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*Factors influencing the induction of immune
responses with DNA vaccines*

Lyn M. Daly, B.Sc.

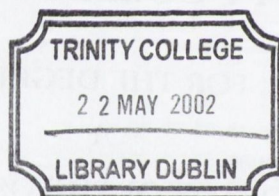


A THESIS SUBMITTED TO
TRINITY COLLEGE DUBLIN

FOR THE DEGREE
OF
DOCTOR OF PHILOSOPHY

Supervisor: Professor Kingston H.G. Mills
Head of Department: Professor Clive Williams
Department of Biochemistry,
Trinity College Dublin.

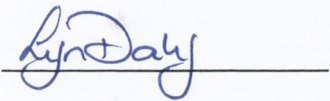
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Declaration of Authorship

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A handwritten signature in blue ink, reading "Lyn Daly", is written over a horizontal line.

Lyn Daly

*This world,
After all our science and sciences,
is still a miracle;
Wonderful, inscrutable,
magical and more,
to whosoever will think of it.*

*Thomas Carlyle (1795-1881)
Scottish Historian*

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Of life and science!!*

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*And finally to Phelim....
Before me goes a shield
To guard me from harm
It is in the shadow of your arms
Between me & danger*

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SUMMARY

Compared with any other medical discovery vaccines have prevented more deaths and suffering. Emergence of novel pathogens accentuates the requisite for ongoing development and improvement of traditional vaccination strategies. Understanding the role of T helper cells in regulation of the immune response is imperative for manipulation of the host immune response and in the design of novel vaccines. Concerns regarding safety and poor immunogenicity of conventional vaccines has provoked the development of safer more immunogenic vaccines, such as those based on naked DNA. Injection of plasmid DNA coding for the protective antigen of a pathogen can generate antigen-specific cellular and humoral responses. Immunogenicity of protein and DNA vaccines may be influenced by several factors, including route of administration, dose, immunization schedule, type of antigen and presence of an adjuvant. This project focuses on characterizing the immune responses induced by plasmid DNA encoding the gp120 surface glycoprotein of HIV-1 virus and the transmembrane hemagglutinin protein of influenza virus. Induction of virus-specific CD4⁺ Th1 and CD8⁺ CTLs are recognized as ideal objectives in the design of many viral vaccines. Modulation of the immune response induced with gp120pDNA towards a Th1-type profile was achieved by co-administration of plasmids coding for IL-12 cytokine and cationic liposomes. The use of prime/boost immunization schedule demonstrated that priming with gp120pDNA followed by a gp120-alum could augment type-1 responses. Multivalent vaccines are possible with DNA vaccine technology and this study found that responses to individual plasmids were not adversely affected by the presence of supplementary plasmids. This study also demonstrated that encoded antigen and plasmid composition influenced the induction of immune responses. Encoding gp120 protein in distinct bacterial plasmid backbones augmented gp120-type-1 responses, emphasizing the importance of selecting an appropriate plasmid backbone depending on the target pathogen. Collectively these findings indicate that many factors influence the induction of immune responses with DNA vaccines. Knockout mice were used to demonstrate the importance of IL-4, IL-10 and IL-12 in directing the response, which appeared to be independent of antigen-type. HIV gp120 protein encoded by plasmid DNA was found to influence BMDC maturation, chemokine and cytokine production. HA-primed APC were detected in secondary lymphoid organs following immunization with HApDNA. Furthermore, *in vitro* HApDNA-stimulated BMDC activated a HA-specific Th1 clone to secrete type-1 cytokines, establishing a key role for DC in the induction of immune responses with DNA vaccines.

This study has identified several factors that influence the immunogenicity of DNA vaccines. Exploring the dynamics of this technology will allow a more logical approach to designing effective novel vaccines. DNA vaccines represent a safe, immunogenic, inexpensive and rapid means of generating immunity against several pathogens and may contribute to eradicating the devastation caused by HIV and many other infectious microorganisms.

ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AKP	Alkaline Phosphatase
APC	Antigen Presenting Cell
BMDC	Bone marrow derived dendritic cell
bp	base pair
BSA	Bovine Albumin Serum
CD	Cluster Designation
CMV	Cytomegalovirus
CpG	Cytosine-phosphate-guanosine motif
cpm	counts per minute
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DMRIE-c	1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide
EDTA	Ethylenediaminetetracetic Acid
ELISA	Enzyme Linked Immunoabsorbant Assay
<i>env</i>	envelope protein of HIV
FACS	Fluorescence Activated Cell Scanning
FCS	Foetal Calf Serum
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HA	Haemagglutinin
HCV	Hepatitis C Virus
HBV	Hepatitis B Virus
HIV	Human Immunodeficiency Virus
HRP	Horse Radish Peroxidase
HSV	Herpes Simplex Virus
i.m.	intramuscular
i.n.	intranasal

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ISCOMs	Immune Stimulating Complexes
Lgp120	gp120 protein coupled to latex microspheres
LT	<i>E. coli</i> heat labile enterotoxin
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemotactic Protein-1
MHC	Major Histocompatibility Complex
MIP	Macrophage Inhibitory Protein
MW	Molecular Weight
NA	Neuraminidase
ng	nanogram
NK	Natural Killer cell
NMS	Normal Mouse Serum
NP	Nucleocapsid Protein
OD	Optical Density
ODN	synthetic oligodeoxynucleotide
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
PBA	PBS/BSA/Azide
pDNA	plasmid DNA
PE	Phycoerythrin
pg	picogram
PMA	Phorbol Myristic Acid
RANTES	Regulated on Activation Normal T-cell Expressed
RPMI	Rosewell Park Memorial Institute
SE	Standard Error
TBS	Tris Buffered Saline
TCR	T Cell Receptor
Th	T helper cell

TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
TMB	Tetramethylbenzidine
UV	Ultra-Violet
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 GENERAL INTRODUCTION

In the late 18th century, Edward Jenner observed how milkmaids were unaffected by smallpox, a disease which caused high mortality in children. He challenged his theories and infected a young boy with cowpox, then smallpox and to his surprise the boy failed to develop symptoms associated with smallpox virus. Jenner had stumbled upon the vaccine for smallpox and ultimately began the vaccine revolution. Most current vaccines are based on attenuated or killed microorganisms and were designed with little knowledge of their mechanism of action or potential side effects. Advances in immunology and molecular biology have facilitated the identification of protective mechanisms against infectious pathogens and have allowed safer and more rational approaches to vaccine design.

At present, one of the world's greatest challenges is to produce an effective vaccine against human immunodeficiency virus (HIV). However, this is proving a difficult task due to the virus's ability to change its 'identity' by genetic switches and evade the body's immune defenses. The worldwide figure for HIV infected individuals is estimated at approximately 20 million, with 16,000 new cases each day. This underlines the necessity for an effective HIV vaccine. Immunization with nucleic acid vaccines (DNA vaccines) is an exciting vaccine technology that provides a novel approach for development of a vaccine against HIV and other infections. The DNA vaccine concept arose from studies on gene transfer in humans to replace missing or faulty genes. The flexibility of this technology was soon realized when plasmid DNA was injected into animals and the encoded protein was expressed. The discovery that DNA coding for viral proteins could induce immune responses and protect animals when challenged with live viruses paved the way for a new era of vaccination.

1.2 THE IMMUNE SYSTEM

The immune system functions to protect a healthy individual from microorganism invasion and is classified into two basic categories: innate immunity and adaptive immunity.

1.2.1 INNATE IMMUNITY

The innate system provides the first line in host defense by rapidly recognizing a pathogen and signaling this danger to the adaptive immune system. Macrophages, dendritic cells (DC), neutrophils and other phagocytic cells are key elements with the ability to recognize non-processed antigens, such as lipopolysaccharide (LPS), through a variety of pattern recognition receptors common between pathogens (Fearon and Locksley, 1996). Natural Killer (NK) cells, complement and interferon function as mediators of the innate system. An effective physiological barrier to most microorganisms is also provided by the body surface through skin, temperature and pH.

Classical NK cells and recently discovered NKT cells are some of the first cells to respond following activation in response to innate cytokine production during viral infection. They mediate defense through both antiviral and immunoregulatory pathways. A cross-regulatory pathway exists between IFN- α/β and IL-12 produced by infected cells, which regulates NK cell responses. Lysis and cell mediated cytotoxicity by NK cells are enhanced by type I interferons (IFN- α/β). NK cell activity is directly modulated by IL-12 and IL-18, which play a role in IFN- γ production by NK cells early in infection. As IL-12 is secreted by a small subset of cells such as macrophages or DCs, it appears that these early infected cells might influence the type of NK function that is activated (Biron and Brossay, 2001). The IL-18 receptor is expressed on NK cells (McInnes *et al.*, 2000). NK cells also stimulate production of tumor necrosis factor (TNF) and chemokines e.g. MIP-1 α . Major histocompatibility complex (MHC)-binding inhibitory receptors expressed by NK cells and activating receptors, NKR-P1, can trigger IFN- γ secretion and cell killing (Bendelac and Fearon, 1997). Cells expressing low levels of MHC class I prevent these receptors binding and so evade NK attack. NKT cells become activated when antigen is bound by their T cell receptor (TCR). Conditions where low level innate cytokine induction occurs activates the NKT pathway that in turn triggers the classical NK defense mechanism (Biron and Brossay, 2001). $\gamma\delta$ T cells also serve innate function by recognizing conserved components of microorganisms and secrete cytokines involved in immune responses to viral infection (Bendelac and Fearon, 2000).

Reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) are toxic molecules of the immune system capable of being produced by activated phagocytic cells, such as macrophages or polymorphonuclear neutrophilic leukocytes (PMN). The phagocytosis process involves the binding of the particle to the cell surface followed by its internalization, destruction and release of toxic reactive intermediates. ROI's generated by neutrophils act as antimicrobial effector molecules against extracellular bacteria, whereas macrophages produce RNI's to combat intracellular pathogens (Bogdan *et al.*, 2000).

In addition to its importance as the first line of defense, the innate system also plays an instructive role to the adaptive responses through the nature of innate recognition. Non-self structures or endogenous signals are both suggested as signals mediating activation of innate immunity (Bendelac and Fearon, 2000). The 'Danger Model' postulates that the immune system responds to damage causing substances (endogenous signals) rather than foreign antigens (Gallucci and Matzinger, 2001).

1.2.2 ADAPTIVE IMMUNITY

Non-specific host defense mechanisms essentially control the initial spread of infection by microorganisms in a healthy individual. However, if the pathogen evades innate immunity, specific adaptive immunity is triggered to eliminate the pathogen and prevent re-infection. Immunological memory is a key feature of adaptive immunity that develops by clonal recognition of pathogenic antigen triggered through T cell receptors (TCR) and B cell receptors, resulting in a cascade of immunological events leading to lymphocytes differentiating into effector and memory cells. Therefore, adaptive immunity is primarily mediated by lymphocytes that specifically recognize epitopes on the pathogen. The lymphocyte population can be divided into two basic categories: B lymphocytes and T lymphocytes.

1.2.2.1 B-LYMPHOCYTES

B cells are generated exclusively in the bone marrow from non-lymphoid stromal cells. B cells are the primary cell type involved in production of an antigen-specific product, antibodies (Ab). B cells secrete antibody when stimulated by specific antigen that is recognized by membrane bound immunoglobulin (Ig) molecules that act as antigen receptors. Antibodies are heterodimeric molecules composed of a generic

structure of four polypeptide chains, 2 of each identical light (L) and heavy (H) chains with each chain possessing constant (C) and variable (V) regions that determines antibody specificity. Collectively antibodies form a family of plasma proteins known as immunoglobulins, subdivided into five isotypes with individual functional and biochemical attributes: IgM, IgG, IgA, IgE and IgD.

Primary antibody responses are mediated by the pentameric IgM. Due to its large structure and inability to diffuse efficiently, it is predominantly located in the bloodstream where it can recognize antigen with low affinity resulting in agglutination of antigen and activation of complement. T cell derived cytokines switch IgM to IgG production soon after an initial antibody response. IgG antibodies are the most abundant class of Ig in the serum and are composed of four distinct subclasses, IgG1, IgG2a, IgG2b and IgG3 in the mouse. IgG mediates a variety of functions such as activation of complement, opsonization of antigen and plays a role in protecting the developing fetus, due to its ability to cross the placenta. IgA is the principal antibody isotype found at mucosal surface, in saliva and other mucosal secretions. Pathogenic entry into a host is prevented by IgA-mediated immune exclusion through effective binding of specific antigen and inhibiting attachment of pathogenic antigen to the mucosal surfaces. IgE antibodies bound by specific receptors on both mast cells and basophils secrete mediators of allergic reactions, such as histamine, when cross-linked with antigen. Therefore, IgE mediates hypersensitivity reactions associated with allergic type responses.

Before B cell activation and subsequent antibody production can occur, mature naïve B cells must encounter antigen that contains epitopes recognized by its cell surface molecule, Ig. This activation of B cells can be direct or indirect where direct activation is T cell independent and requires cross-linkage of the B cell membrane molecule Ig with multivalent antigens. However, the majority of antigen is T cell dependant (indirect activation) and requires cooperation of CD4⁺ T helper cells. Foreign antigen bound by B cell specific antigen receptor Ig, is processed and presented in association with the MHC class II molecule. The CD4⁺ T cell receptor specific for class II peptide, binds the B cell through surface molecules (Parker, 1993). Contact between B cell and T cells is mediated through T cell ligands CD40L and CD28 binding to CD40 and B7-1/B7-2 on B cells respectively (Clark and Ledbetter, 1994). Activated T cells produce an array of cytokines that induce B cell proliferation and antibody production. B cell antibody isotype production is

influenced by T cell derived cytokines. Murine Th1 cells promote isotype switching through IFN- γ , a potent inducer of IgG2a antibody production and inhibitor of IgG1 and IgE production. In contrast, IL-4 secreted by murine Th2 cells promotes IgG1 and IgE production but suppresses IgG2a and IgM (Coffman *et al.*, 1993). The Th2 cytokine IL-6, also secreted by macrophages promotes final maturation of IgA (Yamamoto *et al.*, 1996).

1.2.2.2 T-LYMPHOCYTES

T Lymphocytes or T cells develop and differentiate in the thymus. They can be divided into two distinct lineages dependant on the expression of a unique antigen-binding cell-surface receptor known as the T cell receptor (TCR). The TCR contains a polypeptide complex termed CD3, involved in activation of the T cell signaling cascade. The majority of T cells express TCR composed of α and β heterodimeric chains, while a smaller population express γ and δ heterodimeric chains. The α/β T cells can be subdivided based on surface expression of a co-receptor CD4 or CD8. T cells require antigenic peptide association with the MHC molecule to allow TCR recognition of the foreign antigen. MHC molecules are divided into two classes: MHC class I molecules are expressed by most nucleated cells and MHC class II molecules are confined to cells of the immune system (macrophages, monocytes, DCs and B cells). CD8 and CD4 expressing T cells are restricted to recognizing peptide antigen bound to class I or class II MHC molecules respectively. CD8⁺ class I restricted T cells predominantly function as cytotoxic T cells (CTL), whereas, CD4⁺ class II restricted T cells are responsible for providing helper function to immune responses (CD4⁺ Th cells).

The expression of peptides on MHC molecules is determined by endogenous or exogenous routes of processing and presenting antigen, carried out by professional antigen presenting cell (APC). Internally synthesized peptides such as viral proteins are presented by MHC class I peptide complexes. The antigens are degraded into peptides via proteasome-mediated cleavage followed by transportation to the endoplasmic reticulum (ER), where they are loaded onto class I MHC molecules. The complex is then carried to the cell surface and presented to CD8⁺ T cells. In contrast, MHC class II molecules present antigen that utilize the endocytic route. The exogenous antigen is internalized by endocytosis or phagocytosis and proteolytically

degraded into peptides in the early endosomes. The invariant chain (Ii) associates with the class II molecule and undergoes proteolytic destruction prior to class II MHC peptide loading. The class II associated invariant chain peptide (CLIP) is released to interact with the antigenic peptide and forms the MHC class II peptide complex. This complex is transported to the plasma membrane where it can be recognized by CD4⁺ T cells.

CD4⁺ and CD8⁺ T cells have distinct immunological functions. CD4⁺ T cells are referred to as T helper (Th) cells that provide help for B cell antibody production and regulate immune responses by secreting a selection of cytokines that activate immune cells. CD8⁺ T cells are designated CTL as they are cytotoxic to cells that are infected with viruses and intracellular bacteria. The CTL response comprises of a cascade of organized cellular processes that cumulate in destruction of the infected target cell. CD8⁺ T cells mediate cytotoxic activity by two proposed mechanisms: perforin or Fas based mechanisms. The perforin pathway is characterized by lytic protein perforin and serine esterases (granzymes) stored in cytosolic granules of the CD8⁺ T cell. CTL-target cell recognition triggers the release of perforin that forms pores in the host cells. Granzymes enter through these pores inducing cytoplasmic and nuclear damage leading to apoptosis of the target cell. The Fas-mediated killing of target cells requires cross-linking of CTL surface bound Fas ligand (FasL) and a death receptor (Fas) on target cells. This interaction induces the activation of a cascade of proteolytic enzymes involved in programmed cell death resulting in apoptosis and lysis of the infected cell (Kuwano and Arai, 1996).

