-10.

52. Flynn, N. M., D. N. Forthal, C. D. Harro, F. N. Judson, K. H. Mayer, M. F. Para, and r. H. V. S. Group. 2005. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. The Journal of infectious diseases 191:654-665.

53. Franchi, L., and G. Núñez. 2008. The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1β secretion but dispensable for adjuvant activity. Eur J Immunol 38:2085-2089.

54. Francis, D. P., T. Gregory, M. J. McElrath, R. B. Belshe, G. J. Gorse, S. Migasena, D. Kitayaporn, P. Pitisuttitham, T. Matthews, D. H. Schwartz, and P. W. Berman. 1998. Advancing AIDSVAX to phase 3. Safety, immunogenicity, and plans for phase 3. AIDS research and human retroviruses 14 Suppl 3:S325-331.

55. Frankel, A. D., and J. A. Young. 1998. HIV-1: fifteen proteins and an RNA. Annu Rev Biochem 67:1-25.

56. Frazer, I., M. Quinn, J. Nicklin, J. Tan, L. Perrin, P. Ng, V. O'Connor, O. White, N. Wendt, J. Martin, J. Crowley, S. Edwards, A. McKenzie, S. Mitchell, D. Maher, M. Pearse, and R. Basser. 2004. Phase 1 study of HPV16-specific immunotherapy with E6E7 fusion protein and ISCOMATRIX™ adjuvant in women with cervical intraepithelial neoplasia. Vaccine 23:172-181.

57. FRAZER, I., M. QUINN, J. NICKLIN, J. TAN, L. PERRIN, P. NG, V. OCONNOR, O. WHITE, N. WENDT, and J. MARTIN. 2004. Phase 1 study of HPV16-specific immunotherapy with E6E7 fusion protein and ISCOMATRIX? adjuvant in women with cervical intraepithelial neoplasia. 23:172-181.

58. Fuller, D. H., and J. R. Haynes. 1994. A qualitative progression in HIV type 1 glycoprotein 120-specific cytotoxic cellular and humoral immune responses in mice receiving a DNA-based glycoprotein 120 vaccine. AIDS research and human retroviruses 10:1433-1441.

59. Fynan, E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson. 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proceedings of the National Academy of Sciences of the United States of America 90:11478-11482.

60. Gilbert, P. B., M. L. Ackers, P. W. Berman, D. P. Francis, V. Popovic, D. J. Hu, W. L. Heyward, F. Sinangil, B. E. Shepherd, and M. Gurwith. 2005. HIV-1 virologic and immunologic progression and initiation of antiretroviral therapy among HIV-1-infected subjects in a trial of the efficacy of recombinant glycoprotein 120 vaccine. The Journal of infectious diseases 192:974-983.

61. Gilbert, P. B., M. L. Peterson, D. Follmann, M. G. Hudgens, D. P. Francis, M. Gurwith, W. L. Heyward, D. V. Jobes, V. Popovic, S. G. Self, F. Sinangil, D. Burke, and P. W. Berman. 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. The Journal of infectious diseases 191:666-677.

62. Girard, M., M. P. Kieny, A. Pinter, F. Barre-Sinoussi, P. Nara, H. Kolbe, K. Kusumi, A. Chaput, T. Reinhart, and E. Muchmore. 1991. Immunization of chimpanzees confers protection against challenge with human immunodeficiency virus. Proceedings of the National Academy of Sciences of the United States of America 88:542-546.

63. Gorse, G. J., L. Corey, G. B. Patel, M. Mandava, R. H. Hsieh, T. J. Matthews, M. C. Walker, M. J. McElrath, P. W. Berman, M. M. Eibl, and R. B. Belshe. 1999. HIV-1MN recombinant glycoprotein 160 vaccine-induced cellular and humoral immunity boosted by HIV-1MN recombinant glycoprotein 120 vaccine. National Institute of Allergy and Infectious Diseases AIDS Vaccine Evaluation Group. AIDS research and human retroviruses 15:115-132.

64. Graham, B. S., M. C. Keefer, M. J. McElrath, G. J. Gorse, D. H. Schwartz, K. Weinhold, T. J. Matthews, J. R. Esterlitz, F. Sinangil, and P. E. Fast. 1996. Safety and immunogenicity of a candidate HIV-1 vaccine in healthy adults: recombinant glycoprotein (rgp) 120. A randomized, double-blind trial. NIAID AIDS Vaccine Evaluation Group. Annals of internal medicine 125:270-279.

65. Graham, B. S., T. J. Matthews, R. B. Belshe, M. L. Clements, R. Dolin, P. F. Wright, G. J. Gorse, D. H. Schwartz, M. C. Keefer, D. P. Bolognesi, and et al. 1993. Augmentation of human immunodeficiency virus type 1 neutralizing antibody by priming with gp160 recombinant vaccinia and boosting with rgp160 in vaccinia-naive adults. The NIAID AIDS Vaccine Clinical Trials Network. The Journal of infectious diseases 167:533-537.

66. Guy, B. 2007. The perfect mix: recent progress in adjuvant research. Nat Rev Immunol 5:505-517.

67. Hancock, G. E., D. J. Speelman, P. J. Frenchick, M. M. Mineo-Kuhn, R. B. Baggs, and D. J. Hahn. 1995. Formulation of the purified fusion protein of respiratory syncytial virus with the saponin QS-21 induces protective immune responses in Balb/c mice that are similar to those generated by experimental infection. Vaccine 13:391-400.

68. Hayday, A. C., and M. Peakman. 2008. The habitual, diverse and surmountable obstacles to human immunology research. Nat Immunol 9:575-580.

69. Heeney, J., L. Akerblom, S. Barnett, W. Bogers, D. Davis, D. Fuller, G. Koopman, T. Lehner, P. Mooij, B. Morein, C. De Giuli Morghen, B. Rosenwirth, E. Verschoor, R. Wagner, and H. Wolf. 1999. HIV-1 vaccine-induced immune responses which correlate with protection from SHIV infection: compiled preclinical efficacy data from trials with ten different HIV-1 vaccine candidates. Immunol Lett 66:189-195.

70. Heeney, J. L. 2002. The critical role of CD4(+) T-cell help in immunity to HIV. Vaccine 20:1961-1963.

71. Heeney, J. L., V. J. Teeuwsen, M. van Gils, W. M. Bogers, C. De Giuli Morghen, A. Radaelli, S. Barnett, B. Morein, L. Akerblom, Y. Wang, T. Lehner, and D. Davis. 1998. beta-chemokines and neutralizing antibody titers correlate with sterilizing immunity generated in HIV-1 vaccinated macaques. Proceedings of the National Academy of Sciences of the United States of America 95:10803-10808.

