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Humoral and Cellular Immune Responses in RNA Viral Infections:
Immunogenicity of HIV-1, HCV and SARS-CoV Candidate Vaccines in Animal Models

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Humoral and Cellular Immune Responses in RNA Viral Infections: Immunogenicity of HIV-1, HCV and SARS-CoV Candidate Vaccines in Animal Models

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By

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ABSTRACT

It is difficult to induce protective immunity against most RNA viruses. However, there is strong evidence that humoral and especially cellular immune responses play crucial roles in the control of established RNA viral infections. Thus, an ideal vaccine should be able to induce strong specific antibody titer as well as a robust T-helper and T-cell cytotoxic response. Here, correlates of protective immunity against HIV-1, HCV and SARS-CoV were assessed.

Monocistronic and polycistronic DNA constructs containing structural HIV-1, and SARS genes were designed. The structural proteins (HIV- gp120, gag, pol, HCV-core, E1/E2 and SARS-NC) were also expressed, purified and characterized in mammalian and bacterial cell lines. HLA-A2.1 and B6 mice were immunized with different combinations of DNA constructs, recombinant proteins and novel adjuvants. Humoral responses were measured by titrating of specific antibodies and cell-mediated immune responses were identified by Th1/Th2 cytokine expression, lymphocyte proliferation, intracellular cytokine staining, HLA-peptide dimer assay, and ELISPOT. The first study in HIV-1 showed that a combination of DNA single constructs, protein and adjuvant induce a higher immune response compared to the DNA or/and protein alone. In the second HIV-1 approach, a synergistic effect between HIV/HCV antigens was detected that may lead to induction of multi-specific immune responses against both HIV and HCV. In the third study (SARS project), a high level of specific SARS-CD8+ T-cell response was demonstrated in mice that received DNA encoding the SARS-nucleocapsid, protein and XIAP (X-link inhibitor of apoptosis) as an adjuvant.

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LIST OF ABBREVIATIONS

ADCC-Antibody Directed Cell Cytotoxicity
AIDS-Acquired Immunodeficiency Syndrome
Ags-Antigens
APCs-Antigen Presenting Cells
ART-Antiviral Therapy
BSA-Bovine Serum Albumin
cDNA- Complementary Deoxyribonucleic Acid
CAF- CD8+ Antiviral Factor
CEF: Chick Embryo Fibroblast
CHO-Chinese Hamster Ovary
CMV-Cytomegalovirus
CoV-Coronavirus
CpG-Cytosine phosphate Guanosine
CCR-Chemokine Receptor
CTL-Cytotoxic T Lymphocytes
CRT- Calreticulin
DCs-Dendritic Cells
E-Envelope
EBV-Epstein-Barr Virus
ELISA-Enzyme Linked Immunosorbent Assay
ELISPOT-Enzyme Linked Immunospot Assay
FCS-Fetal Calf Serum
FITC- Flourescein-5-Isothiocyanate
GM-CSF-Granulocyte Macrophage Colony Stimulating Factor
HCV-Hepatitis C Virus
HIV-Human Immunodeficiency Virus
HLA-Human Leukocyte Antigen
HPV-Human Papillomavirus
IBV- Infectious Bronchitis Virus
ICS-Intracellular Cytokine Staining
IFA- Incomplete Freund's Adjuvant
IFNγ-Interferon Gamma
Ig-Immunoglubolin
IL-Interleukin
ION-Ionomycin
IMDM-Iscoves Modified Dulbecco's Medium
LCMV-Lymphocyte Choriomeningitis Virus
LPS-Lipopolysaccharide
LTNP-Long Term Non Progressor
LTR-Long Terminal Repeat
M-Matrix
Mab-Monoclonal Antibodies
MHC-Major Histocompatibility Complex
MHV-Mouse Hepatitis Virus

MIP-Macrophage Inflammatory Protein
MVA-Modified Vaccinia Ankara
Nabs-Neutralizing Antibodies
NK-Natural Killer
NC-Nucleocapsid
ODN-Oligodeoxynucleotide
OPD-O Phenylendiamine Dihydrochloride
PBMC-Peripheral Blood Mononuclear Cells
PCR-Polymerase Chain Reaction
PLC-Phospholipase C
PMA-Phorbol Myristyl Acetate
RANTES-Regulated upon Activation, Normal T cell Expressed and Secreted
RT-Reverse Trascriptase
S-Spike
SARS-Severe Acute Respiratory Syndrome
SDS page-Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis
SHIV-Simian Human Immunodeficiency Virus
SI-Stimulation Index
SIV-Simian Immunodeficiency Virus
SFC-Spot Forming Cells
Th-T Helper
Treg-Regulatory T cell
TCLA-T Cell Line Adapted
TEM-Transmission Electron Microscope
TCR-T cell receptor
TLR-Toll Like Receptor
TNF-Tumor Necrosis Factor
XIAP-X Linked Inhibitor of Apoptosis
VLPs-Viral Like Particles
VV-Vaccinia Virus
WHO-World Health Organization

Chapter 1:

Introduction

1.1 A Short History of Vaccination

The first evidence of vaccination traces back to the 7th century, when Indians used snake venom for vaccination against snake venom. In the 11th century in China, children were inoculated by inhalation of dry scabs from the smallpox pustules of infected people or by rubbing the dried material into a scratch. In 1721, Mary Wortley Montagu traveled to Constantinople and found that people applied dried pus from smallpox pustules onto the skin to prevent smallpox (Grundy, 2000). She later introduced this technique to Europeans. Edward Jenner's vaccine against cowpox was the first scientific attempt to control a disease and he published his work Variolae Vaccinae in 1798 (Katscher, 1999). However, the modern concept of vaccine development was introduced by Louis Pasteur. He used an attenuated form of chicken cholera itself rather than a related organism as Edward Jenner's had done. In 1881, Pasteur left a culture on the bench that was later inoculated into chickens but did not cause disease. He then made a fresh culture and inoculated the same chickens. He found that the chickens were resistant to the infection, and found that the old culture had rendered them immune. He hypothesized that pathogens could be attenuated by high temperature and chemicals. He confirmed his hypothesis by studying rabies and anthrax.

The essential concepts in vaccine research had been discovered by the 19th century, and the research of the early 20th century would bring refinements to these theoretical underpinnings when Calmette and Guerin used passage in media to attenuate

Mycobacterium bovis (Behr & Small, 1999;Oettinger, Jorgensen et al., 1999). Similarly, cell culture was used to grow viruses, which also permitted the selection of mutants by isolation of single clones and by incubation at temperatures below the host temperature, resulting in the generation of attenuated viruses.

1.2 Active and Passive Immunization

Transfer of antibodies to a recipient, such as the transfer from mother to fetus or the transfusion of a recipient with immune sera, is called passive immunization. However, this type of immunization provides only a temporary protection and induces many complications which may lead to systemic anaphylaxis or hypersensitivity reactions. The antibodies are not permanent and disappear over months and the recipient is no longer protected. However, recently, passive immunization in primates offered evidence that neutralizing monoclonal antibodies are suitable tools to control lentiviral infections. In a very interesting study, combinations of four neutralizing monoclonal antibodies could prevent infection in neonatal monkeys challenged orally with SHIV virus (Reimann, Li et al., 1996). In another primate study, a group of pregnant rhesus macaques were immunized with monoclonal antibodies five days before a caesarean section. Three days after, antibody treatment was repeated followed by intravenous challenge with SHIV. All newborn macaques from the immunied macaques were protected (Ruprecht, Ferrantelli et al., 2003). In an *in vitro* study in PBMC, groups of antibodies against well-characterized HIV envelope epitopes were isolated. They neutralized laboratory adapted HIV-strains and a number of primary isolates (Xu, Smith-Franklin et al., 2001). These antibodies may also be used for passive immunization.

Active immunization is a type of immunization that induces humoral and/or cellular immunity. Unlike passive immunization, active immunization may induce memory cells which usually last for many years. This immunization can be acquired naturally after an infection or by administration of antigens such as vaccines. Following exposure of the immune system to the same antigen, memory cells are activated and initiate replication, triggering an anti-microbial response. Active immunization plays a crucial role in protection from infectious diseases.

1.3 Classification of Viral Vaccines

1. Live attenuated vaccines

This type of vaccine stimulates long-lasting protection after a single or multiple immunizations and it has some of the criteria for an ideal vaccine. Attenuated viruses are modified in the laboratory usually by repeated culturing. They retain the ability to replicate and produce immunity but do not cause disease. For example, the measles vaccine strain which is used today was isolated from an 11 year old boy (David Edmonston) in 1954 and was passaged for a long period of time (1980). So far, several attenuated virus vaccines such as polio (Sabin type), rubella and mumps were developed by these methods (Saliou, 1995).

The immune response to a live attenuated vaccine is in many ways identical to natural infection and in general, the immune system can not differentiate between a weakened vaccine and an infection with a wild virus. However, this type of vaccine may cause severe or even fatal reactions in individuals with immunodeficiency (e.g. leukemia or HIV infection). In addition, the risk of reversion to the wild type phenotype, is now an

important issue (Cann, Stanway et al., 1984). Another disadvantage is that live vaccines are less stable and may be damaged by heat or light and have a short shelf life.

2. Inactivated vaccines

The idea for using inactivated microorganisms in a vaccine was first introduced by Daniel Salmon and Theobald Smith. The first strategy for the development of inactivated vaccines was directed against the typhoid and cholera bacilli. Today, many vaccines against human (killed polio vaccine and the hepatitis A vaccine) or animal infections are based on whole organisms, which have been inactivated but retain their immunogenicity (Balli, Di Biase et al., 1996;Murdin, Barreto et al., 1996a). Many of these vaccines were developed empirically and were designed without having a clear understanding of either the protective antigens or the immune response mechanisms. These vaccines are manufactured by inactivating microorganisms with heat and/or chemicals.

One of the advantages in this type of vaccine is that they never cause disease from infection, even in immunodeficient individuals. On the other hand, they do not represent a risk for immunosupressed contacts. In addition, the cost of inactivated vaccines are lower than live or subunit vaccines. However, inactivated vaccines always need multiple injections and are generally associated with a high incidence of side effects. The first shot does not produce protective immunity and immune responses develop after the second or third vaccination. In contrast to live vaccines that closely resemble natural infection, the immune response to a killed vaccine is mostly humoral and not cellular(Gendon, 2004). Antibody titers might be down regulated over time and consequently some inactivated vaccines require booster doses (Murdin, Barreto et al., 1996b;Gregoriadis, 1998;Melnick,

1978).

As mentioned above, these vaccines are usually inactivated by heating or chemical components. Formaldehyde was the first component used for inactivation. However, this chemical may affect the immunologic properties of proteins in the inactivated microorganisms. The use of colicin E2, a potent DNA endonuclease and phage lysis system has been also used for bacterial inactivation (Evans, Jr., Evans et al., 1988).

3. Subunit Vaccines: They are vaccines that contain essential antigens for the pathogenesis of infection; effector immune responses against them result in protection from infection or disease.

A-Polysaccharide vaccines

Carbohydrates are the major components on the surface of bacteria and they are important virulence factor in various bacteria. Several studies showed that immunity against these components confers protection against the infection.

Polysaccharide vaccines are a unique type of subunit vaccines containing long chains of carbohydrates that make up the capsule of some bacteria. However, development of an effective vaccine based on polysaccharides is difficult. The main disadvantage of this type of vaccine is that they are T-cell independent and consequently cannot activate Th cells (Weintraub, 2003). These vaccines are able to activate B cells without the assistance of Th2 cells. Boosting or repeated administration with polysaccharide vaccines does not cause a higher immune response and usually causes

development of a limited amount of memory cells. Another problem with this type of vaccine is that the produced antibody has less functional activity because the predominant antibody is IgM and not IgG (Aaberge & Lovik, 1995). Pure polysaccharide vaccines are available for *pneumococcus, meningococcus* and *Haemophilus influenza* type b. More recently, conjugation of polysaccharide vaccines with proteins has resulted in better memory cell and antibody responses (Ada & Isaacs, 2003).

B-Recombinant proteins:

The finding of specific immunity (humoral and cellular) that reacts with key regions of pathogens has led scientists to design vaccines based on protein components that result in protective immune responses. These proteins could be isolated or secreted from the organism. Alternatively, such proteins can be made by recombinant DNA technology. This technology presents an immunologic part of the pathogen to the immune system, and solves the problem of culturing the organism with possible reversion of the mutation in an attenuated virus (Netesova, Belavin et al., 2004;Gupta, Arora et al., 2000). Currently, many HIV-1, HCV and SARS recombinant proteins are under investigation for their protective effect. The difficulty in growing organisms such as HBV and malaria have prompted the development of vaccines based on recombinant protein. The recombinant HBV surface antigen is an example of a highly effective vaccine approach, the virus surface antigen is synthesized by DNA-transfected yeast or mammalian cells (Corradi, Tata et al., 1992).

Some recombinant proteins have low immunogenicity and thus, they have to be mixed with immunologic adjuvants or cytokines, such as montanide, aluminum salt,

interleukin 2 (IL-2) and interferon gamma (IFN-γ). In one study, combination of HIV-1 gp120 protein and IFN-γ was used for immunization of mice and this combination increased antibody responses to gp120. In addition, proliferative responses and IFN-γ production was strongly enhanced (McCormick, Thomas et al., 2001).

C-Synthetic peptides:

Synthesis of peptides for use in vaccines requires an understanding of T and B cell epitopes in the microorganism's proteins and their interaction with major histocompatibility complex (MHC) or HLA complexes. A successful peptide-based vaccine must include immunodominant T and B cell epitopes (Agadjanyan, Ghochikyan et al., 2005;Udhayakumar, Anyona et al., 1995). However, the epitopes of many important proteins from microorganisms are still unknown. Few viral proteins containing B cell epitopes, that may mediate neutralization antibodies, have been subjected to crystallography and consequently their three-dimensional structure is unknown. Therefore, it is difficult to develop a rational approach for vaccine design. The ability to predict peptides- binding to MHC is becoming valuable to vaccinology. A peptide with an MHC binding motif has potential to elicit a T cell response. Several methods have been recognized for predicting MHC binding of peptides (Sung & Simon, 2004;Segal, Cummings et al., 2001). The main benefit of vaccination with vaccine-based peptides is the ability to minimize the amount and complexity of a well-defined antigen. An appropriate peptide-based vaccine would also decrease the chance of stimulating a response against self-antigens, thus providing a safer vaccine by avoiding autoimmunity. However, synthetic peptides may have low immunogenicity compared with

immunization with large numbers of epitopes in the whole recombinant protein. In general, the peptide vaccine strategy needs to dissect the specificity of antigen processing, the presence of both B and T cell epitopes and the MHC restriction of the T cell responses. Other important issues are that cellular immunity is restricted to HLA type. The HLA has a polymorphic structure even within the same animal species and thus the level of T cell responses to peptides could be variable between each individual. On the other hand, individual peptides are probably not effective immunogens in a large population segment. This problem can be solved by the use of several peptides corresponding to various HLA types in each population (Arnon & Ben Yedidia, 2003). This approach allows the optimal efficacy according to geographical distribution. It is also possible to design a multiple-epitope vaccine that induces multi-specific immune response against several viral strains (Hewer & Meyer, 2005b;Fikes & Sette, 2003;Gao, Peng et al., 2004). Today, several research groups, including our own, are developing peptide-based vaccines for chronic viral infections such as HIV and HCV. Immunogenicity of peptide based vaccines also can be increased by the use of adjuvants, cytokines and macromolecular carriers (Hamajima, Fukushima et al., 1997;Goldberg, Shrikant et al., 2003;Miconnet, Coste et al., 2001).

4. Engineering vector-based vaccines

Today, genetic engineering and molecular biology has had significant effects on the progress of vaccine development. For instance, Hepatitis B vaccines are produced by ligation of its gene (S) into the genome of yeast cells (Kniskern, Hagopian et al., 1988;Kniskern, Hagopian et al., 1994). Viruses and bacteria also may be used as live

vectors, for example, attenuated adenoviruses can be used as the carrier for viral genes and has been employed in an effort to develop HIV and malaria vaccines. The production of some viral proteins in expression vectors may resemble virus-like particles (VLPs). These particles are immunogenic and have led to effective vaccines(Parez, Fourgeux et al., 2006;Deml, Speth et al., 2005). A new technique in vaccinology is the use of DNA plasmids containing genes from viral pathogens. This technique is currently being used into the manufacturing of influenza vaccines. Recently, combinations of vaccines have become more necessary as part of routine vaccination. For example combinations of diphtheria, tetanus, pertusis and hepatitis B are used in Europe(Godfroid, Denoel et al., 2004;Curran & Goa, 2003). Most of the current vaccines are prophylactic but serious attempts are being made to develop therapeutic vaccines. The basic idea is to induce cell-mediated immune responses that kill viral pathogens even when the host is not able to induce these responses naturally.

A-Viral vectors:

Vaccines based on live viruses are highly effective and easy to produce. This approach offers several advantages such as high-level immune production, potential adjuvanting effects from the viral delivery system itself and possible delivery of antigens to antigen presenting cells. Several organisms have been used as vector vaccines such as poxvirus vectors, vaccinia virus, canarypox virus, adnoviruses, etc. (Siemens, Crist et al., 2003). These viruses can be engineered to carry one or multiple genes.

One of the most common tested vectors in human and non-human primates are vaccinia virus vectors that are derived from smallpox vaccine strains.They are double-stranded DNA and their replication cycle is entirely within the cytoplasm of the infected

cells. They have a potency to infect a broad range of cells, replicate and induce strong immune responses. These factors make them especially well-suited as vaccines for the prevention and treatment of viral diseases and cancers. Other advantages of this vector include: broad host range, high-level of gene expression, and high capacity for inserting multiple genes (Essajee & Kaufman, 2004;Pastoret & Vanderplasschen, 2003). A number of clinical trials using this vector have shown promising results for treating infectious diseases and cancer (Guo & Bartlett, 2004). The elimination of smallpox infection was accomplished through mass vaccination with a vaccine containing vaccinia. One highly attenuated vaccina virus is derived from the Ankara strain by several passages in primary chick embryo fibrobalsts (CEF). This modified vaccine Ankara (MVA) has been tested in several non-human primate and clinical studies, with promising results in Epstein-Barr virus (EBV), human cytomegalovirus (CMV) and human papilloma virus candidate vaccines. These studies raised concerns about the use of vaccinia vectors in immunosupressed individuals and people with pre-existing immunity from previous vaccination with smallpox (Drexler, Staib et al., 2004;McCurdy, Larkin et al., 2004).

Another widely tested viral vector in pre-clinical studies is the adenovirus-based vector. This vector is easily manipulated and is highly infectious *in vivo*. It induces both innate and adaptive immune responses in mammals. The most widely used adenovirus vector is replication-incompetent due to the deletion of the E1 region. This vector has been used in cancer therapy or therapeutic infectious disease vaccine candidates (Cao, Koehler et al., 2004;Cao, Koehler, & Hu, 2004;Tatsis & Ertl, 2004). Two promising studies in macaques showed that priming with DNA vectors and boosting with adenoviral vectors could augment the antiviral immunity against Ebola and HIV (Sullivan, Geisbert

et al., 2003;Reyes-Sandoval, Fitzgerald et al., 2004). Generally, this viral vector is a promising platform for further studies in clinical trials. However, one of the limitations in this viral vector is previous immunity to adenoviruses and that multiple repeat administrations could limit its efficacy due to anti-adenovirus immunity, which could be an important issue in cancer immunotherapy

Another viral vector used in mice and non-human primates is the Poliovirus vector. This vector is derived from an attenuated Sabin vaccine strain, which minimizes safety issues. Although previous studies showed that this vector is a potentialy potent agent, however it has some weaknesses including limitation in the size and stability of inserted genes, and high level of pre-existing immunity to polio vaccine in the population (Girard, Martin et al., 1993).

B-DNA vaccines:

DNA vaccines can induce cellular and humoral immune responses and therefore, they have been used during the last decade to develop novel vaccines against a variety of infections. Bacterial DNA contains unmethylated Cytosine-phosphate-Guanosine (CpG) sequences that are recognized by Toll-like receptor 9 (TLR9). This CpG motif within the DNA vectors has the ability to induce both innate and specific adaptive immunity by enhancing antigen presentation, upregulating co-stimulatory molecules and increasing cytokine production (Vollmer, Weeratna et al., 2004b;Vollmer, Weeratna et al., 2004a).

DNA vaccines have several advantages; they are inexpensive to produce; are manipulated easily; are non-infectious and produce both cellular and humoral immunity. DNA immunization may be especially useful to induce long lived memory cells against

viral disease that also require cell-mediated immune responses. The achievement in DNA immunization depends on different factors such as time of immunization, type of antigen, route of administration, codon optimization, usage of adjuvants etc. Therefore, different strategies have been developed for modulating the induced immune responses.

A: Time of injection and route of DNA administration. For instance, injection of DNA vaccines to muscles induces stronger specific immune responses than other routes like intraperitoneal or mucosal. In addition, some studies showed that 3-4 weeks interval between each DNA immunization can increase specific immune responses.

B: Genetic adjuvants can also be used to enhance antigen presentation. Genetic adjuvants are genes for cytokines, growth factors, co-stimulatory molecules and transcription factors that are inserted into DNA vectors and are able to enhance the immune response to an immunogen(Ryan, McCarthy et al., 1998). Some studies also show that genetic adjuvants encoded on the same DNA plasmid as the immunogen, result in stronger immunity than genetic adjuvants encoded on a separate vector. A bicistronic vector encoding gp120 and GM-CSF improved CD4+T cell responses in mice compared with injection of a plasmid encoding gp120 and a plasmid expressing GM-CSF (Barouch, Santra et al., 2002a).

C: Modification in the structure of DNA vectors (Carriere, Escriou et al., 2003). For instance, conjugation of CpG motifs to the major short ragweed allergen Amb α 1 may offer a more effective and safer strategy for allergen immunotherapy and increase Th1 immunogenicity (Tighe, Takabayashi et al., 2000).

D: Combining DNA with recombinant proteins, peptides, viral vectors or other immunization strategies. Several studies showed that DNA vaccination followed by viral

vector expressing the antigen of interest can increase IFN-γ producing CD8+T cells (Otten, Schaefer et al., 2005;Gomez, Abaitua et al., 2004).

DNA vaccines have shown efficacy in small-animal models such as mice, rats, guinea pigs and rabbits. However, the results on larger animals like monkeys and human are not consistent and they require multiple immunization with high doses of DNA constructs. In one study, 80% of monkeys were protected from measles virus infection by injection of a DNA construct (Premenko-Lanier, Rota et al., 2004) and in another research study, 60% of macaques were protected from SIV infection by high doses of DNA vector (Busch, Abel et al., 2005).

DNA vaccines present antigens to the immune system by three different mechanisms:

A: Somatic cells, such as myocytes or keratinocytes, express protein to the immune system and elicit cell-mediated immune responses after immunization with DNA vectors. There are controversial results about the mechanism of Ag represented by somatic cells. Some studies indicated that somatic cells have this ability to activate CD8+T cells directly. However, other studies showed that these cells do not express some co-stimulatory molecules and thus cannot directly activate CTLs and they may have a role in duration of Ag-specific responses by serving as Ag reservoirs (Nichols, Ledwith et al., 1995;Torres, Iwasaki et al., 1997).

B: Following injection of DNA vaccines, APCs at the site of immunization are able to process proteins and express them as peptides that form complexes with MHC/HLA; these complexes are then recognized by T-cell receptors, resulting in activation of CD4+ and CD8+T cells. Activation of B cells occurs after proteins or peptides interact with B

cell receptors and complexes are then internalized and presented by MHC class II. Several studies indicated that epidermal Langerhan's cells (LCs), macrophages and interstitial DCs contain DNA plasmid following immunization (Pardoll & Beckerleg, 1995).

C: Cross-priming is another mechanism for Ag presentation to the immune system. Recent studies showed activation of the immune response by APCs that process peptides or proteins from apoptotic bodies. However, the mechanism is not still clear. A subset of APCs is able to internalize and process Ags in a distinct way from MHC class I pathway. Thus, both direct transfection and cross-presentation of professional APCs are able to stimulate immune response following DNA immunization (Harding & Song, 1994).

1.4 Immunologic Adjuvants

Adjuvants are substances that non-specifically increase the immunogenicity of the vaccine antigens. The term adjuvant is derived from "adjuvare" which means help or aid. The first developed vaccines had less purity but often contained "intrinsic adjuvants" which enhanced immune responses and could increase the immunogenicity of other antigens delivered with them.

Both innate and adaptive immune responses were considered two independent limbs of the immune system. Now, this view has changed. Researchers showed that innate immunity is a key for initiation and activation of adaptive responses(Iwasaki & Medzhitov, 2004). This new perception was fruitful in explaining the adjuvanticity of certain compounds. Innate immunity controls adaptive immune responses by two mechanisms. First, professional APCs, which are part of the innate response, take up

antigen, process and present it as peptide epitopes to lymphocytes. Second, innate cell receptors recognize infection; cells become activated and then provide secondary signals necessary for effective lymphocyte activation. The secondary signals are delivered by specialized ligand-receptor interactions such as B7-CD28 and the regulatory cytokines like TNF, IL-18 and IL-12. Most of the time in vaccination, antigens alone can not induce an effective adaptive immune response. Instead, the addition of adjuvant components to antigen elicits the desired response. Today, new technology is leading to the development of purified antigens for use in vaccines but this purification sometimes eliminates intrinsic adjuvants, which can decrease the immunogenicity. The use of adjuvants to increase the immunogenicity of purified antigens is a promising strategy to improve specific immune responses.

Adjuvants have four distinct mechanisms of action:

1-Prolonging the time of antigen presentation: Mineral salts or emulsion-based adjuvants improve delivery of antigens to APCs and to the secondary lymphoid organs as well as the immunogenicity of soluble antigens that otherwise would disappear rapidly from the site of injection.

2-Improving the delivery of immunogens to APCs. Adjuvants like liposomes release antigens into the cytoplasm of APCs, and consequently, antigens are treated through endogenous pathways and processed through the MHC class I and this can lead to the induction of cellular immune responses. Liposomes have also been shown to increase MHC class II presentation by macrophages and increase humoral immunity (Leserman, 2004;Reddy, Nair et al., 1992).

3-Production of cytokines: Adjuvants can assist T cell subsets to produce various cytokines and chemokines. Adjuvants such as alum can lead the immune response towards a predominant Th2 bias, enhancing the production of IgG1, IgA and IgE antibodies (Lindblad, 2004). A novel adjuvant is unmethylated CpG oligodeoxynucleotides (CpG ODN) which triggers TLR9 activation and consequently leads to a Th1 immune response (Segal, Chang et al., 2000). IL-12 is a promising cytokine that plays a crucial role in cell activation by several adjuvants (Villinger, 2003). There are other adjuvants such as tumor-necrosis factor-α (TNF-α) or incomplete Freund's adjuvant (IFA) that have been used to enhance immune response in a desired bias direction (Usinger, 1997).

4-Effect on antigen-presenting cells (APCs) maturation: The maturation of antigen presenting cells, especially dendritic cells, is a critical step for optimal presentation of antigens and induction of immune responses. Previous studies showed that the synthetic adjuvant R-848 induces the maturation of dendritic cells. R-848 increases the secretion of some cytokines such as IL-12 from dendritic cells (Burns, Jr., Ferbel et al., 2000). Maturation of APCs and their uptake of antigens are critical steps in the design of an effective vaccine.

1.5 Overview of Humoral and Cellular Immune Responses to Vaccines

Humoral immunity against a pathogen refers to the antigenic stimulation of B cells that result in the production of antibodies against a microbe. A mature B cell is activated by two different mechanisms. The first activation process may involve the direct cross-linking of surface Ig to antigens in a process called T-cell independent activation. In this process, B cells are directly activated without signals from T-cells. This mechanism is a major protective immune response generated against encapsulated microbes. In the second mechanism, B cell activation is dependent on T helper cells. The complex of the APC-MHC II-peptide on Ag-specific B cells can be recognized and bound by the TCR on the surface of the Th cells. After the binding, Th cells bind to the CD40 molecule on the specific B cells through the CD40 ligand (CD-40L)(Bergwelt-Baildon, Maecker et al., 2004;Bishop & Hostager, 2003). This ligation results in secretion of cytokines that can activate the production of Igs by the B cells. The activation of B cells leads to the production of IgM. Subsequently, B cells can further differentiate into effector cells secreting a different Ig isotype (IgG, IgA or IgE) or they become memory cells. Memory B cells are responsible for the rapid recognition and efficient generation of antibodies against organisms that have previously invaded the host (Ales-Martinez, Cuende et al., 1991;Parker, 1993). Antibodies participate in host defence by various protective mechanisms such as opsonization, Ab-dependent cellular cytotoxicity (ADCC), agglutination and neutralization. In some cases, polyclonal antibodies are ineffective in passive transfer studies, and these data downplay the value of antibody induction in vaccine design. Recent data indicates that antibodies can be effective against many

pathogens previously regarded insensitive to antibody(Dubois, Yoshihara et al., 2005;Casadevall, 1998). For these infections, we need to design vaccines that do more than mimic natural infection, and are able to induce more potent antibody responses. For example, the discovery of new B cell antigenic epitopes could assist scientists in production of more effective antibody responses. Several studies showed a weak or nonexistent protective antibody response against viruses that cause chronic infections. For example, HIV-1-neutralizing antibody titers in infected individuals are low against HIV-1 isolates (Arendrup, Nielsen et al., 1992).

The functional antibody responses against some viruses can be improved by modification of antigens. For instance, removal of carbohydrate moieties in SIV enhances neutralizing antibody responses(Cheng-Mayer, Brown et al., 1999). Furthermore, some studies showed that targeting APCs, particularly dendritic cells, enhance antibody responses. The study of synthetic peptides is another approach that has been pursued to produce neutralizing antibody responses against viruses such as respiratory syncytial virus (RSV), measles, HIV and HCV (Chargelegue, Obeid et al., 1997;El Kasmi, Fillon et al., 2000;Hewer & Meyer, 2005a;Puntoriero, Meola et al., 1998). This strategy can be expected to progress rapidly in the near future. These approaches could be combined to improve vaccine efficacy.