1.2.2.3 CD4⁺ T CELL SUBSETS

The control of immune responses during infection is highly dependant on the induction of CD4⁺ T helper cell function during initial exposure to foreign antigen. CD4⁺ Th cells can be divided into two distinct subsets of effector cells based on their functional capabilities and their mutually exclusive production of cytokines. These subsets were designated Th1 and Th2 cells and have classically been considered central in mediating cellular and humoral immunity respectively.

Murine Th1 cells produce IL-2, IFN- γ and TNF- β , whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 (Mosmann and Coffman, 1989). A similar pattern is observed in human Th1 and Th2 cells. However IL-2, IL-6, IL-10 and IL-13 synthesis

are not as tightly restricted to a single subset, as is the case in murine T cells. Each Th cell subset produces cytokines that function as autocrine growth factors, so that once a T cell is initiated to differentiate towards a subset of Th cells, these factors promote further differentiation to that subset. The main Th1 cytokine, IFN- γ , has a number of key functions including macrophage activation, upregulation of MHC class II expression and inhibition of viral replication. Th1 cells provide B cell help through IFN- γ secretion by stimulating the preferential production of the IgG2a subclass of immunoglobulin, the principal antibody subclass involved in the opsonization and phagocytosis of microbes.

Th2 cells have been implicated in host defense against extracellular pathogens such as helminthes and nematodes. Cytokines produced by these cells such as IL-4 and IL-5, can activate mast cells and eosinophils and stimulate B cells to produce IgE, resulting in allergic and anti-inflammatory immune responses. Switching of antibody production to the IgE isotype is induced by IL-4 and establishes a key role for IL-4 in mast cell mediated reactions. The principal eosinophil-activation cytokine, IL-5, plays an important role in immunity to helminth and other extracellular pathogens. Th2 cells provide additional B cells help by promoting production of IgM and IgG1, a non-complement fixing IgG subtype.

A third subset of CD4⁺ T cells that produces a mixture of two patterns of cytokine secretion exhibited by Th1 and Th2 cells, have been defined as Th0 cells (Firestein *et al.*, 1989). A mixed population of CD4⁺ T cell subset rather than an individual Th subset may explain the overlapping cytokine profile associated with this Th0 subset. Th1 and Th2 cytokines appear to be exclusively produced by individual cells with IFN- γ synthesis appearing later than IL-4 production (Openshaw *et al.*, 1995). In addition, regulatory Th cells that maintain tolerance and active suppression play a key role in controlling the immune response. These include Th3 cells that secrete TGF- β which demonstrate immune-regulatory functions and provide protection in a murine model of EAE inflammation (Chen *et al.*, 1994). Furthermore, a Th clone that could suppress antigen-specific Th1 responses and actively downregulate pathology in a murine model of colitis, was termed T regulatory (Tr1); these cells secrete high levels of IL-10 and low levels of IFN- γ and IL-4 (Groux *et al.*, 1997).

1.3 FACTORS INFLUENCING THE DEVELOPMENT OF TH SUBSETS

The ability of a host to effectively eradicate an invading organism is dependant on the development of a particular Th subset, as most pathogens are more susceptible to one type of host immunity. Type-1 and type-2 immunity involve fundamentally distinct and opposing effector functions mediated by T cells and the cytokines they secrete. The central role played by Th1/Th2 cells in controlling *Leishmania major* infection demonstrates the dual regulatory and effector roles of these CD4⁺ T cell subsets, where resistance is conferred by Th1 cells and susceptible hosts develop a Th2 response and succumb to infection. Naïve T cells secrete IL-2 following initial stimulation and selectively differentiate into either Th1 or Th2 cells depending on a number of factors. The cytokines present in the cellular microenvironment play a major determining influence in the development of these Th subsets. However, studies have indicated other factors including co-stimulation, nature and dose of antigen and route of entry into the host can influence the selective induction of one Th subset over another, therefore, favoring the progression towards humoral or cell mediated immunity (O'Garra and Murphy, 1994).

1.3.1 THE CYTOKINE ENVIRONMENT

Cytokines are central regulators of host defense and key players in activation of the immune system. The local cytokine environment influences the differentiation of CD4⁺ T cells after initial polarization due to antigenic encounter and subsequent DC1 and DC2 maturation, into mature effector and memory T cell subsets (Kourilsky and Truffa-Bachi, 2001). Definitive evidence for the role of cytokines in the induction of distinct Th subsets has been provided by studies using knockout mice deficient in cytokine, cytokine receptors or molecules required for their signal transduction pathway. IL-12, IL-4 and IL-10 cytokines enhance Th1, Th2 and Tr1 differentiation respectively.

1.3.2 FACTORS INFLUENCING DEVELOPMENT OF TH1 CELLS

The major cytokines involved in differentiation of CD4⁺ T cells to the Th1 subset include IL-12, IFN- γ and IFN- γ -inducing factor (IL-18) (Seder *et al.*, 1993), (Bellinghausen *et al.*, 1999). IL-12 is the most potent Th1 inducing cytokine, produced by DCs and macrophages (Gately *et al.*, 1998). IL-12 enhances

proliferation, cytotoxic function by T cells, B and NK cells and importantly IFN- γ secretion (Takeda *et al.*, 1998). CD4⁺ Th1 cells are primed for IFN- γ production and further expansion of Th1 subtype by both IL-12 and IL-12-induced IFN- γ . The co-existence of IL-12 and functional IL-12 receptor (IL-12R) are essential for the development of competent Th1 cells. IFN- γ enhances IL-12R β 2 expression (Szabo *et al.*, 1997) and sequential exposure of macrophages to both IL-12 and IFN- γ improves their *in vivo* APC function (Grohmann *et al.*, 2001). Myeloid DC and murine macrophages express a surface receptor for IL-12 and IL-12R β 1. IL-12R β 2 genes in macrophages are identical to those expressed by T cells (Grohmann *et al.*, 2001). Similarly, the differential expression of the IFN- γ receptor on Th1 and Th2 cells regulates the ability of Th subsets to respond to cytokines, however, prolonged expression of IFN- γ R2 inhibits Th1 differentiation (Tau *et al.*, 2000). Studies have suggested early IFN- γ is produced by a small subset of CD8⁺ T cells, which is not dependant on MHC class II 1b molecules (Das *et al.*, 2001). IFN- γ initiates and enhances IL-12 effects in macrophages and the Th1 response is stabilized by an autocrine IL-12 positive feedback loop. IFN- γ also stabilizes the Th1 phenotype by suppressing further IFN- γ production by Th1 cells (Zhang *et al.*, 2001).

Th1 responses are most effective in mediating protection against intracellular pathogens. IL-12 is therefore considered to play a key role in the resolution of intracellular infections as it potentiates the development of Th1 cells. Susceptibility to intestinal parasite *Tichuris muris* is promoted by attenuation of the Th1 response through elimination of IL-12 dependant IFN- γ production, indicating a compulsory role for IL-12 in the establishment of a Th1 type response (Bancroft *et al.*, 1997). Protection against progressive *Leishmania major* infection in susceptible BALB/c mice was demonstrated by IL-12 treatment at the time of infection enhancing IFN- γ and diminishing IL-4 production (Heinzel *et al.*, 1993). Therefore, IL-12 may promote Th1 development indirectly through IFN- γ induction, however, whether IFN- γ directly promotes Th1 differentiation during primary Th stimulation remains controversial. Reduction of IFN- γ producing cells in a culture of naïve T cells occurred following addition of anti-IFN- γ antibody (Macatonia *et al.*, 1993). Similarly, the effects of IL-12 in mediating the development of a chronic parasitic infection in mice, was abrogated with anti-IFN- γ antibody (Bancroft *et al.*, 1997). In

contrast, induction of NK cell-derived IFN- γ in a pulmonary infection model indicated that IFN- γ can independently regulate the development of Th1 cells (Mountford *et al.*, 1999). Thus, IL-12 can act directly on Th cells and cause them to secrete IFN- γ or indirectly through NK cell-derived IFN- γ . In addition, IFN- γ and IL-12 can indirectly promote Th1 differentiation by inhibition of IL-4 production. During infection with *L major* in IL-12p40^{-/-} mice, IL-12 was crucial for generation and maintenance of effector Th1 cells (Park *et al.*, 2000). Similarly, maintenance of vaccination-induced immunity to *L major* is mediated by IL-12 (Gurunathan *et al.*, 2000).

IL-18 was first described in 1989 as IFN- γ inducing factor (Nakamura *et al.*, 1989). IL-18 synergizes with IL-12 to promote Th1 subset development and enhance IFN- γ production and NK cells responses (McInnes *et al.*, 2000). Murine Th1 and not Th2 clones express IL-18 receptor (Hunter and Reiner, 2000). IL-12 treatment of T cells, up-regulates IL-18 receptor (IL-18R) and augments IFN- γ production (Yoshimoto *et al.*, 1998). However, Th2 responses may be promoted by IL-18 in the absence of IL-12 (McInnes *et al.*, 2000). Recent evidence has shown IL-18R is downregulated by IL-4 implicating the regulation of IL-18R as an important factor in Th differentiation (Smeltz *et al.*, 2001).

1.3.3 FACTORS INFLUENCING DEVELOPMENT OF TH2 CELLS

The development of CD4⁺ Th2 subset from naïve precursors is regulated primarily by IL-4; evidence for this has been provided by studies using IL-4^{-/-} knockout mice (Kopf *et al.*, 1993). Although Th2 cells are a major producer of IL-4, the early source of IL-4 remains unclear. However, naïve CD4⁺ T cells have been considered as initial producers of IL-4 that can lead to induction of Th2 polarization in the absence of IFN- γ and IL-12 (Noben-Trauth *et al.*, 2000), (Gollob *et al.*, 1996). A number of different cell types including mast cells, basophils, eosinophils and NK1⁺ T cells have been suggested as other sources of early IL-4. Stimulation of NK1⁺ T cells through the TCR triggers rapid production of IL-4 (Kawamura *et al.*, 1998). Cells producing Th2 cytokines retain surface expression of IL-4 receptor (IL-4R) and lose responsiveness to IL-12R β 2 (Kourilsky and Truffa-Bachi, 2001). However, studies involving transgenic mice expressing IL-12 β 2 revealed that combined IL-4 and IL-12 signaling induced Th2 differentiation (Nishikomori *et al.*, 2000).

The importance of STAT6 for IL-4 signaling is demonstrated in mice deficient in the STAT6 gene where IL-4 mediated processes are inhibited (Takeda *et al.*, 1996). However, STAT6-independent pathway associated with expression of GATA-3 can also lead to Th2 development (Ouyang *et al.*, 2000). Furthermore, pathogenic infection in STAT6^{-/-} and IL-4R^{-/-} deficient mice, provide evidence of a population of CD4⁺ T cells with the capacity to generate Th2 responses (Jankovic *et al.*, 2000), (Finkelman *et al.*, 2000). IL-13 transgenic and IL-13-deficient mice have shown that IL-13 is an antagonist of protective Th1 responses and promotes Th2 responses during *L major* infection (Matthews *et al.*, 2000). In addition, IL-13 and IL-4 mediated functions of macrophages depend on identical signaling pathways (Takeda *et al.*, 1996). Human IL-11 has been found to block Th1 polarization through IL-12 inhibition and therefore induces Th2 polarization of human CD4⁺ T cells (Curti *et al.*, 2001).

1.3.4 NATURE AND DOSE OF ANTIGEN

There is evidence to suggest that the nature, dose and availability of antigen can affect the strength, duration and commitment of CD4⁺ T cells to polarized pattern of cytokine production. Prolonged exposure of antigen to the immune system following pathogenic insult promotes a stronger and more persistent T cell response. Furthermore, the time period of CD4⁺ T cell differentiation also plays a major factor in Th1/Th2 differentiation (Rogers and Croft, 1999). A universal model of differentiation based on antigen dose is contradicted by studies demonstrating that both low and high antigen dose favor Th2 responses. However, *in vitro* evidence implicated antigen dose as an important regulator of T cell effector functions where a wide range of antigen doses can switch immune responses between Th1 and Th2 polarization (Mosmann and Sad, 1996).

An early *in vitro* model demonstrated that high and low concentrations of antigen primed DTH responses, whereas moderate concentrations stimulated antibody production (Parish and Liew, 1972). Continuous release of low dose pathogenic antigen polarizes toward a Th2 phenotype (Guery *et al.*, 1996), (Rask *et al.*, 2000). Intranasally delivered HIV reverse transcriptase (RT) induced Th1 responses or antibody production dependant on administration of low or high dose of recombinant RT respectively (Pacheco *et al.*, 2000). Recently it has been proposed that high doses of antigen induce Th1 differentiation mediated by up-regulation of CD40 ligand

(CD40L), whereas CD40L induction is not stimulated with low doses (Ruedl *et al.*, 2000). In contrast to these studies it has also been demonstrated that BALB/c mice primed with ovalbumin (OVA) antigen in a murine model of asthma, induce high IgE/low IgG2a (Th2 profile) at low doses of OVA, whereas low IgE/high IgG2a were induced at higher OVA doses (Sakai *et al.*, 1999). In addition, the influence of antigen dose on Th development is also dependant on the mouse strain, where BALB/c mice are primed toward Th1 and C57BL/6 mice toward Th2 polarization at low antigen dose (Morokata *et al.*, 2000). It is also possible that recognition of peptide by different class II MHC molecules may lead to distinct cellular signals toward Th1 or Th2 differentiation (Svirshchevskaya *et al.*, 2001).

The dose of antigen yields contradictory conclusions, however, the nature of antigen may provide the key difference. A recent study on anterior chamber-associated immune deviation (ACAID), the mechanism of protection in the eye, demonstrates that varying antigen form influences the development of different Th subsets. Soluble but not particulate antigen induced ACAID, which is associated with IL-10 production. Therefore, it appears that the nature of the antigen affects the induction of Th1 and Th2 type responses through interactions with APC (D'Orazio and Niederkorn, 1998). Structural components of antigen alter and influence the selection of T cell responses, as chemical modifications of antigens have been reported to influence the development of a particular Th cell subtype. Chemical treatment of OVA produced a particulate form of the antigen, which induced a Th1 response compared to the Th2 induction favored by the soluble form (Yang *et al.*, 1993).

1.3.5 ROUTE OF ADMINISTRATION

The route of antigen administration influences the differentiation of the Th cell phenotype and has important implications for protection against infection and vaccine development. Intramuscular immunization with a plasmid encoding HA from influenza virus generated a potent Th1 response and protection following influenza virus respiratory challenge (Johnson *et al.*, 2000). However, DNA vaccines administered by gene gun induce Th2 responses (Vahlsing *et al.*, 1994), (Tuting *et al.*, 1998). Intraperitoneal and subcutaneous administration of a pertussis protein subunit vaccine induced predominant Th1 and Th2 responses respectively (Barnard *et al.*, 1996). Furthermore, mucosal immunization with pertussis antigens favors the

induction of Th2 cells (Conway *et al.*, 2000). Induction of specific T cell responses may reflect the role played by APC or the type of immune cells present at the site of injection (DeKruuff *et al.*, 1992), (Finkleman *et al.*, 1990), (Girad and Springer, 1995). Lymphocyte infiltration is determined partially by the actions of distinct chemokines. Recently characterized MDC/STCP-1 chemokine is involved in chemotaxis of activated T cells, NK cells, monocytes and monocyte-derived DCs (Nelson and Krensky, 1998).

1.3.6 CO-STIMULATORY MOLECULES

T cell activation requires TCR signaling through CD3-associated TCR $\alpha\beta$ complex binding to peptide-MHC on the surface of the APC. TCR engagement provides the first activation signal to the naïve T cell. Optimal activation requires a second antigen-independent signal referred to as co-stimulation for T cells to proliferate and produce IL-2. The absence of this second signal results in a state of non-responsiveness or T cell anergy (Mueller *et al.*, 1989). Therefore, the development of both Th1 and Th2 cells is dependent on co-stimulation. The B7 family is the most characterized of the co-stimulatory molecules. They interact with specific ligands (CD28 and CTLA-4) in a two-signal model of T cell activation (Chambers, 2001).

The B7 family of co-stimulatory molecules includes B7-1 (CD80), B7-2 (CD86) and B7H2 among others. These structurally similar proteins are expressed almost exclusively in lymphoid tissues on professional APCs including monocytes, DC and activated B cells. Expression of B7-1 and B7-2 is induced by APC activation signals, however there are significant differences in the kinetics of expression by different co-stimulatory molecules (Hathcock *et al.*, 1994). B7-2 is rapidly modulated, whereas B7-1 is induced slowly and expressed for longer periods. Lenschow *et al.*, (1995) demonstrated that B7-1 and B7-2, in some systems, differentially regulate the differentiation of distinct Th subsets. Studies reveal that B7-2 expression during priming may direct T cell activation towards a Th2 phenotype (Bottomly and Constant, 1997). However, others have reported that these co-stimulatory molecules can result in the opposite effect or have no effect on Th1/Th2 differentiation (Lanier *et al.*, 1995), (Lenschow *et al.*, 1995).