72. Heeney, J. L., C. van Els, P. de Vries, P. Ten Haaft, N. Otting, W. Koornstra, J. Boes, R. Dubbes, H. Niphuis, M. Dings, M. Cranage, S. Norley, M. Jonker, R. E. Bontrop, and A. Osterhaus. 1994. Major histocompatibility complex class I-associated vaccine protection from simian immunodeficiency virus-infected peripheral blood cells. J Exp Med 180:769-774.

73. Heeney, J. L., M. E. van Gils, P. van der Meide, C. De Giuli Morghen, C. Ghioni, M. Gimelli, A. Raddelli, D. Davis, L. Akerblom, and B. Morein. 1998. The role of type-1 and type-2 T-helper immune responses in HIV-1 vaccine protection. Journal of medical primatology 27:50-58.

74. Helling, F., S. Zhang, A. Shang, S. Adluri, M. Calves, R. Koganty, B. M. Longenecker, T. J. Yao, H. F. Oettgen, and P. O. Livingston. 1995. GM2-KLH conjugate vaccine: increased immunogenicity in melanoma patients after administration with immunological adjuvant QS-21. Cancer research 55:2783-2788.

75. Hofmann-Lehmann, R., J. Vlasak, R. A. Rasmussen, S. Jiang, P. L. Li, T. W. Baba, D. C. Montefiori, B. J. Bernacky, T. A. Rizvi, R. Schmidt, L. R. Hill, M. E. Keeling, H. Katinger, G. Stiegler, L. A. Cavacini, M. R. Posner, and R. M. Ruprecht. 2002. Postnatal pre- and postexposure passive immunization strategies: protection of neonatal macaques against oral simian-human

immunodeficiency virus challenge. Journal of medical primatology 31:109-119.

76. Hofmann-Lehmann, R., J. Vlasak, R. A. Rasmussen, B. A. Smith, T. W. Baba, V. Liska, F. Ferrantelli, D. C. Montefiori, H. M. McClure, D. C. Anderson, B. J. Bernacky, T. A. Rizvi, R. Schmidt, L. R. Hill, M. E. Keeling, H. Katinger, G. Stiegler, L. A. Cavacini, M. R. Posner, T. C. Chou, J. Andersen, and R. M. Ruprecht. 2001. Postnatal passive immunization of neonatal macaques with a triple combination of human monoclonal antibodies against oral simian-human immunodeficiency virus challenge. J Virol 75:7470-7480.

77. Hornung, V., F. Bauernfeind, A. Halle, E. O. Samstad, H. Kono, K. L. Rock, K. A. Fitzgerald, and E. Latz. 2008. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol 9:847-856.

78. Hu, S. L., K. Abrams, G. N. Barber, P. Moran, J. M. Zarling, A. J. Langlois, L. Kuller, W. R. Morton, and R. E. Benveniste. 1992. Protection of macaques against SIV infection by subunit vaccines of SIV envelope glycoprotein gp160. Science 255:456-459.

79. Hurst, S. M., T. S. Wilkinson, R. M. McLoughlin, S. Jones, S. Horiuchi, N. Yamamoto, S. Rose-John, G. M. Fuller, N. Topley, and S. A. Jones. 2001. Il-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation. Immunity 14:705-714.

80. Hutnick, N. A., D. G. Carnathan, S. A. Dubey, G. Makedonas, K. S. Cox, L. Kierstead, S. J. Ratcliffe, M. N. Robertson, D. R. Casimiro, H. C. Ertl, and M. R. Betts. 2009. Baseline Ad5 serostatus does not predict Ad5 HIV vaccine-induced expansion of adenovirus-specific CD4+ T cells. Nature medicine 15:876-878.

81. Jones, S. A. 2005. Directing transition from innate to acquired immunity: defining a role for IL-6. J Immunol 175:3463-3468.

82. Kaplanski, G., V. Marin, F. Montero-Julian, A. Mantovani, and C. Farnarier. 2003. IL-6: a regulator of the transition from neutrophil to monocyte recruitment during inflammation. Trends Immunol 24:25-29.

83. Karasavvas, N., E. Billings, M. Rao, C. Williams, S. Zolla-Pazner, R. T. Bailer, R. A. Koup, S. Madnote, D. Arworn, X. Shen, G. D. Tomaras, J. R. Currier, M. Jiang, C. Magaret, C. Andrews, R. Gottardo, P. Gilbert, T. J. Cardozo, S. Rerks-Ngarm, S. Nitayaphan, P. Pitisuttithum, J. Kaewkungwal, R. Paris, K. Greene, H. Gao, S. Gurunathan, J. Tartaglia, F. Sinangil, B. T. Korber, D. C. Montefiori, J. R. Mascola, M. L. Robb, B. F. Haynes, V. Ngauy, N. L. Michael, J. H. Kim, and M. S. de Souza for the MOPH TAVEG Collab. 2012. The Thai Phase III HIV Type 1 Vaccine Trial (RV144) Regimen Induces Antibodies That Target Conserved Regions Within the V2 Loop of gp120. AIDS research and human retroviruses 28:1444-1457.

84. Karnasuta, C., R. M. Paris, J. H. Cox, S. Nitayaphan, P. Pitisuttithum, P. Thongcharoen, A. E. Brown, S. Gurunathan, J. Tartaglia, W. L. Heyward, J. G. McNeil, D. L. Birx, M. S. de Souza, and T. Thai Aids Vaccine Evaluation Group. 2005. Antibody-dependent cell-mediated cytotoxic responses in participants enrolled in a phase I/II ALVAC-HIV/AIDSVAX B/E prime-boost HIV-1 vaccine trial in Thailand. Vaccine 23:2522-2529.

85. Keefer, M. C., M. Wolff, G. J. Gorse, B. S. Graham, L. Corey, M. L. Clements-Mann, N. Verani-Ketter, S. Erb, C. M. Smith, R. B. Belshe, L. J. Wagner, M. J. McElrath, D. H. Schwartz, and P. Fast. 1997. Safety profile of phase I and II preventive HIV type 1 envelope vaccination: experience of the NIAID AIDS Vaccine Evaluation Group. AIDS research and human retroviruses 13:1163-1177.