Currently, most commercial vaccines focus on humoral or B cell mediated responses. B cells produce specific antibodies to the antigens and the persistence of these cells ensures enduring antigen-specific protection. B cell-based vaccines provide effective immunity against pathogens, however do not eliminate pathogens (Slifka & Ahmed, 1998). Protective immunity and elimination of infections is mediated

predominately by cell-mediated immune responses. Basically, T-cell vaccines depend on CD4+ and CD8+T cells. CD8+T cells are frequently referred to as cytotoxic T lymphocytes (CTLs) which recognize and kill infected cells by different mechanisms, such as secreting antiviral cytokines. CD4+T cells are referred to helper cells (Th) and provide cytokines with signals for generation and maintenance of CD8+T cells as well as B cells. Activated T-cells produce cytokines and chemokines that can affect infection-specific mechanisms and may interfere with the replication of pathogens. T cells recognize epitopes derived from viral proteins that are presented by the MHC antigens. CD4+T cells recognize endosome-derived antigens on MHC class II molecules, and CD8+T cells recognize peptides in association with MHC class I, which usually present antigen derived from the cytosolic compartment (Oliveira-Ferreira & Daniel-Ribeiro, 2001;Blattman, Antia et al., 2002;Bousso, Wahn et al., 2000).

-Subsets and effector function of CD8+T cells

Induction of long-lived memory CD4+ T and CD8+T cells is the main goal of T cell based vaccines (Del Giudice, 2003). Memory T cells are characterized by their ability to rapidly respond to infection. They also have a long lifetime. In a primary T cell response, naïve T cells encounter antigens in lymphoid tissue, where their unique T cell receptor (TCR) recognizes one MHC-peptide complex in the context of a professional antigen-presenting cell. The signalling through the TCR as well as through co-stimulatory molecules initiates the expansion and differentiation into effector T cells that secrete antiviral cytokines such as IFN-γ. As antigen is cleared, the T cell subsets differentiate into long-lived memory cells. For instance, in the lymphocytic choriomeningitis virus

(LCMV) mouse model, most effector antiviral T cells undergo apoptotic cell death after infection, but a small fraction of T cells differentiate into memory cells (Jacob & Baltimore, 1999), which after secondary exposure to LCMV are able to expand and efficiently protect against disease. Although the molecular mechanism of memory T cell generation is still unclear, memory CD4+ and CD8+T cells can persist for decades in the absence of antigen and this persistence is assisted by the IL-2 family (IL-7 and IL-15) of cytokines (Prlic, Lefrancois et al., 2002).

A number of surface molecules associated with memory and naïve CD8 T cells are involved in cell adhesion and chemotaxis, which is consistent with distinct trafficking patterns of different T cell populations. For instance, CD44low and CD62Lhi are used to define naïve cells and they become CD44hi and CD62Llow upon activation (Cerwenka, Morgan et al., 1999;Kurepa, Su et al., 2003). In addition, memory CD8+T cells can be divided into effector memory (CD62Llow CCR7-) and central memory (CD62Lhi CCR7+) subsets. Effector memory subsets express receptors for migration to inflamed tissues and display immediate effector function. In contrast, central memory subsets efficiently stimulate dendritic cells and differentiate into CCR7- effector cells upon secondary stimulation (Sallusto, Lenig et al., 1999).

The expression of CD45 (lymphocyte common antigen) isoforms is also a marker used to differentiate between CD8+T cell stages. CD45 is expressed on all leucocytes, and plays a crucial role in the function of these cells. This marker regulates the tyrosine kinase activation in the complex of CD3-TCR and also phosphorylates essential substrates such as phospholipase C gamma 1 (PLC1). However, CD45 is a relatively unpredictable marker for memory T cell definition (Altin & Sloan, 1997). More recently,

co-receptors CD27 and CD28 have been used for the classification of T cells into naive, effector and memory cells. CD27+ CD28+ and CCR7 are expressed highly in naïve T cells but they are down regulated on the surface of effector T cells. Memory cells also lose these markers and maintain CD45 at low levels on their surface. The differentiation pathway from memory CD8+T cells into activated memory/effector CD8+T cells is not well understood (Tomiyama, Matsuda et al., 2002;Tomiyama, Takata et al., 2004).

-Subsets and effector function of CD4+T cells

Naïve CD4+T cells differentiate into at least three subsets (Th0, Th1 or Th2) based on their cytokine secretion. Th0 cells have not differentiated into a functional Th subset yet. The type and amount of antigen, cytokine and the APC subset during the first antigen exposure is important in the differentiation of Th0 into a Th1 or Th2 phenotype.

Many viruses and bacteria induce IL-12 and IFN-γ which lead the naïve CD4+T cells to differentiate into Th1 cells. In contrast, Th2 subsets are associated with inflammatory responses that produce IL-4, leading naïve CD4+T cells to differentiate into Th2 cells. A subpopulation of Th1 cells is characterized as regulatory (T reg). During the immune response, they prevent overzealous clonal expansion of antigen-specific T cells and inhibit proliferation of autoreactive T cells (Sakaguchi, 2000). Activation and inactivation of T reg is important in T cell based vaccine studies. For example, the efficacy of TLR ligands such as CpG and LPS as adjuvants is due in part to the release of factors from activated APCs that temporarily suppress T reg functions (Pasare & Medzhitov, 2003).

CD4+T cell memory is not well studied and the generation and maintenance of

CD4+T cells is not well recognized. Memory CD4+T cells do not have the ability to produce cytokines in the absence of antigen, however, upon re-encounter and stimulation with antigen, they rapidly produce the cytokines elicited during their primary activation. Memory CD4+T cells have a lower activation threshold than naïve CD4 cells and respond much faster to antigens than naïve CD4 cells. However, the factors that are involved in differentiation of memory to effector T cell are still not clear (Stockinger, Kassiotis et al., 2004). Remarkably, in the absence of cytokines IL-2, IL-4 and IFN-γ, CD4+T cell memory is still generated. The cytokine requirements for survival of memory CD4 cells are different than those for CD8+T cells. For example, in the absence of IL-15, the frequency of memory CD8+T cells is highly down regulated but the amount of memory CD4+T cells is not completely dependent on IL-15 (Judge, Zhang et al., 2002;Geginat, Sallusto et al., 2001).

1.6 Examples of Antiviral Immune Effector Mechanisms

-Antibody Immunity in HIV

HIV is a single stranded RNA virus that belongs to the lentivirus subfamily of retroviruses. HIV like other retroviruses, contains a major envelope protein, the nucleocapsid protein, the diploid single-stranded RNA genome, and the viral enzymes protease, reverse transcriptase, and integrase (Barre-Sinoussi, 1996). HIV has cellular tropism for CD4+T cells. However, it also infects other cells such as dendritic cells and macrophages.

The classical approach to develop an effective vaccine against a virus is induction of neutralizing antibodies (Nabs) but whether this approach is possible for HIV remains unclear. Neutralizing antibodies have the ability to block viral entry into cells. Other antibodies are involved in Ab-directed cell cytotoxicity (ADCC). However, antibodies may also enhance virus entry into cells and kill infected cells. In HIV-1, infected individuals may produce some Nab responses but their reactivity is not strong and viral mutants can escape from this response. Although some but not all long-term non-progressors (LTNPs) have shown strong broadly Nabs(Carotenuto, Looij et al., 1998).

Induction of anti-HIV Nabs by different immunogens was evaluated in animal models and clinical trials. The results raised several concerns. First, a very low amount of Nab was induced by tested immunogens. Second, Nabs were more effective against T-cell adapted laboratory strains and not primary HIV-1 strains isolated from patients. Third, the raised Nabs have a lack of cross-clade neutralizing activity and cannot neutralize other strains of HIV except used strain used in the vaccination (Srivastava,

Ulmer et al., 2004;Li, Gao et al., 2005;Sarmati, d'Ettorre et al., 2001;Locher, Grant et al., 1999).

HIV-gp120 is functionally the most important tested immunogen for induction of Nabs. However, the structure of gp120 contains variable loops (V1-V5) which hide the critical epitope sites and contribute to the lack of Nabs(Lauder, Lin et al., 1996). The crystallography of gp120 structure indicates why it is difficult to induce a broadly cross-reactive Nabs. The envelope is a combination of a trimer of gp120-gp41 which is covered by carbohydrates (Kwong, Wyatt et al., 2000;Wyatt, Kwong et al., 1998). Previous studies showed that these carbohydrates facilitate viral escape from Nabs. A recent study showed the protection of macaques against SIV infection based on induced antibodies against viral envelope (Quinnan, Jr., Yu et al., 2005a). However, the number of animals was limited and the study may have restricted application to SIV in non-human primates.

In another study, a group of macaques was immunized with DNA plasmid and recombinant adenoviral vector encoding HIV-1 89.6 envelope protein glycoprotein. This strategy elicited high levels of antibodies that were able to neutralize some heterologous virus isolates. However, the breadth of the response was limited and did not neutralize primary isolates of HIV-1 (Mascola, Sambor et al., 2005).

-Cellular Immunity in HIV

Many viral infections are not cleared completely but are controlled by effective cellular immune responses such as Epstein-Barr virus (EBV) and cytomegalovirus (CMV) (Moss, Cobbold et al., 2003;Cavazzana-Calvo, Durandy et al., 1997a;Cavazzana-Calvo, Durandy et al., 1997b). Both CD4+ and CD8+ cytotoxic T lymphocytes (CTLs)

play crucial roles in controlling viral replication. Upon presentation of a viral peptide by MHC class II, specific CD4+ T helper cells become activated and they induce cytokines and clonally expand in response to antigenic stimulation. The correlation between CD4 and CD8 is demonstrated in human CMV and LCMV infections. In mice depleted of CD4+T cells, CTL function waned over time and mice could not control viremia while in infected mice with intact CD4+T cells, CTL were able to control viremia. These data suggest essential evidence that CD4 function is important in cellular immunity (Kitchen, Whitmire et al., 2005;Sinclair, Black et al., 2004;Harari, Zimmerli et al., 2004).

One of the defects in HIV-1 patients is absent or weak CD4+Th cells in different stages of disease. Furthermore, CTL responses wane over time and disease progresses in HIV-1 infected individuals. Although no correlation was found between lack of Th cells and decline in CTL function, other models of chronic viral infection support this correlation. An inverse correlation between gag-specific T helper cells and HIV-1 RNA plasma virus load has been shown. The strongest gag-p24 specific proliferative responses were associated with lower viral load, while a high viral load was observed with weak response to p24 stimulation (Klein, Veenstra et al., 1997). In another independent study, a reverse correlation between induced CTLs directed against a gag epitope and viral load has been shown (Goepfert, Bansal et al., 2000). These observations suggest that both CD4 and CD8 are crucial for the control of HIV-1 infection.

A deficiency in CD4+T helper cell number is one indication of HIV-1 infection progression. The mechanism is still not clear but one explanation is that CD4+T cells undergo clonal deletion at the early stages of infection. During HIV-1 infection, CD4+T cells become activated in responses to stimulation by HIV virus and then undergo clonal

expansion. A high level of virus is available to infect specific CD4+T cells. A high level of viremia during a critical time for the expansion of CD4+T cells deletes T cell clones from the immunologic repertoire prior to the establishment of a memory population. Another explanation for the functional loss of CD4+T cells is apoptosis. Some of HIV-1 proteins increase activation-induced apoptosis of HIV-1-specific CD4+T cells, which contributes to a loss of control of HIV-1 replication. Some studies show that HIV-1 gp120 has the ability to induce different cytokines in PBMCs and it downregulates macrophage CD4 expression *in vitro* (Karsten, Gordon et al., 1996;Jassoy, Heinkelein et al., 1997). HIV-1 vpr is another important protein which interferes with mitochondrial function, as it releases cytochrome c and activates the caspase 9 pathway culminating in the activation of caspase 3 and consequently induces apoptosis (Muthumani, Choo et al., 2003). However, it is not known why LTNP individuals do not lose CD4+T cells despite not receiving treatment during acute HIV-1 infection.

CD8+T cells have the ability to produce cytokines and chemokines to kill infected cells. CD8+T cells produce a protein named perforin which is present in granules. Perforin and granzymes are important in triggering target-cell death. In one study, perforin-knockout mice did not recover from infection with LCMV. Lysis is mediated predominantly by perforin and granzymes, however, some CTLs induce apoptosis by Fas ligand in the target cells that also express the Fas receptor. It is not clear which CTL function is more important in control of HIV-1 infection. CTL produce cytokines such as IFN-γ and tumour-necrosis factor- α (TNF) that can affect viral replication. CTLs also produce chemokines such as MIP-1α, MIP-1B and RANTES, which compete with HIV-1 for cell receptors and/or downregulate CCR5, CXCR4 and consequently, inhibit virus

cell entry and virus replication. Studies showed that CD8+T cells secrete a protein named CAF (CD8+ antiviral factor). CAF blocks LTR-mediated transcription in infected cells and down regulates virus production(Mackewicz, Blackbourn et al., 1995). In addition, it facilitates the development of latency, where no RNA and protein are expressed (Kajino, Kajino et al., 1998;Doherty, Topham et al., 1997;Gulzar & Copeland, 2004a).

The pattern of cytokine secretion is similar in both HIV and CMV specific CD8+T cells. However, a striking difference is observed in the level of secreted perforin. Less than 15% of HIV-specific CD8+T cells produce perforin, while more than 50% of CMV-specific CD8+T cells induce perforin. Jansen et al. showed that CMV-specific CD8+T cells express less CD27 while HIV-specific CD8+T cells are phenotypely CD27+. It is interesting to note that a correlation between the percentage of CD27^{-}T cells and secretion of IFN-γ has been already shown (Jansen, Piriou et al., 2004;Zhang, Shankar et al., 2003). Thus, HIV- specific CD8+T cells may be less efficient than expected in their antiviral effector function.

Recently, vaccines designed to stimulate cell-mediated immune responses (CD4 and CD8) have protected macaques after challenging with SHIV (Buckner, Gines et al., 2004). The immunized macaques were infected but their viral load was 1000 times less than unvaccinated animals. Someya et al., showed the regimen of DNA encoding gag and pol genes from simian immunodeficiency virus (SIV) and recombinant vaccinia virus expressing SIV gag and pol induced Th1-type immunity in BALB/c mice, which was associated with resistance to viral challenge with wild-type vaccinia virus expressing SIVgag/pol (Someya, Xin et al., 2004).

Protective Immunity in Hepatitis C

Hepatitis C virus (HCV) infects approximately 200 million individuals worldwide. More than 80% of infected individuals develop a chronic infection which can evolve toward cirrhosis and hepatocellular carcinoma. HCV is a member of the virus family *Flaviviridae* and classified into six clades according to genome sequencing and it is further divisible into different subtypes, which is important for epidemiological and clinical studies (Libra, Gasparotto et al., 2005;Hadziyannis, 2000).

Some studies showed that a specific and vigorous CD4+T cell proliferative response plays an important role in successful clearance of HCV(Harcourt, Lucas et al., 2004;Schulze zur, Lauer et al., 2005). The role of Th1 CD4 responses for resolution of hepatitis C was shown in individuals with schistosomiasis who can not control viremia in spite of a strong Th2 bias from this parasitic infection. A comparison between specific CD4+T cells from the peripheral blood of recovered patients and chronic patients showed that the generated cytokines from Th1 in recovered patients is 10 times higher. The strength of HCV specific Th1 cells correlates inversely with viral loads in chronic disease (Park, Yang et al., 2003;Kamal, Bianchi et al., 2001). Different methods for detection of cell-mediated immune responses in HCV showed that CD4+ memory T cells are less frequent in peripheral blood than their CD8+ counterparts. Activation of CD8+T cells by peptide/MHC class I can lead to cytotoxic and non-cytotoxic viral control. During acute infection, a high amount of CD8+ response may be seen in liver which may contribute to its propensity to develop chronic infection. During this step, escape mutations can appear at critical residues in class I-restricted epitopes. The maintenance of effective CD8+T cells in chronic infections is dependant upon specific CD4+T cells which may down-

regulate proliferation with repeated antigen exposure. It seems that early in primary infection, the HCV virus may be able to undermine innate immunity and interfere with specific immune responses. Lack of Th1 response down-regulates CTL which leads to chronic infection. Treatment studies showed that decreasing antigen levels and improving Th1 cells may assist in restoring CTL function which is critical for clearance of HCV virus.

Analysis of HCV specific CD4+T cells showed that the majority of CD4+T cells express the phenotype CCR7+CD45RA-CD27+ which correlates with a central memory phenotype. This type of cell can proliferate and produce IL-2 after exposure to the antigens(Day, Seth et al., 2003). They have a critical role in expansion of memory CTL. In chronic infection, cellular immunity may play a role in control of viremia but cannot eliminate the virus. Generally, functions of CD8+T cells depend on antigen presenting cells, CD4+T cells and appropriate antigen concentration. However, HCV may escape from CD8+T cells because of variation in epitopes recognized by CTL (Kantzanou, Lucas et al., 2003).

Characterization of specific epitopes inducing T cell responses is crucial in defining immune correlates of protection. These epitopes also could be used for design of a candidate vaccine against HCV infection. In one study, HLA-A2.1 transgenic mice were immunized with a group of peptides within the NS3 protein. This strategy was able to induce either CTL and/or IFN-γ producing cells (Martin, Parroche et al., 2004a). In another study, the immunogenicity of candidate DNA vaccine expressing HCV envelope protein 1 (E1) and envelope protein 2 (E2) antigens plus CpG as adjuvant were assessed. Mice receiving CpG adjuvant had a lower level of antibody titers, while this group

showed higher HCV-antigen specific proliferation in splenocytes (Zhu, Liu et al., 2004). Furthermore, Matsui M et al., showed that the prime-boost immunization of mice with DNA vector encoding HCV-core and IL-12 leads to strong induction of CD8+T cell responses (Matsui, Moriya et al., 2003). In addition, Duenas-Carrera et al., showed that a DNA plasmid encoding the HCV structural proteins (Core, E1 and E2) is able to induce a specific humoral and cellular immune response after injection in rabbits and macaques (Duenas-Carrera, Vina et al., 2004). So far, promising results have been obtained with immunization based on DNA vaccines with cytokines and DNA prime/recombinant live vectors or protein/boost regimens.

The role of antibodies in clearance or protection against HCV infection has not been established. Clearance of acute HCV infection in chimpanzees is associated with a CD8+T cell response but not an antibody response(Kakimi, 2003). Recovered patients showed high specific memory T cells while more than 40% did not show any detectable antibody response. Studies of primary clearance and secondary exposure to HCV in both chimpanzees and humans demonstrated that CD4+ and CD8+T cells provide some degree of protective immunity against HCV. However, unlike hepatitis B, neutralizing antibodies may not play an essential role in the control of viral infection (Rosen, 2003;Burioni, Mancini et al., 2003;Koff, 2001).

-Correlates of protective immunity in SARS-CoV infection

SARS coronavirus (SARS-CoV) is a positive-strand RNA virus, the genome is approximately 30 kb which encodes the structural proteins spike (S) which is involved in receptor binding, small envelope (E) which has role in viral assembly , matrix (M) which is important for budding, and nucleocapsid (NC) which is associated with viral RNA packaging (Madariaga & Gotuzzo, 2004;Parashar & Anderson, 2004).

The data on immune protection to SARS needs confirmation and further research studies. Although, it seems that both humoral and cellular immunity play crucial roles in protection against coronavirus infections, neutralizing antibodies and T cells contribute to exacerbation and resolution of the infection but the mechanism is not well characterized. CD4 and CD8+T cells are essential in clearance of bovine coronavirus, however, antibodies are not critical in the control of this infection. In contrast, both humoral and cellular immune responses are involved in the control of Turkey coronavirus infection (Loa, Lin et al., 2001).

Immunization with Spike (S) protein: Spike is a large glycoprotein on the surface of SARS-CoV. Like other members of the coronavirus family, this protein contains two subunits S1 (near the amino) and S2 (C-terminal). The S2 is generally conserved among coronaviruses but S1 has a low level of sequence homology. The S1 binds to cellular receptors and S2 is required for fusion to the host cells (Spiga, Bernini et al., 2003a). A recent research study showed that there is a strong interaction between spike protein and Matrix protein in SARS-CoV and they suggested that the immune response of spike protein might be dependent on this interaction. In another study, rhesus macaques were

immunized intramuscularly with a combination of adenoviral vectors expressing spike protein S1 fragment and nucleocapsid (NC). Vaccinated animals had a high level of antibody responses against the S1 fragment and cellular immunity against the nucleocapsid protein (Gao, Tamin et al., 2003).

Zeng F. et al. have shown that S protein antibody is not able to neutralize SARS-CoV in vaccinated mice. However, the plasmids encoding S1 and S2 subunits showed some neutralization antibody titer (Zeng, Chow et al., 2004). It could be possible that the full length of spike prevents neutralizing antibodies from recognizing epitopes on the protein surface. It might be because of mutation in the structure of spike protein and subsequent change in the exposure of one or some epitopes to neutralizing antibodies. However, subunits S1 and S2 do not make a coil protein and the epitopes would be exposed to the antibodies.

Previous studies in other coronaviruses showed that although spike is not very hypervariable, a single mutation in the structure of the spike protein causes virus escape from neutralizing antibodies. Grosse et al already reported that subunit S1 in MHV (Mouse hepatitis virus, which belongs to the Coronaviridae) is not mutated in the codons that bind neutralizing antibody. However, a single amino acid mutation in the S2 subunit of the protein causes escape of virus from neutralization (Grosse & Siddell, 1994). Dongway Yoo and Dirk Deregt showed that a single point mutation (substitution of C to T at position 1583, results in the change of alanine to valine at amino acid position 528) in the S2 subunit of spike is responsible for the escape of Bovine coronavirus (BCV) from immune response and resistance to neutralizing antibodies (Yoo & Deregt, 2001a).

The immunogenicity of SARS-CoV spike protein was examined by administering mice with the vector encoding full length spike gene, Spike proteins were expressed *in vitro* in two different expression systems (mammalian and baculovirus) and the adjuvant montanide was also studied (The data is not included in the thesis). I showed that spike protein is able to produce a strong antibody titer as well as relatively high lymphocyte proliferation in all vaccinated mice. However, a strong neutralizing antibody titer in immunized groups was not detected.

Immunization with Nucleocapsid (NC) protein: In 1992, Boots et al. induced protective immunity against infectious bronchitis virus (IBV) in chickens that were primed with nucleocapsid protein and boosted with inactivated IBV. A high proliferative response in PBMCs was observed. They also had protection against challenge with live IBV. They concluded that immunization with NC protein had induced protection by activation of CD4+Th or CTL responses (Boots, Benaissa-Trouw et al., 1992b).

Previous studies in other coronaviruses showed that NC-specific CD8+T cells are able to induce protective immunity. After the SARS outbeak, some studies focused on the immunogenicity of NC protein of SARS-CoV as an ideal vaccine target. In a recent study, NC protein was cloned into an adenoviral vector and C57BL/6 mice were immunized with this agent and immune responses were assessed. The results were promising and showed a potent SARS-CoV-specific humoral and T cell-mediated immune response (Zakhartchouk, Viswanathan et al., 2005).

In another study, the NC-SARS-CoV was expressed in Escherichia coli and immune responses induced by intramuscular immunization were evaluated in BALB/c

mice. Antibody titers and splenocyte proliferative responses against NC protein were observed in immunized BALB/c mice. Splenocytes from immunized mice produced high levels of IFN-γ and IL-2 cytokines in response to NC protein. In addition, increased CD8+T cell responses were seen in vaccinated mice. These results showed that NC-SARS-CoV is able to induce a strong humoral and cell-mediated immune response (Zhao, Cao et al., 2005). Kim et al., also showed that DNA vaccination with NC linked to calreticulin (CRT) induced a potent NC-specific humoral and cell-mediated immune response in vaccinated C57BL/6 mice. In addition, vaccinated mice were able to reduce the titer of challenging recombinant vaccinia virus expressing the NC-SARS-CoV (Kim, Lee et al., 2004b). Furthermore, Jin et al., cloned NC, E and M into DNA vectors, immunized different groups of mice intramuscularly and compared the immunogenicity of these constructs between groups. All constructs (E, M and NC) were able to induce high levels of antibody titer, proliferative response and IFN-γ cytokine secretion. However, the highest immune responses were observed by the DNA construct expressing the NC-SARS-CoV protein (Jin, Xiao et al., 2005).

1.7 Hypothesis/Goals: There is growing evidence that humoral and especially cell-mediated immune responses play crucial roles in the control of viral infections. With this goal in mind, candidate vaccines against HIV, HCV and SARS-Co were developed. It has been hypothesized that a combination of DNA vectors as well as recombinant proteins, as a vaccine delivery system might be a good stratgey to activate MHC class I- and class II pathways, and increase antigen-specific T cell and antibody responses. These formulations share many advantages, including ease of construction, low expense of mass production and biological stability.

My goals in these studies were: 1. Isolation, amplification and cloning of HIV-1 and SARS-CoV structural genes into DNA expression vectors (HCV strucutral genes have been cloned by our laboratory before). 2. Expression, purification and characterization of HIV-1, HCV and SARS-CoV proteins in bacteria and mammalian cell lines. 3. Characterization of humoral and cellular immune responses in immunized mice. In the first study, the best combination of HIV-1 structural immunogens for the induction of cell-mediated immune responses was assessed. Data from this model suggested which combination could produce a higher level of IFN-γ, a cytokine that is critical in the induction of cell-mediated immune responses. It has been shown that some combined vaccines might enhance migration of antigen presenting cells to the site of injection, thereby contributing to vaccine efficacy. In the second study, the efficacy of individual HIV/HCV antigens or combined antigens was determined in order to find a synergistic effect between HIV/HCV antigens. In the third study, the best target for induction of a strong humoral and cell-mediated immune response against SARS-CoV was assessed. Further, the level of CD8+T cell responses by XIAP as adjuvant was assessed.

Chapter 2:

Materials and Methods

Construction of DNA plasmids:

Total RNA was purified using RNeasy extraction kit (Qiagen, Mississauga, ON) and the genes (HIV-1-gp120, gag and pol and SARS-CoV-nucleocapsid, spike, matrix and small envelope) were amplified by using the specific primers. The amplicons were purified using the QIAquick gel extraction kit (Qiagen, Mississauga, ON) and cloned into the PCR 2.1 TOPO-TA vectors (Invitrogen, Burlington, ON). After plasmid digestion, bands corresponding to the amplicons were sub-cloned into digested enzyme sites of pVAX-1. The fragments were also sub-cloned into the pEF6-Myc/His and pQE-His vectors (Invitrogen) which are designed for the over-production of recombinant proteins in mammalian and bacterial cell lines.

Cell culture:

Chinese Hamster Ovary (CHO) cells were grown at 37°C, 5% CO_2 in Iscove's Modified Dulbecco's Medium (IMDM: Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS: Life Technologies, Grand Island, NY), 100 U/ml penicillin and 100μg/ml gentamicin.

Transfection and purification of proteins:

CHO cells were transfected by electroporation or lipofectamine with pEF6-myc-

His vector containing specific genes or without insert genes. The proteins were purified after transient transfection. To facilitate gp120 purification, a stable gp120 expressing CHO cell line was established after cloning the envelope gene into a vector encoding blasticidine resistance and using blasticidine-supplemented medium. Cells were harvested, sonicated and lysed in lysis buffer (25mM Tris base, 2mM mercaptoethanol, 1% Triton-X100 and a cocktail of protease inhibitors). Cell pellets were centrifuged and the supernatant was incubated with the TALON metal resin (Clontech, Palo Alto, CA) for an hour. After incubation, the mixture of protein-resin was added to the columns and washed three times with 20 bed volumes of Tris-Cl, NaCl (pH 8). The recombinant protein was eluted with 150 mM imidazole. In this system, recombinant proteins may be purified by affinity chromatography using the poly histidine (6X His) metal-binding tags.

Western blotting:

Protein samples were mixed with Laemmli loading buffer, boiled for 5 minutes, and electrophoresed on a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose membranes by electrophoretic transfer. Membranes were blocked with 5% skim milk in PBS-Tween over night and incubated for 3h at room temperature with a 1/1000 dilution of pooled sera from HIV-1/SARS positive patients, polyclonal p24, protease or polyclonal gp120 antibodies. The blots were washed with PBS-Tween and incubated for 1h at room temperature with secondary antibody (goat anti-human IgG-HRP conjugate) (Bio-Rad, Hercules, CA). The blots were washed, incubated one minute with Luminol reagents (Santa Cruz Biotechnology, Santa Cruz, CA) and exposed to X-ray film (Kodak).

Electron Microscopy:

Transfected CHO cells with the DNA vectors were harvested and washed with PBS. Pellets were then fixed with 2% glutaraldehyde. Cells were rinsed twice in 0.1 M sodium cacodylate buffer at 4°C. The cells were then fixed with 2% Osmium Tetroxide for 2 hr at 4°C. After washing with distilled water, the cells were dehydrated with increasing concentrations of ethanol and embedded in spur resin. Thin sections were stained with uranyl acetate and lead citrate. The sections were screened by using a JEOL 1010 Transmission Electron Microscope (TEM).

Adjuvants:

CpG oligodeoxynucleotide (CpG ODN: 5'-TCCATGACGTTCCTGACGTT-3') was provided by Coley (Ottawa, ON). Montanide ISA-51 mineral oil adjuvant was purchased from Seppic Inc. (Paris, France). The pcDNA3 construct expressing 1.5 kb XIAP gene, which encodes an anti-apoptotic gene product, was a kind gift from Dr. R. G. Korneluk.

Animal immunization:

Six to eight week old HLA-A2.1 mice (Jackson Laboratory, Bar Harbor, ME) and B6/C3/F1 mice (Charles River, St. Constant, PQ) were used in this study. Each mouse was immunized intramascularly (I.M.) or subcutaneously (S.C.) at the base of the tail with one of various combinations of immunogens and adjuvants. Each mouse was

vaccinated four times (One month interval between each immunization). Fourteen days after the last boost, the mice were sacrificed and their spleens, blood and lymph node cells were collected for further testing or long-term storage in cryopreservation medium.

Antibody measurement:

ELISA was used for titration of IgG responses. Briefly, 96-well plates were coated overnight at 4°C with recombinant HIV-1, HCV andSARS proteins. The wells were washed with PBS containing 0.05% Tween 20 and then blocked for 1 h with 1% BSA in PBS. The plates were incubated for 2 h at 37°C with serially-diluted sera. The plates were washed and incubated for 2 h with peroxidase-conjugated affinity-purified goat anti-mouse secondary antibody (Bio-Rad). After washing, color was developed with O-phenylendiamine dihydrochloride (OPD: Sigma).The color reaction was stopped with 1N HCl and absorbance was read at 490 nm with an ELISA plate reader (Bio-Rad).