CD28 is the prototypic T cell co-stimulatory molecule found on both resting and activated T cells. CD28-mediated co-stimulation influences the generation of effective

T cell responses. CD28 ligation facilitates T cell responses, IL-2 secretion, proliferation and generation of cytotoxic activity (Chambers, 2001), (Zhang *et al.*, 1997). It has been suggested that CD28^{-/-} deficient mice can initiate T cell activation through co-stimulatory signals provided by DC (Cassell, 2001). Therefore other co-stimulatory molecules also function in T cell activation. Cytotoxic T Lymphocyte antigen 4 (CTLA-4) is upregulated on TCR-CD28 engagement. CTLA-4-B7 interaction reduces T cell activation and inhibits IL-2 synthesis, thus regulating Th cell differentiation. In the absence of CTLA-4, naïve T cells differentiate into Th2 cells. The polarization of Th differentiation is not due to the strength of TCR interaction as the effect is observed over a wide range of antigen doses (Oosterwegel *et al.*, 1999). Another co-stimulatory molecule, inducible co-stimulator (ICOS), also provides co-stimulatory signals to T cells. This enhances T cell proliferation and influences T cell cytokine production favouring the induction of Th2 type cells (Dong *et al.*, 2001).

CD40-CD40L interactions are essential for initiation and regulation of antigen-specific T cell responses and activation of B cells (Grewal and Flavell, 1996), (Brenner *et al.*, 1997). CD40L is rapidly induced on T cells following activation, whereas CD40 is expressed on DC, macrophages and activated B cells. The CD40-CD40L interaction is important for T cell dependent induction of IL-12, a cytokine known to be crucial for Th1 development (Koch *et al.*, 1996). It could be presumed that such an interaction is partially responsible for Th1 differentiation, however the effects of CD40-CD40L are bi-directional as CD40L-transduced co-stimulation induces naïve T cell production of both Th1 and Th2 cytokines (Peng *et al.*, 1996). Therefore this interaction both amplifies the T cell responses, while the cytokines appear to regulate the T cell activation and differentiation.

The CD4 activation antigen, OX40, is expressed on activated T cells, while OX40L is expressed on APC and plays a role in directing Th differentiation and reactivation of memory T cells (Takasawa *et al.*, 2001). Up-regulation of OX40 by the CD28 signal facilitates synergism between the two molecules to induce rapid expansion of CD4⁺ T cells and induction of Th2 cytokines (Akiba *et al.*, 2000).

1.3.7 ANTIGEN PRESENTING CELLS

Th cell differentiation is influenced by a number of factors that are non-mutually exclusive, however, the type of APC present at the site of T cell activation plays a major role in determining Th1/Th2 commitment. Macrophages, DC and B cells function as APC, providing the naïve T cell with antigen-specific and co-stimulation activation signals. Early studies demonstrated that both macrophages and DC can induce Th1 development, whereas B cell preferentially stimulated Th2 cell development, suggesting that Th1 and Th2 cells are activated in response to distinct APC (Gajewski *et al.*, 1991).

Cytokines produced by APCs can regulate the differentiation of precursor T cells towards either Th1 or Th2 phenotype. The local cytokine milieu may be insufficient to polarize T cell development and therefore the cytokines secreted by APC play a key role in Th differentiation towards a specific T cell subtype. Macrophages are potent inducers of IL-12 and IL-18, which favor the development of Th1 cells (Macatonia *et al.*, 1993), (Doherty and Coffman, 1999). Macrophage-mediated Th1 proliferation can be inhibited by IL-10 (Macatonia *et al.*, 1993) and depletion of macrophages results in the development of a Th2 response (Brewer *et al.*, 1994) suggesting the importance of macrophages in Th1 differentiation. DCs are also capable of secreting IL-12 and stimulating Th1 cell development (Macatonia *et al.*, 1995). Distinct DC subsets have been identified that differentially regulate Th subtype differentiation. Murine DC can be subdivided into lymphoid (DC1) and myeloid (DC2) lineages, where both subsets express different levels of CD11c, CD11b and MHC class II. In addition, CD8 α is expressed as a homodimer on DC1 and is completely absent on DC2. More importantly, DC2 secretes less IL-12 compared to DC1 and does not support IL-12-mediated IFN- γ production. Therefore, DC1 and DC2 cells influence the differentiation of Th1 and Th2 cells respectively (Pulendran *et al.*, 1999), (Liu *et al.*, 2001), but this DC1/DC2 theory is not fully accepted by all researchers in the field. IL-10 can exert negative effects on T cells through APC (Igietseme *et al.*, 2000). IL-10 indirectly down-regulates the expression of MHC class II and B7 on macrophages by suppressing IL-12 production by CD40-CD40L interaction (Ding *et al.*, 1993), (Hino and Nariuchi, 1996). Furthermore, IL-10 secreted by DC can direct differentiation of naïve T cells toward the Tr1 subtype (Mc Guirk, P. *et al* manuscript in preparation).

1.4 MUCOSAL IMMUNITY

Mucosal defense mechanisms are important as they provide chemical and physical barriers preventing diverse pathogenic microbes invading the host. The mucosal immune system comprises of lymphoid tissue associated with mucosal surfaces (gastrointestinal, genitourinary and respiratory tracts) and external secretory glands. Local humoral and cell-mediated responses are associated with each compartment. The interaction of foreign antigen with local lymphoid tissue stimulates precursor B cells to secrete IgA antibodies. Secretory-IgA (s-IgA) is the most abundant antibody isotype in mucosal secretions found in the human digestive tract and milk. Antigens penetrate the epithelium layer and enter the mucosal-associated lymphoid tissues (MALT) by phagocytosis or pinocytosis by specialized microfold cells (M cells) of the Peyer's Patches. These cells efficiently transfer intact antigen to lymphoid tissue containing T and B lymphocytes and APCs. Unprocessed antigen, available for B cell recognition, facilitates the induction of antigen specific IgA (McCluskie and Davis, 1999), (Almeida and Alpar, 1996). DCs and macrophages may also migrate to regional lymph nodes and process antigen where they can present it to T cells and contribute to the enhancement of the immune response. Activated B cells further differentiate, prior to circulating through the common mucosal system, and eventually home to various mucosal surfaces and exocrine glands to produce antigen-specific IgA. Immune complexes are formed when antigen specific IgA binds foreign antigen and prevents further interaction of the antigen with the mucosal surfaces, ultimately leading to immune exclusion.

Generation of effective mucosal immunity against pathogenic microorganisms may not be possible with traditional methods of parenteral immunization. Therefore, targeting recombinant protein or DNA vaccines to mucosal surfaces is currently being explored. For example, mucosal transgene vaccination with a DNA vaccine coding for an M cell ligand directs the vaccine to the inductive tissues of the respiratory tract and generates an effective mucosal-mediated immune response (Wu *et al.*, 2001).

1.5 VACCINATION

Vaccination is the most effective and versatile means of combating infectious disease by pathogens. Lady Mary Montague spread the practice of Chinese variolation that protected against smallpox by intranasally administering lesion scabs from an infected individual. Then in 1796, Edward Jenner of England observed that milkmaids were unaffected by smallpox. He challenged his theories and infected a young boy with cowpox and later with smallpox but no symptoms occurred. He had of course stumbled across an immunogen for smallpox. Vaccination during the last century has been extremely beneficial in reducing infectious disease morbidity, mortality, suffering and healthcare costs. The greatest triumph has been the global eradication of smallpox announced by the World Health Organization (WHO) during the 1980's. A worldwide effort to eradicate poliomyelitis is underway and complete elimination is expected by the year 2003. Most of our current vaccines are based on attenuated or killed microorganisms and were designed with little knowledge of their mechanism of action or potential side effects. Breakthroughs in immunology and genomics offer prospects to define the protective mechanisms against infectious pathogen components and to design safe and effective vaccines using a more rational approach. Recent negative media coverage relating to vaccine issues is weakening public acceptance of vaccination. However, future growth of vaccine technology is certain, as vaccination represents the most cost effective medical intervention available to prevent infection and subsequent need for expensive therapy. All currently licensed vaccines are prophylactic, with the exception of a rabies vaccine, but clinical trials of therapeutic vaccines are underway.

1.5.1 VACCINE DESIGN

Understanding the mechanisms that regulate antibody and T cell induction is important for the manipulation of the host immune response and subsequent vaccine design. Successful vaccination relies on the administration of antigenic material that mimics natural infection and induces an appropriate protective immune response against the infectious pathogen. The immunogenicity of a vaccine is influenced by a variety of factors such as route of administration, dose, immunization schedule, type of antigen and presence of an adjuvant.

Safety and efficacy are among several important criteria for design of an effective vaccine. Potential side effects caused by toxicity or host incompatibility

must be carefully researched during pre-clinical development and in clinical trials. Efficacy should ideally reach 90% following vaccine administration, regardless of species or type of vaccine. The specific requirements vary according to the nature of the infecting organism. Pathogens such as poliovirus infect irreplaceable neuronal cells, so virus neutralizing antibodies are an essential element in its protective mechanism. However, some intracellular pathogens are more effectively targeted by antigen specific CD4⁺ T helper or CD8⁺ CTL responses. An ideal vaccine should provide host defense at the point of entry of the infectious agent, such as the mucosal route of entry. Sustained protection by generating long-lived immunological memory is essential and reduces the need for booster immunizations. Long-term immunity also allows reduction in circulating quantities of pathogen caused by herd immunity. If a vaccination program is relaxed, a resurgence of infection or reduction in the resistance of a population to the vaccine can arise. This was the case during the break up of the former Soviet Union, which witnessed a diphtheria epidemic, or withdrawal of the pertussis whole cell vaccine in Sweden during the 1980's and the end of the 1990's. The potential to develop multivalent combination vaccines would improve vaccine coverage and reduce the cost of vaccination. However, interference between multiple antigens can hinder the development of multicomponent vaccines. Vaccines as a general rule must be genetically and thermally stable. In the case of attenuated vaccines there should be minimum possibility of reversion to virulence. Biological stability of a vaccine is often dependant on cold storage, which increases cost. Therefore, increased vaccine efficacy and reduced cost for developing countries would dramatically improve if vaccines could be developed that do not require cold storage.

1.5.2 CURRENT VACCINE STRATEGIES

The vaccine revolution has surged in the past 20 years due to significant improvements in technological and immunological approaches to vaccine design. Basic types of current vaccines include attenuated, inactivated, subunit and recombinant vaccines. A number of factors influence the decision to develop any one of these vaccines including minimal reactogenicity, a critical factor if the vaccine is targeted towards an infant population.

Attenuated vaccines are essentially a weakened form of the live pathogen, generated by selecting mutants that lack virulence and therefore possess reduced

ability to cause disease. Under-attenuation can result in the potential for reversion to a virulent strain of the pathogen. Targeting a gene that is non-essential for replication but important for virulence is a more rational approach. This was used to develop an attenuated typhoid vaccine. The gene should be completely deleted and other pathways that may facilitate the acquisition of functional DNA from other bacteria should be blocked. Clinical development of a cholera vaccine based on deletion is currently underway. Jonas Salk pioneered inactivated virus vaccines whereby pathogens are inactivated by heat or chemical means. Common problems with these vaccines include poor efficacy with short-lived protection and adverse reactions due to endotoxin in the bacteria. Vaccination against whooping cough using killed *Bordetella pertussis* vaccine (Pw) has been replaced by acellular vaccines (Pa) based on purified proteins from *B. pertussis* with considerably less side effects. A more sophisticated subunit vaccine was introduced in the 1920's against diphtheria and tetanus containing pure or semi-pure detoxified toxins. These vaccines have performed well in terms of safety and efficacy but the cost of purification can be extensive due to the low quantities of protective antigens produced by the infectious agent. However, it is more economical to generate bulk quantities that can be produced utilizing recombinant DNA technology.

Several viruses (Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) and HIV), bacteria (*Mycobacterium leprae*) and parasites (*Plasmodium*) pose a problem in vaccine development due to the difficulty of *in vitro* propagation. Genetic technology allows the transfer of genes from these agents to easily grown bacteria and yeast where recombinant products can be expressed. A prime example is the surface protein of HBV, produced in yeast cells and prepared as a protective vaccine. However, the native conformation of the recombinant product may not always mimic the natural conformation, which can pose problems in the induction of functionally relevant antibodies.

1.6 DNA VACCINATION

DNA vaccines were developed from observations that direct i.m. injection of plasmid DNA encoding reporter genes could induce protein expression in muscle cells (Wolff *et al.*, 1990). It was then demonstrated that a plasmid encoding human growth hormone could elicit antigen-specific antibody responses in mice (Tang *et al.*, 1992). In 1994, at a meeting of the WHO working group on DNA vaccines, it was decided that DNA vaccines would be the definitive terminology for this novel technology (Robinson, 1997). DNA vaccines provide several important advantages over conventional attenuated, inactivated and protein vaccines (Barry and Johnston, 1997). The structure and antigenicity of the encoded antigen mimics protein produced during natural infection, while other vaccine strategies, especially purified recombinant proteins that undergo modification, may distort the protein conformation. They induce antigen-specific cellular responses comparable to those obtained with live vaccines but without the risk of reversion to virulence. The rapid and cost-effective manufacture process, in conjunction with its independence from cold storage, confers a significant advantage on DNA vaccines over other conventional vaccine approaches.

1.6.1 BASIC ELEMENTS OF A DNA VACCINE

The gene coding for the foreign antigen of interest is preceded by a promoter region which in many cases is the cytomegalovirus (CMV) early promoter sequence required to drive transcription of the encoded antigen, a 3' polyadenylation region for mRNA stability, an antibiotic resistance gene (e.g. ampicillin or kanamycin) to select for transformed *E.coli* bacteria and an origin of replication to allow amplification of the plasmid in the bacteria.

The plasmid may be isolated and purified by various techniques including CsCl density gradient centrifugation and affinity chromatography using tagging i.e. GST and His₆ (Spier, 1995), (Gregoriadis, 1998) or endotoxin-free plasmid purification kits commercially available for laboratory research. Commercial plasmid DNA manufacture process includes a final size-exclusion chromatography (SEC) step, whereby stable supercoiled plasmid is separated from relaxed, linear or denatured plasmid. This is a crucial step as plasmid DNA must be supercoiled to generate a successful DNA vaccine (Gregoriadis, 1998). This purification process also facilitates exclusion of low molecular weight contaminants such as RNA, protein and

endotoxins (Ferreira *et al.*, 2000). Purified plasmid or naked DNA is then dissolved in a simple saline solution and can be administered to the host via i.m. injection or other routes of delivery (Donnelly *et al.*, 1997). Once the DNA vaccine is administered and enters a cell, expression of foreign antigen is initiated in a slow controlled manner and generates antigen-specific immune responses.

As immune response generated *in vivo* reflects the level of gene expression, vector components are carefully selected to yield optimal *in vivo* expression following DNA vaccination. Modification of plasmid backbones or optimization of gene regulatory components can improve gene expression. Selection of a strong promoter region favors virally derived promoters over eukaryotic derived promoters. The CMV enhancer-promoter region consistently induces high transgene expression and positioning Intron A, the first intron of the CMV promoter, further enhances gene expression (Norman *et al.*, 1997). Inclusion of transcription termination signals also influences expression efficiency.