86. Kennedy, J. S., M. Co, S. Green, K. Longtine, J. Longtine, M. A. Neill, J. P. Adams, A. L. Rothman, Q. Yu, R. Johnson-Leva, R. Pal, S. Wang, S. Lu, and P. Markham. 2008. The safety and tolerability of an HIV-1 DNA prime-protein boost vaccine (DP6-001) in healthy adult volunteers. Vaccine 26:4420-4424.

87. Kensil, C. R., and R. Kammer. 1998. QS-21: a water-soluble triterpene glycoside adjuvant. Expert opinion on investigational drugs 7:1475-1482.

88. Kensil, C. R., U. Patel, M. Lennick, and D. Marciani. 1991. Separation and characterization of saponins with adjuvant activity from Quillaja saponaria Molina cortex. J Immunol 146:431-437.

89. Kensil, C. R., J. Y. Wu, and S. Soltysik. 1995. Structural and immunological characterization of the vaccine adjuvant QS-21. Pharmaceutical biotechnology 6:525-541.

90. Kim, S. K., G. Ragupathi, C. Musselli, S. J. Choi, Y. S. Park, and P. O. Livingston. 1999. Comparison of the effect of different immunological adjuvants on the antibody and T-cell response to immunization with MUC1-KLH and GD3-KLH conjugate cancer vaccines. Vaccine 18:597-603.

91. Kong, W.-P., Y. Huang, Z.-Y. Yang, B. K. Chakrabarti, Z. Moodie, and G. J. Nabel. 2003. Immunogenicity of multiple gene and clade human immunodeficiency virus type 1 DNA vaccines. J Virol 77:12764-12772.

92. Kool, M., T. Soullié, M. van Nimwegen, M. A. M. Willart, F. Muskens, S. Jung, H. C. Hoogsteden, H. Hammad, and B. N. Lambrecht. 2008. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J Exp Med 205:869-882.

93. Korsholm, K. S., R. V. Petersen, E. M. Agger, and P. Andersen. 2010. T-helper 1 and T-helper 2 adjuvants induce distinct differences in the magnitude, quality and kinetics of the early inflammatory response at the site of injection. Immunology 129:75-86.

94. Lambrecht, B. N., M. Kool, M. A. M. Willart, and H. Hammad. 2009. Mechanism of action of clinically approved adjuvants. Current opinion in immunology 21:23-29.

95. Ledford, H. 2007. HIV vaccine may raise risk. Nature 450:325.

96. Letvin, N. L., D. C. Montefiori, Y. Yasutomi, H. C. Perry, M. E. Davies, C. Lekutis, M. Alroy, D. C. Freed, C. I. Lord, L. K. Handt, M. A. Liu, and J. W. Shiver. 1997. Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. Proceedings of the National Academy of Sciences of the United States of America 94:9378-9383.

97. Li, H., S. Nookala, and F. Re. 2007. Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1beta and IL-18 release. J Immunol 178:5271-5276.

98. Li, M., F. Gao, J. R. Mascola, L. Stamatatos, V. R. Polonis, M. Koutsoukos, G. Voss, P. Goepfert, P. Gilbert, K. M. Greene, M. Bilska, D. L. Kothe, J. F. Salazar-Gonzalez, X. Wei, J. M. Decker, B. H. Hahn, and D. C. Montefiori. 2005. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J Virol 79:10108-10125.

99. Liu, G., C. Anderson, H. Scaltreto, J. Barbon, and C. R. Kensil. 2002. QS-21 structure/function studies: effect of acylation on adjuvant activity. Vaccine 20:2808-2815.

100. Livingston, P. O., S. Adluri, F. Helling, T. J. Yao, C. R. Kensil, M. J. Newman, and D. Marciani. 1994. Phase 1 trial of immunological adjuvant QS-21 with a GM2 ganglioside-keyhole limpet haemocyanin conjugate vaccine in patients with malignant melanoma. Vaccine 12:1275-1280.

101. Lu, S. 2009. Heterologous prime-boost vaccination. Current opinion in immunology 21:346-351.

102. Lu, S. 2008. Immunogenicity of DNA vaccines in humans: it takes two to tango. Hum Vaccin 4:449-452.

103. Lu, S., J. Arthos, D. C. Montefiori, Y. Yasutomi, K. Manson, F. Mustafa, E. Johnson, J. C. Santoro, J. Wissink, J. I. Mullins, J. R. Haynes, N. L. Letvin, M. Wyand, and H. L. Robinson. 1996. Simian immunodeficiency virus DNA vaccine trial in macaques. J Virol 70:3978-3991.

104. Lu, S., J. C. Santoro, D. H. Fuller, J. R. Haynes, and H. L. Robinson. 1995. Use of DNAs expressing HIV-1 Env and noninfectious HIV-1 particles to raise antibody responses in mice. Virology 209:147-154.

105. Lu, S., S. Wang, and J. M. Grimes-Serrano. 2008. Current progress of DNA vaccine studies in humans. Expert review of vaccines 7:175-191.

106. Luheshi, G., and N. Rothwell. 1996. Cytokines and fever. Int Arch Allergy Immunol 109:301-307.

107. Ma, J., P. A. Bulger, D. R. Davis, B. Perilli-Palmer, D. A. Bedore, C. R. Kensil, E. M. Young, C. H. Hung, J. R. Seals, C. S. Pavia, and et al. 1994. Impact of the saponin adjuvant QS-21 and aluminium hydroxide on the immunogenicity of recombinant OspA and OspB of Borrelia burgdorferi. Vaccine 12:925-932.

108. Macgregor, R. R., J. D. Boyer, K. E. Ugen, K. E. Lacy, S. J. Gluckman, M. L. Bagarazzi, M. A. Chattergoon, Y. Baine, T. J. Higgins, R. B. Ciccarelli, L. R. Coney, R. S. Ginsberg, and D. B. Weiner. 1998. First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1

infection: safety and host response. The Journal of infectious diseases 178:92-100.

109. Maraskovsky, E., M. Schnurr, N. S. Wilson, N. C. Robson, J. Boyle, and D. Drane. 2009. Development of prophylactic and therapeutic vaccines using the ISCOMATRIX adjuvant. Immunol Cell Biol 87:371-376.