Lymphocyte proliferation assay:

Splenocytes from immunized mice were resuspended at 2 x 10^6 cells/ml in RPMI 1640 containing 10% FCS, 50 µM β-mercaptoethanol and 100 U/ml penicillin/streptomycin. A 100 µl aliquot containing 2 x 10^5 cells was added to each well of a 96 well plate. The recombinant proteins (100 µl at 10µg/ml) were added to each well in triplicate. As a positive control, cells were also stimulated with phorbol 12-myristate 13-acetate and ionomycin (PMA/ION: Sigma). After 72h of culture, 1 µCi [^{3}H] thymidine (Amersham, Arlington Heights, IL) was added to each well. Following 16h of incubation, cells were harvested onto glass fibre filtermats and thymidine incorporation

was measured with a Microbeta beta counter (Wallac, Turku, Finland).

Evaluation of Cytokine Expression:

Cytokine levels in the culture supernatants of the splenocytes used for proliferation assays were determined by quantitative ELISA. Briefly, 96-well flat-bottom plates (Nunc, Roskilde, Denmark) were coated with 100μl of monoclonal antibodies specific for IFN-γ, IL-4 and IL-5 (BD PharMingen, San Diego, CA) at 2μg/ml and diluted in 0.1 M Na_2HPO4, pH 9.0. After incubating overnight at 4°C, the wells were washed with PBS-Tween and blocked with 1% FCS in PBS for 2h at room temperature. After washing six times, serially-diluted supernatants were added in duplicate and incubated overnight at 4°C. The wells were washed with PBS-Tween and incubated with biotinylated anti-IFN-γ, anti-IL-4 or anti-IL-5 antibodies (BD PharMingen) diluted in 10% FCS-PBS. After washing six times, wells were incubated with streptavidin-peroxidase (Jackson Immuno Research, West Grove, PA) for 20 minutes at 37°C and developed with OPD. The reaction was stopped with 1N HCl and the absorbance values were measured at 490 nm with an ELISA plate reader (Bio-Rad).

HIV-1 and HCV Peptides:

The HIV-1 and HCV peptide epitopes specific for MHC class I HLA-A2 were searched in the Los Alamos Molecular Immunology Database (http://www.hiv.lanl.gov/content/immunology) and contained the following sequences: Env(120-128):KLTPLCVTL; Gag(77-85):SLYNTVATL; Gag(386-394):VLAEAMSQV; Pol(309-317):ILKEPVHGV and Pol(33-41):ALVEICTEM. The HCV peptide epitopes

contained the following sequences: Core(35-44):YLLPRRGPRL; Core(132-140):DLMGYIPLV; E1(220-229):ILHTPGCV; E2(363-372):SMVGNWAKV and E2(401-411):SLLAPGAKQNV. These peptides (Dalton Laboratories Inc., Toronto, ON) were used to detect CD8+ T-cells with specific activity against HIV-1 and HCV.

Intracellular cytokine staining:

Fresh blood from immunized mice was cultured in IMDM media in the presence of 10μg/ml brefeldin A (Sigma) and stimulated *in vitro* with the antigens (10μg/ml). In every experiment, a negative control (without stimulation), positive control (PMA/ION) and an irrelevant peptide were included to control for spontaneous production of IFN-γ. Sixteen hours after incubation, the cells were washed once (1600 rpm for 5 min) with 3 ml PBS/2% FCS/0.01% azide and surface-stained for 15 min with PE-labeled Ab to mouse CD3 and TC-labeled Ab to mouse CD8α (Caltag, Hornby, ON). The cells were washed as above, fixed and permeabilized using 100 μl each of A and B fixation-permeabilization solution (Caltag). The cells were stained intracellularly with anti-mouse IFN-γ FITC-labeled Ab and incubated for 30 min (in the dark) at 4°C. Following washing, cells were analyzed by FACScan (Becton Dickinson, Mississauga, ON). An increase of 0.1% of IFN-γ producing cells over the unstimulated control was considered as a positive response to vaccination.

HLA 2.1 peptide dimer assay:

The HLA-A2.1 protein domain $\alpha 3$ fused to purified polyclonal mouse IgG was used to identify HIV-1 peptide specific CD8+T cells (BD DimerX MHC:Ig; BD

Biosciences, Mississauga, ON). The HIV-1 peptides were diluted in PBS to a final concentration of 2 mg/ml. The HLA-A2:Ig dimers were incubated overnight at 37°C with gp120, gag and pol peptides. Mouse blood was quickly placed at 37°C and washed with media. Purified polyclonal human IgG (2μg) was added for 10 min at room temperature to block non-specific binding of dimers to surface Fc receptors. Dimer/peptide mixture (2μg) was added to the cells and incubated for 1 hr at 4°C. The cells were then washed with FACS buffer and re-incubated with 2 μg of purified polyclonal human IgG. After 10 min incubation at room temperature, the cells were washed and stained with TC conjugated anti-CD8 Ab, FITC conjugated anti-CD3 Ab, as well as PE-labelled anti-mouse IgG1 secondary Ab (A85-1) to detect dimers. An increase of 0.1% of dimer-positive cells over the unstimulated control was considered as a positive response to vaccination.

ELISPOT assay:

Multiscreen-HTS plates (Millipore, Bedford, MA) were coated with 10μg/ml of anti-mouse IFN-γ antibody (mAb AN18, Mabtech, Mariemont, OH) in PBS over night at 4°C. The plates were then washed with PBS and blocked with IMDM containing 10% FCS and 100U/ml penicillin/streptomycin for 1 h at room temperature. The medium was removed and a 4 x 10^5 cell suspension (100μl/well) including antigens (10μg/ml) were added and incubated for 30 h at 37°C. For detecting HIV-specific IFN-γ, gp120, gag/pol proteins and gag/pol peptides were used. For HCV, the spleen cells were stimulated with Core/E1/E2 proteins and Core peptides. For SARS, the splenocytes were stimulated with NC-SARS protein. After incubation, cells were removed, washed with PBS+0.05%

Tween 20 and incubated with 1μg/ml of biotinylated anti-mouse IFN-γ antibody (mAb R4-6A2-Biotin, Mabtech) for 2 hr at room temperature. After washing, 100 μl/well of 1/1000 Streptavidin-ALP-PQ (Mabtech) in PBS+ 0.5% FCS was added and incubated for 1 hr at room temperature. The plates were washed as above and developed with 100μl per well BCIP/NBT alkaline phosphatase (Moss Inc) for 20 minutes at room temperature. The reaction was stopped with rinsing the plates with tap water. The numbers of spots were analyzed with ELISPOT reader in Health Canada.

Statistical analysis:

Results were expressed as mean ±S.D. or ±S.E. In each experiment four animals were used per group. All experiments were repeated 2-3 times. The student's *t*-test was applied for the statistical analysis of the data. The *p* value equal to or less than 0.05 was considered significant.

Chapter 3:

Potency of Cell-Mediated Immune Responses to Different Combined HIV-1 Immunogens in a Humanized Murine Model

3.1 Abstract

In this study, cell-mediated immune responses were evaluated in HLA-A2.1 mice that received polycistronic vector expressing HIV-1 gp120, gag and pol or single vectors expressing gp120 +gag/pol as well as recombinant structural proteins and adjuvants. Mice primed with the polycistronic DNA/CpG and boosted with the same regimen plus proteins induced a higher T-cell proliferative response to gp120. However, a very high frequency of IFN-γ was detected in mice receiving the mixture of gp120 + gag/pol DNA constructs, recombinant proteins and CpG. Specific CD8+T cells in PBMCs were measured by intracellular cytokine and HLA-A2.1-peptide dimer staining in response to HLA-A2.1-restricted HIV-1 epitopes (gp120, gag and pol). The group that received single gp120+gag/pol DNA constructs, recombinant proteins and CpG had a higher CD8+T cell response to the combination of peptides compared to the other groups that received the polycistronic construct. The present study reveals an optimal combination of immunogens to enhance immune responses against HIV-1.

3.2 Introduction

It has been more than two decades since the discovery of HIV, but efforts to develop an effective vaccine to prevent or treat this infection have not been successful. Although anti-retroviral therapy has slowed the epidemic curve in North America (Blower, Schwartz et al., 2003), anti-retroviral resistance is a growing problem even in recently acquired infections (Noe, Verhofstede et al., 2004;Ogunbodede, 2004). Human efficacy trials with the HIV-1 gp120 envelope protein vaccination failed to protect against HIV infection (Senior, 2003;McCarthy, 2003). One reason for this failure may be due to lack of cellular immunity, which is essential for the control of HIV infection. Ultimately, vaccines containing HIV-1 antigens and stronger adjuvants that induce both humoral and cellular responses may be a better alternative to gp120-based vaccines. Conserved HIV-1 genes such as gag or pol are considered as promising immunogens (Livingston, Crimi et al., 2002) for vaccine development because these proteins contain many T-helper and CTL epitopes and are believed to be important targets for the control of the virus during natural infection. (Frahm, Korber et al., 2004a;zur, Otten et al., 2003c;Yusim, Kesmir et al., 2002b;Singh & Barry, 2004). Previous studies suggest that long-term non-progressors have a high level of gag and pol-specific CTL activity (Balla-Jhagjhoorsingh, Koopman et al., 1999;Gillespie, Kaul et al., 2002;Harrer, Harrer et al., 1996;Harrer, Harrer et al., 1998). Gp120 may also be immunogenic when used as a target in cohorts of infected individuals and it may be possible to generate protective immunity under this condition (Gao, Weaver et al., 2005a;Paoletti & Kennedy, 2002a). However, anti-gp120 responses might be strain or HIV-1 isolate-specific (Zolla-Pazner, Gomy et al., 1999;Lewis, Chen et al., 2002;Hanke, McMichael et al., 2002b). The accessory genes of HIV have also been considered as vaccine candidates (Ayyavoo, Nagashunmugam et

al., 1998) but some of these genes have been associated with untoward effects in the host (Ayyavoo, Mahalingam et al., 1997).

DNA vaccines are able to induce both humoral and cellular immune responses and have demonstrated their efficacy in several experimental models (McMichael, Mwau et al., 2002;Hanke, McMichael et al., 2002a;Wang, Wiley et al., 2003;Stratov, DeRose et al., 2004). There is a need to determine the optimal combination of HIV-1 immunogens to develop efficient HIV-1 vaccine candidates. Vaccination with gp120, gag and pol alone (DNA or protein) in animal models did not generally induce protective immunity (Liu, Mboudjeka et al., 2004;Barnett, Rajasekar et al., 1997;Qiu, Song et al., 1999) and a better immune response was observed following specific changes in their coding sequence or boosting with proteins and adjuvants. To induce optimal cell-mediated immune responses against structural HIV-1 proteins, Different combinations of DNA constructs (polycistronic or monocistronic) encoding gp120, gag, and pol along with recombinant HIV-1 structural proteins and different adjuvants (CpG, montanide) were evaluated in mice expressing human HLA-A2.1. Our results showed that the two single DNA constructs (DNA-gp120 and DNA-gag/pol) induced a better CD8+T-cell response than the polycistronic DNA construct (DNA-gp120/gag/pol) and that the use of CpG as adjuvant induced better cell-mediated responses than montanide.

3.3 Results

Characterization of HIV-1 single and polycistronic DNA constructs

Total RNA was purified from the plasma of a patient seropositive for HIV-1 clade B or from non-syncytium inducing HIV-1 clade B co-cultures. The full-length gag and truncated pol genes (~3.0 kb) and gp120 were amplified, using specific primer pairs (Fig 1.3).

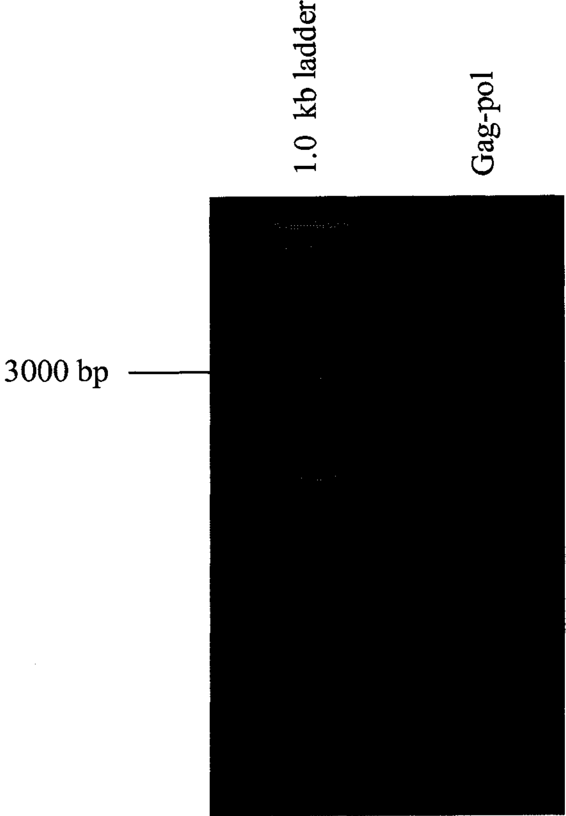
To compare the potency of the anti-HIV response, single DNA constructs (pVAX1-gp120 and pVAX1-gag/pol) and polycistronic (pVAX1-gp120/gag/pol) encoding structural genes of HIV-1 were designed (Fig 2.3 A and B). The pVAX vector was chosen because it has been shown that the expression of proteins was enhanced under control of human cytomegalovirus promoter.

In addition, the amplicon genes of gp120 and gag/pol were cloned into pEF6-myc/His vectors for the expression and purification of the recombinant proteins *in vitro* (Fig 2.3 C). All expression constructs were confirmed and characterized by restriction enzymes and nucleotide sequence analysis.

Fig.1.3. RT-PCR of HIV-1 structural genes. Each amplified PCR product was electrophoresed on a 1% agarose gel. A: To amplify the 3.0 kb gag and truncated pol genes, the following primers were used: forward primer: 5'-atatcttcgagatggggtgcgagagcgtca-3'; reverse primer: 5'-tgaagtctagagggcacccctcgttcttg-3'. The total RNA used as a template was extracted from the sera of an HIV-1 positive patient. B: The 1.6 kb gp120 was amplified by RT-PCR using the following primers: forward primer: 5'-gcaatgaaagtgaaggggaccagga-3'; reverse primer, 5'-tattgaattcagtgacgacgctgcgcccata-3'. The total RNA used as a template was extracted from a non-syncytium inducing HIV-1 co-culture.

Fig 1.3

A



B

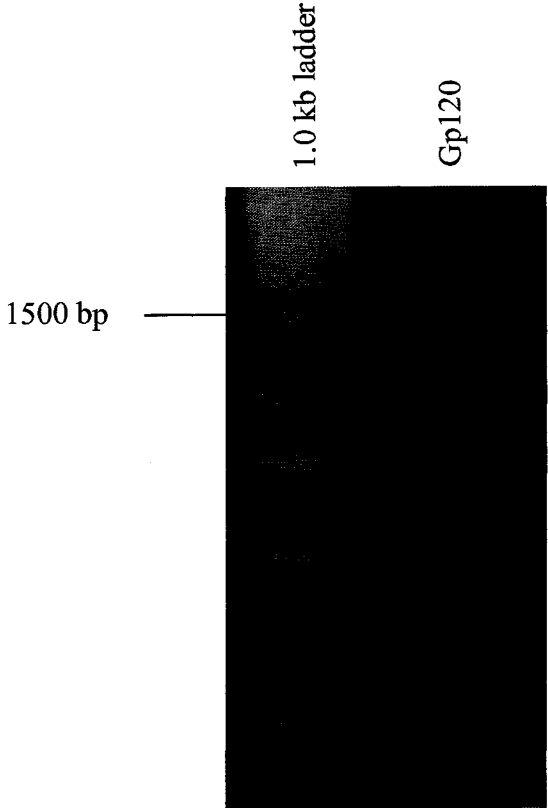


Fig.2.3. HIV-1 structural genes cloned into pVAX1 and pEF6-myc-His DNA vectors. The gag/pol amplicon was purified using the QIAquick gel extraction kit and cloned into the PCR 2.1 TOPO-TA vector. After plasmid digestion, the 3.0 kb band corresponding to the gag/pol genes was sub-cloned into XhoI- and EcoRI sites of pVAX-1 and pEF6-myc-His. The gp120 fragment was cloned into the TOPO-TA vector and sub-cloned into EcoRI sites of pVAX1-gag/pol; pVAX-1 and pEF6/Myc/His vectors.

A: Two DNA constructs expressing gp120 or gag/pol genes. B: The polycistronic DNA construct expressing gag/pol and gp120 under control of one CMV promoter. C: To purify HIV-1 structural proteins, gp120 and gag/pol amplified genes were sub-cloned into pEF6-myc-His vectors.

Fig 2.3

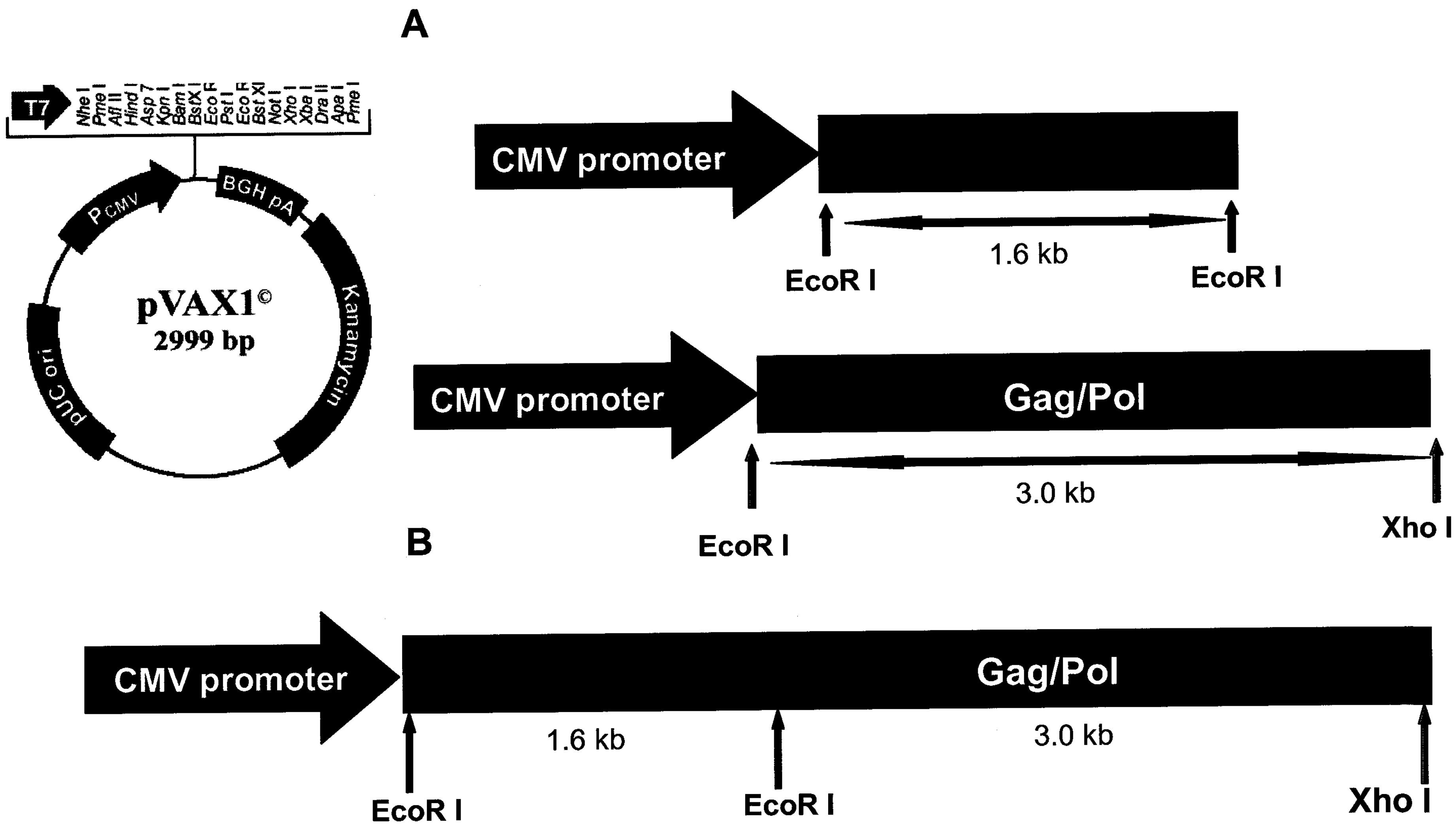
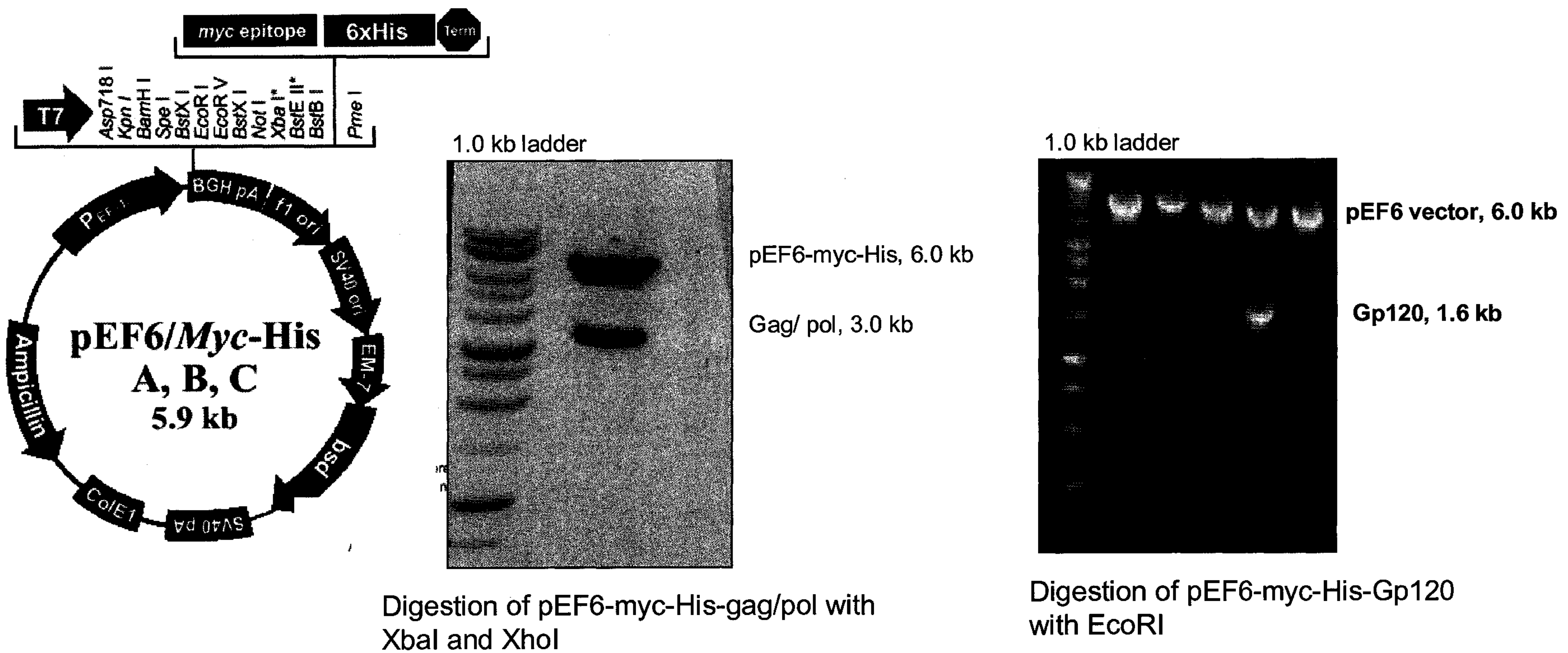


Fig 2.3: C



Digestion of pEF6-myc-His-gag/pol with XbaI and XhoI

Digestion of pEF6-myc-His-Gp120 with EcoRI

Expression of HIV-1 structural proteins in eukaryotic cells

To express the recombinant HIV-1 structural proteins, CHO cells were transfected with recombinant pEF6-Myc/His plasmid expressing the gag/pol or gp120 genes.

In contrast to the gag/pol construct, the gp120 construct was poorly expressed in transiently-transfected CHO cells. It was shown that gp120 has the ability to kill mammalian cells by apoptosis. To solve this problem, a stable CHO cell line was created by using a blasticidine resistance gene. The production of gp120 protein was increased more than 20-fold in this cell line, allowing for its efficient purification. Transfected cells were harvested, lysed and the recombinant proteins were purified by affinity chromatography. The expression of recombinant HIV-1 structural proteins in transfected cells was determined by western blotting (Fig 3.3 A and B). To demonstrate the expression of HIV-1 genes in our polycistronic construct, CHO cells were transfected with this construct, lysed and western blotting was performed (Fig 3.3 B. lane 5).

Vaccination strategy

Six groups of HLA-A2.1 mice (four per group) were immunized with different combinations of HIV-1 immunogens and CpG or montanide as adjuvants. Each mouse was immunized I.M. (Table 1.3) consisting of 50 μg DNA, 5.0 μg of recombinant gp120, gag and pol proteins and with either 30 μg CpG oligodeoxynucleotide (Vollmer, Jurk et al., 2004) *(5"-TCCATGACGTTCCTGACGTT-3")* (Coley, Ottawa, ON) or with 50 μl montanide ISA-51 (Seppic, France). Animals were immunized four times at one month intervals and immune responses were determined 2 weeks after the last injection. In contrast to the negative control group which was primed and boosted with empty vector

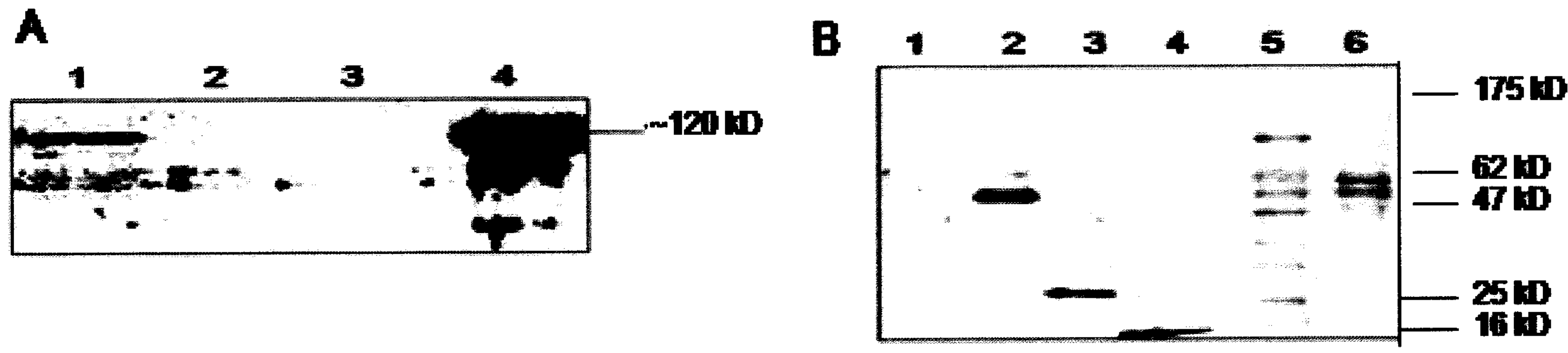
and CpG (group A), the groups that received polycistronic (Groups C, D, E), single DNA constructs (group F) or recombinant proteins (group B) demonstrated a differential specific HIV-1 immune response.

	Priming	**Boosting**
Group A	**Vector alone + CpG**	**Vector alone + CpG**
Group B	**Proteins + CpG**	**Proteins + CpG**
Group C	**Polycistronic DNA + proteins + CPG**	**Polycistronic DNA + proteins + CPG**
Group D	**Polycistronic DNA+proteins+ Montanide**	**PolycistronicDNA+proteins+Montanide**
Group E	**Polycistronic DNA + CPG**	**Polycistronic DNA + proteins + CPG**
Group F	**Gp120 + Gag/pol DNA + proteins + CpG**	**Gp120+Gag/pol DNA + proteins + CpG**

Table 1.3. Vaccination strategies. Each mouse was vaccinated four times with one month intervals between each immunization.

Fig.3.3. Western blot analysis of expressed recombinant HIV-1 proteins. A: Lane 1: purified gp120 protein expressed in CHO cells. Lane 2 and 3: cell lysates from CHO cells transfected with vector alone. Lane 4: control gp120 protein expressed in a baculovirus system obtained from NIH. The blot was probed with polyclonal gp120 antibody. B: Lane 1: cell lysates from CHO cells transfected with vector alone. The lanes 2, 3 and 4 are p55, p24 and protease (10kd), respectively, which were obtained from NIH. Lane 5: cell lysates from CHO cells transfected with polycistronic DNA construct expressing gp120, gag and pol. Lane 6: gag/pol proteins purified from transfected CHO cells. The blot was probed with pooled sera from HIV-1 positive patients.

Fig 3.3



Antibody response in vaccinated HLA-A2.1 mice

In order to determine the binding antibody titre was raised against gp120 and gag/pol proteins, plasma from immunized mice were collected two weeks after the last immunization and analyzed by end-point ELISA titres. 96-well plates were coated overnight at 4°C with HIV-1 gp120 or gag/pol proteins clade B (NIH). The plates were incubated with serially-diluted sera and developed based on Materials and Methods. HIV-specific IgG was detectable in the plasma of all but the negative control mice (Fig 4.3). Groups C, E and F, which received CpG demonstrated a relatively low titre of gp120 and gag/pol antibodies. The highest antibody titres ($p < 0.05$) raised against both gp120 and gag/pol proteins were observed in the group of mice immunized with the polycistronic DNA construct, recombinant proteins and montanide (group D).

Measurement of proliferative immune responses after DNA and/or protein immunization

Proliferative immune responses were evaluated in the spleen cells of immunized mice. Two weeks after the last immunization, animals were sacrificed, and splenocytes were stimulated with gp120, gag/pol proteins. Cells were also stimulated with PMA/ION. After 72 h of culture, 1 μCi [^{3}H] thymidine was added to each well and after 16 h incubation, thymidine incorporation was measured with a Microbeta beta counter.