1.6.2 CONTRIBUTION OF CpG MOTIFS THROUGH TOLL RECEPTOR SIGNALING

DCs and macrophages that function as APC for T cells have evolved a set of non-clonal pattern recognition receptors (PRR), which bind to conserved structures associated with microbial pathogens including collectins, CD14, Toll-like receptors (TLR), mannose binding protein, serum amyloid P, complement receptors, C-reactive protein, Fc receptors, CD11b, CD11c, CD18, and DEC 205 (Fig 1.2) (Medzhitov and Janeway, 1997). The TLR family is composed of conserved proteins essential for specific recognition of pathogenic ligands. The discovery of *Drosophila* TLRs triggered the search for mammalian homologues and to date ten TLRs are reportedly expressed in humans. These receptors are strategically expressed on surface epithelia and APC that encounter pathogens during early stages in infection (Medzhitov and Janeway, 1997). Murine TLR9 was recently identified as the receptor for unmethylated CpG DNA, whereas LPS mediated signal transduction is TLR4 dependant (Hemmi *et al.*, 2000), (Beutler, 2000). Human TLR9 requires distinct CpG motifs for signal transduction suggesting a species-specific recognition of immunostimulatory CpG motifs (Bauer *et al.*, 2001). It has been demonstrated that CpG-DNA induced Th1 responses are abrogated in TLR9^{-/-} mice (Hemmi *et al.*, 2000). Signal transduction triggered by microbial molecules binding to TLRs, is dependant on a common adaptor, protein myeloid differentiation factor 88 (MyD88)

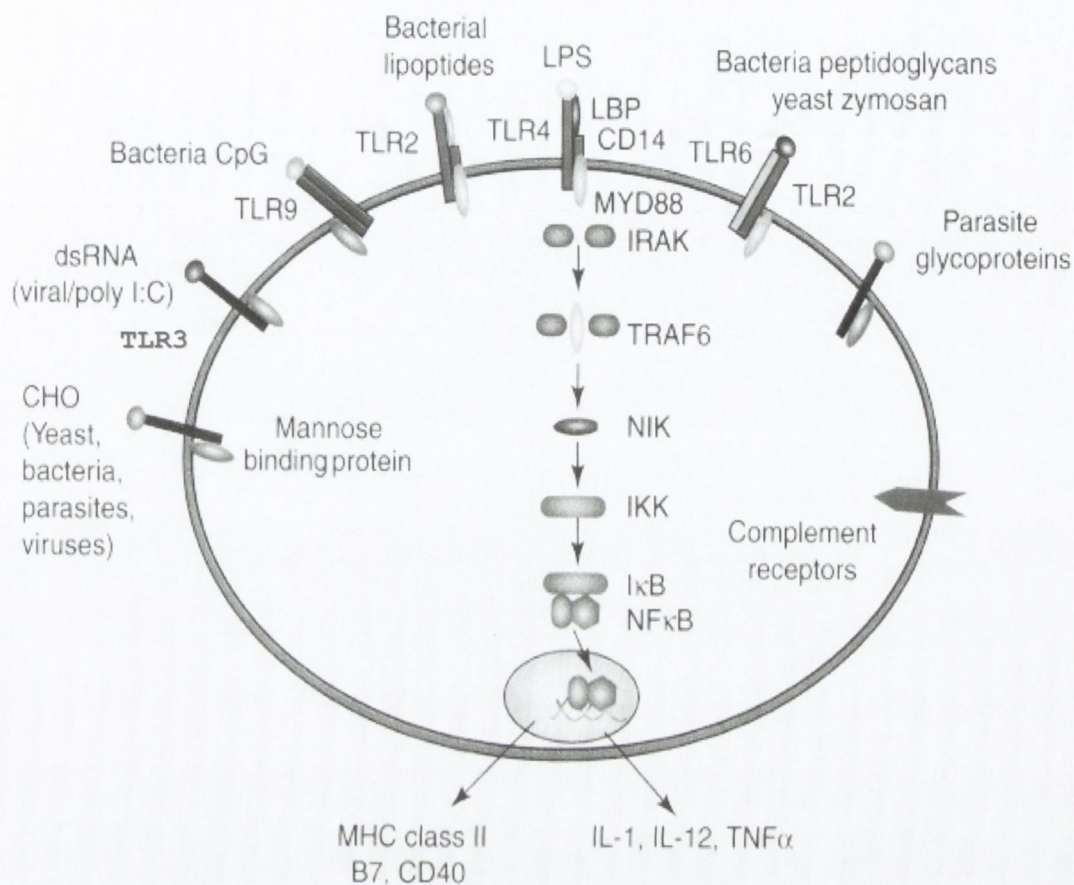


Figure 1.1 Toll-like receptor signaling pathway through pathogen-derived immunomodulatory molecules. CpG motifs present in bacterial DNA and DNA vaccines constructed using bacterial plasmids can trigger signal transduction through the newly identified TLR9 receptor (Hemmi *et al*, 2000). Taken from Ryan *et al*, (2001).

and nuclear factor κ B (NF κ B) (Kopp and Medzhitov, 1999), (Hatada *et al.*, 2000). DC cytokine production and maturation in response to CpG-ODN are MyD88-dependant (Kaisho and Akira, 2001), (Jankovic *et al.*, 2001). Furthermore, mice deficient in DNA-protein kinase catalytic subunit (DNA-PKcs) demonstrated a lack of CpG-ODN-mediated induction of proinflammatory cytokines. Therefore, NK- κ B signal transduction is dependant on CpG-ODN activation of DNA-PKcs (Chu *et al.*, 2000).

Microbial ligation of PRR on APC enhances the presentation of antigen and subsequent stimulation of T cell activation. CpG motifs have been shown to act as adjuvants for Th1 responses through DC1 maturation and induction of IL-12 and inflammatory chemokine secretion, both necessary for priming of Th1 cells (Behboudi *et al.*, 2000). *In vitro* CpG- oligodeoxynucleotides (ODN) can trigger TNF- α , IL-1 β , IL-6, IL-12, IL-10 cytokine and chemokine MIP-1 α , MIP-1 β and MIP-2 secretion from macrophages and TNF- α , IL-6 and IL-12 from bone-marrow derived DC (BMDC) and upregulation of surface markers associated with DC maturation (Sparwasser and Lipford, 2000), (Sparwasser *et al.*, 1998), (Lipford *et al.*, 1997). Furthermore, they can induce murine B cell activation (Yi *et al.*, 1996) and stimulate NK secretion of IFN- γ in an IL-12 dependant manner (Bohle *et al.*, 1999), which can enhance and favor Th1 cell differentiation and further IFN- γ production (Takeshita and Klinman, 2000). *In vitro* studies have demonstrated CpG-ODN mediated secretion of IL-12 from DC and therefore enhanced DC activation of MHC class-I restricted T cells (Warren *et al.*, 2000). Recently two structurally distinct groups of CpG-ODN have been identified and designated D, which stimulate NK cells to secrete IFN- γ , and K, which preferentially stimulate cell proliferation and secretion of IL-6 and IgM from monocytes and B cells (Verthelyi *et al.*, 2001).

CpG motifs have been shown to act as adjuvants for Th1 responses and can therefore improve the immunogenicity of plasmid DNA vaccines (Klinman *et al.*, 2000). However, methylation of CpG motifs results in a significant reduction in the immunogenicity of a DNA vaccine (Klinman *et al.*, 1997). Neutralizing CpG motifs present in adenovirus serotype 2 have been shown to inhibit immunostimulatory CpG Th1-inducing function (Krieg *et al.*, 1998). In addition to directly activating professional APCs, CpG motifs present in DNA vaccines were found to induce chemokine and IFN- γ secretion leading to upregulation of MHC class II on muscle

cells, which could efficiently present to CD4⁺ T cells *in vitro* (Stan *et al.*, 2001). CpG-ODN act as potent adjuvants and have been shown to induce Th1 systemic immune responses following i.m, i.p. or i.n. administration of protein antigens (Davis *et al.*, 1998), (Klinman *et al.*, 1999), (McCluskie *et al.*, 2000). However, further enhancement in the immunogenicity of plasmid DNA vaccines is not possible by coadministering CpG-ODN as an adjuvant, as competition for binding sites on APC reduces antigen expression from plasmid DNA (Weeratna *et al.*, 1998). Similarly, immunostimulatory sequences and plasmid DNA co-precipitated onto gold particles, failed to enhance DNA induced specific CTL responses (Oehen *et al.*, 2000). However, mice pretreated with bacterial DNA or CpG motifs, were protected from intracellular infection with *Francisella tularensis* live vaccine strain and *Listeria monocytogenes* (Elkins *et al.*, 1999). CpG-ODN can enhance local and systemic antibody responses following mucosal immunization with tetanus toxoid or influenza virus vaccines. It has also been shown that non-CpG "control" ODN had adjuvant effects when delivered at mucosal sites which was unexpected since non-CpG ODN are not immunostimulatory *in vitro* or after parenteral immunization (McCluskie and Davis, 2001).

CpG-ODN directed Th1 cell activation has several potential therapeutic applications. Vaccines formulated with CpG may prevent initiation of a potentially harmful Th2 response associated with allergy and anaphylactic shock. Furthermore, CpG as adjuvants can induce protective immune responses during murine models of infection with the intracellular pathogens where resistance to infection is associated with Th1 responses (Elkins *et al.*, 1999), (Al-Mariri *et al.*, 2001), (Oxenius *et al.*, 1999). Polarization towards Th2 responses is a major concern when developing neonatal vaccines as they are more prone to infections during early development and may require type-1 responses to clear infections cause by viruses. Nevertheless, it has been demonstrated that CpG-ODN can stimulate APC to trigger Th1 type responses to various antigens and override the neonatal Th2 polarization. However, established Th2 responses could not be shifted toward a Th1 response, revealing limitations in this approach for neonatal vaccination (Kovarik *et al.*, 1999). Therefore, CpG-ODN or CpG motifs present in plasmid DNA vaccines demonstrate potential as immune enhancers and therapeutic agents.

1.6.3 MECHANISM OF ANTIGEN PRESENTATION

The mechanism by which encoded antigen expressed by a plasmid DNA vaccine is processed and presented to the immune system has yet to be fully elucidated. The levels of protein expressed *in vivo* following DNA vaccination range between picogram (pg) and nanogram (ng) quantities and are significantly lower than protein levels in subunit or inactivated vaccines. Therefore, the efficiency of antigen presentation may be due to the type of APC transfected and/or adjuvant activity of immune enhancing CpG motifs present in bacterial DNA. Antigen processing and presentation following DNA vaccination may occur by three possible mechanisms: direct priming of somatic cells (muscle or skin cells), direct transfection of professional APC (DC) or cross-priming.

Cells directly transfected at the site of DNA injection can contribute to the generation of an immune response. Intramuscular and intradermal routes of DNA delivery are extensively employed, whereby muscle cells and keratinocytes are likely to become transfected with plasmid DNA. It has been demonstrated that intramuscular (i.m.) immunization can result in transfection of non-professional APC, such as muscle cells and generate protein expression and CTL responses (Wolff *et al.*, 1990), (Ulmer *et al.*, 1993). Skeletal muscle can directly take up plasmid DNA through T tubules and caveolae and persist for at least 30 days (Danko and Wolff, 1994). Furthermore, there is evidence to suggest that nonlymphoid cells can generate antigen for APC following i.m. injection of plasmid DNA (Corr *et al.*, 1999). Moreover, it has been demonstrated that CpG motifs present in DNA vaccines can induce chemokine and IFN- γ secretion leading to upregulation of MHC class II on myocytes, which can efficiently present to CD4⁺ T cells *in vitro* (Stan *et al.*, 2001).

In contrast, there is abundant evidence to support the role of directly transfected professional APC during DNA vaccination. DNA delivery into the muscle recruits APC following local inflammation; the muscle contains only a small population of professional APC (Tighe *et al.*, 1998). Torres *et al.*, (1997) demonstrated that removal of muscle bundles after i.m. injection had no effect on the immune response, however skin biopsy following gene gun immunization abrogated the response completely, indicating that APC such as keratinocytes were directly transfected. It has been suggested that the highly developed skin immune surveillance, mediated by specialized DCs, Langerhans cells, plays a major role in induction of

immune response following DNA immunization. Klinman *et al.*, (1998) carried out similar studies and found that immune responses were abrogated if vaccinated skin was removed, however the immune response improved the longer the skin remained intact, demonstrating the influence of the non-migratory cells on the magnitude of the immune response. Furthermore, plasmid DNA containing DCs and macrophages can be detected in draining lymph nodes and spleen of immunized mice (Bouloc *et al.*, 1999), (Akbari *et al.*, 1999).

Cross-priming occurs when antigen or peptides generated by somatic cells are taken up and presented by professional APCs. This mechanism was based on observations that exogenous sources of peptides could present through MHC class I molecules which were usually exclusive to endogenously derived antigen (Ulmer *et al.*, 1997), (Harding and Song, 1994). Somatic cells, including myocytes, keratinocytes or non-lymphoid cells have been shown to transfer antigen to professional bone marrow derived APCs (Ulmer *et al.*, 1996), (Corr *et al.*, 1999), (Akbari *et al.*, 1999), (Fu *et al.*, 1997). A detailed study examining the distribution of plasmid DNA after thigh muscle injection into mice revealed that labeled DNA could be detected near the site of injection in bundles of muscle cells, whereas mononuclear cells (MNC) gradually accumulated the labeled DNA in the draining popliteal lymph node. Expression of both CD80 and CD86 were detected on MNC but not muscle cells; muscle cells express MHC class I, but lack CD80 or CD86 co-stimulatory molecules that are key factors for priming of T cells. However transgene expression, determined by RT-PCR, was only found in muscle cells and not MNC. This suggests that non-professional cells are responsible for generating the antigen directly or for cross-priming (Gurunathan *et al.*, 2000). Despite clear evidence that DC are key players in initiating an immune responses following DNA immunization, somatic cells may mediate augmentation and maintenance of the responses by acting as reservoirs for antigen.

1.6.4 INDUCTION OF IMMUNE RESPONSES BY DNA VACCINATION

DNA vaccines have been shown to be potent inducers of both CD4⁺ and CD8⁺ T cells. Induction of CD4⁺ T cells have three major functions: promotion of B cell survival and antibody production, activation of macrophages and other elements of cellular immunity and as helper function for CD8⁺ T cells. CD8⁺ CTLs are required for clearance of many intracellular infections. DNA vaccines are effective at inducing

CD8⁺ CTL and CD4⁺ Th1 cells that play a major role in protection during a number of intracellular infections (Johnson *et al.*, 2000), (Leclerc *et al.*, 1997), (Gurunathan *et al.*, 1997). As previously discussed, this is predominantly due to immunostimulatory CpG motifs present in the bacterial plasmid backbone that trigger IL-12 secretion from APC and generation Th1 responses (see section 1.6.2). There are numerous studies demonstrating Th1-type immune response induction following DNA immunization including: Needle-free PowderJect delivery of a HBV DNA vaccine induced IFN- γ producing CD4⁺ T cells in hepatitis-naïve human volunteers (Roy *et al.*, 2000). Intradermal immunization with a plasmid encoding β -gal of Th2 immune polarized *Schistosoma*-infected mice was found to generate a strong β -gal-specific type-1 response (Ayash-Rashkovsky *et al.*, 2001). Similar type-1 IFN- γ production was produced following i.m. immunization with a plasmid co-expressing hepatitis D and B surface antigens (Huang *et al.*, 2000). Furthermore, DNA vaccines can also generate Th2 responses dependant on the type of antigen and route of immunization (see section 1.6.6).

DNA vaccines have been assessed in a number of animal models and in clinical trials. Mice immunized with a DNA vaccines encoding HIV-1 *env* gp120 induced strong antigen-specific antibody responses, IFN- γ cytokine secretion and CD8⁺ CTL (Shiver *et al.*, 1997). Furthermore, HIV-1 regulatory genes expressed by plasmid DNA were shown to induce HIV-1 specific CTL responses composed predominantly of MHC class I restricted CD8⁺ T cells (Calarota *et al.*, 1998). In the progression to non-human primates, HIV-1 *env* plasmid DNA vaccine generated IFN- γ -secreting MHC class II-restricted CD4⁺ T cells in a rhesus monkey model (Lekutis *et al.*, 1997). These promising results were not translated to the first human clinical trial of plasmid DNA encoding HIV-1 *env* and *rev* genes which induced weak specific CTL activity (MacGregor *et al.*, 1998). DNA vaccination has been effective at inducing immune responses against influenza virus antigens. Influenza nucleoprotein (NP) plasmid DNA administered intramuscularly generated NP specific CTL responses (Corr *et al.*, 1996) and hemagglutinin (HA) encoded plasmid induced strong Th1 responses in mice (Johnson *et al.*, 2000). DNA vaccines have also been shown to confer protection in a number of disease models. BALB/c mice injected with the plasmid DNA coding for the surface glycoprotein, gp63, of *L. major* developed resistance to infection mediated by production of antigen specific IL-2 and IFN- γ (Xu

and Liew, 1995). Furthermore, CD8⁺ mediated protection was induced against malaria in a murine model following i.m. immunization with circumsporozoite protein encoded by a plasmid DNA (Sedegah *et al.*, 1994).

Immunization with plasmid DNA has proven to be an effective means of inducing humoral responses specific for viral, bacterial and parasitic encoded proteins (Strasser *et al.*, 2000), (Tanghe *et al.*, 2000), (Lobo *et al.*, 1999). Mice immunized with plasmid DNA encoding the surface glycoprotein gp120 of HIV, developed strong gp120-specific antibody responses (Shiver *et al.*, 1997). DNA vaccination of mice with the Pfs25 gene of *Plasmodium falciparum* generated potent transmission-blocking antibodies (Lobo *et al.*, 1999). Furthermore, a tuberculosis DNA vaccine composed of secreted protein, Ag85B, induced high titers of antigen-specific IgG (Kamath *et al.*, 1999). The induction of neutralizing antibodies is indicative of correct native conformational protein expression by the DNA vaccine. Influenza virus hemagglutination inhibition (HI) antibodies have been detected in rhesus macaques immunized with HA encoding DNA. The macaques were protected from wild-type influenza virus challenge and protection correlated with induction of antigen specific neutralizing antibodies (Polack *et al.*, 2000).

1.6.5 INDUCTION OF MUCOSAL IMMUNITY WITH DNA VACCINES

DNA vaccines have been shown to be effective at eliciting strong systemic responses and protection in a variety of viral, bacterial and parasitic preclinical infection models. The favored route of DNA delivery is by i.m. injection which generates systemic T cell and antibody responses (Gurunathan *et al.*, 2000) but has been reported not to enhance local IgA production or to protect against an entero-invasive pathogen at the mucosal surface (Noll *et al.*, 1999). Therefore, considerable interest has been generated in targeting DNA vaccines to respiratory, digestive and genitourinary tracts to enhance the local immune responses at mucosal surfaces.