110. Maraskovsky, E., S. Sjölander, D. P. Drane, M. Schnurr, T. T. T. Le, L. Mateo, T. Luft, K.-A. Masterman, T.-Y. Tai, Q. Chen, S. Green, A. Sjölander, M. J. Pearse, F. A. Lemonnier, W. Chen, J. Cebon, and A. Suhrbier. 2004. NY-ESO-1 protein formulated in ISCOMATRIX adjuvant is a potent anticancer vaccine inducing both humoral and CD8+ t-cell-mediated immunity and protection against NY-ESO-1+ tumors. Clin Cancer Res 10:2879-2890.

111. Marichal, T., K. Ohata, D. Bedoret, C. Mesnil, C. Sabatel, K. Kobiyama, P. Lekeux, C. Coban, S. Akira, K. J. Ishii, F. Bureau, and C. J. Desmet. 2012. DNA released from dying host cells mediates aluminum adjuvant activity. Nature medicine:1-8.

112. Mascola, J. R., M. G. Lewis, G. Stiegler, D. Harris, T. C. VanCott, D. Hayes, M. K. Louder, C. R. Brown, C. V. Sapan, S. S. Frankel, Y. Lu, M. L. Robb, H. Katinger, and D. L. Birx. 1999. Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. J Virol 73:4009-4018.

113. Mascola, J. R., S. W. Snyder, O. S. Weislow, S. M. Belay, R. B. Belshe, D. H. Schwartz, M. L. Clements, R. Dolin, B. S. Graham, G. J. Gorse, M. C. Keefer, M. J. McElrath, M. C. Walker, K. F. Wagner, J. G. McNeil, F. E. McCutchan, and D. S. Burke. 1996. Immunization with envelope subunit vaccine products elicits neutralizing antibodies against laboratory-adapted but not primary isolates of human immunodeficiency virus type 1. The National Institute of Allergy and Infectious Diseases AIDS Vaccine Evaluation Group. The Journal of infectious diseases 173:340-348.

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

115. Mata-Haro, V., C. Cekic, M. Martin, P. M. Chilton, C. R. Casella, and T. C. Mitchell. 2007. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science 316:1628-1632.

116. McAleer, W. J., E. B. Buynak, R. Z. Maigetter, D. E. Wampler, W. J. Miller, and M. R. Hilleman. 1984. Human hepatitis B vaccine from recombinant yeast. Nature 307:178-180.

117. McClements, W. L., M. E. Armstrong, R. D. Keys, and M. A. Liu. 1996. Immunization with DNA vaccines encoding glycoprotein D or glycoprotein B, alone or in combination, induces protective immunity in animal models of

herpes simplex virus-2 disease. Proceedings of the National Academy of Sciences of the United States of America 93:11414-11420.

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

119. Mckee, A. S., M. W. Munks, M. K. L. Macleod, C. J. Fleenor, N. Van Rooijen, J. W. Kappler, and P. Marrack. 2009. Alum Induces Innate Immune Responses through Macrophage and Mast Cell Sensors, But These Sensors Are Not Required for Alum to Act As an Adjuvant for Specific Immunity. J Immunol 183:4403-4414.

120. McKenzie, A., M. Watt, and C. Gittleson. 2010. ISCOMATRIX™ vaccines: Safety in human clinical studies. Hum Vaccin 6:237-246.

121. Michel, M. L., H. L. Davis, M. Schleef, M. Mancini, P. Tiollais, and R. G. Whalen. 1995. DNA-mediated immunization to the hepatitis B surface antigen in mice: aspects of the humoral response mimic hepatitis B viral infection in humans. Proceedings of the National Academy of Sciences of the United States of America 92:5307-5311.

122. Middleton, D., S. Rockman, M. Pearse, I. Barr, S. Lowther, J. Klippel, D. Ryan, and L. Brown. 2009. Evaluation of vaccines for H5N1 influenza virus in ferrets reveals the potential for protective single-shot immunization. J Virol 83:7770-7778.

123. Mitchell, T. C. 2012. Effects of differences in lipid A structure on TLR4 pro-inflammatory signaling and inflammasome activation. Front Immunol:1-8.

124. Montefiori, D. C. 2005. Evaluating neutralizing antibodies against HIV, SIV, and SHIV in luciferase reporter gene assays. Current protocols in immunology / edited by John E. Coligan ... [et al.] Chapter 12:Unit 12 11.

125. Montefiori, D. C., C. Karnasuta, Y. Huang, H. Ahmed, P. Gilbert, M. S. de Souza, R. McLinden, S. Tovanabutra, A. Laurence-Chenine, E. Sanders-Buell, M. A. Moody, M. Bonsignori, C. Ochsenbauer, J. Kappes, H. Tang, K. Greene, H. Gao, C. C. LaBranche, C. ANDREWS, V. R. Polonis, S. Rerks-Ngarm, P. Pitisuttithum, S. Nitayaphan, J. Kaewkungwal, S. G. Self, P. W. Berman, D. Francis, F. Sinangil, C. Lee, J. Tartaglia, M. L. Robb, B. F. Haynes, N. L. Michael, and J. H. Kim. 2012. Magnitude and Breadth of the Neutralizing Antibody Response in the RV144 and Vax003 HIV-1 Vaccine Efficacy Trials. The Journal of infectious diseases 206:431-441.

126. Morein, B., B. Sundquist, S. Höglund, K. Dalsgaard, and A. Osterhaus. 1984. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature 308:457-460.

127. Morelli, A., D. Becher, S. Koernig, A. Silva, D. Drane, and E. Maraskovsky. 2012. ISCOMATRIX adjuvant: A novel adjuvant for use in prophylactic and therapeutic infectious disease vaccines. J Med Microbiol.

128. Neuzil, K. M., J. E. Johnson, Y. W. Tang, J. P. Prieels, M. Slaoui, N. Gar, and B. S. Graham. 1997. Adjuvants influence the quantitative and qualitative immune response in BALB/c mice immunized with respiratory syncytial virus FG subunit vaccine. Vaccine 15:525-532.

129. Newman, M. J., J. Y. Wu, B. H. Gardner, C. A. Anderson, C. R. Kensil, J. Recchia, R. T. Coughlin, and M. F. Powell. 1997. Induction of cross-reactive cytotoxic T-lymphocyte responses specific for HIV-1 gp120 using saponin adjuvant (QS-21) supplemented subunit vaccine formulations. Vaccine 15:1001-1007.