Groups B-F demonstrated a high level of proliferative responses after stimulation with gp120 and gag/pol in contrast to group A that received vector alone and CpG (Fig5.3). Based on the *t*-test, there was no significant difference in proliferative response

to gag/pol proteins between groups C, D and F that received various combinations of HIV-1 vaccines. However, groups E (primed with DNA/CpG and boosted with DNA/proteins and CpG) had the best T helper response to gp120 compared to other immunized groups ($p \leq 0.05$).

Fig.4.3. Antibody titers were determined in mice (n=4) two weeks after the last immunization. The 96-well plates were coated with HIV-1 structural proteins and mouse sera were serially diluted in wells for the endpoint titration of anti-gp120 and anti gag/pol antibodies. Results are shown as mean concentration ± S.D. Symbol * indicates a significant difference when compared with groups B, C, E and F ($p < 0.05$).

Fig 4.3

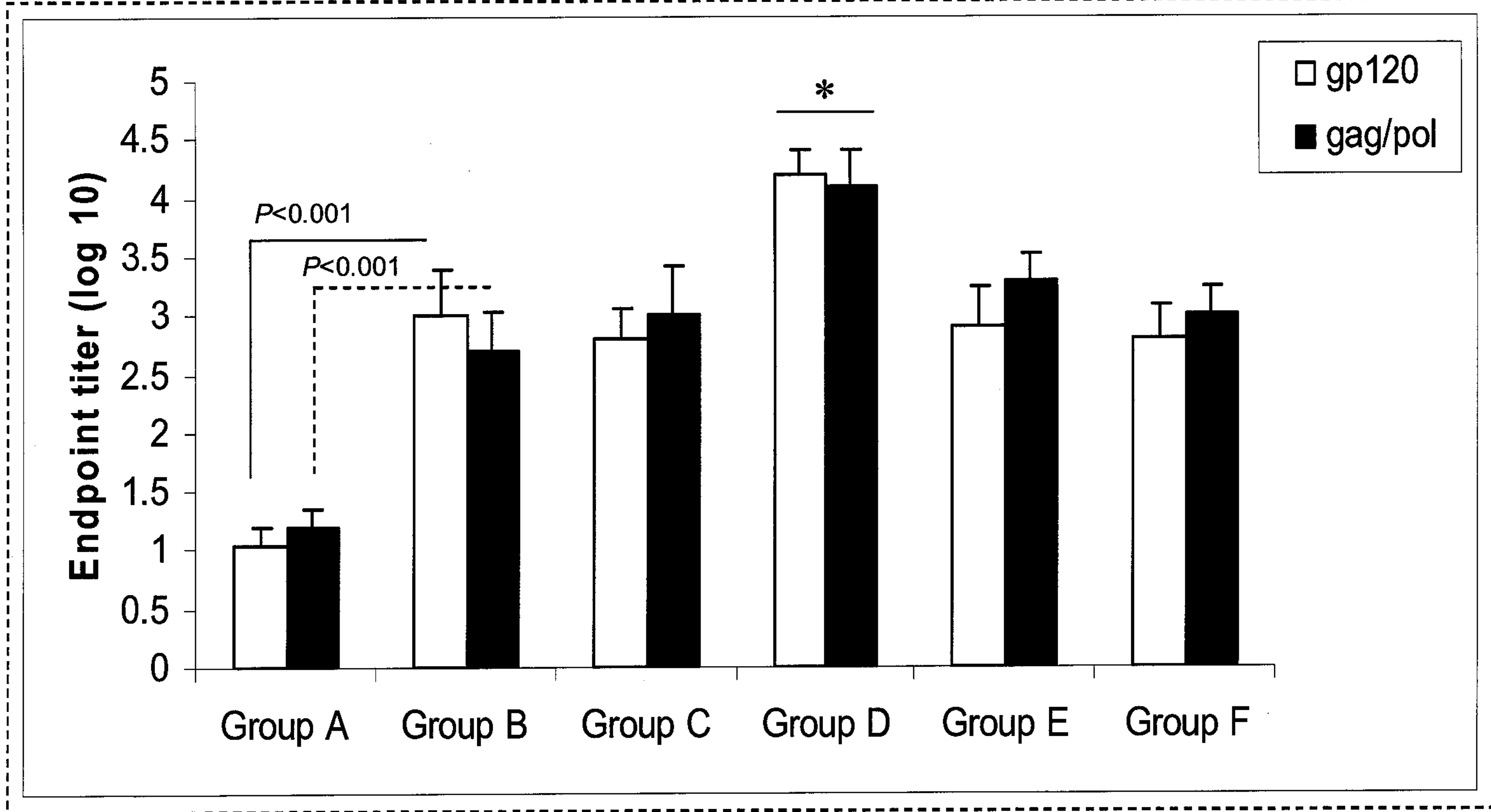
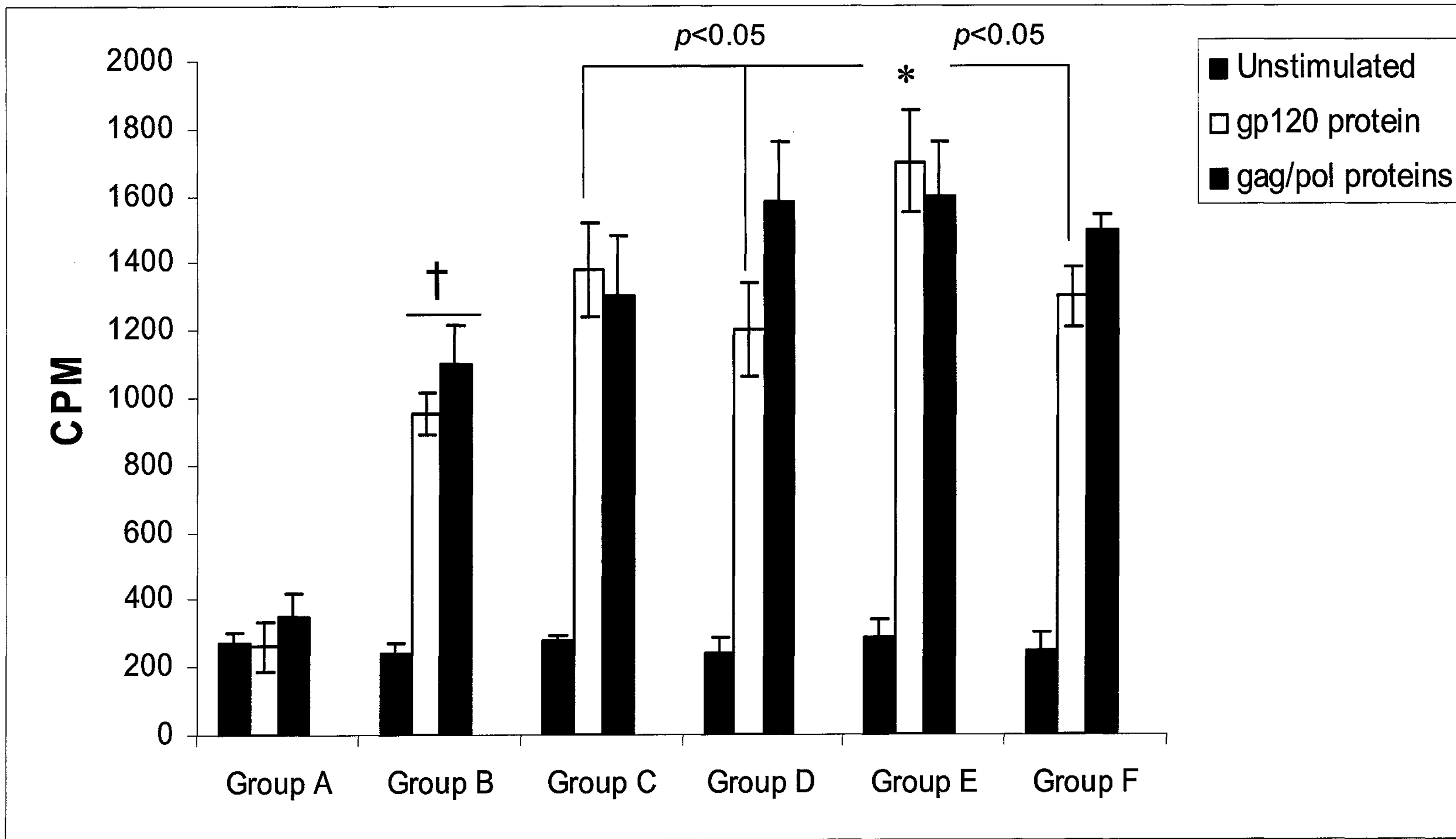


Fig.5.3. T-cell proliferation results in HLA-A2.1 mice immunized with the candidate HIV vaccines. Splenocytes were cultured and stimulated with 10 μg/ml gp120 and gag/pol proteins. Three days later, ^{3}[H]-thymidine was added and after 18h the incorporated radioactivity was measured in harvested splenocytes. The mean counts per minute (CPM) ±S.E. is shown for each group. Symbol * indicates a significant difference when compared with groups C, D and F ($p \leq 0.05$). Symbol † indicates a significant difference between group B and groups C, D, E and F ($p < 0.05$).

Fig 5.3



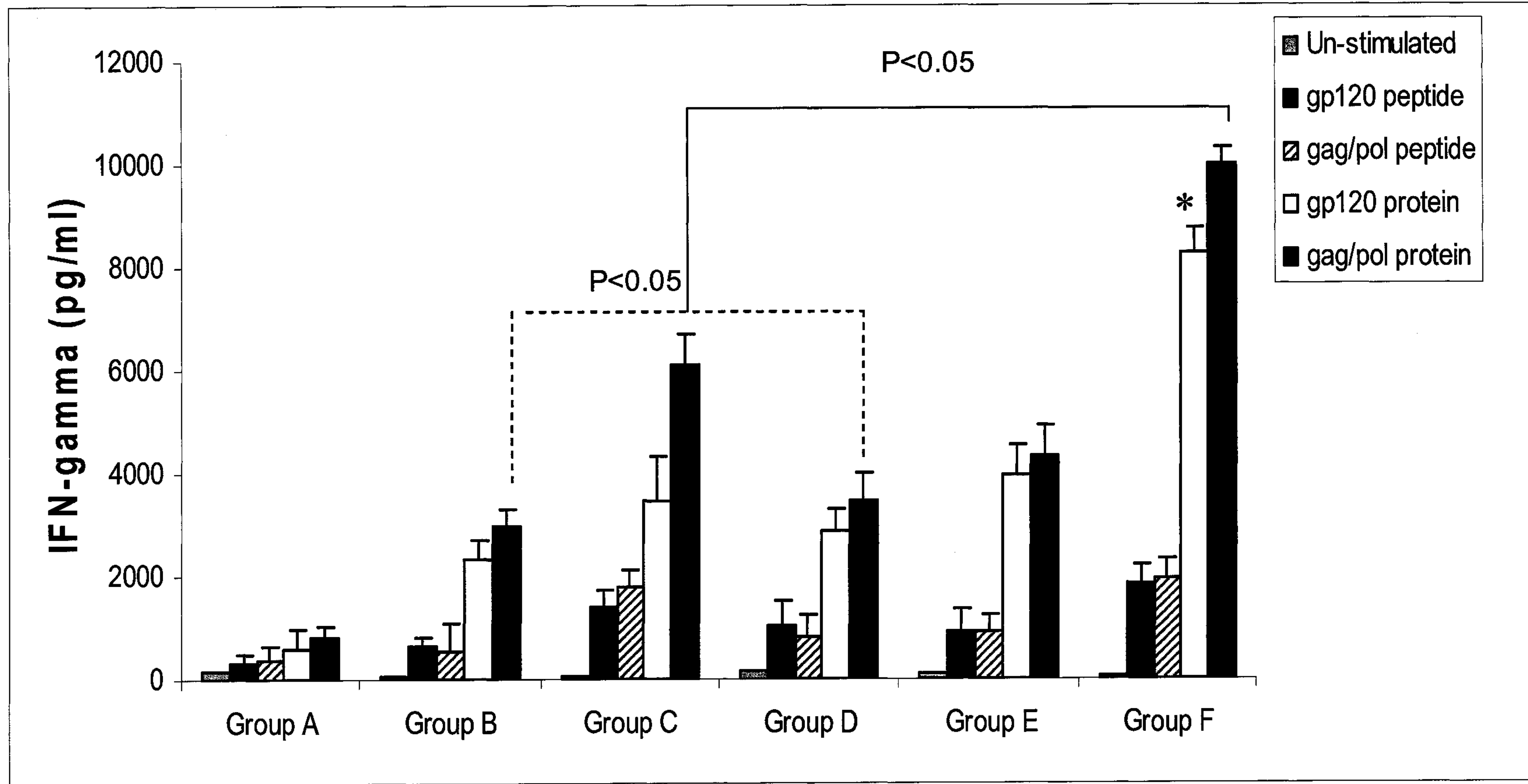
Combinations of single constructs, proteins and CpG induced the highest level of Th1 cytokine secretion in immunized groups

Supernatants from the stimulated cultures were also collected and assayed for IL-4, IL-5 and IFN-γ production (Fig 6.3). While IL-4/IL-5 was not detected, there was IFN-γ production in response to gp120, gag/pol proteins in all groups of immunized mice with the exception of the negative control mice (group A). However, mice receiving CpG and single DNA constructs (group F) produced the highest level of IFN-γ to gp120 ($p<0.05$) and gag/pol ($p<0.05$-0.001) in comparison with the other immunization groups.

The level of IFN-γ response was further assessed with gp120, gag and pol CTL epitopes. Stimulation with HLA-A2.1-binding HIV-1 peptides resulted in IFN-γ production in immunized mice. IFN-γ levels were similar in groups B, D and E, with a trend for higher levels in groups C and F immunized and boosted with DNA, recombinant proteins and CpG. In these two groups, gag/pol induced higher level of IFN-γ than gp120.

Fig.6.3. IFN-γ production in HLA-A2.1 mice immunized with the candidate HIV vaccine. Two weeks after the last immunization, splenocytes were restimulated in vitro with gp120 and gag/pol recombinant proteins or specific peptides that recognize HLA-A2.1. The level of IFN-γ production was measured by quantitative ELISA in supernatants removed 72h after stimulation. Results are shown as mean ±S.D. Statistically significant difference between indicated groups is shown. Symbol * indicates a significant difference when compared with groups B, C, D and E ($p \leq 0.05$).

Fig 6.3



Combination of single constructs, proteins and CpG induce higher amount of specific CD8+ T- cell immune responses in HLA-A2.1 mice

To determine whether this vaccination strategy induced HIV-1-specific T-cell effector responses, intracellular cytokine staining (ICC) and HIV peptide loaded HLA-A2.1 dimer were performed. CD8+T cells producing IFN-γ and/or cells that specifically bound HIV-1 epitopes were measured with these assays. Mice were bled 14 days after the last immunization and cells were cultured with the HIV-1 peptides corresponding to HLA-A2.1. The ability of the DNA vaccine candidates to induce a CD8+T cell response to each or a mixture of the HIV-1 peptides was compared. The group of mice immunized with the mixture of DNA constructs, recombinant proteins and CpG (group F) demonstrated a higher amount of HIV-1 specific CD8+T cells to most peptides but peptides #3 and #5 (Table 2.3). Mice immunized with the polycistronic DNA construct had a predominant response to gag/pol peptides, while mice immunized with the single DNA constructs had a predominant response to gp120.

The HLA-peptide dimer assay confirmed the results obtained by intracellular cytokine staining by identifying increased anti-HIV-1 CD8+T-cell responses in the vaccinated groups. The HLA-A2:Ig dimers were incubated overnight at 37°C with gp120, gag and pol peptides. Mouse blood was quickly placed at 37°C and washed with media. Dimer/peptide mixture was added to the cells and incubated. The cells were washed and stained with mouse TC conjugated anti-CD8 Ab, FITC conjugated anti-CD3 Ab, and PE labelled A85-1 mAb, which is an anti-mouse IgG1 to detect the dimers and analyzed by flow cytometry.

The highest CD8+ response was observed (Fig 7.3) in mice immunized with the single DNA constructs, proteins and CpG (group F). These results are especially significant because the assays were performed in peripheral blood without further T cell expansion *in vitro*.

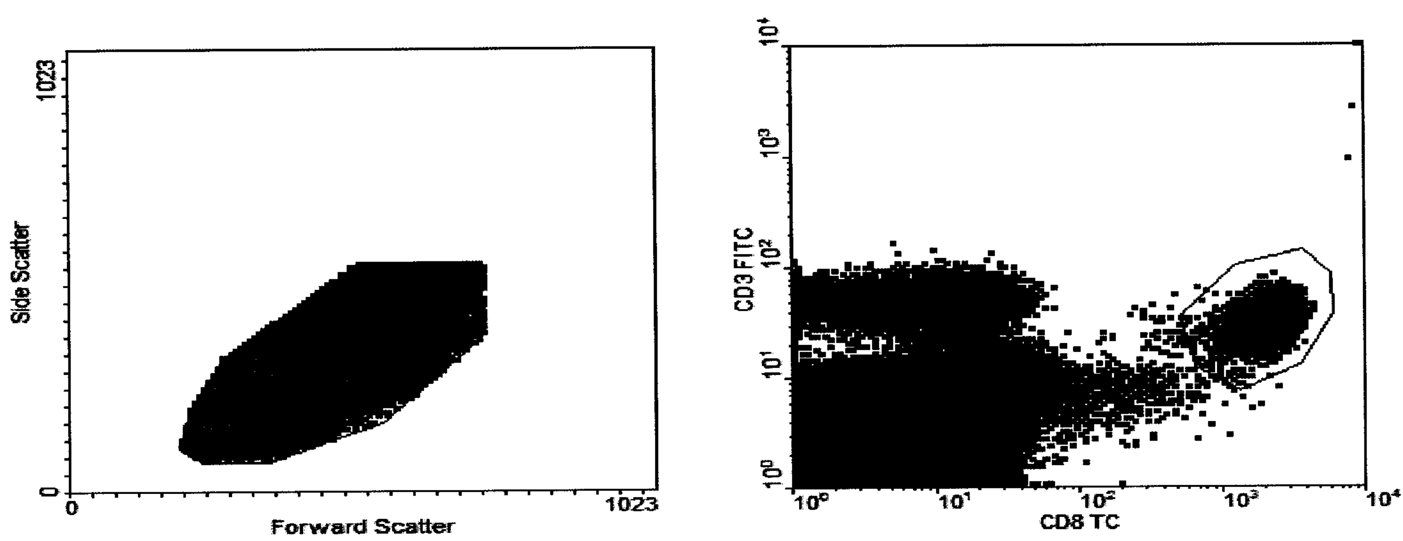
Table 2.3

Peptides			Percentages of CD8/IFNγ				
Number	**Region**	**Sequence**	**Group B**	**Group C**	**Group D**	**Group E**	**Group F**
1	Env (120-128)	KLTPLCVTL	ND	0.43	0.51	0.58	1.34
2	Gag (77-85)	SLYNTVATL	ND	0.68	0.27	0.37	1.53
3	Gag (386-394)	VLAEAMSQV	0	0.69	0.75	0.37	0.32
4	Pol(309-317)	ILKEPVHGV	0	ND	0.45	0.48	0.64
5	Pol (33-41)	ALVEICTEM	0	0.38	0.68	0.45	0.16
6	Mixture of peptides		0.27	0.44	0.38	0.39	1.18

Table 2.3. Frequecies of IFN-γ secreting CD8+T lymphocytes is shown in the immunized groups after stimulation with HIV-1 peptides. Peripheral blood from immunized mice was stimulated with single or a mixture of HIV-1 peptides. A negative control (HCV-core peptide) was included to control for the spontaneous production of IFN-γ (data not shown). The cells were surface and intracellularly stained and analyzed by FACS. All values are reported after subtraction from the control group (group A).

Fig.7.3. HIV-1 specific CD8+T cells in HLA.A2.1 immunized mice detected by a HLA-peptide dimer assay. Frozen blood was incubated with dimer/peptide mixture and HIV-specific CD8+T cells were detected by flow cytometry as described in *Materials and Methods*. A. Dot plots show results from individual representative animals from each mouse group. The numbers shown in the right corners of the pictures are the percentages of HIV-specific CD8+T cells in a representative mouse from each group. B. Quantification of dimer assay in the immunized mice. Results are shown as mean ±S.D. Statistically significant difference between indicated groups is shown.

Fig 7.3: A



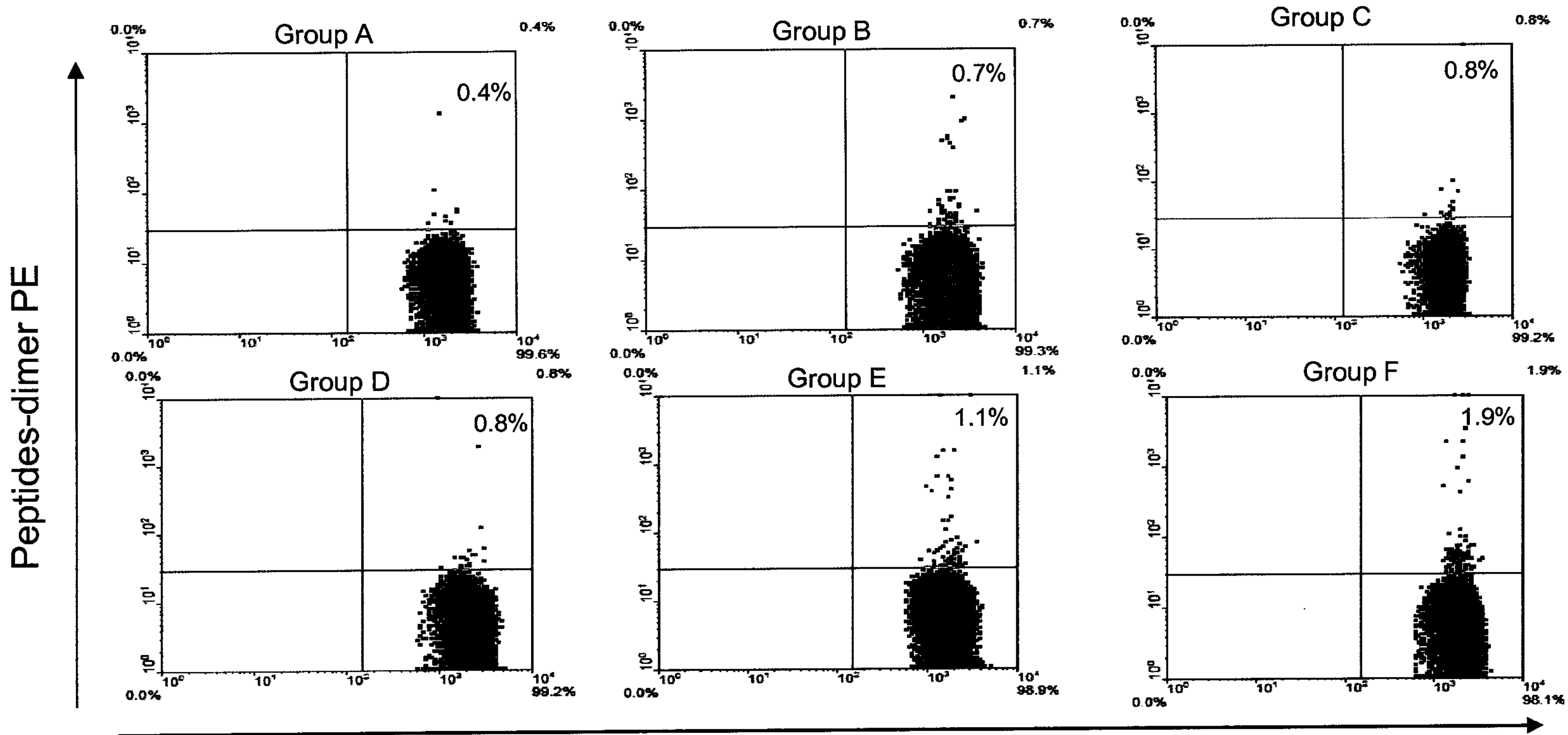
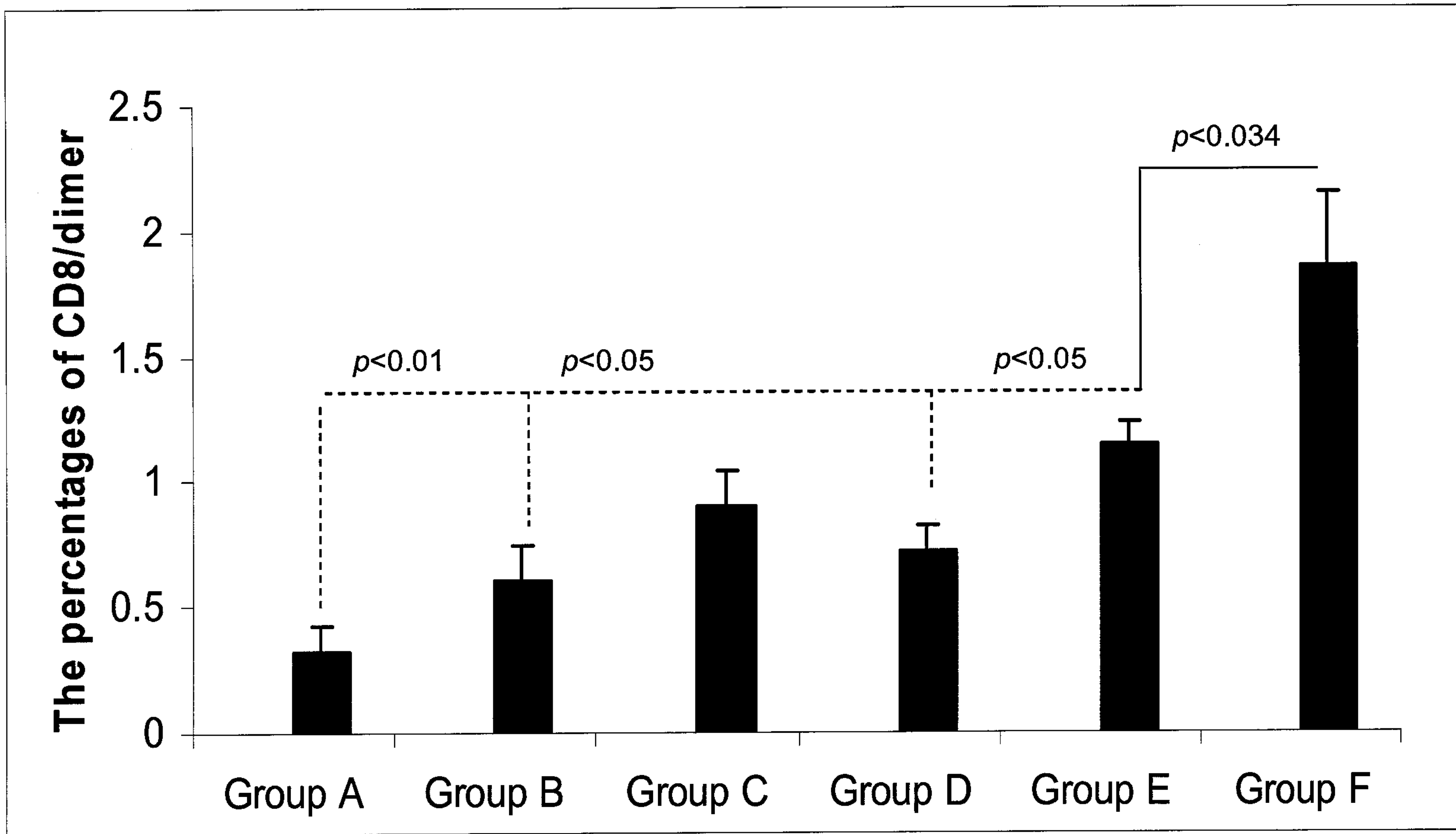


Fig 7.3: B



3.4. Discussion

The ideal vaccine should be able to induce both strong humoral and cell-mediated immune responses, especially Th1 and CTL responses. DNA vaccines as well as recombinant proteins have advantages in the induction of specific immune responses and the optimized combination of DNA/protein regimens may have good potential for development of an effective vaccine for controlling HIV-1 infection. The present study compared the immunogenicity of two DNA constructs (polycistronic and monocistronic) along with recombinant proteins. The rationale for using a polycistronic construct was based on the number of Th and CTL epitopes present in the structural viral genes and the immunodominance reported for some of these epitopes, which are able to induce a polyspecific and robust immune response (Betts, Yusim et al., 2002;Nabel, 2002a;zur, Otten et al., 2003b).

In this study, the HLA-A2.1 murine model was chosen to represent the potential human CTL response to these candidate vaccines. This animal model may predict the efficacy of the CD8+T cells immune responses induced by each vaccine. Woodberry T et al., described an HIV-1 CTL polyepitope comprising seven HLA-A2 CD8 epitopes. HLA A2-1 transgenic mice were immunized and each epitope in the polytope construct was processed and presented in the HLA-A2.1 mouse model (Okazaki, Pendleton et al., 2003;Woodberry, Gardner et al., 1999).

Furthermore, the vaccine immunogenicity can be changed by the type of adjuvant used. CpG and montanide ISA-51 are among the potential experimental adjuvants. Montanide, a mineral oil-based adjuvant, has been previously tested in clinical trials (Aucouturier, Dupuis et al., 2002;Sanderson, Scotland et al., 2005) and it has a good

reactogenicity profile, which makes it a good adjuvant for human use. CpG is a strong inducer of Th1 responses and it stimulates dendritic cells through TLR9, inducing cell maturation and enhancing antigen presentation and Th1 responses (Moss, Diveley et al., 2000;Moss, Diveley et al., 2001;Vinner, Nielsen et al., 1999;Schlaepfer, Audige et al., 2004).

The candidate HIV-1 vaccines induced specific antibody titer in immunized mice compared to the control (<50). However, the regimen containing montanide as adjuvant induced the highest level of antibody response against both gp120 and gag/pol (>8100). These results are in agreement with previous HIV vaccine studies in which high antibody titres were induced when montanide was combined with synthetic peptides (Peter, Men et al., 2001;Gomez, Navea et al., 1999) or recombinant proteins (Kirkley, Goldstein et al., 1996). We were not able to test the ability of our vaccines to induce neutralizing antibodies because of the inherent problem of high background in mouse sera (Newton, Acierno et al., 2002). However, it would be ideal to immunize other animal models such as macaques, rabbits or hamsters for further studies.