It has been reported that nasal immunization of mice with plasmid DNA encoding for a 15kDa antigen of *Cryptosporidium parvum* induces systemic and intestinal antibody responses (Sagodira *et al.*, 1999). Furthermore, IgA responses were detected in lung and vaginal washes following intranasal instillation with a DNA vaccine encoding HIV antigens (Asakura *et al.*, 1999). Following intravaginal delivery of a similar DNA vaccine to pregnant chimpanzees, anti-HIV-1 IgG was detected in the saliva, vaginal and rectal washes and IgA was detected in the milk

(Bagarezzi, 1999). Although naked DNA can induce immune responses at mucosal surfaces, the immunogenicity does not appear to equal that observed by the intramuscular route. Consequently, novel injection methods and formulations have been used to enhance the efficacy of DNA vaccines delivered to mucosal surfaces. Targeted salivary gland immunization with plasmid DNA elicited specific salivary IgA and IgG (Kawabata *et al.*, 1999). DNA vaccine delivery using an intra-oral jet injection technique in the cheek has been shown to be a simple rapid way of administering DNA in solution and can induce specific IgA responses (Lundholm *et al.*, 1999).

1.6.6 FACTORS INFLUENCING INDUCTION OF IMMUNE RESPONSES WITH DNA VACCINES

Induction of an antigen specific immune response following DNA vaccination can be influenced by a number of factors including the plasmid backbone, nature of encoded antigen, CpG motifs, dose, route of administration and immunization schedule. A wide variety of routes for the delivery of DNA vaccines have been evaluated including i.m., intravenous, intradermal, subcutaneous, intraperitoneal (Fynan *et al.*, 1993), (Bohm *et al.*, 1998), non-invasive skin-targeted plasmid vaccination (Shi *et al.*, 1999), topical ocular administration (Daheshia *et al.*, 1998), intranasal (i.n.) (Klavinskis *et al.*, 1997) and gene gun (Vahlsing *et al.*, 1994), (Tuting *et al.*, 1998). A broad overview of studies to date suggests that, i.m. immunization induces a Th1 response but requires large quantities of plasmid DNA compared to a polarized Th2 response resulting from gene gun immunization, requiring just 0.1-1µg of plasmid DNA (Fynan *et al.*, 1993), (Gurunathan *et al.*, 2000). These distinct profiles may result from the effective delivery of gold or tungsten particles coated with plasmid DNA directly into the cell. Therefore these two methods may prime different DC subsets and trigger differentiation of distinct T cell subsets.

The type and magnitude of the immune response induced by DNA vaccination however, is influenced by the antigen type: secreted, membrane bound, or cytosolic bound. It has been reported that plasmid DNA encoding secreted or cell-associated antigens induced antigen-specific IL-4 and IFN-γ-secreting cells respectively (Lewis *et al.*, 1999). Activated T cells produce an array of cytokines that induce B cell proliferation and antibody production and antigen-specific IgG subclass induction is

indirectly influenced by antigen type, whereby secreted antigen generates predominantly IgG1 subclass and cytosolic antigen generates mixed IgG1/IgG2a subclass profile (Haddad *et al.*, 1998). Lewis *et al.*, (1999) examined an expression plasmid coding for membrane anchored, secreted or intracellular versions of the glycoprotein D from bovine herpes virus type-1 (BHV-1) and found that they all induced distinct serum IgG subclasses and T cell subsets in the lymph nodes. The type of antigen encoded by the plasmid affected T cell induction, with the intracellular protein generating the greatest level of antigen specific IFN- γ production .

DNA vaccination can be further improved by using recombinant proteins to boost immune responses primed with plasmid DNA (prime/boost immunization schedule). It has been demonstrated that priming with plasmid DNA and boosting with recombinant protein enhances antibody production, whereas reversal of this immunization schedule abrogated the enhancing effect (Barnett *et al.*, 1997). Immune responses induced with a DNA vaccine coding for structural and nonstructural proteins (capsid/E1/E2/NS2/NS3) from HCV was improved with a canarypox virus boost encoding the same HCV genes (Pancholi *et al.*, 2000). Similarly, macaques primed with a HIV-1 DNA vaccine and boosted with recombinant fowlpox virus induced dramatically stronger specific T helper and CTL responses and a concurrent decline in antigen specific antibody titers (Kent *et al.*, 1998). The vaccine regimen protected macaques from an intravenous HIV-1 challenge, with the resistance most likely mediated by T-cell responses. These studies suggest a safe strategy for enhancing the generation of T-cell-mediated protective immunity to HIV-1. Interestingly, the strength of the immune response following DNA priming is influenced by the route by which the boosting is administered, as demonstrated by studies where the intravenous route was significantly better than intradermal and i.m. routes (Schneider *et al.*, 1998).

1.6.7 ADJUVANTS AND DELIVERY SYSTEMS

Novel methods of vaccine delivery need to be developed whereby conventional or DNA vaccines can be optimized to induce strong long lasting immune responses and enhance immunogenicity by mucosal routes. Aluminum salts (alum) and MF-59 are currently the only vaccine adjuvants licensed for human use and may have limited efficiency for inducing protective immune responses against all pathogens.

Immunomodulators (CpG DNA), monophosphoryl lipid A (MPL), saponins, immune stimulating complexes (ISCOMS), cholera toxin (CT) and *E. coli* heat labile toxin (LT) containing conserved pathogenic sequences recognized by the innate immune system, can trigger an adaptive immune response to co-administered antigen or plasmid DNA. Furthermore, vaccine delivery systems (liposomes, polylactide-co-glycolide (PLG) microspheres, chitosan, or live attenuated vectors) can protect the antigen or plasmid DNA from host enzymatic degradation and increase antigen uptake by APC resulting in improved vaccine immunogenicity. A variety of these systems are effective for mucosal delivery of protein vaccines and have been successfully used to enhance the immunogenicity of mucosally delivered DNA vaccines (Gurunathan *et al.*, 2000).

Microparticles primarily composed of biodegradable and biocompatible polyesters such as poly-(lactic-coglycolic acid) (PLGA) or polylactic acid (PLA) have been used for oral delivery of encapsulated plasmid DNA. The particles prevent plasmid degradation upon entry into the cell, facilitate uptake by APCs and generate systemic and mucosal antibody responses (Jones *et al.*, 1997). Furthermore, mucosal immunization with proteins formulated in microparticles induced protective immunity in animal models of infection, such as *B. pertussis* (Conway *et al.*, 2000). Chitosan is a cationic biopolymer prepared by deacetylation of chitin. Oral administration of chitosan nanoparticles complexed to DNA encoding peanut allergen, resulted in transduced gene expression in the intestinal epithelium of mice, the induction of secretory IgA and serum IgG2a responses, and modulation of IgE and anaphylaxis in a murine model of allergen-induced hypersensitivity (Roy *et al.*, 1999). Several other methods for optimizing DNA delivery have been studied: virosomes (Cusi *et al.*, 2000), cocheltes (Gurunathan *et al.*, 2000), and attenuated organisms (Sizemore *et al.*, 1997), (Dietrich *et al.*, 2001).

When a DNA vaccine encoding gp160 env protein from HIV-1 was administered orally in PLG microparticles, virus specific Th1, CTL and IgA immune responses were induced and mice were protected against intrarectal challenge with HIV env-expressing recombinant vaccinia virus (Kaneko *et al.*, 2000). Formulation of DNA vaccines into liposomes (Okada *et al.*, 1997), lipid complexes (Klavinskis *et al.*, 1997), (Etchart *et al.*, 1997) or macroaggregated albumin conjugates (Orson *et al.*, 2000) is efficient at targeting DNA to mucosal surfaces, especially the respiratory tract. Furthermore, co-administration of DNA and cationic lipids or CT by the

intragastric route induced CTL responses against an encoded measles virus antigen (Etchart *et al.*, 1997). Bacterial vectors are also being explored for the mucosal delivery of DNA coding for heterologous antigens. Intranasal inoculation with attenuated *Shigella* vector containing a measles DNA vaccine induces a strong response of virus specific Th1 and CD8⁺ CTLs (Fennelly *et al.*, 1999). Another approach has been the use of replication-defective recombinant Semliki Forest Virus (SFV) expressing a variety of heterologous foreign antigens. These are highly immunogenic following systemic delivery and also generate potent immune responses by mucosal routes (Malone *et al.*, 1997).

1.6.8 LIPOSOMES

Liposomes are bilayered membrane vesicles consisting of amphipathic molecules such as phospholipids. Liposomes function as effective antigen delivery systems and exhibit immunoadjuvant activity and therefore represent an attractive alternative to other viral gene delivery systems. The ability of APCs to efficiently take up present liposome entrapped DNA may be enhanced by improvements in the liposome composition (Perrie *et al.*, 2001). Furthermore, liposomes protect DNA from host degradation and possess structural versatility to improve transfection (Gregoriadis, 1998). The addition of a disulphide bond to a DNA-liposome complex can enhance responses by inducing selective destabilization in the cytosol of the cell (Tang and Hughes, 1998). Moreover, combinations of liposomes can influence transfection efficiency, whereby DOGSDSO/DOPE liposome combination reportedly resulted in approximately 50 times greater transfection efficiency than DOTAP/DOPE (Smith, 1994).

It has been proposed that DNA/cationic liposome complexes enter the cell by associating with the cell surface and internalizing via endocytic pathway (Xu and Szoka Jr., 1996). Endosomal membranes become destabilized by the complex and disrupt anionic lipids located within the cell. Neutral ion pair formation between anionic and cationic lipids enables plasmid DNA displacement from the complex and facilitates entry to the cell cytoplasm, where expression of the encoded antigen can occur (Xu and Szoka Jr., 1996). It has been reported that i.m. and intraperitoneal administration of plasmid DNA formulated with cationic liposomes generates stronger immune responses compared to subcutaneous or intradermal routes (Ishii *et al.*, 1997). Moreover, incorporation of plasmid DNA into cationic lipids

DMRIE/DOPE can increase the duration of antigen expression (Barnfield *et al.*, 1997). As prolonged antigen-specific immune responses may be necessary to control HIV infection, it has been demonstrated that liposome/plasmid DNA combination could induce HIV-specific CTL response that remained detectable 4 months following immunization (Ishii *et al.*, 1997). Recent studies have reported injection of immunostimulatory CpG-ODN and OVA protein encapsulated in sterically stabilized liposomes into mice, generated a 2-fold increase in the duration of protective immune responses against pathogenic challenge and significantly enhanced both antigen-specific IFN- γ and IgG antibody titres (Gursel *et al.*, 2001).

Formulation with cationic liposomes has been shown to enhance the immunogenicity of i.m. delivered plasmid DNA with significantly increased antibody titres and long lasting CTL HIV-1 specific activity (Ishii *et al.*, 1997). Liposomes composed of a various lipid ratios and cholesterol can also increase the effectiveness of mucosally delivered vaccines. Various liposome formulations have shown stability in acidic solutions, bile and pancreatin solutions, which suggests that they may be suitable as oral vaccine delivery vehicles (Han *et al.*, 1997). The nasal administration of *Streptococcus mutants* antigen formulated into liposomes was shown to enhance local secretory IgA in human volunteers (Childers *et al.*, 1999). Recently, it was shown that intranasal immunization with liposome-formulated *Yersinia pestis* vaccine enhanced mucosal immune responses (Baca-Estrada *et al.*, 2000). Liposomes, however are most commonly used in combination with other adjuvants. The conjugation of recombinant CTB or CT to liposomes greatly enhances their effectiveness (Harokopakis *et al.*, 1998).

1.6.9 MODULATION OF IMMUNE RESPONSES INDUCED WITH DNA VACCINES

A distinct advantage of DNA vaccination, is the ease with which genes for immunomodulatory molecules can be included to both modulate and selectively enhance distinct arms of the immune response (Kim *et al.*, 1998), (Gurunathan *et al.*, 2000). Custom made vaccines composed of plasmids coding for Th1-directing immune molecules are beneficial in viral vaccine design where Th1 cells are of paramount importance. Herpes simplex virus (HSV) DNA plasmid administered with an IL-12 expression plasmid enhanced survival rate of animals challenged with a lethal dose of HSV-2, whereas co-administration with a Th2 cytokine plasmid (IL-4

or IL-10), resulted in increased mortality and morbidity (Sin *et al.*, 1999). Differentiation of the CD4⁺ T cell population following DNA vaccination can be enhanced by the co-delivery of GM-CSF and IL-2 expression plasmids, whereas the efficacy of DNA vaccines against extracellular bacteria can be improved by coadministering IL-4, IL-5 and IL-10 expression plasmids, that direct Th2 responses. A HBV DNA vaccine was shown to produce divergent Th1 and Th2 immune profiles, depending on the co-administered cytokine plasmid, where a Th1 response was induced following co-administration with IL-12 or IFN- γ plasmid and Th2 responses with IL-2 or GM-CSF plasmids (Chow *et al.*, 1998). Co-administration of HIV-1 or HSV plasmid with an IL-7 plasmid resulted in improved antigen specific Th2 and CTL responses respectively (Sin *et al.*, 2000). Furthermore, rhesus macaques generated enhanced HIV antigen-specific T cell-mediated immune responses following co-administration of expression plasmids coding for IL-2 and IFN- γ (Kim *et al.*, 2001). Intranasal immunization of a DNA vaccine in liposomes with plasmids encoding IL-12 and GM-CSF induces strong mucosal and cell-mediated responses against HIV-1 antigens (Okada *et al.*, 1997). Furthermore, coinjection of a plasmid coding for the chemokine, RANTES, dramatically enhanced HSV-specific Th1 type cellular immune responses and generated protective immunity from lethal HSV-2 challenge (Sin *et al.*, 2000).

Enhancement of co-stimulation molecule expression on APC can also be used to augment the induction of T cell responses following DNA vaccination. B7-1 and B7-2 co-stimulatory molecules interact with specific ligands in a two-signal model of T cell activation (Chambers, 2001). Co-linear expression of B7-2 co-stimulatory molecule and a mutant version of NP from influenza generated significantly enhancement of NP-specific CTL responses (Iwasaki *et al.*, 1997). CD40-CD40L interactions are also essential for initiation and regulation of antigen-specific T cell responses and activation of B cells (Grewal and Flavell, 1996), (Brenner *et al.*, 1997). Subcutaneous immunization of mice with a CD40L trimer plasmid improved T cell induction, CTL and IgG2a responses and could successfully control the parasitic infection induced with the *L. major* antigen (Gurunathan *et al.*, 1998). Similarly, cellular immune responses generated following mucosal administration of a HIV-1 DNA vaccine were enhanced by the inclusion of a plasmid expressing CD40L (Ihata *et al.*, 1999). This approach is also being used as a therapy for Th1-mediated

inflammatory diseases: mucosal administration of plasmid DNA encoding IL-10 was shown to inhibit Th1 cytokines in mice infected with HSV (Chun *et al.*, 1999).

1.6.10 SAFETY ISSUES

Despite several benefits of over conventional vaccines, there are a number of major safety concerns that require careful consideration during clinical evaluation of DNA vaccines. The Center for Biological Evaluation and Research (CBER) has produced guidelines for vaccine development and have reported a number of key concerns in DNA vaccine development (Smith, 1994). Safety concerns include integration of plasmid DNA into the host chromosome, activation of oncogenes or inactivation of tumor suppressor genes and the induction of autoimmunity and immunotolerance.

Integration of plasmid DNA may result from injected plasmid DNA persisting at the site of injection and other tissues for many months, and result in mutagenesis through activation of an oncogene or inactivation of tumor suppressor genes. Furthermore, integrated DNA could also lead to chromosomal breaks and rearrangements, which have the potential to cause mutagenesis and carcinogenesis. Random integration occurs 50-10,000 times more frequently than integration due to homologous recombination with DNA vaccines (Donnelly *et al.*, 1997). Integration studies using PCR, have demonstrated that only 1×10^6 individuals will encounter malignancies as a result of DNA vaccination compared to a 1 in 1000 incidence of developing a spontaneous tumor (Gregoriadis, 1998). Additional concerns focus on the immature immune system of newborns that has a higher risk of developing neonatal tolerance as the neonate may recognize the encoded antigen as 'self' rather than foreign. This concern was demonstrated when neonates developed tolerance rather than immunity, following DNA vaccination encoding the circumsporozoite protein of malaria (Mor *et al.*, 1996). Furthermore, adults with mature immune systems, but repeatedly exposed to small quantities of the encoded antigen, may also develop tolerance. Immune responses to specific antigens may be enhanced following DNA vaccination due to the immunostimulatory activity of CpG motifs in the plasmid backbone and this could result in development of autoimmune diseases, such as systemic lupus erythematosus (SLE), where individuals generate anti-antibodies that react with self- cell components such as nucleic acid released from normal breakdown of skin and other tissues (Donnelly *et al.*, 1997), (Pisetsky, 1997). Experiments have shown that purified double stranded DNA does not induce anti-DNA antibodies. One

final safety issue relates to modifications of DNA vaccines to induce excessive cytokine production that may create an imbalance in immune Th1/Th2 homeostasis. The overproduction of particular cytokines could disrupt host susceptibility to infection. Nevertheless, secretion of cytokines from transfected cells may only affect the local environment and is unlikely to have a systemic effect.