130. Newman, M. J., J. Y. Wu, B. H. Gardner, K. J. Munroe, D. Leombruno, J. Recchia, C. R. Kensil, and R. T. Coughlin. 1992. Saponin adjuvant induction of ovalbumin-specific CD8+ cytotoxic T lymphocyte responses. J Immunol 148:2357-2362.

131. Nicholaou, T., W. Chen, I. D. Davis, H. M. Jackson, N. Dimopoulos, C. Barrow, J. Browning, D. MacGregor, D. Williams, W. Hopkins, E. Maraskovsky, R. Venhaus, L. Pan, E. W. Hoffman, L. J. Old, and J. Cebon. 2011. Immunoediting and persistence of antigen-specific immunity in patients who have previously been vaccinated with NY-ESO-1 protein formulated in ISCOMATRIX™. Cancer Immunol Immunother 60:1625-1637.

132. Nitayaphan, S., P. Pitisuttithum, C. Karnasuta, C. Eamsila, M. de Souza, P. Morgan, V. Polonis, M. Benenson, T. VanCott, S. Ratto-Kim, J. Kim, D. Thapinta, R. Garner, V. Bussaratid, P. Singharaj, R. el-Habib, S. Gurunathan, W. Heyward, D. Birx, J. McNeil, A. E. Brown, and A. V. E. G. Thai. 2004. Safety and immunogenicity of an HIV subtype B and E prime-boost vaccine combination in HIV-negative Thai adults. The Journal of infectious diseases 190:702-706.

133. Norrby, E., and S. B. Prusiner. 2007. Polio and Nobel prizes: looking back 50 years. Annals of neurology 61:385-395.

134. O'Brien, K. L., J. Liu, S. L. King, Y. H. Sun, J. E. Schmitz, M. A. Lifton, N. A. Hutnick, M. R. Betts, S. A. Dubey, J. Goudsmit, J. W. Shiver, M. N. Robertson, D. R. Casimiro, and D. H. Barouch. 2009. Adenovirus-specific immunity after immunization with an Ad5 HIV-1 vaccine candidate in humans. Nature medicine 15:873-875.

135. Ogilvie, A. C., C. E. Hack, J. Wagstaff, G. J. van Mierlo, A. J. Erenberg, L. L. Thomsen, K. Hoekman, and E. M. Rankin. 1996. IL-1 beta does not cause neutrophil degranulation but does lead to IL-6, IL-8, and nitrite/nitrate release when used in patients with cancer. J Immunol 156:389-394.

136. Okemoto, K., K. Kawasaki, K. Hanada, M. Miura, and M. Nishijima. 2006. A potent adjuvant monophosphoryl lipid A triggers various immune responses, but not secretion of IL-1β or activation of caspase-1. J Immunol 176:1203-1208.

137. Pal, R., V. S. Kalyanaraman, B. C. Nair, S. Whitney, T. Keen, L. Hocker, L. Hudacik, N. Rose, I. Mboudjeka, S. Shen, T.-H. Wu-Chou, D. Montefiori, J.

Mascola, P. Markham, and S. Lu. 2006. Immunization of rhesus macaques with a polyvalent DNA prime/protein boost human immunodeficiency virus type 1 vaccine elicits protective antibody response against simian human immunodeficiency virus of R5 phenotype. Virology 348:341-353.

138. Pal, R., S. Wang, V. S. Kalyanaraman, B. C. Nair, S. Whitney, T. Keen, L. Hocker, L. Hudacik, N. Rose, A. Cristillo, I. Mboudjeka, S. Shen, T.-H. Wu-Chou, D. Montefiori, J. Mascola, S. Lu, and P. Markham. 2005. Polyvalent DNA prime and envelope protein boost HIV-1 vaccine elicits humoral and cellular responses and controls plasma viremia in rhesus macaques following rectal challenge with an R5 SHIV isolate. Journal of medical primatology 34:226-236.

139. Pal, R., S. Wang, V. S. Kalyanaraman, B. C. Nair, S. Whitney, T. Keen, L. Hocker, L. Hudacik, N. Rose, A. Cristillo, I. Mboudjeka, S. Shen, T.-H. Wu-Chou, D. Montefiori, J. Mascola, S. Lu, and P. Markham. 2005. Polyvalent DNA prime and envelope protein boost HIV-1 vaccine elicits humoral and cellular responses and controls plasma viremia in rhesus macaques following rectal challenge with an R5 SHIV isolate. Journal of medical primatology 34:226-236.

140. Pal, R., Q. Yu, S. Wang, V. S. Kalyanaraman, B. C. Nair, L. Hudacik, S. Whitney, T. Keen, C.-L. Hung, L. Hocker, J. S. Kennedy, P. Markham, and S. Lu. 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.

141. Pearse, M. J., and D. Drane. 2004. ISCOMATRIX adjuvant: a potent inducer of humoral and cellular immune responses. Vaccine 22:2391-2395.

142. Pitisuttithum, P., P. W. Berman, B. Phonrat, P. Suntharasamai, S. Raktham, L. O. Srisuwanvilai, K. Hirunras, D. Kitayaporn, J. Kaewkangwal, S. Migasena, H. W. Sheppard, E. Li, M. Chernow, M. L. Peterson, R. Shibata, W. L. Heyward, and D. P. Francis. 2004. Phase I/II study of a candidate vaccine designed against the B and E subtypes of HIV-1. Journal of acquired immune deficiency syndromes 37:1160-1165.

143. Pitisuttithum, P., S. Nitayaphan, P. Thongcharoen, C. Khamboonruang, J. Kim, M. de Souza, T. Chuenchitra, R. P. Garner, D. Thapinta, V. Polonis, S. Ratto-Kim, P. Chanbancherd, J. Chiu, D. L. Birx, A. M. Duliege, J. G. McNeil, A. E. Brown, and A. V. E. G. Thai. 2003. Safety and immunogenicity of combinations of recombinant subtype E and B human immunodeficiency virus type 1 envelope glycoprotein 120 vaccines in healthy Thai adults. The Journal of infectious diseases 188:219-227.

144. Pitisuttithum, P., S. Rerks-Ngarm, V. Bussaratid, J. Dhitavat, W. Maekanantawat, S. Pungpak, P. Suntharasamai, S. Vanijanonta, S. Nitayapan, J. Kaewkungwal, M. Benenson, P. Morgan, R. J. O'Connell, J. Berenberg, S. Gurunathan, D. P. Francis, R. Paris, J. Chiu, D. Stablein, N. L. Michael, J.-L. Excler, M. L. Robb, and J. H. Kim. 2011. Safety and Reactogenicity of Canarypox ALVAC-HIV (vCP1521) and

HIV-1 gp120 AIDSVAX B/E Vaccination in an Efficacy Trial in Thailand. PLoS One 6:e27837.