In the current study, the proliferative immune response was assessed *in vitro* in splenocytes as an indication of CD4+T cell responses. Comparison of the proliferative immune responses among the immunized groups demonstrated that administration of proteins and CpG (group B) developed a lower proliferative response to gp120 and gag/pol compared to the other groups that received the combined administration of protein and DNA, suggesting that the DNA/protein regimens induces a more robust CD4 response. The level of CD4+ response to gag/pol protein was not significantly different between groups D, E and F. However, the vaccine regimen consisting of DNA priming

and DNA/protein boosting (group E) induced stronger specific gp120-CD4 immune responses.

Thl immune responses play crucial roles in protection against viral infections, including HIV-1 (Kim, Befus et al., 2004;Ahlers, Belyakov et al., 2001;Kim, Yang et al., 2001). The activity of dendritic cells could be increased by Thl cytokines and it is possible that these dendritic cells induce production of memory CD8+T cells. Previous studies in long terminal non-progressor individuals (LTNP) showed that this group of infected people respond to HIV-1 gag epitopes with a Thl-like response, characterized by IFNγ production(Migueles & Connors, 2001). In addition, a shift from Thl bias to Th2 cytokine production occurs during the period of HIV-1 disease. Thus, we addressed which prime-boost candidate vaccine could promote stronger Thl immune responses. The evaluation of the T helper cytokine responses in the immunized groups suggests that the various combinations of DNA constructs, proteins and adjuvant induce predominantly a Thl response. However, the highest level of IFN-γ was achieved after immunization with the single DNA constructs, proteins and CpG (group F). In contrast to Thl immune responses, IL-4 and IL-5 which are indicators for Th2 responses were not detected in the lymphocyte culture supernatants.

In this study, peptide-loaded dimer complexes and intracellular IFN-γ staining were used to measure the CD8+T cell response in immunized groups. The mice that received single DNA constructs expressing gp120 or gag/pol genes (group F) demonstrated a higher amount of CD8+T cell immune responses than the groups that received polycistronic construct. It is possible that the ability of single constructs to induce CD8+T cells is attributed to the presence of unmethylated CpG motifs in the DNA

plasmids. In addition, the enhanced immune response could be associated with the level of protein expression in the different plasmids (Kjerrstrom, Hinkula et al., 2001). These results suggest that it might be better to manipulate cell-mediated immune responses through the use of different multigenic DNA constructs. The induction of a strong HIV-1 specific CD8+T cells is the goal for most current vaccines strategies. Previous studies suggest that HIV-1 specific CTL responses may prevent or slow the onset of disease in infected individuals (Benito, Lopez et al., 2004b;Ganusov, 2003b). However, a high CTL response may dampen antibody levels, resulting in lower antibody titers (Gog, Rimmelzwaan et al., 2003). An interesting observation in this study is that the group of mice with the highest level of CD8+T cell responses (group F) had one of the lowest gp120 antibody titers.

The mechanism behind the optimal immunization regimen is not clear and needs further investigation. However, it is possible that endogenous antigens (DNA constructs) as well as exogenous antigens (recombinant proteins) can allow access to alternative processing pathways and can give rise to an extended repertoire of antigenic epitopes. The combined immunization regimens (DNA/protein/adjuvant) may increase the repertoire of antigenic epitopes and consequently elicit stronger specific humoral and cellular immune responses than DNA-protein regimen alone. In summary, the highest level of specific Th1 and anti-HIV-1 CD8+T cell responses were demonstrated in mice receiving a mixture of single gp120+gag/pol constructs, recombinant structural proteins and CpG. This new combined regimen may add breadth to the antiviral immune response and facilitate the development of an effective vaccine against HIV-1.

Chapter 4:

Synergistic effect of combined HIV/HCV immunogens: A Combined HIV-1/HCV Candidate Vaccine Induces a Higher Level of CD8+T cell-Immune Responses in HLA-A2.1 Mice

4.1 Summary

Dual infections with HIV-1 and Hepatitis C virus (HCV) may proceed in concert to cause severe disease. Individuals with both infections advance faster to AIDS than those that are infected with a single virus (van Asten & Prins, 2004b).

In this study, HLA-A2.1 mice were immunized with HIV/HCV immunogens alone or in combination where immunogens refers to DNA and protein. Mice immunized with the combined vaccine had similar gp120 and core antibody titers as the group receiving either HIV-1 or HCV immunogens. Proliferative responses showed that mice receiving the combined vaccine (HIV/HCV group) exhibit a three fold higher stimulation index (SI) to gp120 than mice immunized with HIV alone (HIV group). To determine whether our vaccine strategies induced Th1 or Th2 immune responses, levels of two cytokines, IFN-γ and IL-5, were measured. The combined vaccine induced a higher level of Th1 responses to gag protein compared with other groups. In contrast, no groups showed detectable IL-5 responses neither to the HIV-gag protein or the HCV-core protein. Interestingly, detection of IFN-γ by ELISPOT assay demonstrated that the HIV/HCV group had increases in spot forming cells (SFC) to HIV-gp120 peptide when compared to the HIV-1 group. The HIV/HCV group also showed an increase in SFC to HCV-core peptide in comparison with the group receiving HCV immunogens alone. Intracellular IFN-γ staining was also performed, confirming the

ELISPOT results and demonstrating that the HIV/HCV group had significantly higher percentages of HIV and HCV-specific CD8+T cells in comparison to groups receiving HIV or HCV immunogens alone. These results open a new approach to maximizing vaccine efficacy against HIV and HCV.

4.2 Introduction

One of the major complications in individuals infected with HIV is liver disease caused by HCV infection. It is estimated that 30% of HIV patients are infected with HCV (Tossing, 2005). These viruses share the same route of transmission; however, HCV unlike HIV is more easily transmitted vertically than through sexual contact (Balestra, Aquaro et al., 2003). Currently, there is no effective vaccine due to lack of knowledge of HIV/HCV pathogenesis, lack of animal models and high viral mutation rate (Shibata, 2002;Nathanson & Mathieson, 2000;Sela, 2002;Chan, Bye et al., 1996).

Previous studies show that individuals infected with both HIV and HCV progress faster to AIDS and death than HIV patients who are HCV negative (van Asten & Prins, 2004a;Hershow, O'Driscoll et al., 2005). HCV effects have limited ability to undergo antiretroviral therapy due to the toxic effects of HCV on the liver (Uberti-Foppa, De Bona et al., 2003). HCV may also accelerate HIV progression to AIDS by acting as a cofactor to increase the replication of HIV-1 (Greub, Ledergerber et al., 2000), and/or by down-regulating the amount of CD4+T cells, thus inhibiting immune responses(Carlos, Castilla et al., 2004).

Cell-mediated immune responses play an important role in the control of HIV/HCV infections (Benito, Lopez et al., 2004a;Gulzar & Copeland, 2004b;Zhu & Eckels, 2002b). Studies show an inverse correlation between viral load and amount of CD4+ and CD8+T cells. Although previous studies indicated that specific CD8+T cells can be induced against viruses in the absence of CD4+ cells, the role of Th1 CD4+ cells in maintaining CD8+ cells (Barker, Mackewicz et al., 1995;Mooij, Nieuwenhuis et al., 2004a) is essential. CD4+Th1 cells secrete cytokines that could shift immunity to the

cellular immune responses and consequently increase the level of CD8+T cells. Thus, a candidate vaccine must elicit strong Th1 cytokines, such as IFN-γ, as well as broad CD8+T cell immune responses (Wang, Epstein et al., 2004). Genetic immunization is a good strategy to mimic viral infection with regard to mode of antigen production and consequent induction of immune responses. In order to develop an effective vaccine, conserved region nucleocapsid protein containing T-helper cell and CTL epitopes were considered as ideal targets (gag and core in HIV and HCV respectively) (Ikeda-Moore, Tomiyama et al., 1997;Buseyne, McChesney et al., 1993;Nakamura, Kameoka et al., 1997;Hu, Wang et al., 1999). In addition, to generate robust protective immunity against infection, the immunogenic HIV and HCV envelope proteins (gp120 and E1/E2) were also utilized in this study(Kim, Han et al., 2003;Lee, Cho et al., 1998).

So far, few studies have been performed on combined vaccines and the efficacy of individual antigens or combined antigens in the generation of an immune response is not well recognized. A previous study showed that the combination of the small envelope of Hepatitis B (HBV) and hypervariable region of E2 in HCV induces higher immune responses in combination than vaccination with either antigen alone (Netter, Macnaughton et al., 2001;Jin, Yang et al., 2002). Antibody titers and cellular immune responses in immunized mice with HIV/HCV immunogens alone or in combination were measured. A synergistic effect between HIV/HCV antigens was detected that may lead to induction of multi-specific immune responses against both HIV and HCV.

4.3. Results

DNA constructs and vaccination strategy

Two polycistronic DNA constructs were included in this study (Fig1). The polycistronic HIV-1 DNA was described in section 3. The other DNA construct encoded HCV-Core, E1 and E2 has been already constructed in our laboratory (Ghorbani, Nass et al., 2005). Briefly, HCV-Core, E1, and truncated E2 gene fragment (encoding the amino acids 1-683), was amplified by RT-PCR from the serum of a patient seropositive for HCV-1b (forward primer: 5'-accatgagcacgaatcctaaacctc-3'; reverse primer: 5'-tggtagggttgtgaaggaacacg-3'). The amplified gene was TA-cloned into the PCR 2.1 plasmid using the TOPO-TA Cloning kit. The 2.1 kb fragment was then sub-cloned into pVAX-1 and pEF6/myc/His plasmids. All expression constructs were confirmed and characterized by restriction enzymes and nucleotide sequence analysis.

The empty vector (pVAX1) without any HIV or HCV inserts was included as the negative control. In order to express the recombinant HIV-1 and HCV structural proteins, CHO cells were transfected with recombinant pEF6-Myc/His plasmid expressing HIV-1 (Gag/Pol or gp120 genes) and HCV (Core, E1 and E2) genes. Transfected cells were harvested, lysed and the recombinant proteins were purified. The expression of recombinant HIV-1 and HCV structural proteins was confirmed by western blotting and IFA staining (Naas, Ghorbani et al., 2005). Five groups of HLA-A2.1 mice (four per group) were immunized with HIV/HCV viral immunogens alone or in combination as described in Table 1.4. Animals were immunized four times at one month intervals and immune responses were measured 2 weeks after the last immunization.

Fig 1.4. Schematic representation of immunization DNA vectors. (A) The polycistronic DNA construct expressing HIV-1 structural genes (gag/pol and gp120) under control of one CMV promoter. The 1.6 kb HIV-1 gp120 clade B was fused in frame with 3.0 kb HIV-1 gag/pol amplicon. (B) The polycistronic DNA construct expressing HCV structural genes (core, E1 and E2). The core, E1, and truncated E2 gene amplicons (amino acids1-683) was amplified from the serum of a patient seropositive for HCV-1b and cloned into pVAX-1.

Fig 1.4

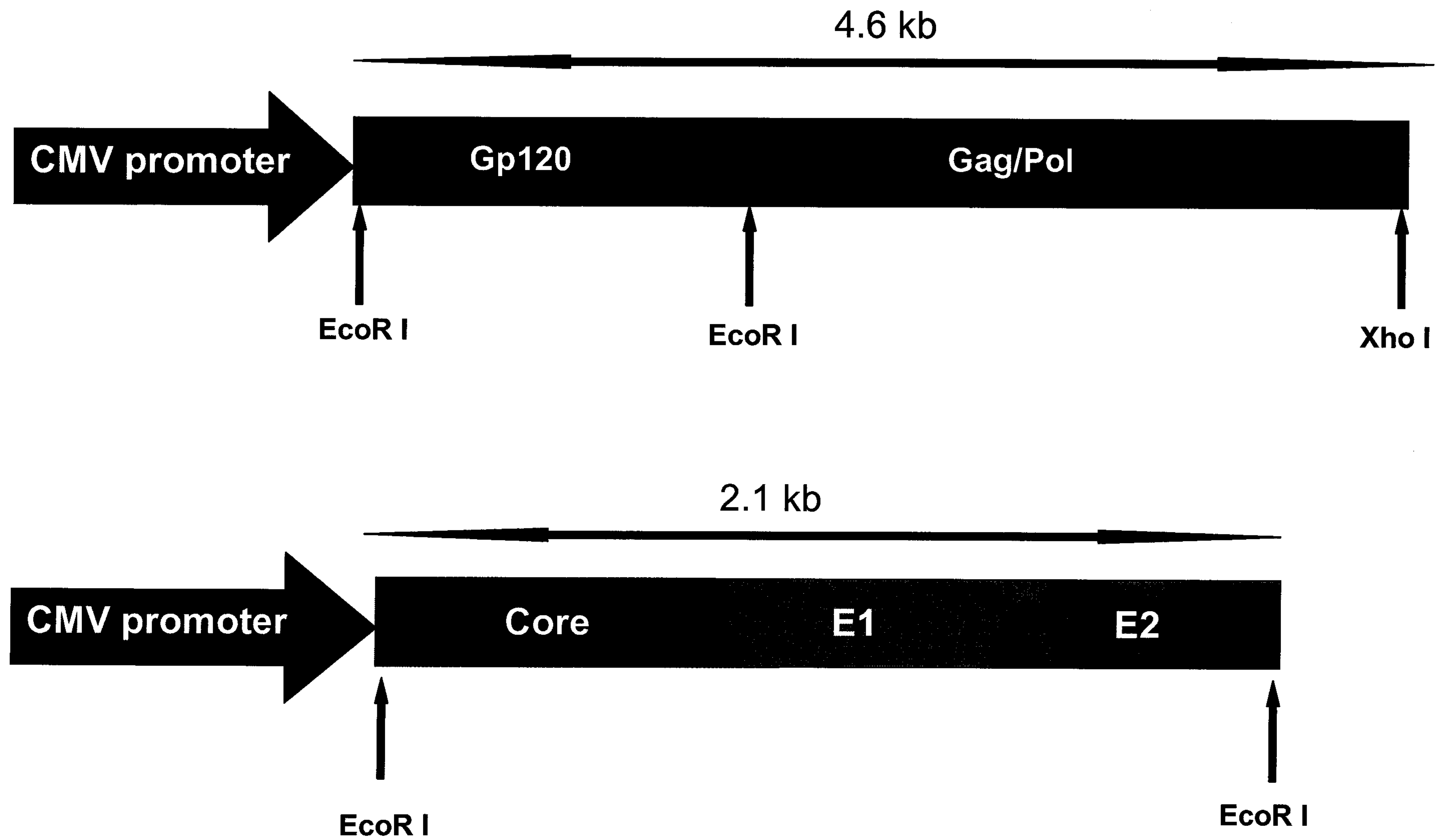


Table 1.4. Immunization strategies

Group A	*Vector alone + Montanide/CpG*
Group B	*HIV-1 immunogens + Montanide/CpG*
Group C	*HCV immunogens + Montanide/CpG*
Group D	*HIV-1/HCV immunogens + Montanide/CpG*
Group E	*HIV-1/HCV proteins + Montanide/CpG*

Each mouse was immunized I.M. with 50 μg polycistronic DNA construct or 10 μg of recombinant proteins. All mice received 30 μg CpG and 50 μl montanide ISA-51. Each mouse was vaccinated four times.

Combined HIV/HCV vaccination elicited similar humoral response to groups immunized with single immunogens

In order to analyze the binding antibody titer against gp120 and core proteins, plasma from immunized mice were collected two weeks after the last immunization and analyzed by end-point ELISA titers. 96-well ELISA plates were coated overnight at 4°C with gp120 protein (MN, SF162) or core protein obtained from the NIH. The plates were washed and incubated for 2h at 37°C with serially-diluted sera. The plates were washed, incubated with peroxidase-conjugated affinity-purified goat anti-mouse secondary antibody and developed with OPD. The color reaction was stopped with 1N HCl and absorbance was read at 490 nm with an ELISA plate reader (Bio-Rad).

The group D that received both HIV/HCV immunogens showed a slightly higher mean IgG antibody titer to core protein in comparison to the group C that received only HCV immunogens. The group A (control), group B (received only HIV-1 immunogens) and E (received only HIV/HCV proteins) produced a relatively low antibody titers to core protein (Fig 2.4 A). In addition, the antibody titer to gp120 clades A, B and E was measured (only clade B is shown) (Fig 2.4 B). However, there was no significant antibody titer difference between the immunized groups receiving HIV immunogens alone or in combination with HCV immunogens. These results suggest that addition of HCV or HIV immunogens does not significantly boost the antibody responses.

Fig 2.4. Antibody titers were determined in mice (n=4) two weeks after the last immunization. The 96-well plates were coated with (A) HCV-core and (B) HIV-1-gp120 SF162 protein. Mouse sera were serially diluted in wells and absorbance was read at 490 nm with an ELISA plate reader. Results are shown as mean titers of four mice.

Fig 2.4:A

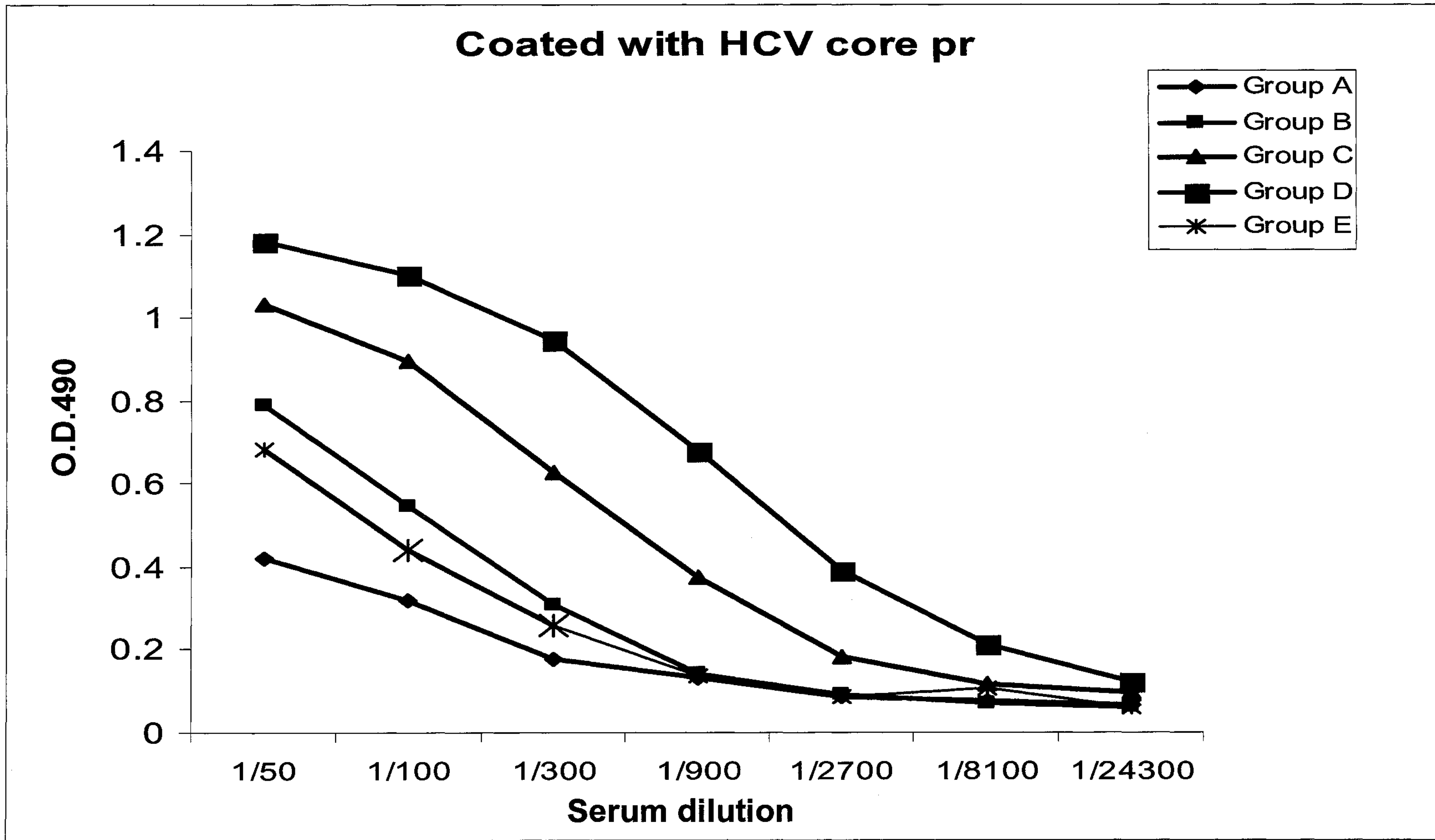
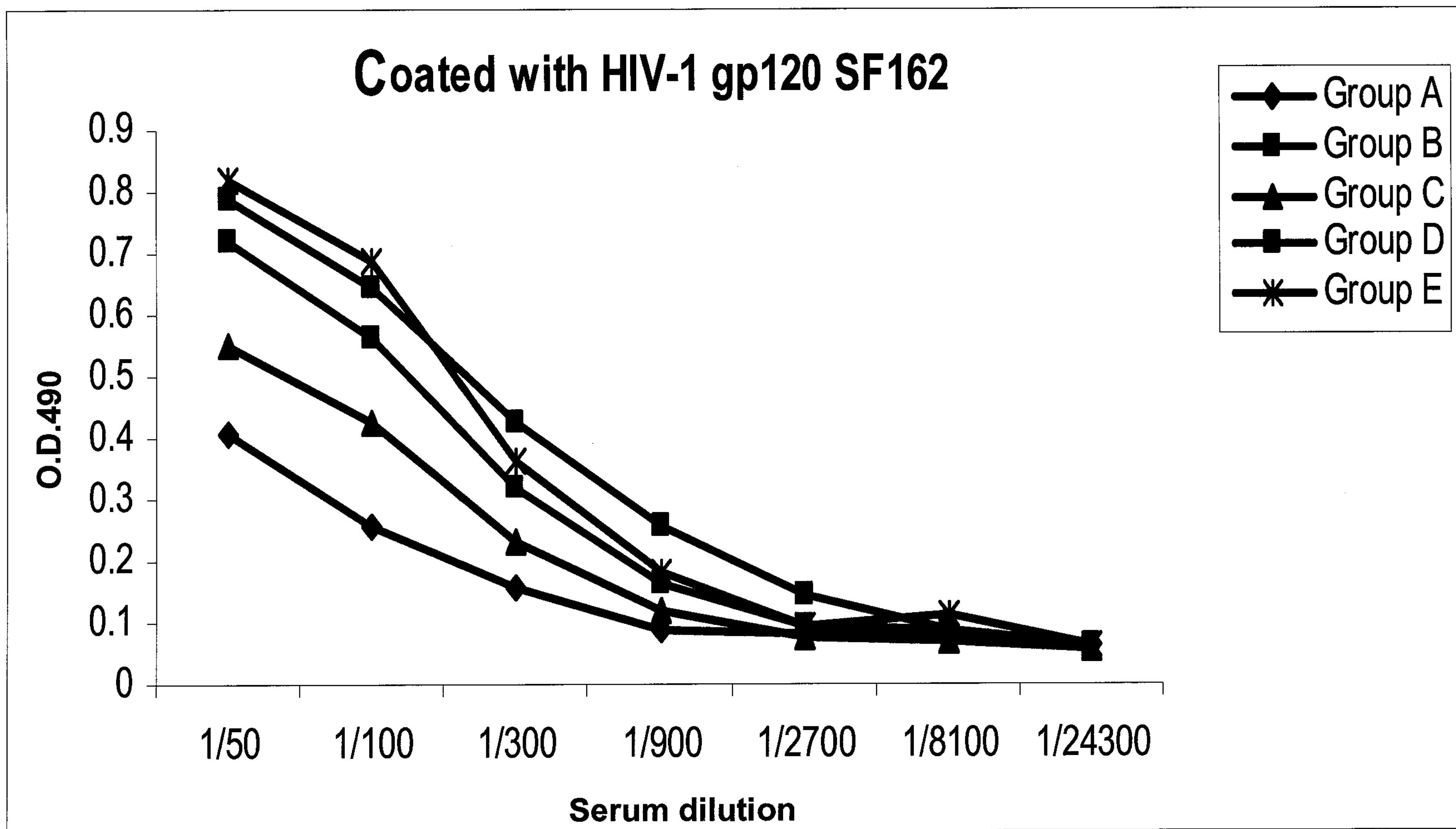


Fig 2.4:B

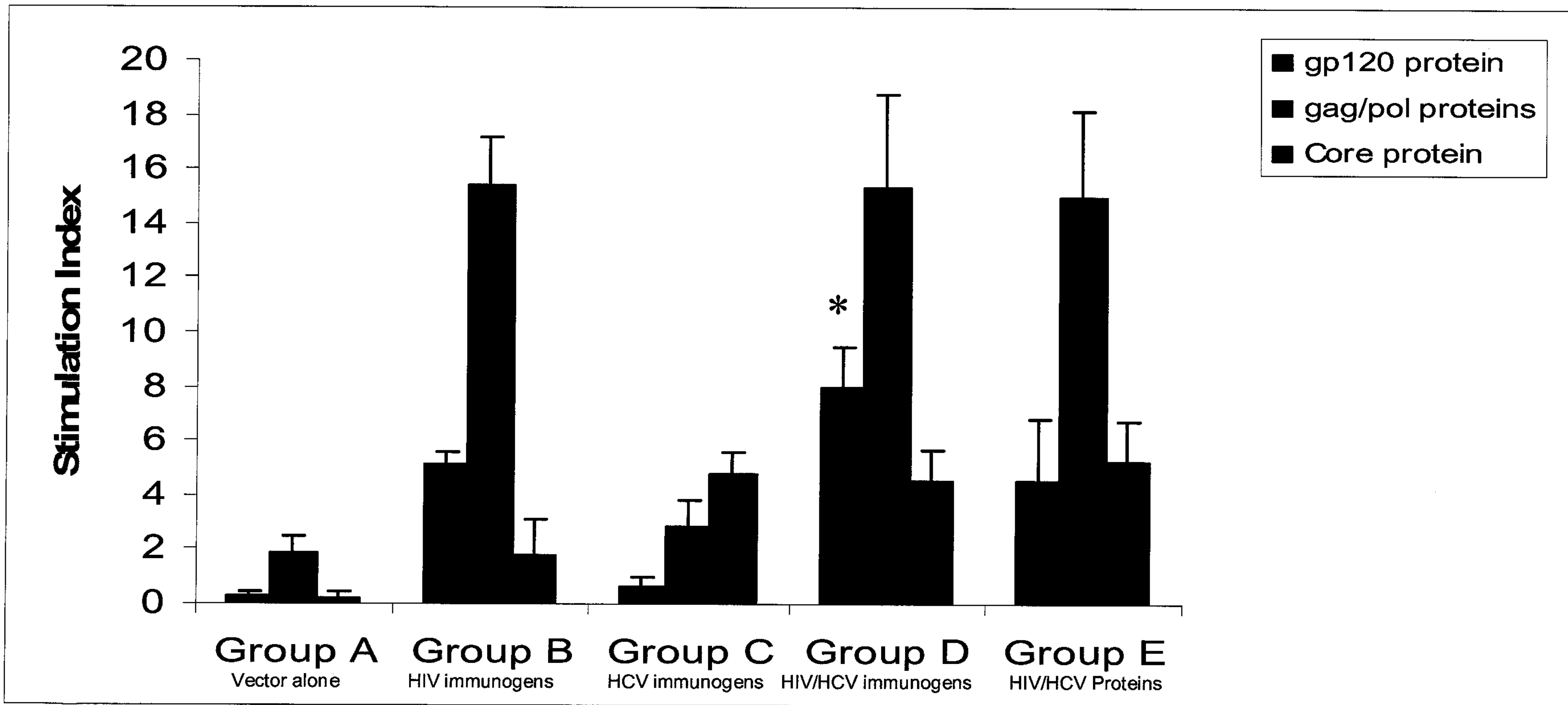


Measurement and comparison of proliferative responses in immunized groups

We next determined whether the combination of HIV and HCV immunogens would affect the proliferative immune response. Fourteen days after the final immunization, animals were sacrificed and splenocytes were stimulated with gp120, gag/pol and core proteins. Cells were harvested and thymidine incorporation was measured by a beta counter. The group B (HIV immunogens), D (HIV/HCV immunogens) and E (HIV/HCV proteins) induced strong proliferative response to gag/pol protein in contrast to group A (control) and group C (HCV immunogens). However, based on p value, there was no significant difference in proliferative response to gag/pol protein (Fig 3.4, red bars) between the above groups. For further analysis, splenocytes from immunized mice were also stimulated with gp120 protein (Fig 3.4, blue bars). The immunized groups showed a lower level of proliferation to gp120 in comparison with gag/pol. However, the combined vaccine (group D) showed a higher proliferative response to gp120 protein compared to groups B and E ($p<0.05$). The proliferative response to HCV-core protein (Fig3.4, green bars) was next assessed. Some minor variations in proliferative immune responses were observed between groups C, D and E. However, single HCV or combined immunogens showed comparable immune response to the core protein. These results suggest that co-delivery of HIV/HCV immunogens may lead to a synergistic proliferative response to gp120 but not to gag/pol or core.

Fig 3.4. T-cell proliferation results in HLA-A2.1 mice immunized with the candidate vaccines. Splenocytes were cultured and stimulated with 10 μg/ml HIV-1 gp120, HIV-1 gag/pol and HCV-core proteins. Three days later, ^{3}H-thymidine was added and after 18h the incorporated radioactivity was measured in harvested splenocytes. The stimulation index (SI) ± S.E. is shown for each group. Symbol * indicates a significant difference between HIV-1-gp120 in group D (combined HIV/HCV vaccines) with HIV-1-gp120 protein in groups B (HIV-1 vaccine) and E (HIV/HCV proteins) ($p \leq 0.05$).