1.7 HIV

The first cases of Acquired Immunodeficiency Syndrome (AIDS) were reported in 1981 and characterized by susceptibility to opportunistic pathogens (e.g. *Candida albicans*), Kaposi sarcoma, B cell lymphoma and most importantly the depletion of the CD4⁺ T cell population. Human Immunodeficiency Virus was first isolated in 1983 and is responsible for the progression to AIDS. HIV has shared virological and pathogenic characteristics with two other human retroviruses (LAV and HTLV-III). HIV is a retrovirus belonging to the lentivirus family, with a genomic size of ~9.8Kb. The surface of the virion consists of envelope protein arranged as trimers or tetramers. The precursor protein, gp160 (MW 169KD), cleaves to yield gp120 external protein and the transmembrane gp41 protein. The HIV core is composed of viral p24 protein and contains two identical RNA strands required for reverse transcriptase, integrase and ribonuclease. HIV cases in the European Union, in particular Ireland, have risen steadily over the past decade as seen in the figure 1.3 provided by the Central Statistics Office of Ireland (Fig 1.3 and 1.4).

1.7.1 HIV CELLULAR INFECTION AND REPLICATION MECHANISM

The main portal of HIV entry into the host is via mucosal surfaces where M cells facilitate uptake and transportation of HIV to lymphoid tissues (Amerongen *et al.*, 1991). HIV primarily targets CD4⁺ T cells through the envelope glycoprotein gp120 on the viral surface binding to the surface CD4 receptor on T cells. CXCR4 and CCR5 chemokine receptors function as co-receptors for gp120. The V3 loop of gp120 mediates effective chemokine receptor binding and deletions or anti-V3 antibodies can block HIV binding. The viral envelope undergoes conformational changes due to initial binding events and facilitates fusion to the cell plasma membrane and virus internalization. The virus exists as a nucleocapsid and integrates into the host's genome as a provirus. The core proteins (RT and RNase) attached to the viral RNA

Cases of and Deaths from AIDS, 1982 to 1998

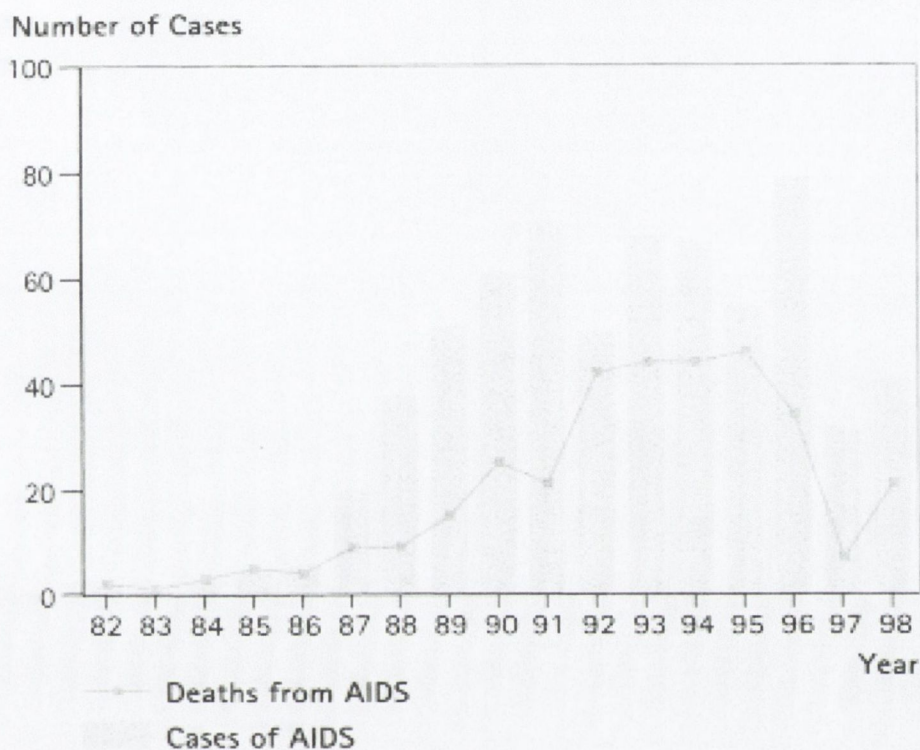


Figure 1.2. Cases of and Deaths from AIDS in Ireland from 1982 to 1998.

During the early 1980's Ireland presented few cases of AIDS although these numbers grew steady during the 1990's (*Central Statistics Office of Ireland, Dublin, 2001*).

Incidence of AIDS in 1997 per 100,000 Population:
EU Countries

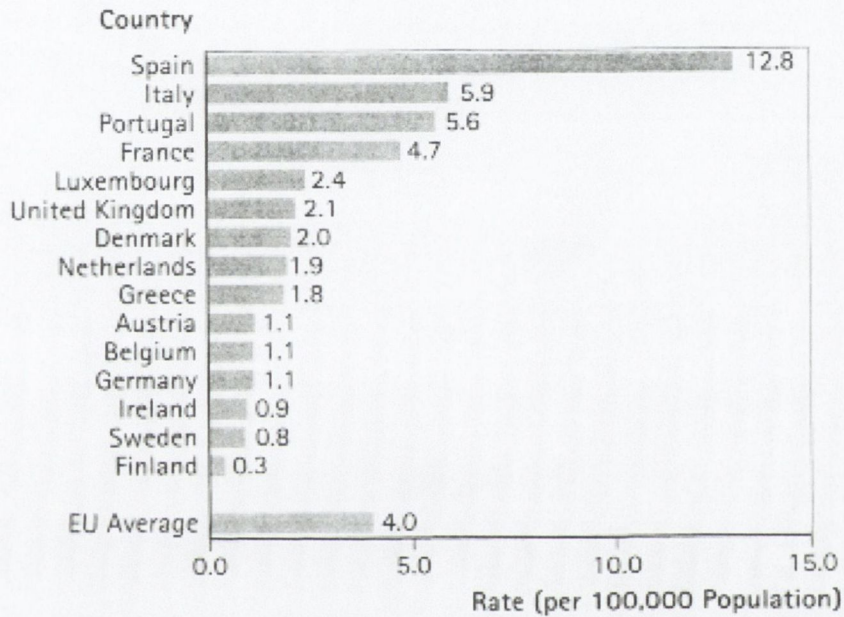


Figure 1.3. Incidence of AIDS in EU countries during 1997.

The incidence of AIDS per 100,000 was highest in Spain during 1997, followed closely by Italy, Portugal and France. Ireland was ranked third lowest in EU countries for incidence of AIDS cases during 1997 (*Central Statistics Office of Ireland (CSO), Dublin, 2001*).

mediate reverse transcriptase to generate double stranded DNA. This newly formed cDNA is transported to the nucleus and integrated (integrase-mediated) into the host chromosome, a key process required for efficient completion of the viral replication mechanism. Early phase transcription events utilize regulatory Tat and Rev genes, whose products mediate viral mRNA entry into the cell cytoplasm and begin the late phase of the viral RNA process respectively. The full-length primary transcript of viral mRNA is then translated into Gag, Pol and Env proteins. Gag generates viral proteins, including core p24, whereas Pol precursor is cleaved into RT, protease and integrase proteins. Protease is involved in viral particle maturation during viral budding. Recent research in HIV CD4⁺ infected T cells have shown that the blocking the proteasome-ubiquitin system interferes with the processing of Gag proteins and therefore plays an important role in viral replication (Perk, 2001).

1.7.2 HOST IMMUNITY AND CORRELATES OF PROTECTION TO HIV

Resistance to HIV infection is reported to be naturally occurring in a limited selection of exposed HIV individuals. Although cell mediated and humoral immune responses have been extensively studied during HIV infection, the exact correlates of protection remain undefined. Non-human primate models and human infection suggest that suppression of HIV replication is predominantly mediated by CD8⁺ T cells and neutralizing antibodies (Schmitz *et al.*, 1999), (Parren *et al.*, 1999). Evidence that CD4⁺ T cells both coordinate and regulate antibody and CD8⁺ effector functions (see section 1.2.2.3), suggest a role for CD4⁺ T cells in anti-HIV immunity. The negative correlation between CD4⁺ and viral load confirms the impact of CD4⁺ T cells in immunity to HIV. Furthermore, progression to AIDS is associated with loss of CD4⁺ T cells and reduced CTL function. Maintenance of effective CTL function in a murine chronic viral infection was attributed to virus specific CD4⁺ T helper cell responses (Altfeld and Rosenberg, 2000). Recent advances in flow cytometric analysis have facilitated the detection of *gag*-specific memory CD4⁺ Th1 cells, previously thought not to exist in HIV-1 infected individuals (Pitcher *et al.*, 1999).

HIV specific CD8⁺ T cells can control initial viremia following HIV exposure (Borrow *et al.*, 1992). HIV-specific CTL responses are maintained throughout asymptomatic phase and recognize a large number of epitopes from several HIV proteins including the immunodominant epitope Gag and subdominant epitopes Env and Pol. Disease progression is associated with reduced HIV-specific CD8⁺ CTL

activation partially as a result of a weakened immune system, with a depleted CD4⁺ T cell population and associated cytokine production such as IL-12, essential for CTL activation and differentiation.

Apoptosis facilitates the depletion of both CD4⁺ and CD8⁺ T cell populations during HIV infection. T cells become primed for activation-induced apoptosis following engagement of CD4 and co-receptors CXCR4 and CCR5 with gp120 envelope glycoprotein (Mahlknecht and Herbein, 2001). This process is mediated by upregulation of Fas and FasL expression on uninfected T cells and macrophages (Mahlknecht and Herbein, 2001). Studies have suggested that as macrophages can harbor HIV virus they may play a role in modulating T cell apoptosis during HIV infection (Badley *et al.*, 1994). It appears that macrophages can recruit resting CD4⁺ and CD8⁺ T cells through secretion of MIP-1 α and MIP-1 β chemokines in responses to HIV *nef* expression in the infected cell. This Nef-mediated mechanism of T cell attraction significantly enhances the rate of HIV spread (Mahlknecht and Herbein 2001). A role for neutralizing antibodies in protection against HIV has been suggested by studies where gp120 immunized chimpanzees were protected through the induction of V3-specific neutralizing antibodies (Berman *et al.*, 1990). However, chimpanzees do not develop disease following HIV infection, therefore humoral responses alone may not be sufficient for protection against infection or disease progression in humans. Passive antibody immunotherapy did not improve survival and antibodies that neutralize one strain may promote infection against another strain (Fust *et al.*, 1995). Nevertheless, the potential of a mucosal antibody response to protect against HIV challenge was demonstrated in macaques immunized with whole virus encapsulated in PLG microparticles (Marx *et al.*, 1993).

Resistance and control of intracellular pathogens is predominantly mediated by Th1 cytokines such as IL-12, IFN- γ and IL-18 (see section 1.3.2). It has been suggested that type 1 responses may be more protective against HIV infection (Clerici *et al.*, 1992) as IL-10 mediates immune dysfunction (Clerici *et al.*, 1994). Disruption of immune homeostasis by CD4⁺ Th1 depletion and subsequent increase in Th2 cells correlates with progression to AIDS (Clerici and Shearer, 1993).

1.7.3 HIV VACCINE STRATEGIES

An ideal HIV vaccine should generate cross-clade (A,B,C,D and E), neutralizing antibodies, broad CD4⁺ and CD8⁺ T cells responses, chemokines, mucosal immunity and memory. Rational design of a prophylactic vaccine is dependant on understanding the correlates of protection against HIV infection. However unlike other vaccine approaches, mimicing natural infection is not an option as the immune systems fails to clear an HIV infection under most circumstances. Several factors pose obstacles to effective HIV vaccine development; rapid mutation rates enable HIV to evade host detection and it can persist as a latent provirus. Replication is mediated by HIV binding to CD4⁺ T cells. Death of infected cells leads to a reduction in the CD4⁺ population, resulting in an impaired immune system that is required for maintenance of an immune responses and successful vaccination. HIV can move between cells and evade neutralizing antibody detection. Effective mucosal immunity is required as transmission frequently occurs via mucosal surfaces. Finally there is a lack of comparable predictable animals models and the high cost of clinical trials.

A variety of HIV vaccine strategies have been explored, including live attenuated, inactivated whole virus, live vector, DNA as well as different therapeutic vaccines. The macaque model has been employed for pre-clinical evaluation of many of these approaches. A high level of protection against SIV challenge has been demonstrated in non-human primates immunized with an attenuated vaccine containing a *nef* deletion (Daniel *et al.*, 1992). However, this strategy may pose serious limitation risks in humans as a result of rapid mutations and the generation of pathogenic variants. Therefore the use of whole inactivated virus delivered with an adjuvant has also been examined and shown to induce protection in non-human primates when macaques were immunized with inactivated whole virus delivered with alum or SAF-1 (Murphy-Corb *et al.*, 1989). However, protection was limited to the peak of vaccine-induced immunity and was dependant on antibodies produced against components of the cells in which the virus had been cultured (Stott *et al.*, 1991).

Poxviruses are widely studied as live vectors expressing HIV antigens and they represent an attractive alternative to whole HIV vaccines. Vaccinia virus priming and recombinant protein boost induced protection in a SIV model (Hu *et al.*, 1989). However, immunosuppressed HIV individuals were susceptible to vaccinia infection (Redfield *et al.*, 1987) and therefore a modified vaccinia strain (Modified Vaccinia Ankara (MVA)) with gene deletions and limited pathogenicity has been assessed.

There have been reports of other viral vectors for HIV vaccine development: adenovirus, herpes simplex virus (HSV), varicella zoster virus, simian virus 40, Sindbis virus (Cairns and Sarver, 1998). Renewed interest in antibody-based vaccines was triggered by the characterization of the gp120/CD4/17Fab 3D complex (Kwong *et al.*, 1998). The design of antibody-based vaccines is hampered by the hidden conformational epitopes exposed briefly during gp120-CD4 interaction. A fusion competent vaccine exposing conformational epitopes was produced by formaldehyde treatment during the fusion process. Alloimmunization has been investigated as an immune-based therapy for HIV as it has the potential to target cells expressing MHC-foreign antigens and circumvents difficulties resulting from antigenic variation mediated by HIV mutations (Shearer *et al.*, 1999). Studies have shown that CD8⁺ T cells induced by repeated allostimulation could inhibit HIV replication (Pinto *et al.*, 1998). Therapeutic approaches composed of highly active antiretroviral therapy (HAART) have demonstrated restoration of cell-mediated immunity and control of viral load. HIV *tat* is also being targeted as a novel vaccine whereby induction of neutralizing antibodies could prevent HIV infected cells secreting transactivating factor associated with the spread of HIV (Gallo, 1999). However *env* proteins gp41 and gp120 have received the most attention as candidate antigens for HIV vaccines. It has been reported that potent HIV specific CD8⁺ CTL and CD4⁺ T cells secreting antigen specific IFN- γ were induced in mice immunized with recombinant gp120 encapsulated in PLG microparticles (Moore *et al.*, 1995). Currently several recombinant forms of gp120 have been evaluated in phase I/II clinical trials (McElrath *et al.*, 2000).

The control of HIV infection is predominantly mediated through CD8⁺ CTL responses. Therefore DNA vaccines are ideal candidate vaccines as potent inducers of both cell mediated and humoral immune responses, without the risk of pathogenicity. HIV-1 infected chimpanzees immunized with DNA vaccine encoding *env* genes developed specific CTL and humoral responses directed against the HIV-1 envelope glycoprotein (Ugen *et al.*, 1997). Anti-HIV-1 immunity was established in murine and non-human primates when gp160 plasmid DNA was coadministered with a bupivacaine-HCL component (Coney *et al.*, 1994). Murine studies have reported successful induction of strong antigen specific antibody, CD8⁺ CTL and Th1 responses following gp120 plasmid DNA (Shiver *et al.*, 1997). In the same study a bivalent vaccine for gp120 and gp160 generated significant CTL induction in rhesus

monkeys (Shiver *et al.*, 1997). As mucosal immunity is a desirable, intranasal delivery of a combination vaccine composed of HIV-1 *rev*, *tat*, *nef* and gp160 genes elicited p24 specific IgA in mice (Hinkula *et al.*, 1997). DNA vaccine immunogenicity can be further improved by inclusion of delivery vehicles such as liposomes (Klavinskis *et al.*, 1997) (see section 1.6.8). Recent developments for HIV-1 vaccines have assessed the formulation of CpG immunostimulatory sequences with gp120-depleted whole killed HIV-1 and incomplete freund's adjuvant (IFA). This approach generated antigen-specific IFN- γ production by lymph node cells from immunized mice (Moss *et al.*, 2001).