145. Plotkin, S. A. 2010. The RV144 Thai HIV vaccine trial. Hum Vaccin 6:159.

146. Polakos, N. K., D. Drane, J. Cox, P. Ng, M. J. Selby, D. Chien, D. T. O'Hagan, M. Houghton, and X. Paliard. 2001. Characterization of hepatitis C virus core-specific immune responses primed in rhesus macaques by a nonclassical ISCOM vaccine. J Immunol 166:3589-3598.

147. Priddy, F. H., D. Brown, J. Kublin, K. Monahan, D. P. Wright, J. Lalezari, S. Santiago, M. Marmor, M. Lally, R. M. Novak, S. J. Brown, P. Kulkarni, S. A. Dubey, L. S. Kierstead, D. R. Casimiro, R. Mogg, M. J. DiNubile, J. W. Shiver, R. Y. Leavitt, M. N. Robertson, D. V. Mehrotra, E. Quirk, and V. S. G. Merck. 2008. Safety and immunogenicity of a replication-incompetent adenovirus type 5 HIV-1 clade B gag/pol/nef vaccine in healthy adults. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 46:1769-1781.

148. Rathinam, V. A., Z. Jiang, S. N. Waggoner, S. Sharma, L. E. Cole, L. Waggoner, S. K. Vanaja, B. G. Monks, S. Ganesan, E. Latz, V. Hornung, S. N. Vogel, E. Szomolanyi-Tsuda, and K. A. Fitzgerald. 2010. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. Nat Immunol 11:395-402.

149. Rathinam, V. A. K., S. K. Vanaja, and K. A. Fitzgerald. 2012. Regulation of inflammasome signaling. Nat Immunol 13:333-332.

150. Rerks-Ngarm, S., P. Pitisuttithum, S. Nitayaphan, J. Kaewkungwal, J. Chiu, R. Paris, N. Premsri, C. Namwat, M. de Souza, E. Adams, M. Benenson, S. Gurunathan, J. Tartaglia, J. G. McNeil, D. P. Francis, D. Stablein, D. L. Birx, S. Chunsuttiwat, C. Khamboonruang, P. Thongcharoen, M. L. Robb, N. L. Michael, P. Kunasol, J. H. Kim, and M.-T. Investigators. 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N Engl J Med 361:2209-2220.

151. Ribi, E. 1984. Beneficial modification of the endotoxin molecule. Journal of biological response modifiers 3:1-9.

152. Ribi, E., J. L. Cantrell, K. Takayama, N. Qureshi, J. Peterson, and H. O. Ribi. 1984. Lipid A and immunotherapy. Reviews of infectious diseases 6:567-572.

153. Richmond, J., S. Lu, J. Santoro, J. Weng, S. Hu, D. Montefiori, and H. Robinson. 1998. Studies of the neutralizing activity and avidity of anti-human immunodeficiency virus type 1 Env antibody elicited by DNA priming and protein boosting. J Virol 72:9092.

154. Rimmelzwaan, G., N. Nieuwkoop, A. Brandenburg, G. Sutter, W. Beyer, D. Maher, J. Bates, and A. Osterhaus. 2000. A randomized, double blind study in young healthy adults comparing cell mediated and humoral immune responses induced by influenza ISCOM (TM) vaccines and conventional vaccines. Vaccine 19:1180-1187.

155. Robinson, H. L., L. A. Hunt, and R. G. Webster. 1993. Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. Vaccine 11:957-960.

156. Robinson, H. L., D. C. Montefiori, R. P. Johnson, K. H. Manson, M. L. Kalish, J. D. Lifson, T. A. Rizvi, S. Lu, S. L. Hu, G. P. Mazzara, D. L. Panicali, J. G. Herndon, R. Glickman, M. A. Candido, S. L. Lydy, M. S. Wyand, and H. M. McClure. 1999. Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. Nature medicine 5:526-534.

157. Robson, N. C., H. Beacock-Sharp, A. M. Donachie, and A. M. Mowat. 2003. Dendritic cell maturation enhances CD8+ T-cell responses to exogenous antigen via a proteasome-independent mechanism of major histocompatibility complex class I loading. Immunology 109:374-383.

158. Roy, M. J., M. S. Wu, L. J. Barr, J. T. Fuller, L. G. Tussey, S. Speller, J. Culp, J. K. Burkholder, W. F. Swain, R. M. Dixon, G. Widera, R. Vessey, A. King, G. Ogg, A. Gallimore, J. R. Haynes, and D. Heydenburg Fuller. 2000. Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine 19:764-778.

159. Rts, S. C. T. P., S. T. Agnandji, B. Lell, J. F. Fernandes, B. P. Abossolo, B. G. Methogo, A. L. Kabwende, A. A. Adegnika, B. Mordmuller, S. Issifou, P. G. Kremsner, J. Sacarlal, P. Aide, M. Lanaspa, J. J. Aponte, S. Machevo, S. Acacio, H. Bulo, B. Sigauque, E. Macete, P. Alonso, S. Abdulla, N. Salim, R. Minja, M. Mpina, S. Ahmed, A. M. Ali, A. T. Mtoro, A. S. Hamad, P. Mutani, M. Tanner, H. Tinto, U. D'Alessandro, H. Sorgho, I. Valea, B. Bihoun, I. Guiraud, B. Kabore, O. Sombie, R. T. Guiguemde, J. B. Ouedraogo, M. J. Hamel, S. Kariuki, M. Oneko, C. Odero, K. Otieno, N. Awino, M. McMorrow, V. Muturi-Kioi, K. F. Laserson, L. Slutsker, W. Otieno, L. Otieno, N. Otsyula, S. Gondi, A. Otieno, V. Owira, E. Oguk, G. Odongo, J. B. Woods, B. Ogutu, P. Njuguna, R. Chilengi, P. Akoo, C. Kerubo, C. Maingi, T. Lang, A. Olotu, P. Bejon, K. Marsh, G. Mwambingu, S. Owusu-Agyei, K. P. Asante, K. Osei-Kwakye, O. Boahen, D. Dosoo, I. Asante, G. Adjei, E. Kwara, D. Chandramohan, B. Greenwood, J. Lusingu, S. Gesase, A. Malabeja, O. Abdul, C. Mahende, E. Liheluka, L. Malle, M. Lemnge, T. G. Theander, C. Drakeley, D. Ansong, T. Agbenyega, S. Adjei, H. O. Boateng, T. Rettig, J. Bawa, J. Sylverken, D. Sambian, A. Sarfo, et al. 2012. A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. N Engl J Med 367:2284-2295.