Fig 3.4



Evaluation of Th1/Th2 immune responses in immunized groups

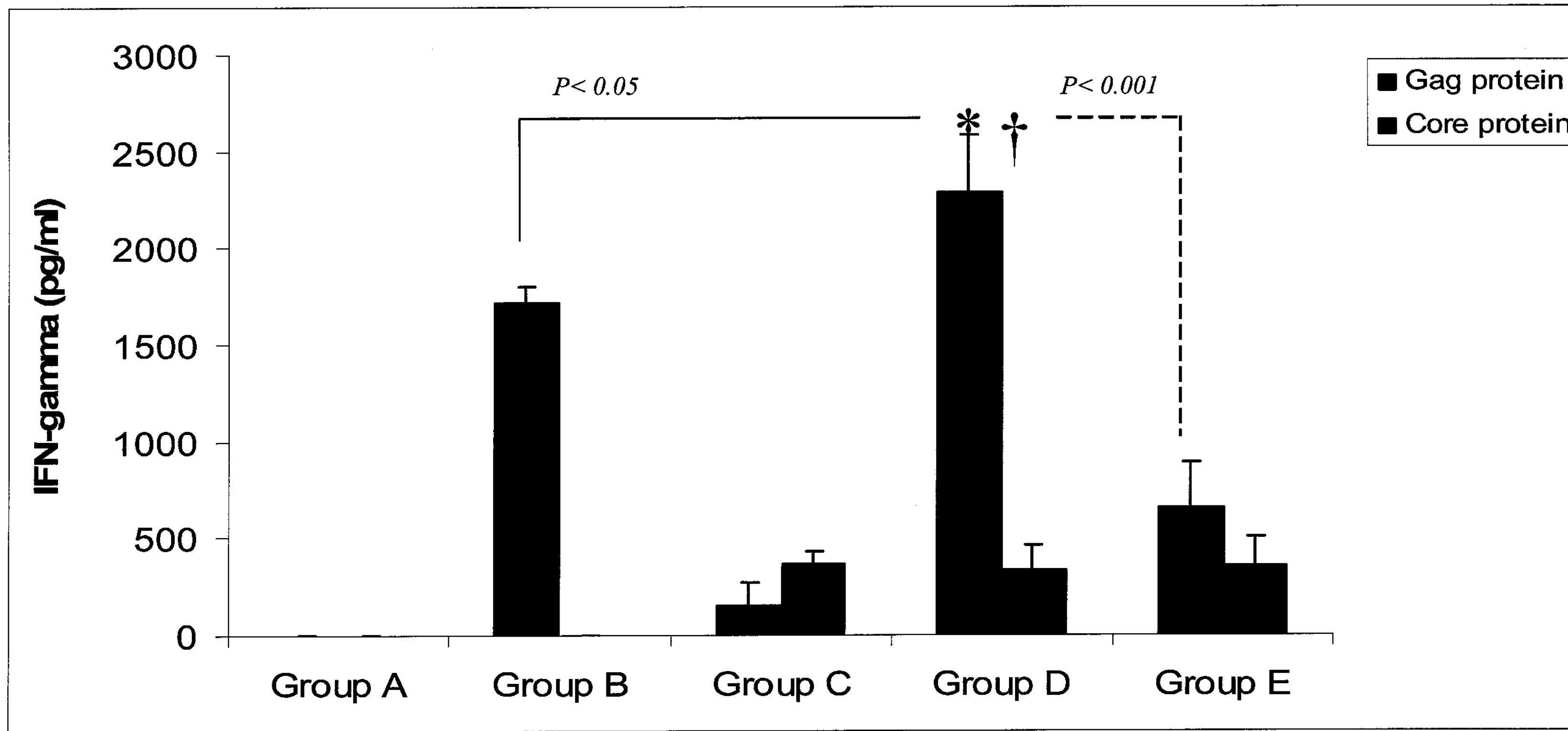
Supernatants from the stimulated cultures were also collected and assayed for IL-5 and IFN-γ production (Fig 4.3). Control animals that received vector alone and adjuvants did not show any gag or core Th1/Th2 immune responses at any time point. As shown in Fig 4, immunized groups received HIV immunogens (B, D, and E) resulted predominantly in a gag-specific (red bars) Th1 response. However, immunizing with HCV immunogens did not result in a high core-specific Th1 responses in groups C, D and E. Depending on the immunizations, various Th1 bias were detected. The gag-specific Th1 response was stronger in animals immunized with combined immunogens compared to the group B ($p<0.05$) and group E ($p<0.001$). Interestingly, no detectable IL-5 Th2 immune response was detected (data not shown) in immunized groups. Only one of the animals in group C developed a very weak IL-5 specific response to core protein (data not shown).

Combined HIV/HCV immunogens induce higher levels of cell-mediated immune responses than HIV or HCV immunogens alone

Cell-mediated immune responses were evaluated by intracellular cytokine staining and ELISPOT. Splenocytes from immunized mice were prepared and stimulated with pooled HIV/HCV peptides, gag or core peptides. The HIV-1 and HCV peptide epitopes specific for MHC class I HLA-A2 were searched in the Los Alamos Molecular

Fig 4.4. IFN-γ cytokine production in HLA-A2.1 mice immunized with the candidate vaccines. Two weeks after the last immunization, splenocytes were stimulated in vitro with HIV-1 gag and HCV-core recombinant proteins. The level of IFN-γ production was measured by quantitative ELISA in supernatants removed 72h after stimulation. Results are shown as the mean concentrations of triplicate wells ±S.E. of four mice. Statistically significant difference between indicated groups is shown.

Fig 4.4



Immunology Database (http://www.hiv.lanl.gov/content/immunology) and contained the following sequences: Env(120-128):KLTPLCVTL; Gag(77-85):SLYNTVATL; Gag(386-394):VLAEAMSQV; Pol(309-317):ILKEPVHGV and Pol(33-41):ALVEICTEM. The HCV peptide epitopes contained the following sequences: Core(35-44):YLLPRRGPRL; Core(132-140):DLMGYIPLV; E1(220-229):ILHTPGCV; E2(363-372):SMVGNWAKV and E2(401-411):SLLAPGAKQNV. These peptides were used to detect CD8+ T-cells with specific activity against HIV-1 and HCV.

After stimulation, cells were surface stained for CD3, CD8, as well as stained intracellularly with IFN-γ and analyzed by flow cytometry. The flow cytometry results (Fig 5.4) showed that group D (combined vaccine) induced a higher proportion of IFN-γ secreting CD8+T lymphocytes to pooled HIV-1 and gag peptides ($p<0.05$) compared to the group B which received HIV-1 immunogens only. The combined vaccine also elicited higher level of CD8+T cell response to pooled HCV and core peptides in comparison to group C which was immunized with HCV immunogens alone ($p<0.05$).

Cellular immune responses induced in immunized mice were further assessed by ELISPOT assay. The plates were coated with anti-mouse IFN-γ antibody and splenocytes stimulated with both HIV/HCV proteins and peptides were added. Two days after the incubation, plates were developed and the number of spots was measured. As shown in Fig 6.4 A the combined immunogens induced a higher number of spots ($p<0.003$ and 0.05 to core and gag proteins respectively) than group E that received HIV/HCV proteins.

Fig 5.4. HIV-1 and HCV-specific CD8+T cell response detected by intracellular cytokine staining (ICS) in the immunized groups. Splenocytes from immunized mice was stimulated with the cocktail of HIV-1, HCV peptides or/and HIV-1-gag (77-85) (386-394) and HCV-core (35-44) (132-140) peptides specific for HLA-A2.1. The cells were surface and intracellularly stained and analyzed by flow cytometry. The results were re-analyzed with WinMDI program. A: Dot plots show results from individual representative animals from each mouse group. Number 1: represents cocktail of HIV-1 peptides. Number 2: represents HIV-1 gag peptides. Number 3: represents cocktail of HCV peptides. Number 4: represents HCV-core peptides. B: Results are shown as the percentages of IFN-γ+CD8 T cells. Data represent the mean ±S.E. of four mice. Statistically significant difference between indicated groups is shown. Symbol * indicates $p \leq 0.05$.

Fig 5.4: A

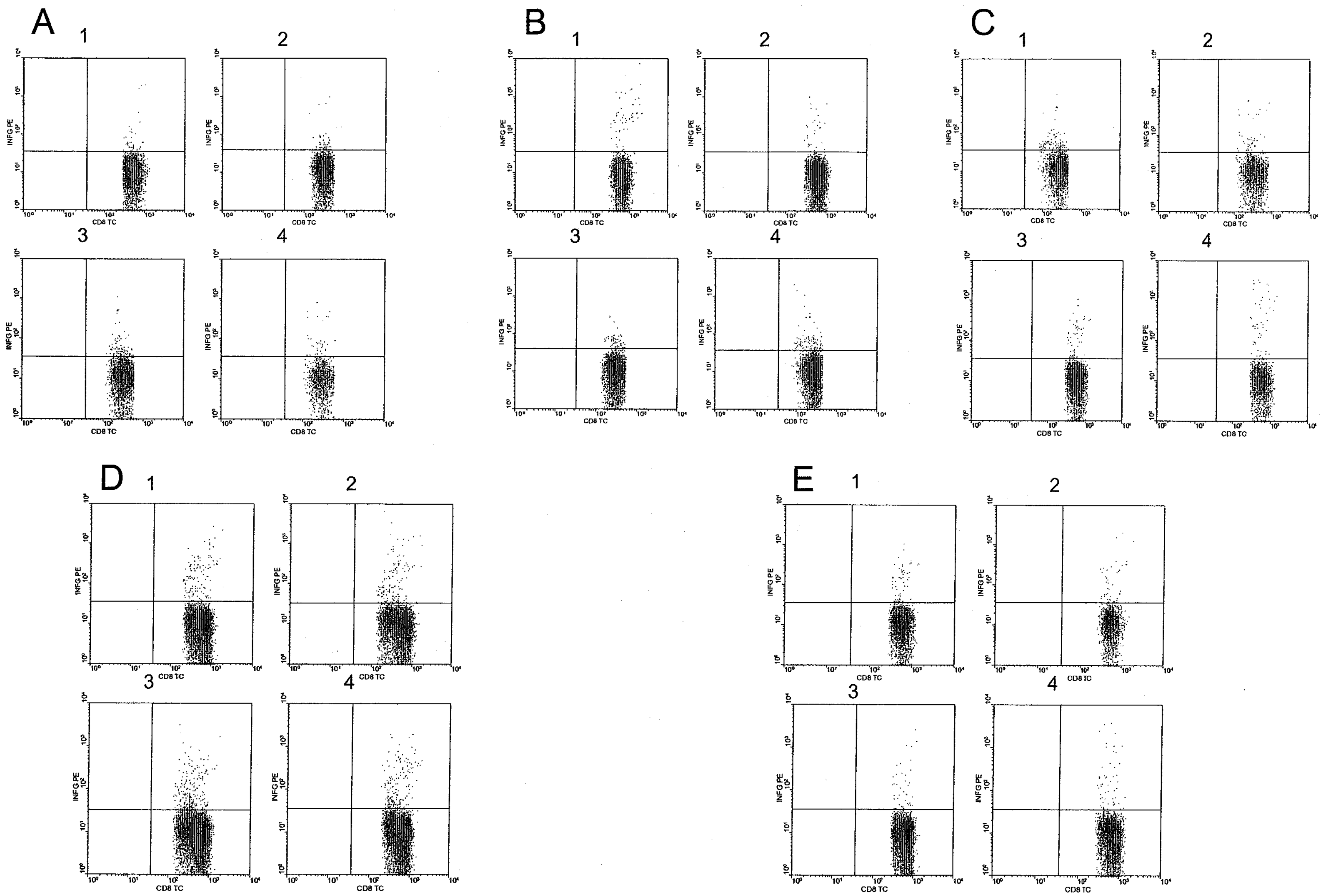
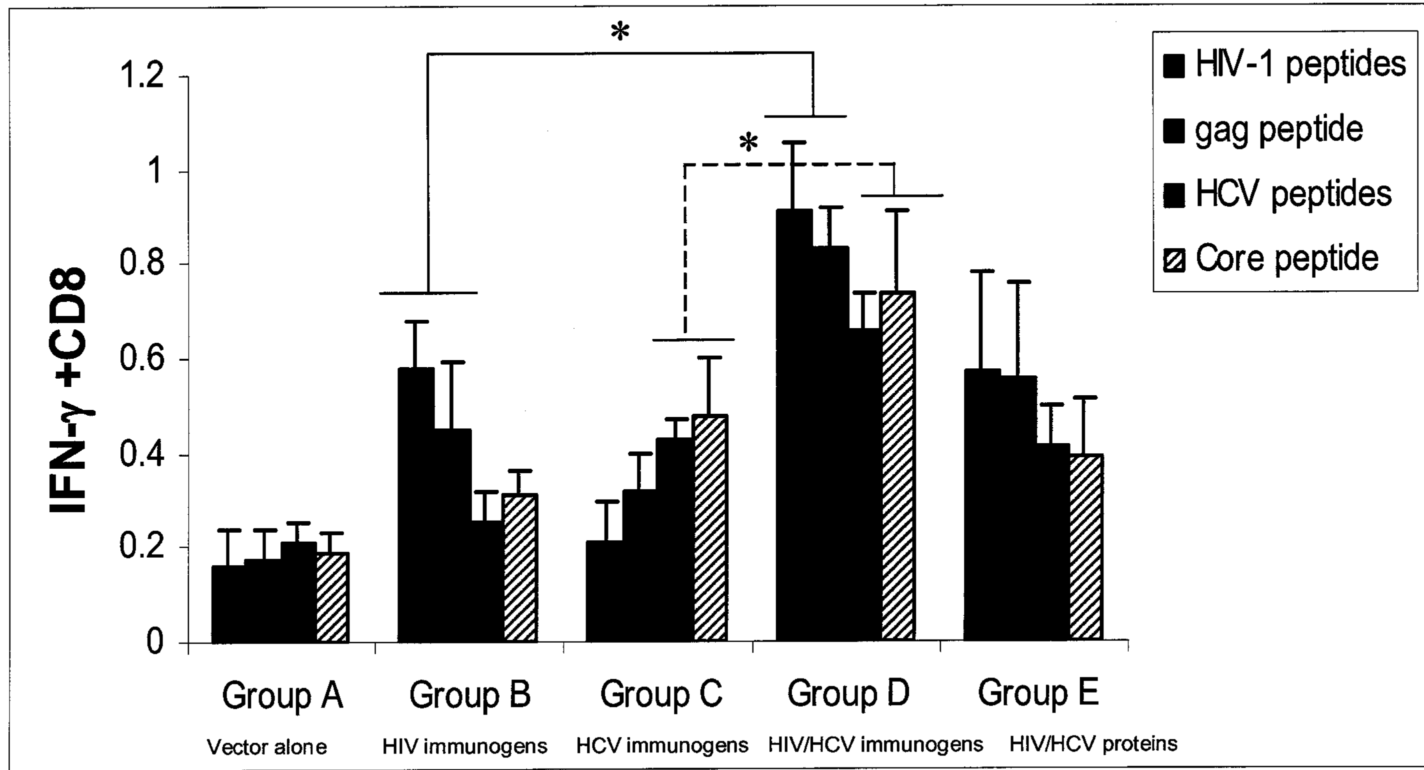


Fig 5.4

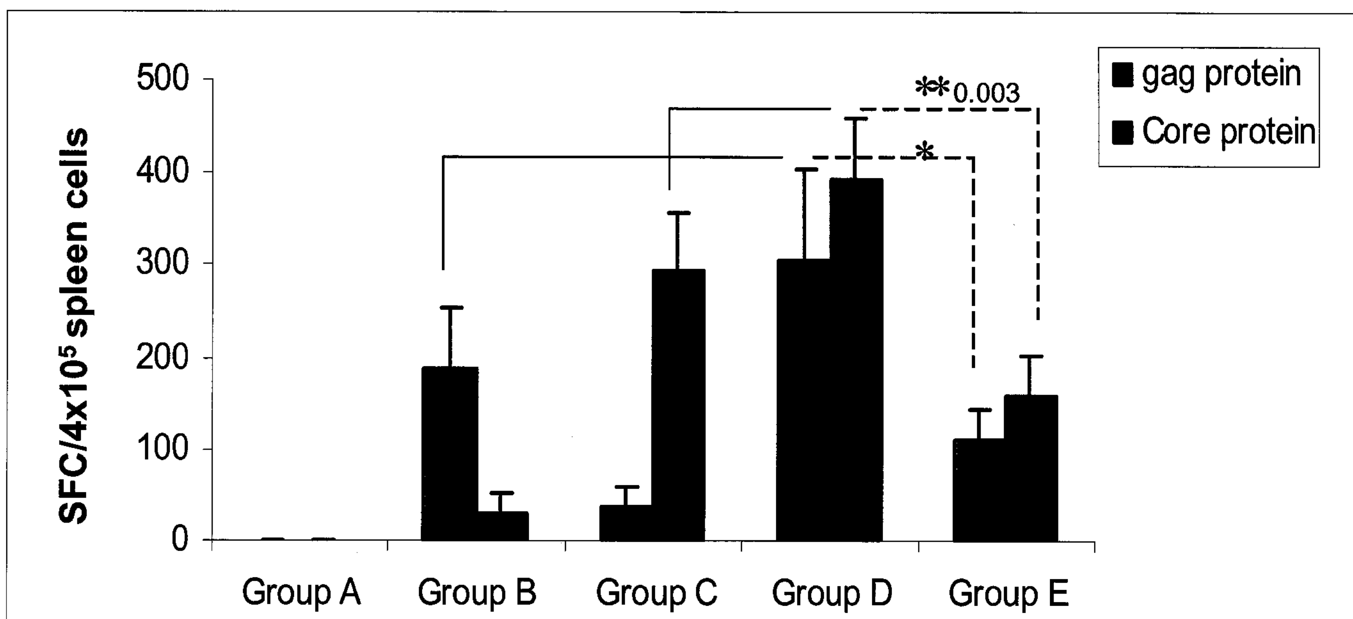


However, based on the p value, there was no significant increase between the combined immunogens and HIV or HCV immunogens alone. The number of spot forming cells (SFC) found in this assay correlated with the total levels of CD4/CD8+T cells. In order to analyze CD8+T cells, splenocytes were also stimulated with the HIV-1 and HCV peptide epitopes specific for MHC class I HLA-A2.1 (Fig 6.4 B). The group D (combined group) had more than 1.9% increase ($p<0.05$) in spot forming cells when compared to the group B (HIV-1 group). The HIV/HCV group showed higher numbers of spots to core peptide in comparison with the group C which received HCV immunogens alone ($p<0.05$). The level of IFN-γ was also higher in group D as compared to immunized mice in group E ($p<0.01$ and 0.05 to HIV and HCV peptides respectively). These responses suggest that a higher cytotoxic anti-HIV/HCV activity develops after immunization with the combined immunogens.

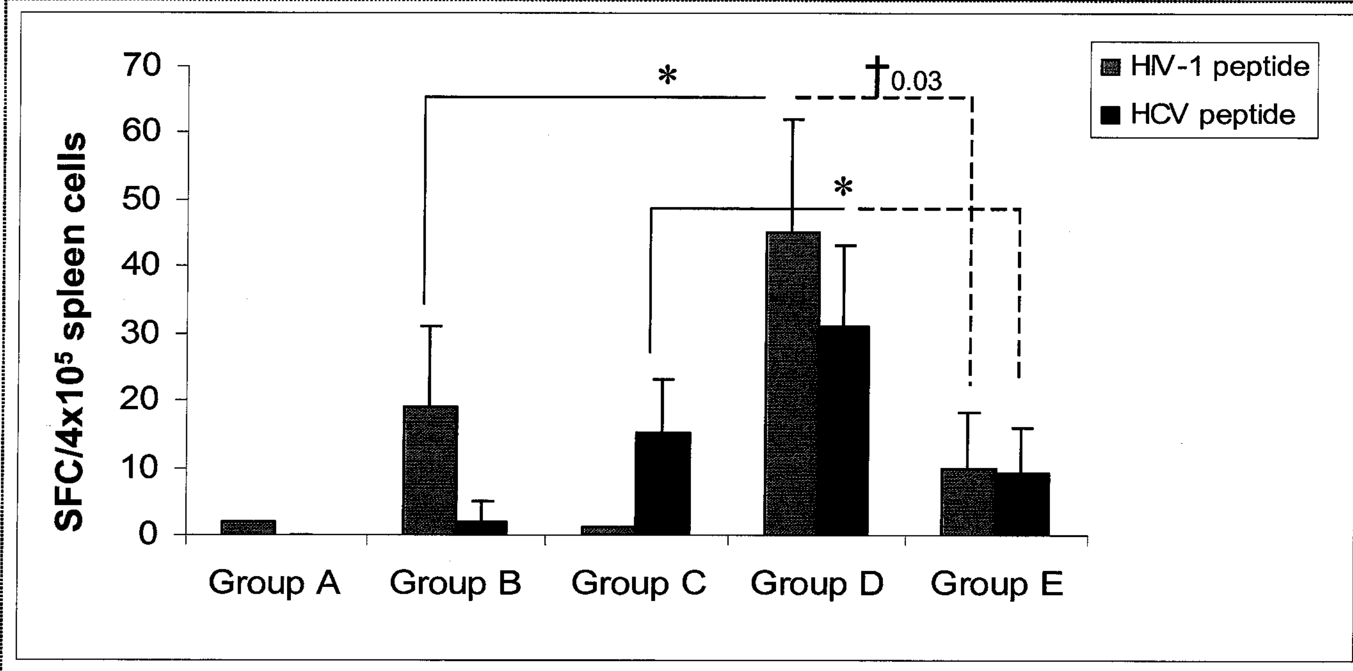
Fig 6.4. HIV/HCV-specific cell-mediated immune responses detected by IFNγ–ELISPOT assay. Splenocytes from immunized mice were cultured in the presence of A: gag/core proteins and B: HIV/HCV peptides specific for HLA-A2.1. The cells were added to the plates were coated with anti-mouse IFN-γ antibody and spot forming cells (SFC) were enumerated. The data are the mean ± S.D values of duplicate samples. Statistically significant difference between indicated groups is shown. Symbol * indicates $P \leq 0.05$.

Fig 6.4

A



B



4.4 Discussion

The optimal combination of immunogens to develop an effective candidate vaccine against HIV/HCV is still unknown. The aim of this study was to compare and evaluate the efficacy of immunogens derived from HIV/HCV alone or in combination in eliciting immune responses against either HIV or HCV. There are some reports evaluating the potential of combined antigens against different diseases (Ovsyannikova, Jacobson et al., 2005;Gessner, Sutanto et al., 2005;Beeching, Clarke et al., 2004;Lasaro, Luiz et al., 2004;Chippaux, Garba et al., 2004). However, to date, there are no reports which compare and evaluate the potential of combined vaccines to deliver HIV and HCV antigens. In this report, antibody titers, Thl/Th2 cytokine secretion, proliferation and CD8+ T-cell responses were compared between different immunized groups. The ability of the combined vaccine to elicit potent HIV/HCV-specific CD8/CD4+T-cell immune responses may be a useful strategy in the development of an effective vaccine. The structural proteins of HIV-1 clade B were cloned, expressed and used as part of a candidate HIV vaccine that included the DNA construct and encoded recombinant proteins. Gag/pol and core were chosen as part of our HIV and HCV vaccines respectively because they are the most conserved viral proteins and are a good source of CTL epitopes in HIV-1 and HCV (Frahm, Korber et al., 2004b;zur, Otten et al., 2003a;Yusim, Kesmir et al., 2002a). Gp120 and E1/E2 were also included in light of several studies which showed that these antigens are strongly immunogenic when used as a vaccine in cohorts of infected individuals (Gao, Weaver et al., 2005b;Paoletti & Kennedy, 2002b;Alvarado & Leroux-Roels, 1999). The use of these antigens may generate protective immunity as they can be processed by endogenous or exogenous

pathways and are presented by antigen presenting cells (APCs), especially dendritic cells, and therefore promote immune responses. In this report, the immune response was enhanced with the adjuvants montanide ISA-51 and CpG. Montanide induces a high level of antibody titers in several animal species and it has been used in human vaccine trials with malaria and HIV vaccines (Miles, McClellan et al., 2005). CpG stimulates dendritic cells through TLR9, inducing cell maturation and enhancing antigen presentation and Th1 responses. Treatment of healthy people in phase I clinical trial with CpG showed the safety of this compound (Becker, 2005). The use of montanide and CpG in combination was recently demonstrated to induce a greater response to vaccines than either adjuvant alone. They showed that mixture of CpG ODN and Montanide ISA induces higher levels of IgG2a and IgG2b antibodies, lead to the induction of Th1 bias which is responsible for the higher degree of protective immunity (Kumar, Jones et al., 2004b). In another study, the immunogenicity of montanide ISA-51, and CpG ODN was assessed in *Plasmodium yoelii*. Interestingly, they did not detect any parasites in the blood of mice immunized with combination of synthetic CpG and montanide and all immunized mice were completely protected (Hirunpetcharat, Wipasa et al., 2003). CD8+ and CD4+T-cells are important factors in the control of viral infections. Previous studies showed that CD4+Th1 plays a critical role (Barouch, Santra et al., 2002b;Ostrowski, Justement et al., 2000;Chamoto, Kosaka et al., 2003d;Chamoto, Kosaka et al., 2003c;Chamoto, Kosaka et al., 2003b;Chamoto, Kosaka et al., 2003a) in the function of CD8+T cells. Here, we demonstrated that immunization with combined HIV/HCV immunogens results in strong gag-Th1 immune responses that facilitates CTL responses and may be protective against progression to AIDS. Despite the strong induced Th1 anti-gag response, a low level of

Th2 immune responses were detected in this study. The presence of CD8+T cells reduces viral load in individuals infected with HIV-1 (Benito, Lopez et al., 2004c;Ganusov, 2003a). Specific activation of CD8+ cells results in secretion of inflammatory cytokines (IFN-γ and TNF-α) (Noble, Leggat et al., 2003a) and other components like perforin and granzymes (Renner, Held et al., 1997a;Pham & Ley, 1997a) which kill infected cells before replication and establishment of infection *in vivo*. The combined HIV/HCV vaccine had the capacity to efficiently promote CD8+T cell responses in comparison with HIV or HCV immunogens alone. The mechanism of combined vaccines in the induction of a high level of CD8+T-cell response is not fully understood. However, it is possible that this strategy reflects the type or capability of antigen presenting cells (APCs) in processing antigens. In addition, the combined vaccine might enhance migration of antigen presenting cells to the site of injection, thereby contributing to vaccine efficacy. This phenomenon could also be explained in part by the influence of CpG motifs in the structure of DNA vectors that potentially activate cellular immune responses (Grifantini, Finco et al., 1998). In summary, it has been demonstrated that the quality of antibody production is similar in single and combined vaccines. The important observation from this work is that combined HIV/HCV antigens act synergistically on cellular immune responses and cooperate in enhancing CD8+T cell responses against specific HIV and HCV peptides in HLA-A2.1 mice. These results may have implications for further testing in non-human primates.

Chapter 5:

Induction of Vigorous SARS-CD8+ T-Cell Immune Responses by a Combined Nucleocapsid Vaccine

5.1 Summary

Several studies have shown that cell-mediated immune responses play a crucial role in controlling viral replication (Okada, Takemoto et al., 2005;Glass, Subbarao et al., 2004;Sylvester-Hvid, Nielsen et al., 2004). As such, a candidate SARS vaccine should elicit broad CD8+ T-cell immune responses. Several groups of mice were immunized alone or in combination with SARS-nucleocapsid (NC) immunogen. A high level of specific SARS-CD8+ T-cell response was demonstrated in mice that received DNA encoding the SARS-NC, NC protein and XIAP as an adjuvant. We also observed that co-administration of a plasmid expressing nucleocapsid, recombinant protein and montanide/CpG induces high antibody titers in immunized mice. Moreover, this vaccine approach merits further investigation as a potential candidate vaccine against SARS-CoV.

5.2 Introduction

The SARS epidemic had a high mortality rate as well as a huge economic impact worldwide. Treatment with antiviral drugs or an effective vaccine is not available for protection against this disease (Buchholz, Bukreyev et al., 2004;Spiga, Bernini et al., 2003b). The SARS-CoV is a single-stranded RNA virus that has been identified as a new type of coronavirus. The genome is approximately 30 kb long and contains four structural proteins: spike, envelope, matrix and nucleocapsid in the same order as other coronaviruses (Lu, Zhao et al., 2004;Egloff, Ferron et al., 2004). However, the sequence analysis of SARS-CoV with other members of the coronavirus family did not show more than 20% nucleotide homology (He, Leeson et al., 2004).

The SARS-NC gene encodes a 46 kDa protein that participates in the replication and transcription of the virus and interferes with the cell cycle of host cells (Surjit, Liu et al., 2004). Previous studies in other coronavirus members suggest that this protein is highly immunogenic and could be a good target for the design of an effective vaccine (Wege, Schliephake et al., 1993b;Wege, Schliephake et al., 1993a;Wege, Schliephake et al., 1993c;Boots, Benaissa-Trouw et al., 1992a). The expression of NC in CHO cells led to the observation that this protein folds spontaneously into VLPs. These particles are effectively incorporated at several stages of the virus life cycle, including assembly, budding from cells, and receptor-binding leading to membrane fusion. The viral particles also present antigens to the immune system in a structure that mimics the infectious virion (Young, Smith et al., 2004;Doan, Li et al., 2004;Takamura, Niikura et al., 2004).

DNA vaccines are able to induce both humoral and cellular immune responses

and have demonstrated their efficacy in several experimental models (Pachuk, McCallus et al., 2000;Davis & McCluskie, 1999). There are several eukaryotic vectors that express recombinant proteins efficiently. However, the uptake and presentation of antigens are critical elements in DNA vaccination strategies. One strategy to increase the potency of DNA vaccines is to prolong the survival of antigen presenting cells (APCs), especially dendritic cells. Previous studies show that survival of dendritic cells is increased in the presence of anti-apoptotic factors such as XIAP (Kim, Hung et al., 2003b;Kim, Hung et al., 2004a). Several antiapoptotic factors such as BCL-XL, BCL-2 and XIAP and etc were tested by Kim et al. They chose human *Papillomavirus* type-16 [HPV-16] E7 as a model antigen because this virus is the agent for a majority of cervical cancers, and E7 is necessary for the induction and maintenance of cellular transformation. Mice were immunized with coadministration of DNA-E7 plus DNA encoding antiapoptotic agents and a high number of antigen-expressing DCs in the draining lymph nodes were observed. In addition, vaccinated mice with DNA-E7 and DNA-BCL-XL demonstrated a lower percentage of apoptotsis in lymph nodes. Their results suggest that the enhancement in the number of DCs in the lymph nodes may contribute to the enhancement of E7-specific CD8+T cell responses.