1.7.4 HIV VACCINE CLINICAL TRIALS

To date more than 30 candidate vaccines have been assessed in clinical trials. As the correlates of protection have yet to be fully elucidated, the design of HIV vaccines is dependant on proteins and peptides with immunodominant T cell and antibody epitopes. Env proteins are widely used as they are recognized by specific CD8⁺ CTL during the course of HIV infection (Hammond *et al.*, 1992). However, strong selective pressure generating mutations comparable to structural and regulatory proteins, such as p24 and *nef*, may have potential as candidate antigens for HIV vaccine design. The V3 loop of the envelope gp120 protein contains the principal neutralizing antibody domain, defines cell tropism and influences CD4 protein modulation through gp120-CD4-chemokine receptor interactions (Choe *et al.*, 1996). Envelope-based vaccines using gp120 have reportedly induced neutralizing antibodies, but not CTL responses (Klein, 2001). A phase III trial in Thailand using genetically modified bivalent gp120 from prevalent subtypes B and E is currently underway and the data is anxiously awaited. Phase I and II trials of this vaccine demonstrated safety and antibody production, however complete protection may require a combination of proteins derived from several HIV strains. HIV phase I trial using a DNA vaccine encoding *env* and *rev* genes from HIV-1 delivered by Genevax Biojector 2000, is underway in the USA. However, Africa's infection rate is bordering on pandemic status, so it is ideally placed for a clade-specific prophylactic human HIV-1 vaccine trial; the first trial is currently being conducted in Uganda.

1.8 INFLUENZA

The three types of Influenza virus, known as A, B and C, were isolated in 1933, 1940, and 1950 respectively, and classified as members of the orthomyxoviridea family. The virus has an enveloped helical structure with a size of ~80-200nm. The external surface of the viron is mainly comprised of two major membrane glycoproteins: the homotrimer haemagglutinin (HA) and the tetrameric neuraminidase (NA). The surface of the spherical viron has approximately 500 HA and 50 NA molecules (10:1 ratio). HA is synthesized as a single rod-shaped glycoprotein and functions as a receptor-binding and membrane fusion molecule and is the major antigen target for neutralizing antibodies. Infectivity of the virus requires cleavage of HA by specific host proteases into two separate peptides: HA₁ and HA₂ attached by a disulphide bond. The functional HA molecule consists of three non-covalently linked HA₁ and HA₂ polypeptides and facilitates subsequent activation of membrane fusion at endosomal pH. The head of the NA molecule is composed of four identical polypeptides linked together by disulphide bonds. This mushroom-like protein mediates budding of the viron progeny from the infected cell surface and prevents clumping of the newly formed virus. The viron genome comprises of eight negative single-stranded viral RNA segments and an additional five viral proteins: NP, M, PA, PB, PB₂. The matrix (M) proteins are identical monomer structures located on the internal lipid bilayer surface and contribute to stability of the viral structure. The nucleocapsid protein (NP) is phosphorylated and associates with RNA transcriptase during viral replication. The three viral polymerases play a role in transcription and translation of viral RNA.

1.8.1 ANTIGENIC VARIATION

Influenza viruses variants emerge continuously as a result of minor antigenic changes in the HA and NA molecules. In the past century, avian influenza virus crossed into humans three times each time initiating a pandemic. Pandemics resulting from reassortment of segmented genome of influenza A subtype between human and animal species is known as antigenic shift. Influenza B is unable to contribute to pandemics due to a lack of animal reservoirs of the B subtype virus. In contrast, annual epidemics emerge from mutations in HA or NA genes of either influenza A or B and is referred to as antigenic drift.

1.8.2 PATHOGENESIS OF INFLUENZA

In mammals and humans, influenza is an acute respiratory illness and replication is restricted to the respiratory epithelial cells. However, birds are the natural hosts of influenza, transmitted via the faeco-oral route and display gastrointestinal infection. Influenza is contagious and spread between humans by aerosol projection and enters the nasopharynx spreading to cells expressing sialic acid receptors (mucoprotein) receptors in the respiratory tract. Mucoproteins present in respiratory secretions bind the virus, however NA can cleave these proteins rendering them ineffective. Influenza virus can modulate the immune effector mechanisms of the host by altering chemotactic, oxidative and killing functions of immune cells during infection (Hartshorn *et al.*, 1996). This controlled killing mechanism does not trigger anti-inflammatory responses and therefore constitutes a mechanism of viral evasion. Dead infected cell are then released through the respiratory epithelium by extensive desquamation.

1.8.3 HOST IMMUNITY TO INFLUENZA

A variety of cellular and humoral mechanisms are involved in recovery from influenza infection, whereas protection against infection requires subtype specificity, mucosal immunity and long-term memory responses. Virus specific CD8⁺ CTLs generated against the immunodominant NP epitope and subdominant epitopes have important effector functions in destroying infected cells; adoptive transfer studies with virus specific CD8⁺ T cells cleared infection in a syngenic murine model (Lukacher *et al.*, 1984). In the absence of CD8⁺ T cells virus can be eliminated in a CD4⁺ T cell dependent mechanism, mediated predominantly through antibody production (Scherle *et al.*, 1992). It is well established that humoral immunity to influenza virus is associated with murine IgG2a (Hocart *et al.*, 1989), (Deck *et al.*, 1997). A DNA vaccine coding for the major B cell epitope and the immunodominant CD4⁺ T cell epitope of A/PR/8/34 influenza virus provided protection against lethal challenge with the virus (Casares *et al.*, 1997). IgG and IgA antibody forming cells have been shown to have a protective role during lethal influenza challenge (Justewicz *et al.*, 1995). However, studies in mice deficient in IgA demonstrated that prevention of influenza virus is not IgA dependant (Mbawuike *et al.*, 1999).

1.8.4 INFLUENZA VACCINE STRATEGIES

Traditional inactivated split influenza vaccines, live viral vaccines and live attenuated vaccines have been extensively studied as strategies against influenza infection. A combination study examined cold-adapted influenza A and inactivated vaccines and both were required to generate effective immune responses and improve efficacy (Treanor and Betts, 1998). It has been demonstrated that live cold-adapted trivalent influenza vaccine induced more effective immune responses compared to heat inactivated or influenza virus vaccines. They were shown to induce significant cytotoxic CD8⁺ CTLs, anti-viral IFN- γ and up-regulation of co-stimulatory molecules on APCs (Blazevic *et al.*, 2000). In contrast, recombinant influenza A vaccines induce protection against viral challenge when several matched viral subtypes are administered in combination (Li *et al.*, 1999). Potent adjuvants and delivery systems have been employed to improve immunogenicity of influenza vaccines administered to the elderly with impaired immune systems. An influenza vaccine formulated with ISCOMs had high immunogenicity and protected aged mice against infection (Sambhara *et al.*, 1998). Intranasal immunization of BALB/c mice with an influenza vaccine and *E. coli* heat labile toxin (LT) combination generated high levels of HA-specific serum IgG and mucosal IgA. Inclusion of a B subunit (LTB) further enhanced these responses (Komase *et al.*, 1998). Inactivated split influenza vaccine administered with a mutant LT, LT-R72, induced significant levels of HA specific IgG2a considered to be important for viral clearance (Barackman *et al.*, 1999). The formulation of an influenza HA vaccine and LT-R72 adjuvant with HYAFFTM bioadhesive microspheres significantly enhanced serum IgG antibodies, neutralizing antibodies and nasal IgA following i.n. immunization in pigs (O'Hagan and Rappuoli, 2001).

DNA vaccines can persist in transfected tissues and cells for many months and maintain slow release of the encoded antigen (Wolff *et al.*, 1992). Therefore they represent an alternative approach to conventional influenza vaccines in generating protective immunity against influenza virus infection. Several investigations have reported specific immune response induction following immunization with plasmid coding for influenza viral genes. Ferrets were protected from A/PR/8/34 (H1N1) influenza virus challenge following immunization with a plasmid DNA expressing influenza virus HA (Webster *et al.*, 1994). Protection against influenza is primarily

mediated by neutralizing antibodies and cytotoxic CD8⁺ CTLs. It has been demonstrated that non-human primates immunized with a DNA vaccine encoding HA and NP of influenza virus generated persistent antigen-specific CD8⁺ CTL, antibodies and neutralizing antibodies (Liu *et al.*, 1997). Furthermore, BALB/c mice immunized with a similar HA or NA plasmid DNA generated significant antigen-specific antibody responses and were subsequently protected against live virus challenge (Chen *et al.*, 1998). DNA vaccination can induce responses over long periods following immunization. This has been shown by the induction of CD4⁺ Th1 cells that persisted for at least 6 months following immunization with HA encoding plasmid DNA (Johnson *et al.*, 2000). Furthermore, HA specific plasma cells have been shown to persist for at least 1 year after HA plasmid DNA gene-gun immunization, indicating maintenance of memory B cells (Justewicz and Webster, 1996).

1.9 AIMS OF THE STUDY

Development of a prophylactic vaccine is dependant on understanding the role of T helper cells in regulation of the immune response and novel vaccine delivery technologies. The aims of this project were to firstly characterize the immune profiles induced with plasmids encoding gp120 surface glycoprotein of HIV-1 virus (gp120pDNA) and the transmembrane hemagglutinin protein of influenza virus (HApDNA). Although the correlates of HIV protection have yet to be fully elucidated, the induction of virus-specific CD4⁺ Th1 and CD8⁺ CTLs are recognized as ideal goals in the design of many viral vaccines. Therefore, modulation and enhancement of immunogenicity of gp120pDNA toward a Th1-type profile was attempted through the addition of plasmids coding for cytokine genes and cationic liposomes to improve delivery to sites of immune induction. Alternative prime/boost immunization schedules were examined to optimize antigen-specific responses following DNA immunization in mice. Since developing countries would benefit from improved vaccine efficacy through the design of effective multicomponent DNA vaccines, the immunization of multiple plasmids in a single vaccine formulation was examined. Furthermore, this study investigates the mechanism of immune response induction following immunization with plasmid DNA, as this knowledge would facilitate a more rational approach to designing novel effective DNA vaccines.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 PHOSPHATE-BUFFERED SALINE (PBS)

80 g Sodium Chloride (NaCl)

11.6 g Sodium hydrogen phosphate (Na_2HPO_4)

2 g Potassium dihydrogen phosphate (KH_2PO_4)

2 g Potassium Chloride (KCl)

Dissolved in 1 L of ddH₂O and pH adjusted to 7.0.

2.1.2 TRIS-BUFFERED SALINE (TBS)

2.42g Trizma base

8.76g Sodium Chloride (NaCl)

Dissolved in 1 L of ddH₂O and pH adjusted to 7.3

2.1.3 DIETHANOLAMINE BUFFER

97 ml Diethanolamine

0.1 g Magnesium Chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)

0.2 ml 1M Sodium Azide (NaN_3)

Dissolved in 1 L with ddH₂O and pH adjusted to 7.2

2.1.4 PBS/BSA/AZIDE (PBA)

500 ml PBS pH 7.2

2.5 g Bovine Serum Albumin (BSA)

0.5 ml 1M Sodium Azide

2.1.5 AGAROSE GEL

Agarose (to the desired percentage) was dissolved in Tris-EDTA (TAE) buffer

27 g Tris

13.75 g Boric Acid

18 g EDTA

Dissolved in 2.5 L dH₂O

2.1.6 LURIA BROTH (LB)

10 g Bactotryptone

5 g Bacto yeast extract

10 g NaCl

Dissolved in 1 L with dH₂O and pH adjusted to 7.5 followed by autoclaving. Ampicillin (Sigma) was added at 100µg/ml final concentration. Luria agar was made by adding 15 g agar to 1 L LB (pH 7.5) which was autoclaved and cooled to 55°C before adding ampicillin (100 µg/ml final concentration).

2.1.7 CELL CULTURE MEDIUM

Complete RPMI medium: Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco BRC, Life Technologies, Paisley, Scotland), was used to culture all cell lines, and primary cells isolated from murine spleens and lymph nodes. The medium was supplemented with 8% heat inactivated (56°C for 30 mins) foetal calf serum (FCS), 100mM L-Glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 50µM 2-Mercaptoethanol.

2.1.8 ANTIBODIES

The specificities and source of monoclonal and polyclonal antibodies used are detailed in Table 2.1

2.1.9 CYTOKINE STANDARDS

Murine cytokines were obtained from the Division of Immunobiology in NIBSC and from commercial sources as outlined in Table 2.2

2.2 ANTIGENS, PLASMID DNA AND CPG-ODN

2.2.1 GP120 ENVELOPE (ENV) PROTEIN

The recombinant Env protein of the HIV-1 isolate W61D, was kindly provided by Dr. Wilfried Dalemans (SmithKline Beecham Pharmaceuticals, Rixensart, Belgium). HIV-1_{W61D} was produced in Chinese Hamster Ovary (CHO) cells and was analyzed for purity and endotoxin levels.

2.2.2 FORMULATION OF ALUM AND PROTEIN ANTIGEN

Alum absorbed antigen was prepared by mixing 5 µg gp120 recombinant protein with 25 µl alum. This mixture was incubated overnight at 4°C with continuous agitation.

2.2.3 PREPARATION OF ANTIGEN COUPLED LATEX MICROSPHERES

The method described by Katrak *et al*, (1992), was used to non-covalently couple latex microspheres to antigen which provide the *in vitro* cell culture with a dense localized population of gp120 antigen versus free antigen. The latex beads (0.8 µm) (Sigma) were prewashed in sterile 0.1 M glycine-NaOH buffer, pH8.6, and pelleted by centrifugation at 15,000 g in a benchtop microfuge at 4°C. 50 µg of antigen, in a volume of 100 µl of the 0.1 M glycine-NaOH buffer, was added to 100 µl of the 10% suspension of latex beads. The antigen-latex bead suspension was incubated overnight at 4°C with continuous rotation and agitation. The antigen coupled latex beads were pelleted as before and resuspended in 1 ml PBS containing 8% FCS to block unbound sites on the beads. The mixture was incubated for 6 hours with continuous agitation at 4°C and then washed twice in PBS with 2% FCS. The antigen coupled latex beads were washed once more with PBS followed by UV sterilization for 20 minutes at 254 nm. The latex coupled protein was finally resuspended in serum free medium and stored at -80°C until required.

2.2.4 PLASMID DNA CONSTRUCTS

The JA4304 plasmid consists of a pUC backbone carrying an ampicillin resistance gene and a bacterial origin of replication for selection and growth. A strong viral promoter pCMV is also present which contains polyadenylation, termination and

intron A sites. The gp120 (W61D, CHO expressed) surface glycoprotein from HIV-1 was cloned in between the HindIII - BamHI sites. The gp120 plasmid DNA (gp120pDNA) was supplied by Dr. Wilfried Dalemans (Smithkline Biologicals, Belgium) (Fig 2.1). A/Sichuan/2/87 (H3N2) full length HA was cloned into plasmid PI.18 which is a pUC-based plasmid containing a bacterial origin of replication, ampicillin resistance gene, truncated enhancer region, full promoter and Intron A gene from human CMV and a CMV terminator sequence (Fig 2.2). To create a plasmid with identical backbone for both the gp120 and HA antigens, the gp120 protein was excised from the gp120pDNA by cleaving it at two restriction sites which was then inserted into the PI.18 backbone in place of the HA gene to create a new plasmid, PI.18sfigp120. A plasmid coding for the IL-12 cytokine had the gene inserted into a PI.19 pUC-based backbone containing a bacterial origin of replication, ampicillin resistance gene, full promoter and Intron A gene from human CMV and a CMV terminator sequence. Both HApDNA and IL-12pDNA were supplied by NIBSC, UK. Expression levels by distinct plasmids can be determined by examining lysate from plasmid/cell cultures using western blot analysis for protein quantities. Plasmid DNA was amplified in *E.coli* K 12 and purified using Qiagen endofree mega and giga kits (Qiagen, Crawley, West Sussex, UK).

2.2.5 GIGA PREPARATION OF ENDOTOXIN -FREE PLASMID DNA

The plasmid DNA was prepared using a Qiagen endotoxin free giga kit ® (Qiagen Ltd., Crawley, West Sussex, UK). Ideally one batch should be used throughout to ensure consistency, however a number of plasmid batches were prepared during this study. Standard checks (purity, endotoxin, concentration and plasmid form) should be performed after each batch. A 10 ml culture of transformed cells was grown at 37°C for 4-6 hours with continuous agitation in LB containing 100µg/ml ampicillin. The mini culture was transferred into 500 ml LB containing ampicillin and grown in similar conditions overnight. The cells were then harvested by centrifugation at 6,000 g for 15 minutes at 4°C in a Sorvall RC5b refrigerated centrifuge (GSA rotor). The bacterial pellet was resuspended in 125 ml of P1 buffer (50 mM Tris.Cl, pH 8.0; 10 mM EDTA; 100 µg/ml Rnase A) and the resulting suspension was transferred into two 150 ml Sorvall tubes. 125 ml of P2 lysis buffer (200 mM NaOH, 1% SDS) was gently added to the tubes and inverted a few times before a 5 minute incubation at

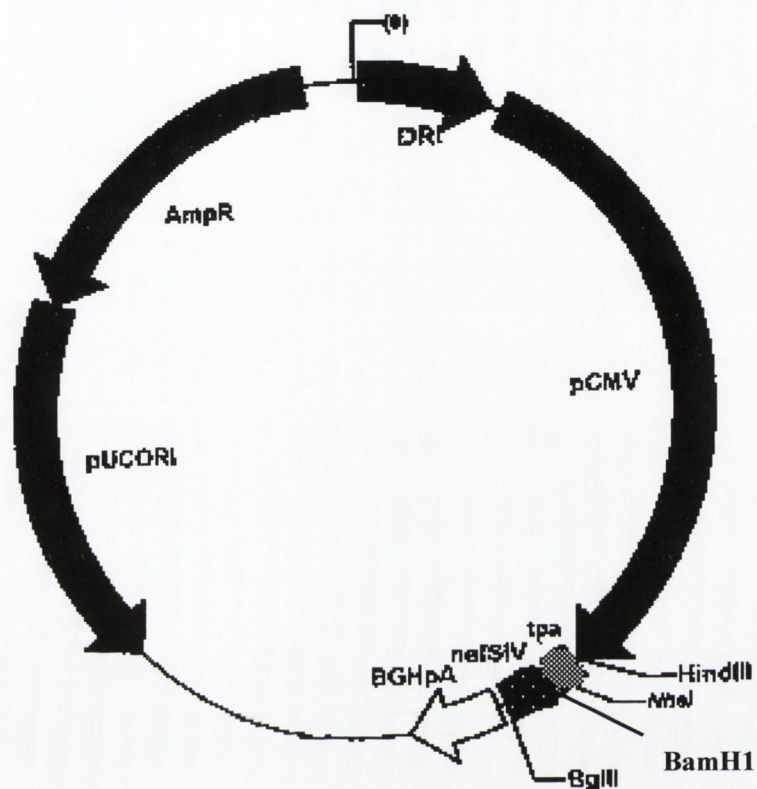


Figure 2.1. Gp120pDNA plasmid map.