160. Sanders, M. T., L. E. Brown, G. Deliyannis, and M. J. Pearse. 2005. ISCOM-based vaccines: the second decade. Immunol Cell Biol 83:119-128.

161. Sasaki, S., K. Sumino, K. Hamajima, J. Fukushima, N. Ishii, S. Kawamoto, H. Mohri, C. R. Kensil, and K. Okuda. 1998. Induction of systemic and mucosal immune responses to human immunodeficiency virus type 1 by a

DNA vaccine formulated with QS-21 saponin adjuvant via intramuscular and intranasal routes. J Virol 72:4931-4939.

162. Sasaki, S., T. Tsuji, K. Hamajima, J. Fukushima, N. Ishii, T. Kaneko, K. Q. Xin, H. Mohri, I. Aoki, T. Okubo, K. Nishioka, and K. Okuda. 1997. Monophosphoryl lipid A enhances both humoral and cell-mediated immune responses to DNA vaccination against human immunodeficiency virus type 1. Infection and immunity 65:3520-3528.

163. Scapini, P., J. A. Lapinet-Vera, S. Gasperini, F. Calzetti, F. Bazzoni, and M. A. Cassatella. 2000. The neutrophil as a cellular source of chemokines. Immunol Rev 177:195-203.

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

165. Schnurr, M. 2005. Tumor antigen processing and presentation depend critically on dendritic cell type and the mode of antigen delivery. 105:2465-2472.

166. Schnurr, M., Q. Chen, A. Shin, W. Chen, T. Toy, C. Jenderek, S. Green, L. Miloradovic, D. Drane, I. D. Davis, J. Villadangos, K. Shortman, E. Maraskovsky, and J. Cebon. 2005. Tumor antigen processing and presentation depend critically on dendritic cell type and the mode of antigen delivery. Blood 105:2465-2472.

167. Schnurr, M., M. Orban, N. C. Robson, A. Shin, H. Braley, D. Airey, J. Cebon, E. Maraskovsky, and S. Endres. 2009. ISCOMATRIX adjuvant induces efficient cross-presentation of tumor antigen by dendritic cells via rapid cytosolic antigen delivery and processing via tripeptidyl peptidase II. J Immunol 182:1253-1259.

168. Seok, J., H. S. Warren, A. G. Cuenca, M. N. Mindrinos, H. V. Baker, W. Xu, D. R. Richards, G. P. McDonald-Smith, H. Gao, L. Hennessy, C. C. Finnerty, C. M. Lopez, S. Honari, E. E. Moore, J. P. Minei, J. Cuschieri, P. E. Bankey, J. L. Johnson, J. Sperry, A. B. Nathens, T. R. Billiar, M. A. West, M. G. Jeschke, M. B. Klein, R. L. Gamelli, N. S. Gibran, B. H. Brownstein, C. Miller-Graziano, S. E. Calvano, P. H. Mason, J. P. Cobb, L. G. Rahme, S. F. Lowry, R. V. Maier, L. L. Moldawer, D. N. Herndon, R. W. Davis, W. Xiao, R. G. Tompkins, Inflammation, and L. S. C. R. P. Host Response to Injury. 2013. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proceedings of the National Academy of Sciences of the United States of America 110:3507-3512.

169. Sharp, F. A., D. Ruane, B. Claass, E. Creagh, J. Harris, P. Malyala, M. Singh, D. T. O'Hagan, V. Petrilli, J. Tschopp, L. A. O'Neill, and E. C. Lavelle. 2009. Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome. Proceedings of the National Academy of Sciences of the United States of America 106:870-875.

170. Sjolander, A., and J. C. Cox. 1998. Uptake and adjuvant activity of orally delivered saponin and ISCOM vaccines. Advanced drug delivery reviews 34:321-338.

171. Sjölander, A., D. Drane, E. Maraskovsky, J. P. Scheerlinck, A. Suhrbier, J. Tennent, and M. Pearse. 2001. Immune responses to ISCOM formulations in animal and primate models. Vaccine 19:2661-2665.

172. Sjolander, A., B. van't Land, and K. Lovgren Bengtsson. 1997. Iscoms containing purified Quillaja saponins upregulate both Th1-like and Th2-like immune responses. Cell Immunol 177:69-76.

173. Stewart, T. J., D. Drane, J. Malliaros, H. Elmer, K. M. Malcolm, J. C. Cox, S. J. Edwards, I. H. Frazer, and G. J. P. Fernando. 2004. ISCOMATRIX adjuvant: an adjuvant suitable for use in anticancer vaccines. Vaccine 22:3738-3743.

174. Sunderkotter, C. 2009. Vasculitis of small blood vessels--some riddles about IgA and about the complexity of transmigration. Exp Dermatol 18:91-96.

175. Thompson, B. S., P. M. Chilton, J. R. Ward, J. T. Evans, and T. C. Mitchell. 2005. The low-toxicity versions of LPS, MPL adjuvant and RC529, are efficient adjuvants for CD4+ T cells. J Leukoc Biol 78:1273-1280.

176. Thompson, M. R., J. J. Kaminski, E. A. Kurt-Jones, and K. A. Fitzgerald. 2011. Pattern recognition receptors and the innate immune response to viral infection. Viruses 3:920-940.

177. Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, and A. Friedman. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259:1745-1749.

178. Vaine, M., S. Wang, E. T. Crooks, P. Jiang, D. C. Montefiori, J. Binley, and S. Lu. 2008. Improved induction of antibodies against key neutralizing epitopes by human immunodeficiency virus type 1 gp120 DNA prime-protein boost vaccination compared to gp120 protein-only vaccination. J Virol 82:7369-7378.

179. Vaine, M., S. Wang, A. Hackett, J. Arthos, and S. Lu. 2010. Antibody responses elicited through homologous or heterologous prime-boost DNA and protein vaccinations differ in functional activity and avidity. Vaccine 28:2999-3007.