Specific CD8+T-cells play an important role in the control of viral infection (Benito, Lopez et al., 2004d;Gulzar & Copeland, 2004c;Zhu & Eckels, 2002a). Activation of specific CD8+ cells results in the secretion of inflammatory cytokines (IFN-γ and TNF-α) (Noble, Leggat et al., 2003b) and the synthesis of effector molecules, such as perforin and granzymes which kills infected cells, thus decreasing virus replication and virus load (Renner, Held et al., 1997b;Pham & Ley, 1997b).

The present study characterizes cellular and humoral immune responses to SARS-CoV in mice receiving a DNA-NC construct alone or in combination with protein and different adjuvants. The combination of DNA-NC, protein and XIAP elicited a significant anti-SARS CD8+ T-cell response independent of CD4+ T-cell immune responses.

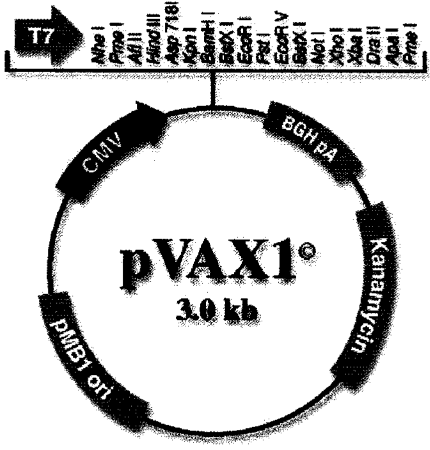
5.3 Results:

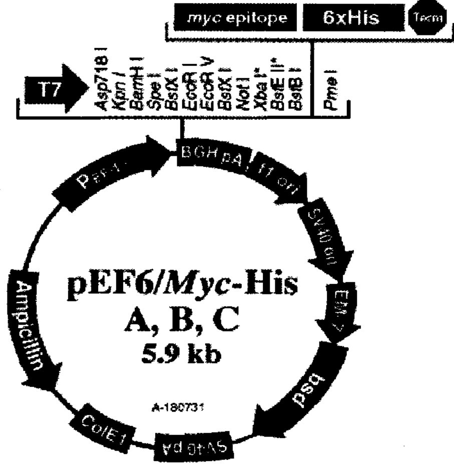
Construction of the DNA vectors and expression of SARS-nucleocapsid protein in mammalian and bacteria cells

Total RNA was purified from the lung tissue of an autopsy sample of a patient who died from SARS. The full-length NC (1.2 kb) gene was amplified using specific primers (forward primer: 5'-ggatccatgtctgataatggaccc-3'; Reverse primer: 5'-gaattcttatgcctgagttgaatc-3'). The amplicon was purified and cloned into the PCR 2.1 TOPO-TA vector according to the manufacturer's instructions. After plasmid digestion, the 1.2 kb band corresponding to the NC gene was sub-cloned into BamHI-and EcoRI sites of pVAX-1 (Fig 1.5) which contains a CMV promoter for high level expression *in vivo*. The fragment was also sub-cloned into the pEF6-myc-His and pQE-His. To express NC protein, CHO cells and E.coli (JM109) were transfected with pEF6 and pQE vectors encoding NC gene, respectively. To increase the yield of recombinant protein, a stable CHO cell line was created using a selective blasticidine resistance gene, allowing for efficient purification of the recombinant protein. Cells were harvested, lysed and the recombinant proteins were purified according to standard methods. The expression of NC protein in transfected cells was determined by western blotting (Fig 2.5) and immunofluorescence staining of CHO cells infected with vector-NC or vector alone. Antibody raised in rabbits to the NC protein expressed in bacteria, strongly reacted to the perinuclear region of the SARS-NC-CHO cell line (Fig 3.5).

Fig 1.5. RT-PCR from SARS-NC gene. SARS-NC gene (1.2 kb) was amplified using specific primers (forward primer: 5'-ggatccatgtctgataatggaccc-3'; Reverse primer: 5'-gaattcttatgcctgagttgaatc-3') and cloned into pVAX-1, pEF6-myc-His and pQE-His DNA vectors.

Fig 1.5





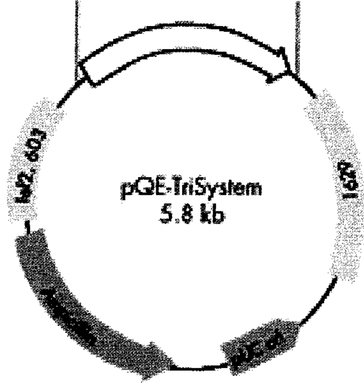


Fig. 2.5. Western blot analysis of recombinant SARS-CoV-NC protein. Lane 1: purified protein from JM 109 cells transfected with pQE vector encoding nucleocapsid gene. Lane 2: represents cells transfected with the vector alone. The blot was probed with sera from a SARS patient.

Fig 2.5

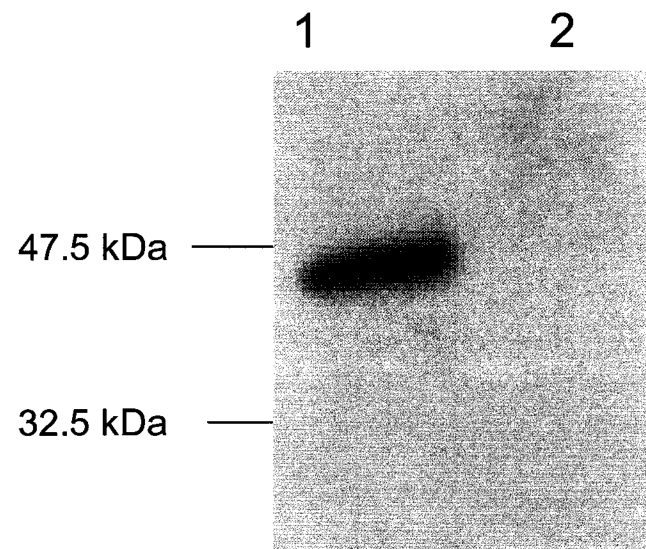


Fig 3.5. Expression of SARS-NC protein. CHO cells transfected with pVAX alone (left) and pVAX expressing SARS-NC protein (right). Rabbit polyclonal antibody to NC was used as primary antibodies. FITC-conjugated antibody was used as a secondary antibody.

Fig 3.5

A

B

The assembly of NC protein into viral like particles (VLPs)

The viral particles present antigens to the immune system in a structure that mimics the infectious virion. Here, CHO cells were transfected with pEF6-myc-His NC or vector alone. Cells were fixed and examined by transmission electron microscopy (TEM). Bundles of VLP of the same morphology as wild type particles inside and outside of the cells infected with pEF6-myc-His NC were observed. However, none of the vector alone or mock transfected cells showed the viral-like particles (Fig 4.5). These observations demonstrate that our construct expressing the NC protein synthesised sufficient protein within infected cells to facilitate the formation of VLPs.

Detection of antibody titer in mice immunized with the candidate vaccine combinations

In order to analyze the antibody titer against SARS-NC, five groups of mice were primed and boosted with SARS-nucleocapsid immunogen alone or in combination (Table 1.5). Two weeks after the last boost, sera were collected and antibody titer was measured by ELISA. The group C that received protein plus montanide/CpG showed higher mean IgG antibody titer compared to the group A and B that received vector alone/XIAP and DNA-NC respectively. The group C also showed a slightly higher antibody titer compared to the group E that received DNA/protein and XIAP. However, the highest SARS-CoV-specific antibody response was detected in mice (group D) immunized with the combination of DNA, protein and montanide/CpG (Fig 5.5).

Table 1.5. Immunization strategies in B6 mice.

Group A	*Vector alone + XIAP*
Group B	*DNA-NC*
Group C	*NC protein + Montanide/CpG*
Group D	*DNA-NC + NC protein + Montanide/CpG*
Group E	*DNA-NC + NC protein + XIAP*

Fig. 4.5. Production of viral-like particles is shown by electron microscopy. The CHO cells were transfected with DNA-NC or vector alone. Arrows indicate VLPs in the transfected cell lines.

Fig 4.5

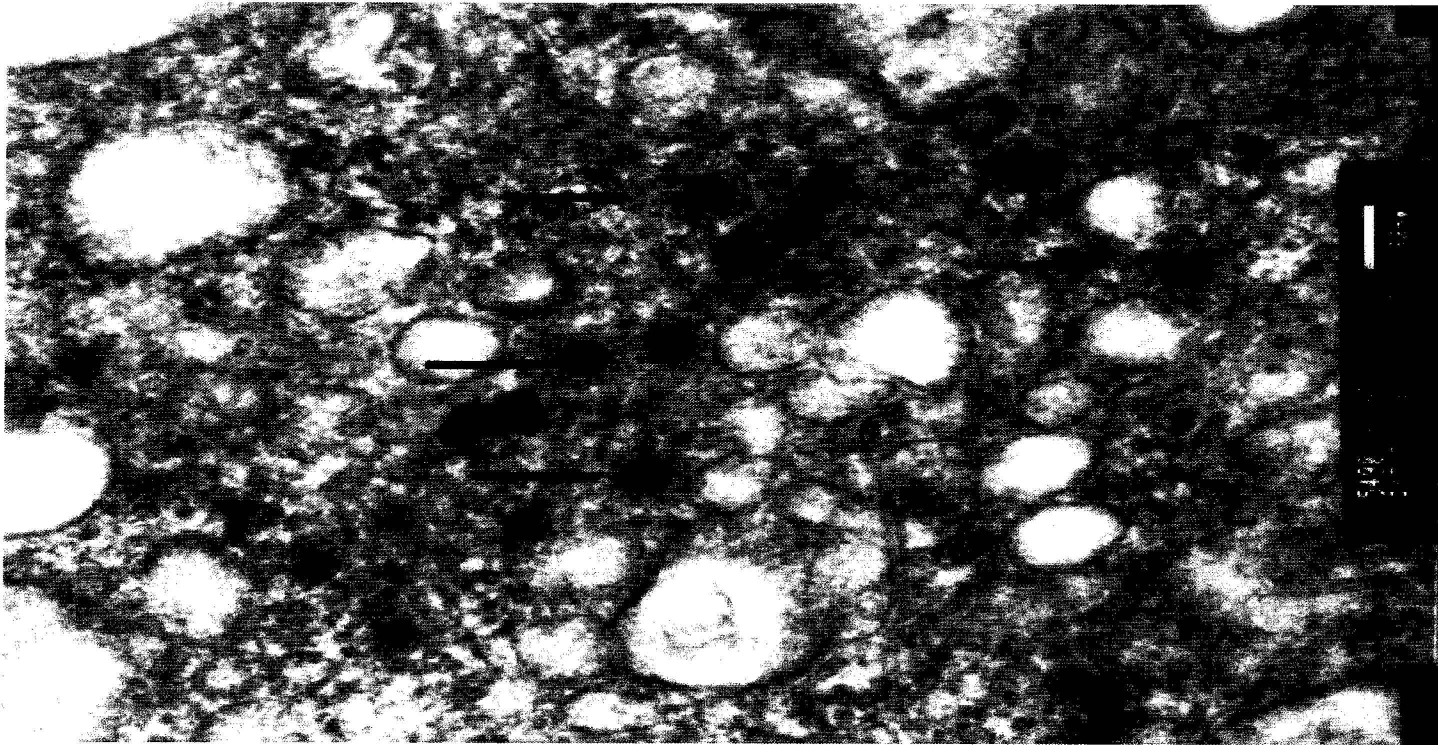
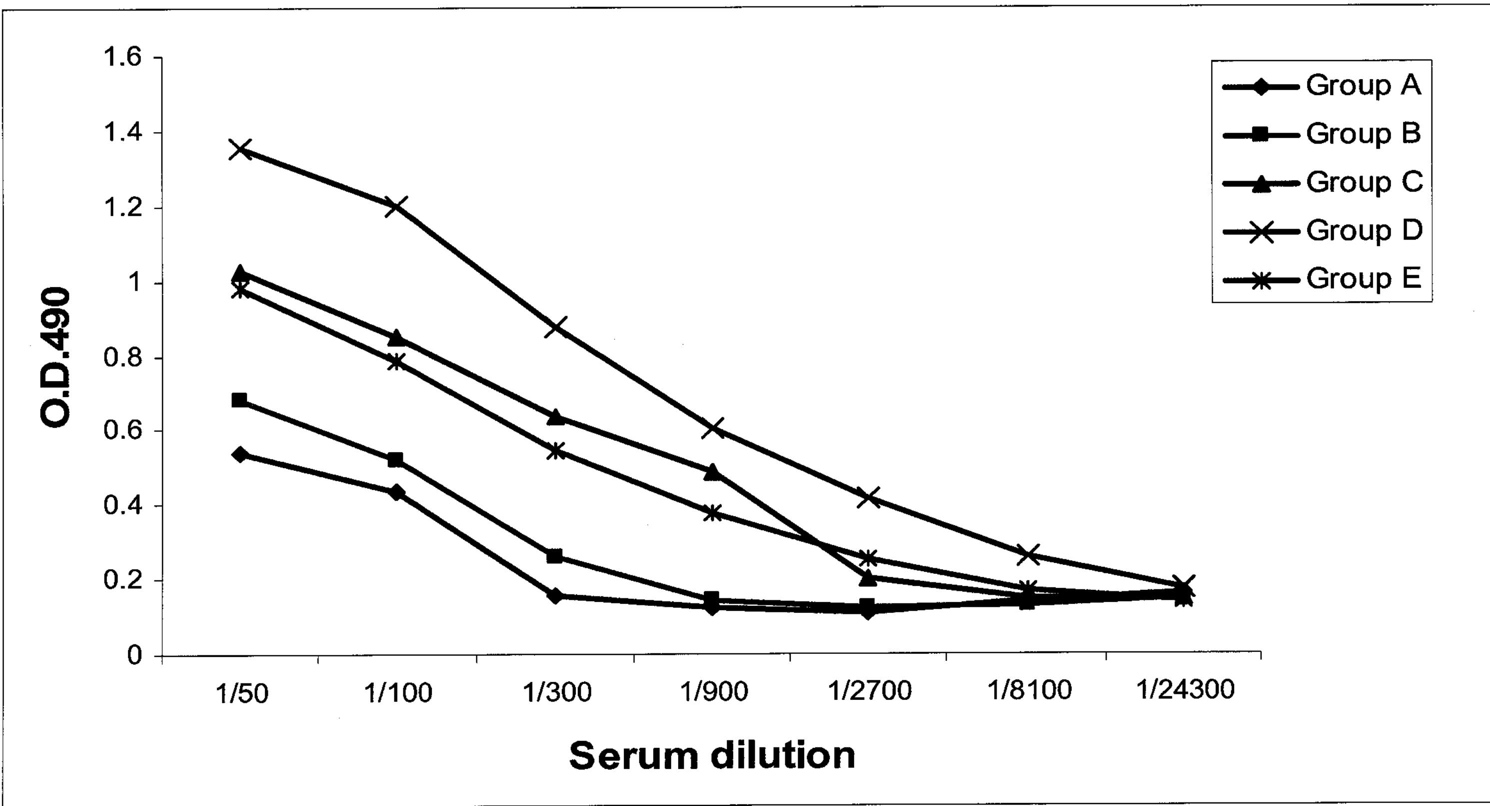


Fig. 5.5. Antibody titer in mice immunized with SARS-CoV-NC immunogens.The levels of anti-NC antibodies were determined by performing ELISA on serum samples collected (Four mice per group) two weeks after the last immunization.

Fig 5.5



Combination of DNA, recombinant protein and XIAP induce higher level of CD8+T cell immune responses

To assess whether the vaccination with nucleocapsid increased the cell-mediated immune response, splenocytes and fresh blood from immunized mice were retrieved, stimulated *in vitro* and stained for surface CD4 and CD8+T cells as well as intracellular interferon gamma. The results for CD4+T cells are shown in Fig 6.5. The level of IFN-γ producing CD4+T cell in immunized mice did not demonstrate a significant CD4+T cell response against the SARS-NC protein. However, the group D receiving DNA, protein and montanide/CpG demonstrated higher level of IFN-γ producing CD4+T cells. Splenocytes were also stimulated with NC protein and CD4 lymphocyte proliferation was performed by tritiated thymidine. However, a high T-cell proliferation was not detected with this assay (data not shown). Cell-mediated immune responses were evaluated by intracellular cytokine staining. The group D elicited higher level of CD8+T-cells to nucleocapsid in comparison to the groups B and C that received DNA or proteins.

However, the highest NC-specific CD8+T cell response was detected in both splenocytes and fresh blood in mice that received the DNA construct, recombinant NC protein and adjuvant XIAP (Fig 7.5). To confirm the results obtained with the intracellular cytokine staining, IFN-γ ELISPOT assay to measure NC-specific T-cell responses of splenocytes from immunized mice was performed. Group A (control), B and C did not show a high number of spots. Potent IFN-γ responses were observed in mice immunized with combination of DNA and protein (group D and E). However, following

substitution of adjuvant montanide/CpG with XIAP, spot forming cells (SFC) were more than two fold higher ($p \leq 0.01$) (Fig 8.5). Although IFN-γ may be produced by both antigen-stimulated CD4+ and CD8+T cells, most likely the observed IFN-γ response was generated by effector CD8+T cells, since flow cytometry demonstrated CD8+T cells as the main producers of IFN-γ.

Fig. 6.5. SARS-CoV-NC specific CD4+T-cell responses in mice immunized with the candidate vaccines. Fresh peripheral blood cells were cultured, stimulated with NC protein and stained for CD4, CD3 and IFN-γ. Flow cytometry was used to analysis the NC-specific CD4+T cells. A negative control (without stimulation) and a positive control (phorbol myristate acetate + ionomycin) were included to control for the spontaneous production of IFN-γ (data not shown). Results are shown as mean ±S.D. The symbol † indicates a significant difference ($P \leq 0.05$) compared with the control group (DNA+XIAP).

Fig 6.5

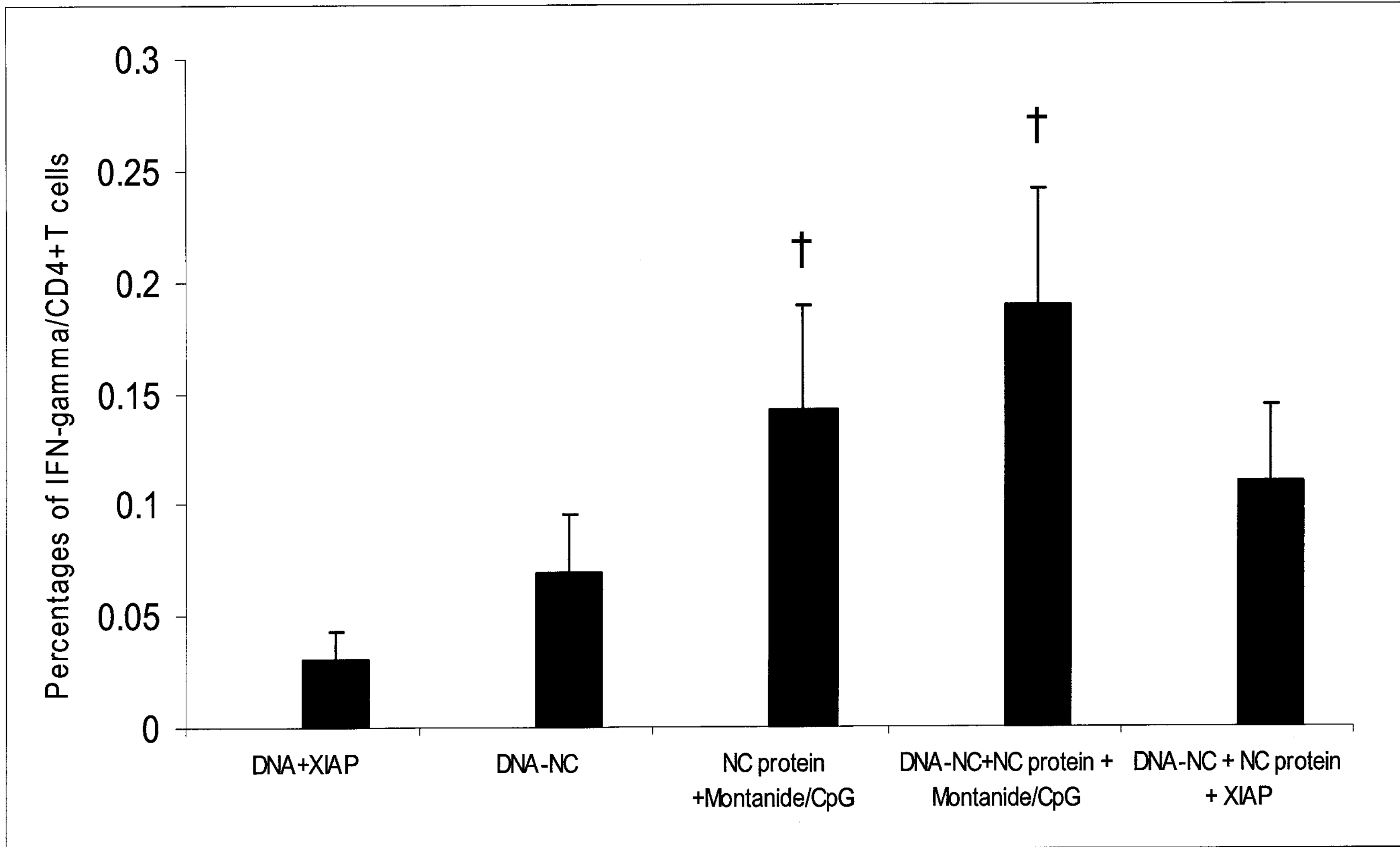
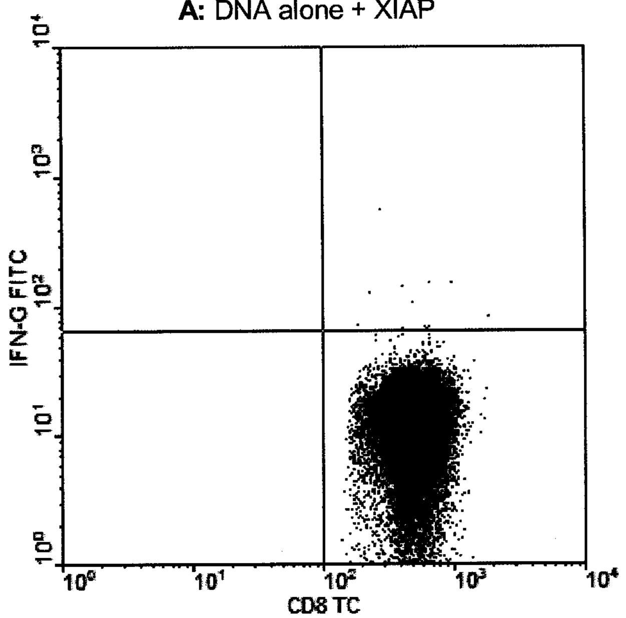


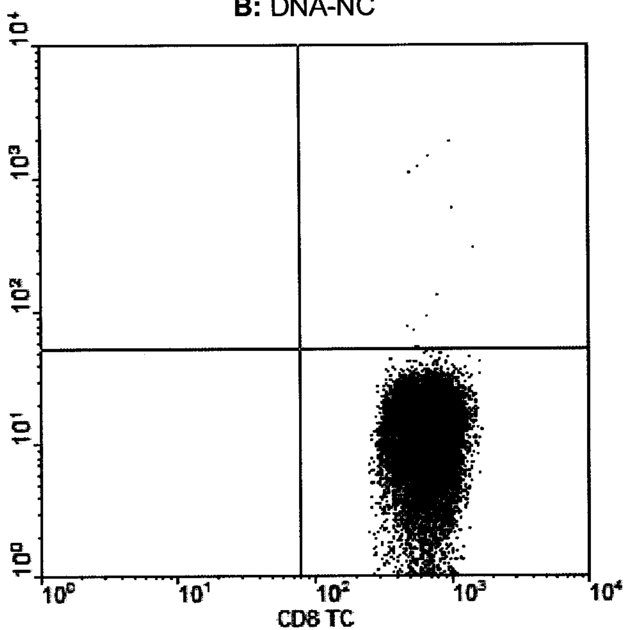
Fig. 7.5. SARS-CoV-NC specific CD8+T cell responses in mice immunized with the candidate SARS vaccines. Fresh peripheral blood cells from immunized mice were stimulated with various antigens and stained for CD8, CD3 and IFN-γ with labeled monoclonal antibodies. After staining, flow cytometry was used to analyze the NC-specific CD8+T cells. A negative control (without stimulation) and a positive control (phorbol myristate acetate + ionomycin) were included to control for the spontaneous production of IFN-γ. Cells were also cultured in the presence of an irrelevant protein, HIV-1 gp120 (data not shown). A: Dot plots show results from individual representative animals from each group of mice. B: Results are shown as mean ±S.D. The symbol * indicates a significant difference ($p \leq$ 0.01-000.1) compared to all other immunized groups. The symbol † indicates a significant difference ($p \leq 0.05$) compared to the control group.