180. Vandepapeliere, P., Y. Horsmans, P. Moris, M. Van Mechelen, M. Janssens, M. Koutsoukos, P. Van Belle, F. Clement, E. Hanon, M. Wettendorff, N. Garcon, and G. Leroux-Roels. 2008. Vaccine adjuvant systems containing monophosphoryl lipid A and QS21 induce strong and persistent humoral and T cell responses against hepatitis B surface antigen in healthy adult volunteers. Vaccine 26:1375-1386.

181. Vernacchio, L., H. Bernstein, S. Pelton, C. Allen, K. MacDonald, J. Dunn, D. D. Duncan, G. Tsao, V. LaPosta, J. Eldridge, S. Laussucq, D. M. Ambrosino, and D. C. Molrine. 2002. Effect of monophosphoryl lipid A (MPL) on T-

helper cells when administered as an adjuvant with pneumococcoccal-CRM197 conjugate vaccine in healthy toddlers. Vaccine 20:3658-3667.

182. Vitoriano-Souza, J., N. Moreira, A. Teixeira-Carvalho, C. M. Carneiro, F. A. Siqueira, P. M. Vieira, R. C. Giunchetti, S. A. Moura, R. T. Fujiwara, M. N. Melo, and A. B. Reis. 2012. Cell recruitment and cytokines in skin mice sensitized with the vaccine adjuvants: saponin, incomplete Freund's adjuvant, and monophosphoryl lipid A. PLoS One 7:e40745.

183. Waite, D. C., E. W. Jacobson, F. A. Ennis, R. Edelman, B. White, R. Kammer, C. Anderson, and C. R. Kensil. 2001. Three double-blind, randomized trials evaluating the safety and tolerance of different formulations of the saponin adjuvant QS-21. Vaccine 19:3957-3967.

184. Wang, B., K. E. Ugen, V. Srikantan, M. G. Agadjanyan, K. Dang, Y. Refaeli, A. I. Sato, J. Boyer, W. V. Williams, and D. B. Weiner. 1993. Gene inoculation generates immune responses against human immunodeficiency virus type 1. Proceedings of the National Academy of Sciences of the United States of America 90:4156.

185. Wang, S., J. Arthos, J. M. Lawrence, D. Van Ryk, I. Mboudjeka, S. Shen, T.-H. W. Chou, D. C. Montefiori, and S. Lu. 2005. Enhanced immunogenicity of gp120 protein when combined with recombinant DNA priming to generate antibodies that neutralize the JR-FL primary isolate of human immunodeficiency virus type 1. J Virol 79:7933-7937.

186. Wang, S., J. S. Kennedy, K. West, D. C. Montefiori, S. Coley, J. Lawrence, S. Shen, S. Green, A. L. Rothman, F. A. Ennis, J. Arthos, R. Pal, P. Markham, and S. Lu. 2008. Cross-subtype antibody and cellular immune responses induced by a polyvalent DNA prime-protein boost HIV-1 vaccine in healthy human volunteers. Vaccine 26:1098-1110.

187. Weiner, D. B. 2010. RV144: old vs. new. Hum Vaccin 6:159-161.

188. Weissburg, R. P., P. W. Berman, J. L. Cleland, D. Eastman, F. Farina, S. Frie, A. Lim, J. Mordenti, T. T. Nguyen, and M. R. Peterson. 1995. Characterization of the MN gp120 HIV-1 vaccine: antigen binding to alum. Pharmaceutical research 12:1439-1446.

189. Wheeler, A. W., J. S. Marshall, and J. T. Ulrich. 2001. A Th1-inducing adjuvant, MPL, enhances antibody profiles in experimental animals suggesting it has the potential to improve the efficacy of allergy vaccines. Int Arch Allergy Immunol 126:135-139.

190. WHO. 2011. Global HIV/AIDS response: Epidemic update and health sector progress towards universal access: progress report 2011. World Health Organization.

191. Wilson, N. S., B. Yang, A. B. Morelli, S. Koernig, A. Yang, S. Loeser, D. Airey, L. Provan, P. Hass, H. Braley, S. Couto, D. Drane, J. Boyle, G. T. Belz, A. Ashkenazi, and E. Maraskovsky. 2011. ISCOMATRIX vaccines mediate CD8+ T-cell cross-priming by a MyD88-dependent signaling pathway. Nat Immunol:1-13.

192. Windon, R. G., P. J. Chaplin, L. Beezum, A. Coulter, R. Cahill, W. Kimpton, D. Drane, M. Pearse, A. Sjolander, J. M. Tennent, and J. Y. Scheerlinck. 2000. Induction of lymphocyte recruitment in the absence of a detectable immune response. Vaccine 19:572-578.

193. Windon, R. G., P. J. Chaplin, P. McWaters, M. Tavarnesi, M. Tzatzaris, W. G. Kimpton, R. N. Cahill, L. Beezum, A. Coulter, D. Drane, A. Sjolander, M. Pearse, J. P. Scheerlinck, and J. M. Tennent. 2001. Local immune responses to influenza antigen are synergistically enhanced by the adjuvant ISCOMATRIX. Vaccine 20:490-497.

194. Wu, J. Y., B. H. Gardner, C. I. Murphy, J. R. Seals, C. R. Kensil, J. Recchia, G. A. Beltz, G. W. Newman, and M. J. Newman. 1992. Saponin adjuvant enhancement of antigen-specific immune responses to an experimental HIV-1 vaccine. J Immunol 148:1519-1525.

195. Yamashiro, S., H. Kamohara, and T. Yoshimura. 1999. MCP-1 is selectively expressed in the late phase by cytokine-stimulated human neutrophils: TNF-alpha plays a role in maximal MCP-1 mRNA expression. J Leukoc Biol 65:671-679.

196. Zak, D. E., E. Andersen-Nissen, E. R. Peterson, A. Sato, M. K. Hamilton, J. Borgerding, A. T. Krishnamurty, J. T. Chang, D. J. Adams, T. R. Hensley, A. I. Salter, C. A. Morgan, A. C. Duerr, S. C. De Rosa, A. Aderem, and M. J. McElrath. 2012. Merck Ad5/HIV induces broad innate immune activation that predicts CD8(+) T-cell responses but is attenuated by preexisting Ad5 immunity. Proceedings of the National Academy of Sciences of the United States of America 109:E3503-3512.

197. Zolla-Pazner, S. 2004. Identifying epitopes of HIV-1 that induce protective antibodies. Nat Rev Immunol 4:199-210.