Fig 7.5: A

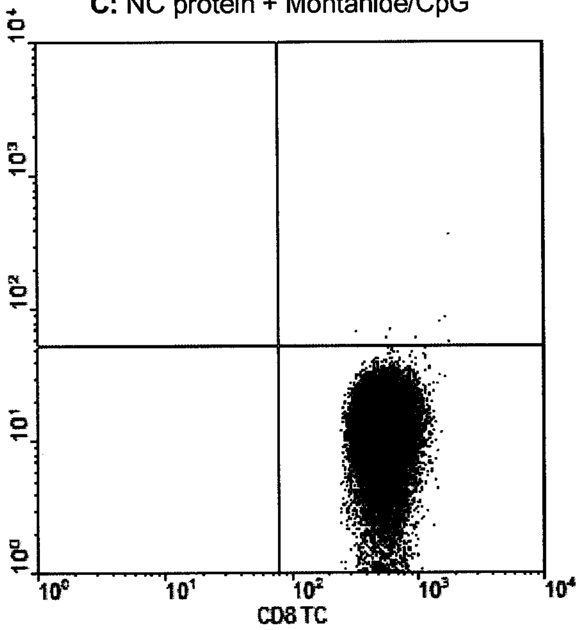
A: DNA alone + XIAP



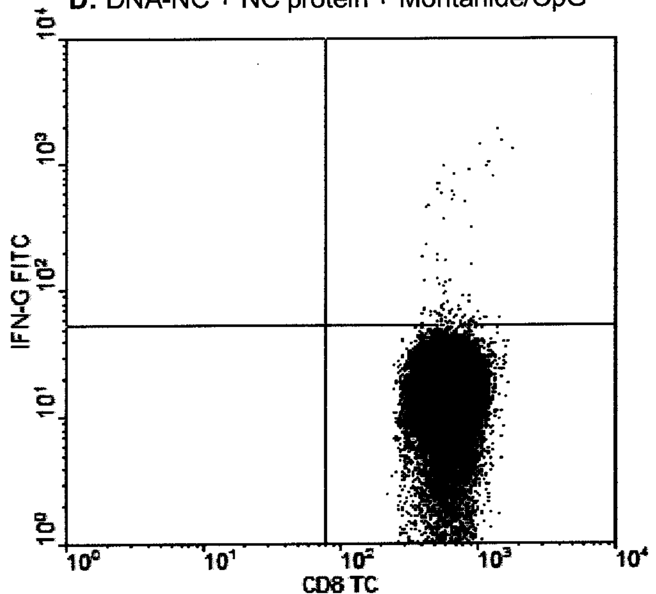
B: DNA-NC



C: NC protein + Montanide/CpG



D: DNA-NC + NC protein + Montanide/CpG



E: DNA-NC + NC protein + XIAP

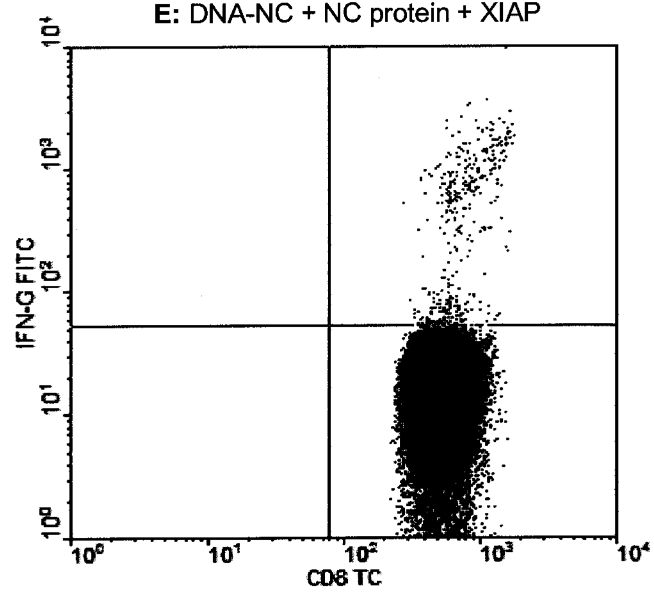


Fig 7.5: B

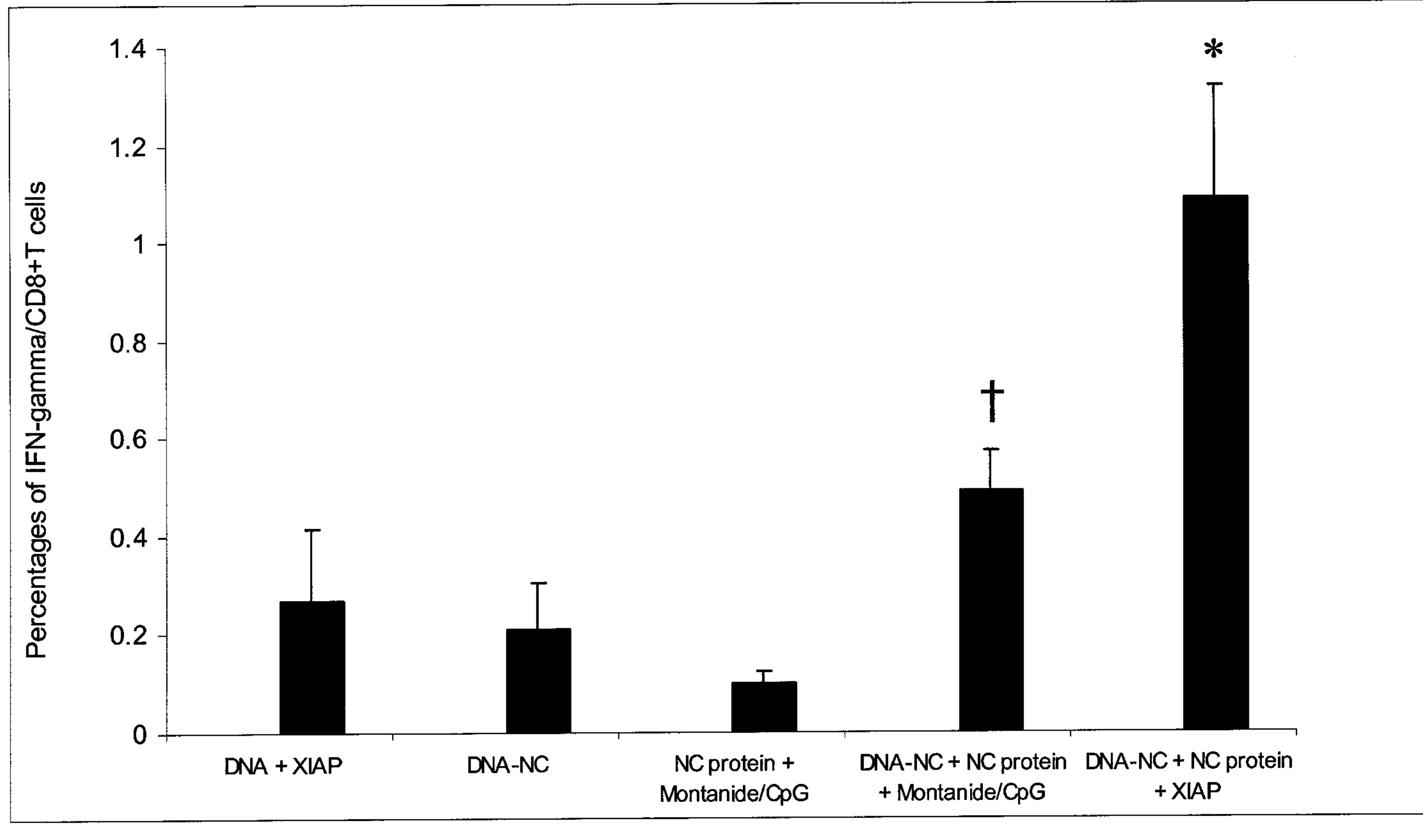
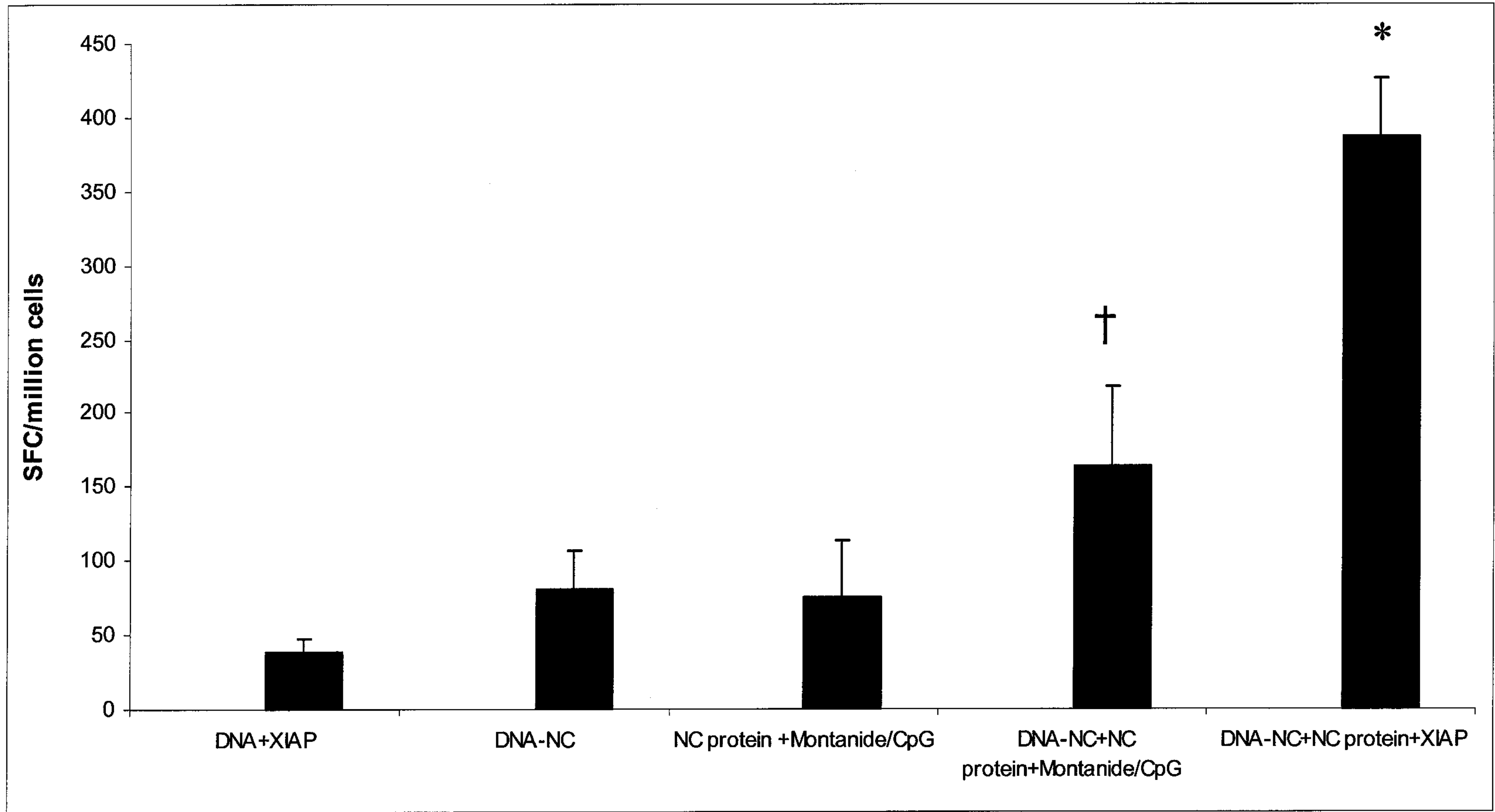


Fig 8.5. The number of IFN-γ producing cells was measured by an ELISPOT assay. The plates were coated with an anti-mouse IFN-γ antibody. The cells were cultured in the presence of recombinant NC protein or an irrelevant antigen (gp120 protein). NC-specific IFN-γ were detected as described in *Materials and Methods*. The mean ±S.D. is shown for each group. The symbol * indicates a significant difference ($p \leq 0.01$-0.001) between the indicated group and all other immunized groups. The symbol † indicates a significant difference ($p \leq 0.05$) between the indicated group and the control group (DNA+XIAP).

Fig 8.5



5.4 Discussion

The SARS pandemic is currently under control. However, the absence of an effective therapeutic agent against this lethal virus, compounded by the threat of its re-emergence, has triggered research efforts to develop an effective vaccine. Previous studies indicate that the spike protein is responsible for the binding of the virus to angiotensin-converting enzyme 2 (ACE2) (Prabakaran, Xiao et al., 2004; Xiao, Chakraborti et al., 2003; Li, Moore et al., 2003). The spike protein contains epitopes that might elicit neutralizing antibodies in the host species thus making it a good target for vaccine development against SARS (He, Zhou et al., 2004; Han, Kim et al., 2004; Zhang, Wang et al., 2004; Yang, Kong et al., 2004). However, mutation of this protein could affect the virulence by allowing the virus to escape from specific immune response (Yoo & Deregt, 2001b;Wang, Xu et al., 1997). Other research groups have made efforts to develop vaccines based on viral nucleocapsids since these viral proteins have conserved regions. Milich and McLachlan showed that the viral nucleocapsid contains T cell dependent and independent epitopes responses (Milich, McLachlan et al., 1987). Nude (athymic) mice immunized with HBV-nucleocapsid alone develop high titers of IgM, IgG2a and IgG2b antibodies which are the predominant antibodies in the Th1. There is evidence that the specific structure folding of viral nucleocapsids is responsible for its high immunogenicity (Noad & Roy, 2003).

The success of immunization depends on several factors, such as type of antigen, route of administration and usage of adjuvants. Mittal et al. showed (Mittal, Aggarwal et al., 2000) that mice immunized intramuscularly, intraperitoneally or subcutaneously have higher antibody titers than mice immunized orally or intranasally. In this study, mice

were immunized subcutaneously as this route of administration has been used successfully in the past (Tobiasch, Kehm et al., 2001; Du, Jia et al., 2003; Cui & Mumper, 2003).

The immune response with adjuvants montanide ISA-51/CpG or XIAP was assessed. In an HIV vaccine candidate study, I showed that montanide can induce strong antibody titers against HIV-1 structural genes (gp120, gag and pol). CpG is also among the most frequently used experimental adjuvants; this adjuvant stimulates dendritic cells through Toll-like receptor 9 (TLR9), inducing cell maturation and enhancing antigen presentation and Th1 responses (Jiao, Wang et al., 2004;Lin, Gerth et al., 2004;Zhang, Palmer et al., 2003). The combination of montanide and CpG was investigated in light of a recent study demonstrating that this combination is more effective than the use of any of the adjuvants alone (Kumar, Jones et al., 2004a). A group of mice received XIAP as adjuvant based on the finding by Kim et al. that mice immunized with DNA encoding XIAP exhibit a strong cell mediated immune response against melanoma. Kim et al. hypothesize that this strong response may be due to increased survival of dendritic cells or T cells *in vivo* (Kim, Hung et al., 2003a;Kim, Hung et al., 2004b). Several antiapoptotic factors such as BCL-XL, BCL-2, XIAP were tested. They also chose human *Papillomavirus* type-16 [HPV-16] E7 as a model antigen because this virus is the agent for a majority of cervical cancers, and E7 is necessary for the induction and maintenance of cellular transformation. Mice were immunized with coadministration of DNA-E7 plus DNA encoding antiapoptotic agents and consequently, an increased number of antigen-expressing DCs in the draining lymph nodes were observed. In addition, vaccinated mice with DNA-E7 and DNA-BCL-XL demonstrated a lower percentage of apoptotsis in

lymph nodes. Their results suggest that the enhancement in the number of DCs in the lymph nodes may contribute to the enhancement of E7-specific CD8+T cell responses.

Nucleocapsid has a fundamental role in the viral life-cycle and could be a potential target for enhancing the immune responses. It is also of interest as a particulate carrier for conserved CD8+T cell epitopes that might be suitable for the development of an effective vaccine for SARS-CoV. In order to characterize specific immune responses in our candidate SARS vaccines, it was decided to use a recombinant protein expressed in bacteria for *in vitro* assays to detect CD4+ and CD8+T cell responses, while the vaccine candidates contained a recombinant protein expressed in CHO cells. Ideally, peptides are used to stimulate CD8+effector responses, however, this is not yet feasible since NC CTL epitopes are not yet characterized in this strain of mice. It is likely that VLPs are processed by antigen presenting cells and that epitopes are presented in an MHC I context, as suggested by the increased CD8+ T-cell responses observed post-vaccination.

Several studies have assessed the SARS-CoV-NC protein as a candidate vaccine. Wang et al (Wang, Yuan et al., 2005) compared immunogenicities of various SARS-CoV DNA vaccines encoding SARS structural proteins (S, M, and NC proteins) in BALB/c mice. They found that pcDNA-M had higher specific lymphoproliferative responses compared with other DNA constructs. They showed a low proliferative response in BALB/c mice that receive a DNA vector expressing NC protein. A weak CD4+T cell response was also observed in our study.

Two more studies analyzed humoral and cell-mediated immune responses in mice immunized with DNA vaccines expressing NC (Kim, Lee et al., 2004a;Zhu, Pan et al., 2004a). Kim at al. showed that linkage of NC protein to calreticulin (CRT: A 46-kDa

Ca^{2+}-binding protein located in the endoplasmic reticulum and belongs to the family of heat shock proteins) increased humoral and cellular immune responses in vaccinated mice compared to mice receiving DNA-NC alone. A high level of CD8+T cell immune response in mice immunized with DNA-NC or NC protein alone was not detected. However, the immunogenicity of our candidate DNA vaccine encoding NC was improved with the co-administration of the recombinant nucleocapsid protein and adjuvants. In another study, Zhu MS et al. showed a high level of antibody titer in mice after three injections of DNA-NC (Zhu, Pan et al., 2004b). They immunized C3H/He mice intramuscularly with 100 mg of DNA-NC for three times at 0, 2nd and 4th week. Antibody titer was measured six weeks after the first immunization. To test cellular immunity, cytolysis activity of the splenocytes was measured. They did not find any significant difference between groups that received two versus three injections and both groups showed a higher CTL response than the control group. In contrast, a high level of antibody titer in mice immunized with DNA-NC alone was not detected and the humoral response was increased with adding recombinant NC protein.

In summary, our results indicate that immunization with different adjuvants could influence the type of immune response. Mice that received DNA, protein and montanide/CpG showed a high level of specific antibody titer against NC. However, vaccination with combinations of DNA-NC, recombinant NC protein and XIAP may add breadth to cell-mediated immune responses. These results suggest a novel approach to produce an effective vaccine against SARS infection.

Chapter 6:

General discussion

It is difficult to induce protective immunity against most RNA viruses. The difficulty is mainly due to lack of knowledge of the specific immunity that can block infection. It is well known that many RNA viruses evade the immune mechanisms of host species due to their high mutation rate of the genome, which is a result of transcription errors (Fiers, De Filette et al., 2004). Humoral immunity may prevent infectivity; however, induction of cell-mediated immune responses with a large repertoire of specificities is essential for viral clearance. Cellular immunity has emerged as a major factor to control most RNA viral infections. Here, correlates of protective immunity of three RNA viral infections (HIV-1, HCV and SARS-CoV) were studied. However, it has been mainly focused on development of effective candidate vaccines against HIV-1.

In the first study, the best combination of HIV-1 structural immunogens for the induction of cell-mediated immune responses in HLA-A2.1 mice was assessed. The results showed that the combination of DNA constructs, recombinant HIV-1 proteins and adjuvant induced a higher CD8+T cell response than the proteins and adjuvant. Furthermore, it was shown that the vaccine immunogenicity can be changed by the type of adjuvant. Thus, adjuvants play an essential role in the optimization of candidate HIV-1 vaccines and could lead immunity towards a predominance of humoral or cellular responses.

In the second study, the efficacy of individual HIV/HCV antigens or combined antigens in the generation of a specific immune response was studied. This research was

to our knowledge, the first that characterized additive or synergistic effects between HIV/HCV antigens in immunized mice. In the third study, cellular and humoral immune responses to SARS-CoV in mice receiving a DNA-NC construct alone or in combination with protein and different adjuvants were characterized. In addition, anti-apoptosis inhibitor XIAP exhibited a strong cell-mediated immune response against SARS which could be due to increased survival of dendritic cells or T cells.

Several approaches have been undertaken to enhance specific cell-mediated immune responses against HIV-1. In the first and second HIV studies, HIV-1 gag, pol and gp120 structural genes were considered for induction of specific immunity against HIV-1. Gag is one of the most sought after target genes for the development of an HIV vaccine. Several conserved CTL epitopes have been identified in the structure of gag protein which plays important roles in clearing primary viremia and in controlling later viral replication, resulting in the slow progression of the disease. HIV-1 pol was also considered because of its degree of conservation. One study showed that removal of the frameshift between gag and pol, makes a negative influence and decreases the expression of gag protein (Nabel, 2002b). Previous studies also reported strong gag and pol specific cell-mediated immune responses in nonhuman primates after immunization (Seth, Ourmanov et al., 1998;Someya, Ami et al., 2006;Quinnan, Jr., Yu et al., 2005b). Furthermore, HIV-1 gp120 has been included in our vaccine candidates even though neutralizing activity was not measured. Mice are known to have a high background, making it difficult to assess neutralizing activity in mice sera. Gp120 is the only HIV-1 component exposed to the external humoral environment and thus, it represents the sole legitimate target for neutralizing antibodies. Recombinant gp120 protein has been used as

the first candidate immunogen in phase III trials against HIV-1 infection. It is interesting to note that current HIV-1 gp120 candidate vaccines elicit neutralizing antibody responses against T cell labortaory adapted (TCLA) strains and not primary HIV-1 isolates (Graham & Mascola, 2005;Billich, 2004).

DNA vaccine strategies have been chosen for its ability to induce humoral and in particular cellular immune responses. There are several studies that showed DNA-based immunization could control and slow disease progression (Giri, Ugen et al., 2004;Radaelli, Nacsa et al., 2003;Buge, Murty et al., 1999).

We constructed and developed a number of different DNA vectors for use as HIV-1 vaccines *in vivo* or for expression and purification of HIV-1 proteins *in vitro*. These vectors had the ability to express recombinant HIV-1 structural proteins. Previous studies showed that specific immune responses by DNA vaccines could be enhanced by boosting with viral vectors or recombinant proteins. Thus, to induce optimal cell-mediated immune responses, different combinations of DNA constructs along with recombinant HIV-1 structural proteins was evaluated. Recombinant proteins can present viral antigens to B cells, CD4 and CD8+T cells. HIV-1 gp120, gag and pol proteins originating from clade B HIV-1 isolates were purified and characterized. A gp120 glycoprotein derived from CHO cells is more likely to posses the natural conformations of the peptide epitopes when compared to proteins derived from yeast or bacterial cells. It seems that a recombinant gp120 protein which mimics native structure and glycosylation patterns is crucial in generating specific humoral and cellular immune responses(Srivastava, Stamatatos et al., 2002;Jones, McBride et al., 1995a). In addition, the production of neutralizing antibodies against important binding sites of gp120-CD4

may need the preservation of structural integrity through the whole molecule (Jones, McBride et al., 1995b). One of the preliminary pitfalls in the HIV-1 study was the expression of recombinant gp120 protein. It was shown that gp120 has the ability to kill mammalian cells by apoptosis. To circumvent the low yield of gp120 expression, a cell line that stably expressed gp120 was developed. This was accomplished by cloning gp120 into an expression plasmid expressing the selective blasticidine resistance gene. This cell line had a 20-fold increase in the expression of gp120 when compared to the expression of acutely transfected cells. DNA expressing HCV-core, E1 and E2 and HCV structural proteins (core, E1 and E2) have previously been expressed and purified in our laboratory (Ghorbani et al. Viral Immunol, in press). These antigens have been used in our second study for the development of a combined HIV/HCV vaccine.

Development of an effective vaccine depends on the ability to mimic human HLA-restricted T cell responses. HLA-A2.1 mice are a good model of vaccine efficacy. It has been shown that HLA-A2.1 mice recognize HIV and HCV epitopes identical to those recognized by human CD8+T cells from individuals possessing this haplotype(Martin, Parroche et al., 2004b;Pancholi, Perkus et al., 2003;McKinney, Skvoretz et al., 2004).

In the first HIV-1 study, the potential for vaccination of HLA-A2.1 mice by using polycistronic and monocistronic DNA constructs when combined with recombinant proteins and adjuvants CpG and montanide was investigated. In the second HIV study, a synergistic effect between HIV-1 and HCV was studied by immunizing mice with HIV/HCV immunogens alone or in combination.

The type of adjuvant used in a vaccine formulation is a key factor in induction and control of antigen-specific immune responses. At present, there is a limited selection of

adjuvants in clinical use. Two potential adjuvants that have been used in previous clinical trials were chosen. Montanide is produced by SEPPIC Inc. and is a mixture of mineral oils that has given good activity in animal trials and has been approved for human clinical trial use. Synthetic non-methylated CpG has also been shown as a promising adjuvant for animal and human clinical trials. It has the capacity to directly activate B cells, macrophages, and dendritic cells. Adjuvant formulations control antibody isotypes and as a result, elicit Thl or Th2 immune responses. Antibody responses were evaluated by ELISA against wild type gp120 and gag/pol proteins. The addition of montanide ISA-51 as adjuvant increased antibody titers against both gp120 and gag proteins. These results were similar as other studies from our laboratory. IgG antibody titers in mice receiving montanide ISA-51 as adjuvant were higher than in mice received other types of adjuvants such as CpG, Alum and MPL. Two recent studies showed that the mixture of montanide and CpG induced higher levels of IgG2a and IgG2b antibodies and led to the induction of Thl bias which is responsible for the higher degree of protective immunity. Thus, this combination of adjuvants was used in the HIV/HCV (second approach) and SARS-CoV studies. The immunized mice showed higher level of antibody titers compared to the groups receiving CpG alone (HIV study).

Although it has been shown that T cell responses play an important role in the control of viral infections, it is not yet known what combination of phenotypes and functions, and what frequencies of specific T cells supply protection from viral infections. In my research studies, several critical features of cellular immunity such as Thl/Th2 cytokine secretion, lymphocyte proliferation and frequency of specific CD8+T cell responses were examined. Previous studies showed that the maintenance of

qualitative Th responses, especially IFN-γ is fundamental in preventing progression to AIDS (Mooij, Nieuwenhuis et al., 2004b;Egan, Chong et al., 2004). By measuring cytokine secreting CD4+T cells in response to viral specific antigens, we were able to discern the ability of candidate vaccines in induction of Th1 or Th2 like responses.

Depending on the immunizations, various Th1 biases were detected in our studies. In the first HIV-1 study, a high level of IFN-γ cytokine secretion was observed in response to gp120, gag/pol proteins in all immunized groups but control mice. Generally, in the second study (HIV/HCV approach), the amount of gag-specific Th1 response was lower and the level of HCV-core Th1 responses was relatively weak. It seems that CpG alone causes a stronger shift toward Th1 immune bias than CpG and montanide. Interestingly, no detectable IL-4/IL-5 Th2 immune response was detected in HIV studies. Our findings confirmed previous studies that showed a highly significant reduction of Th2 bias and a shift in the general immune background towards Th1 immune responses by administration of CpG and montanide.

The lymphocyte proliferation assay is used to determine the activation of lymphocytes after encounter with specific antigens. After stimulation, B and T cells are undergoing proliferation and increase their rate of protein and DNA synthesis. The increase in DNA synthesis can be measured by adding [^{3}H] thymidine, to the media. The amount of tritium taken up by the dividing cells is correlated to the level of cellular proliferation. In the first HIV-1 study, splenocytes from immunized mice were stimulated with gp120 and gag/pol proteins and we found that the administration of recombinant proteins + CpG, without any DNA construct (polycistronic or single DNA vectors) resulted in the development of lower proliferative responses compared to the other groups

that received the combined administration of proteins, DNA, and CpG. It suggests that administration of DNA is essential to enhance proliferative immune responses against HIV-1 antigens. However, we have to consider that this technique is an integrative assay and depends on several factors such as: (1) antigen processing and presentation, (2) *in vitro* culture conditions, and (3) the initial frequency of antigen-specific T cells. In the second study (HIV/HCV project), the proliferative immune responses were studied against HIV and HCV proteins and interestingly, the combined HIV/HCV immunized mice showed a higher proliferative response to HIV-gp120 protein compared to the group receiving only HIV immunogens. However, no synergistic proliferative responses were observed to HIV-gag and HCV-core proteins.

In the first HIV-1 study, intracellular cytokine (ICS) and HLA-A2.1 peptide dimer staining were used to detect CD8+T cell subsets by measuring production of intracellular IFN-γ following antigenic stimulation. For the ICS, whole blood for the detection of CD8+T cells was used. Analysis of CD8+T cells in whole blood has some advantages over PBMCs or frozen cells. For instance, in whole blood, cells are stained directly and remain intact, thus they are unaltered by the Ficoll separation which reduces background in the analysis of CD8+T cells. In cryopreserved PBMCs, permeabilization of cells may be incomplete, which may lead to erroneous observations. It has been shown that the staining of the same population from whole blood and cryopreserved PBMC results in totally different observations, with a different distribution of cytokines in the CD8+T-cell subsets (Appay & Rowland-Jones, 2002). The analysis of samples in the first HIV study by ICS showed that the group of mice that received single DNA constructs expressing gp120 or gag/pol genes, recombinant proteins and CpG demonstrated a higher amount of

CD8+T cell immune responses compared to the other groups. It is possible that the ability of single constructs to induce CD8+T cells is attributed to the presence of unmethylated CpG motifs in the DNA plasmids. In addition, the level of protein expression in the single DNA constructs *in vivo* might be different with the polycistronic construct.

The CD8+T cells from immunized mice were further analyzed by HLA-A2.1 peptide dimer assay. This novel technique facilitates the detection and characterization of antigen-specific T cells by flow cytometry. Soluble dimer MHC-Ig molecules can be easily loaded with any HLA restricted peptide by incubating them together over night. One of the advantages in this technique is that peptide-loaded MHC-Ig complex is completely stable and can be stored for several months without any loss in activity (Oelke & Schneck, 2004). Although the frozen blood samples without any T-cell expansion were used, the analysis of CD8+T cells by HLA-A2.1 peptide dimer assay was promising. These results confirmed the consequences obtained by intracellular cytokine staining.

In the second study, the peptide dimer assay was replaced with ELISPOT, which measure cytokine secretion by splenocytes or PBMCs. This technique could quantitively measure the functional readout of each specific single-cell. In both ICS and ELISPOT techniques, splenocytes from immunized mice were prepared and stimulated with HIV-1 and HCV peptide epitopes specific for MHC class I HLA-A2.1. It has been shown that immunization with HIV/HCV immunogens induces stronger cellular immune responses to HIV and HCV peptides. Although both ICS and ELISPOT assays showed higher percentages of specific CD8+T cells in the combined group, no correlation was found between the amount of CD8+ IFN-γ by ICS and the level of generated IFN-γ by ELISPOT. It should be noted that although these assays measure cytokine responses after

stimulation with specific antigens, they differ in several aspects. The flow cytometer is the instrument used for the detection of ICS, whereas the readout for ELISPOT is microscopy or an ELISPOT reader. In addition, the incubation times, number of parameters analyzed, and sample compatibility are different between the assays (Maino & Maecker, 2004). In summary, knowledge gained from this research may also be applied in non-human primate animal models and may help us to develop new combined vaccines against other diseases.

In another study, development of an effective vaccine against SARS-CoV was examined. This lethal disease affected Canada in March 2003 and had a huge economic impact in some countries. It was first reported in some Asian countries such as China, Taiwan, Indonesia, Thailand and then, spread to other regions, including Canada. Since the outbreak, research has focused on the induction of an effective SARS vaccine approach in animal models. Initially the following questions were considered.

(i) Which SARS-CoV immunogen is the best target for induction of a strong humoral and cell-mediated immune response?

(ii) Could we promote the level of CD8+T cell immunity by antiapoptotic factors such as XIAP?

Immunity against SARS-CoV was studied in mice receiving DNA constructs alone or in combination with proteins and different adjuvants. The SARS structural genes (S, M, E and NC) have been constructed in different expression vectors with an ultimate goal to develop DNA vaccines and purify proteins in two expression systems (mammalian and bacterial). The immunogenicity of these constructs and proteins were tested in different groups of mice (Appendix). Several of these groups immunized with

DNA constructs and proteins demonstrated increased humoral and cellular immunity when compared to the control groups. It seems that both humoral and cellular immune responses contribute to protection against SARS. Like HIV and other RNA viruses, SARS-CoV may escape from immunity by mutation, a phenomenon that had been already described in the S protein of other coronaviruses (De Groot, 2003). Here, NC-SARS-CoV was studied as a main target due to higher CD8+T cell immune responses compared to the other structural genes and less antigenic variation compared to Spike.

Five groups of mice were primed and boosted with SARS-nucleocapsid DNA alone or in combination. Our results demonstrated that a combination of DNA + protein and adjuvant is more immunogenic in mice than the DNA vaccine alone and proteins/adjuvants. However, the type of adjuvant plays a critical role in the strength of humoral and cellular immune responses. Mice receiving montanide/CpG developed detectable IgG titers, but mice receiving XIAP as adjuvant did not show a high IgG after immunization. On the other hand, the results of ICS and ELISPOT in the vaccinated mice suggested that XIAP may enhance cellular immune response. It has to be noted that the amount of proliferative immune responses and the level of IFN-γ producing CD4+T cells was very low in immunized mice, leading to the hypothesis that most likely the IFN-γ response was generated by effector CD8+T cells which is independent of CD4+T cells.

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Appendix 1

	Priming	Boosting
Group A	pVAX + CpG	pVAX + Montanide
Group B	pVAX/S + CpG	pVAX/S + S protein + Montanide
Group C	pVAX/S + XIAP	pVAX/S + S protein + XIAP
Group D	pVAX/S + /NC +/M +/E + XIAP	pVAX/S +/NC +/M +/E + S/NC/M/E proteins + XIAP
Group E	pVAX/S +/NC +/M +/E + S/NC/M/E proteins + XIAP	pVAX/S +/NC +/M +/E + S/NC/M/E proteins + Montanide
Group F	pVAX/ E+/M +CpG	pVAX/E+/M +E/M proteins + Montanide
Group G	pVAX/ E+/M + XIAP	pVAX/E+/M +E/M proteins + XIAP
Group H	S/NC/M/E proteins + CpG	S/NC/M/E proteins + CpG
Group I	S/NC/M/E proteins + alum	S/NC/M/E proteins + alum
Group J	S/NC/M/E proteins + Montanide	S/NC/M/E proteins + Montanide

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Group D	pVAX/S + /NC +/M +/E + XIAP	pVAX/S +/NC +/M +/E + S/NC/M/E proteins + XIAP
Group E	pVAX/S +/NC +/M +/E + S/NC/M/E proteins + XIAP	pVAX/S +/NC +/M +/E + S/NC/M/E proteins + Montanide
Group F	pVAX/ E+/M +CpG	pVAX/E+/M +E/M proteins + Montanide
Group G	pVAX/ E+/M + XIAP	pVAX/E+/M +E/M proteins + XIAP
Group H	S/NC/M/E proteins + CpG	S/NC/M/E proteins + CpG
Group I	S/NC/M/E proteins + alum	S/NC/M/E proteins + alum
Group J	S/NC/M/E proteins + Montanide	S/NC/M/E proteins + Montanide