This pUC based plasmid, JA4304 (5132bp), contains a pCMV promoter, ampicillin resistance gene and origin of replication. The gp120 (W₆₁D, CHO expressed) protein was inserted into the multiple cloning site located between HindIII and BamHI, to create gp120pDNA. This plasmid was supplied by Smithkline Biologicals.

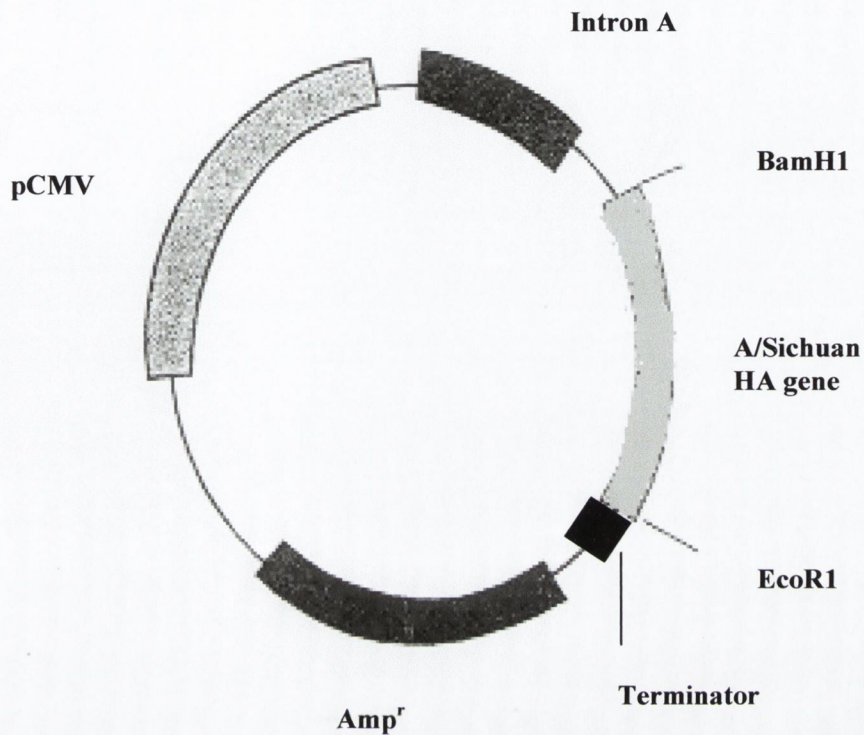


Figure 2.2. HApDNA plasmid map.

This pUC19 (PI.18) based plasmid (2.6kbp) contains an ampicillin resistance gene and full promoter and intron A from human CMV and a CMV terminator sequence. The HA gene from A/sichuna/87 influenza virus was cloned into the multiple cloning site between the EcoR1 and BamH1 restriction enzyme sites, to create HApDNA (~6kbp). This plasmid was supplied by NIBSC, England.

room temperature. The mixture was then neutralized with 125 ml of chilled P3 buffer (3.0 M potassium acetate, pH 5.5) and mixed well until a fluffy material formed. The lysate was poured onto the QIAfilter cartridge and incubated for 10 minutes at room temperature. A vacuum pump drew the preparation through the cartridge, which captured the bacterial waste and allowed the DNA to pass. The resulting solution was run through an equilibrated Qiagen-tip 10,000 with endotoxin treating buffer and washed through with 300 ml of wash buffer (1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol). The DNA was eluted from the column with 75 ml of elution buffer (1.6 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol) and the mixture collected in SS34 Sorvall tubes. Isopropanol was added to precipitate the eluted DNA and centrifuged immediately at 15000 g in a SS34 Sorvall rotor for 30 minutes at 4°C. The supernatant fluid was decanted carefully and the DNA pellet washed with 70% ethanol. The pellet was harvested by a further centrifugation at 15000 g for 10 minutes. The pellet was left to air-dry overnight in sterile conditions and was resuspended with a small volume of endotoxin free Tris buffer. To quantify the DNA a sample of the preparation was run through a spectrophotometer using UV and visible light at 254 nm. The results yielded information on the concentration and purity of the plasmid DNA.

2.2.6 DETECTION OF ENDOTOXIN LEVELS IN PLASMID DNA PREPARATION

The *Limulus* amoebocyte lysate (LAL) test, obtained commercially as E-TOXATE kit® (Sigma) was used to assess the endotoxin levels present in the plasmid DNA prior to immunization. The samples were tested according to the procedure in the E-TOXATE kit that analyzed the presence (positive for endotoxin) or absence of a hard gel. The endotoxin levels can be semi-quantified by a simple equation to give endotoxin result in EU/ml.

2.2.7 AGAROSE GEL ELECTROPHORESIS

The integrity of the plasmid DNA was examined by running it on a 1% agarose gel (dissolve 0.5 g agarose in 50 ml TAE) containing 3.5 µl of EB to visualize the DNA. The plasmid DNA (10 µl with 2 µl of loading buffer (0.2595 W/V Bromophenol Blue and 40% W/V sucrose) and DNA molecular weight marker of known concentration (used to quantify the sample of DNA) were loaded into the wells. The gel was run in

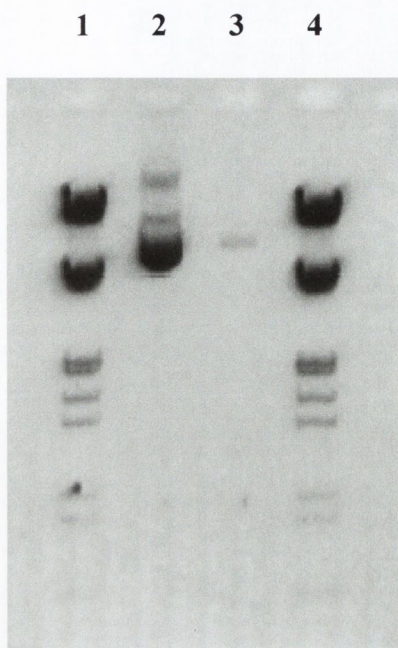


Figure 2.3. Integrity of Gp120pDNA.

The integrity of the pUCORI based plasmid expressing the gp120 gene from HIV-1_{WD61} was examined by agarose gel electrophoresis before immunization. The naked form of gp120pDNA predominantly existed in the supercoiled and relaxed circular form (lanes 2 and 3). Lanes 1 and 4 are 1kb DNA ladder consisting of 13 fragments from 250 to 10,000bp.

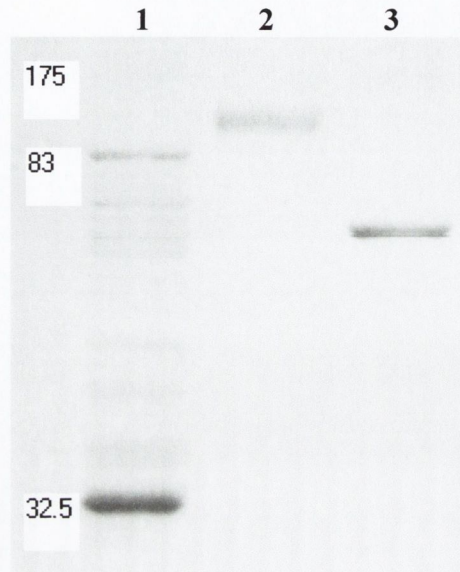


Figure 2.4. Integrity of gp120 recombinant protein.

The integrity of the gp120 recombinant protein derived from HIV-1_{W61D} was examined by SDS gel electrophoresis before immunization and use in analysis. The gp120 has a molecular weight of 120kDa (lane 2). BSA was used as a comparative protein (lane 3). The proteins were read from a molecular weight marker (lane 1).

TAE buffer at 55 volts for 2 hours and a UV trans-illuminator visualized the DNA bands (Fig 2.4).

2.2.8 SDS-POLYACRYLAMIDE ELECTROPHORESIS GEL

The integrity of the recombinant gp120 protein was assessed by running an SDS gel (Fig 2.5). This electrophoresis gel required the preparation of two gels, a 10% SDS resolving gel (7.9 ml dH₂O, 6.7 ml of 30% acrylamide mix, 5.0 ml of 1.5 M Tris – pH 8.8, 0.2 ml of 10% SDS, 0.2 ml of 10% Ammonium persulphate, 0.0008 ml TEMED to make 20 ml of gel) and stacking gel (3.4 ml ddH₂O, 0.83 ml of 30% acrylamide mix, 1.0 M Tris – pH 6.8, 0.05 ml of 10% SDS, 0.05 ml of Ammonium persulphate, 0.005 ml TEMED to make 5 ml of gel). The samples were resuspended in 1X SDS (500 µl 2X SDS, 25 µl β-mercaptoethanol, 475 µl ddH₂O). This was then heated at 90°C for 7 minutes to dissociate the proteins. The sample was run in TAE buffer for 2 hours at 60 volts and later stained with Coomassie Blue stain (0.25 g Coomassie Blue dye, 90 ml methanol: H₂O, 10 ml glacial acetic acid) for 2 hours followed by destain solution (40% methanol, 10% glacial acetic acid) overnight. Images of gels were prepared using Eagle Eye ® image analysis.

2.2.9 OLIGONUCLEOTIDES CONTAINING CpG MOTIFS (CpG-ODN)

CpG-ODN were custom made by Sigma Genosys, UK. The sequences used were adapted from Lipford *et al*, (1997) 3'-t c c a T G A C G T T C C T G a t g c T-5'. The anti-sense of these sequences were used as control CpG motifs. The CpG-ODN had been purified by desalting.

2.2.10 LIPOSOMES PREPARATION

DMRIE-c reagent (GibcoBrl) is a 1:1 (M/M) formulation of the cationic lipid DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide) and cholesterol. Vaccine formulations of 50 µg Gp120pDNA with 10 µg DMRIE-c were prepared just prior to immunization according to manufacturers specifications.

2.2.11 BIO-RAD PROTEIN MICROASSAY

Protein concentrations were determined by the use of the Bio-Rad protein assay (Bio-Rad, Herts, UK). 160 µl of the test sample or a serially diluted protein standard

Bovine serum albumin (BSA), or PBS were mixed with 40 µl of Bio-Rad dye reagent in 96-well microtitre plates. After 10 minutes, absorbance at 595nm was measured against the reagent blank. The protein concentration of the test sample was determined from a standard curve prepared using BSA in the range of 0 to 200 µg/ml.

2.3 METHODS

2.3.1 ASSESSMENT OF CELL VIABILITY

Equal quantities (0.1 mg) of Acridine Orange (AO) and Ethidium Bromide (EB) (Sigma) were dissolved in 100 ml of PBS to prepare a staining solution (AO/EB) which was stored in the dark at 4°C. To assess cell viability, 20µl of cell suspension was mixed with the appropriate quantity of AO/EB stain (depending on cell density) and placed in a haemocytometer. The number of viable cells (green) and non-viable cells (orange) were counted using a fluorescent microscope with a combination of UV and visible light.

2.3.2 CELL CULTURE

Primary cell cultures or cell lines were cultured under sterile conditions in RPMI-1640 medium at 37°C and 95% humidity in a CO₂ incubator. The cells were seeded at a variety of densities depending on cell type and passaged every two to three days depending on cell viability and rate of growth.

2.3.3 CELL LINES

The adherent murine macrophage cell line J774 was obtained from the American Type Culture Collection (ATCC) Rockville, USA.

2.3.4 PREPARATION OF ANTIGEN PRESENTING CELLS (APC)

Whole spleens from BALB/c (H-2^d) mice were placed in vials containing ice-cold sterile PBS supplemented with 8% FCS. These vials were inserted into the irradiation chamber of the Nordian Gammacell 3000 Elan Irradiator and irradiated at 30 Gy over 2 minutes. This dose was found to prevent cell proliferation. The irradiated spleens were then homogenized on sterile grids in fresh sterile RPMI-1640 (8%) FCS. The

cell suspension was washed twice and resuspended in fresh medium, and cell viability count was preformed.

2.3.5 T CELL CLONES

A T cell clone specific for the HA protein from A/Sichuan/87 influenza virus was established by Dr. Margaret Conway. The clone HDS 2.6 was maintained following recovery from liquid nitrogen by restimulation at the initial concentration of 1×10^5 cells/ml with influenza virus (10 HAU/ml) and APC (2×10^6 /ml) every 10 days. IL-2 at 5 U/ml (Table 2.2) was added 4-5 days after antigen stimulation.

2.3.6 CRYOPRESERVATION OF CELLS IN LIQUID NITROGEN

Cells were frozen in RPMI-1640 medium containing 10% (v/v) dimethylsulphoxide (DMSO) and 20% FCS (v/v). The cells to be cryopreserved were first pelleted by centrifugation, resuspended with the freezing mix at 4°C and then transferred to cryo tubes in 1.2 ml volumes. Cells were frozen in freezing plug over liquid N₂ tank, conditions that permitted a gradual reduction of temperature (1°C/min) before being transferred to liquid nitrogen. Recovery of viable cells from liquid nitrogen was performed by thawing the cells quickly in a 37°C water bath. The cells were then transferred to 10 ml centrifuge tubes and washed 3 times in ice-cold RPMI-1640 to ensure that all traces of DMSO were removed from the cell suspension. A viability count was performed and cells resuspended in complete medium.

2.3.7 UV-STERILIZATION

Protein antigens or other preparations were sterilized by exposure to UV-irradiation at 254nm for 20 minutes at a distance of 8 cm from the light source. Only preparations in medium free reagents were subjected to UV-sterilization as the phenol red dye present in RPMI-1640 medium generates toxic free radicals upon exposure to UV light.

2.4 MOUSE IMMUNIZATION

2.4.1 MOUSE STRAINS

Specific pathogen-free mice were purchased from various sources: BALB/c (H-2^d) and C57BL/6 (H-2^b) from Harlan UK Ltd, Bicester, Olac, UK, mutant C57/BL6 mice deficient in the IL-4 gene (IL-4^{-/-}) from B & K Universal, IL-12 knockout (IL-12^{-/-}) and IL-10 knockout (IL-10^{-/-}) from Jackson Laboratories, USA. All mice were bred and maintained under the guidelines of the Irish Department of Health and were 8-12 weeks old at the initiation of experiments.

2.4.2 IMMUNIZATION PROCEDURE

Mice immunized either by the i.m. route with a total volume of 125 µl into the biceps and quadriceps, subcutaneous (s.c.) into the footpad with 50 µl or i.n. by dropping 20 µl of vaccine preparation onto both nares of the mouse without the aid of anesthesia. In different experiments various doses of gp120 DNA (50, 5, 0.5, 0.05 µg) formulated with or without 10 µg of DMRIE-c were administered i.m. or i.n.. Mice were boosted at four weeks post primary immunization and sacrificed two weeks later.

2.5 DETECTION OF IMMUNE RESPONSES

Immunized mice were sacrificed usually two weeks after a single or booster immunization. Whole spleen or lymph nodes (Fig 2.6) from BALB/c (H-2^d) mice were placed in vials containing ice-cold sterile RPMI supplemented with 8% FCS. The spleens and nodes were then homogenized on sterile grids in fresh sterile RPMI-1640 (8%) FCS. The cell suspension was washed twice and resuspended in fresh medium, and cell viability count was preformed.

2.5.1 DETECTION OF T CELL PROLIFERATION AND CYTOKINE PRODUCTION

Spleen mononuclear cells (2×10^6 /ml) or lymph node cells (1×10^6 /ml) were stimulated *in vitro* with various concentrations of antigens (gp120 or influenza virus), mitogens (positive control) or medium alone (negative control). Supernatants were removed after 72h and stored at -20°C, to be used later to assess the concentrations of the cytokines IL-4, IL-5 and IFN-γ. Cells were cultured for a further 24hrs and pulsed with [³H]-thymidine for the final 4-6hrs of culture. The cells were harvested onto

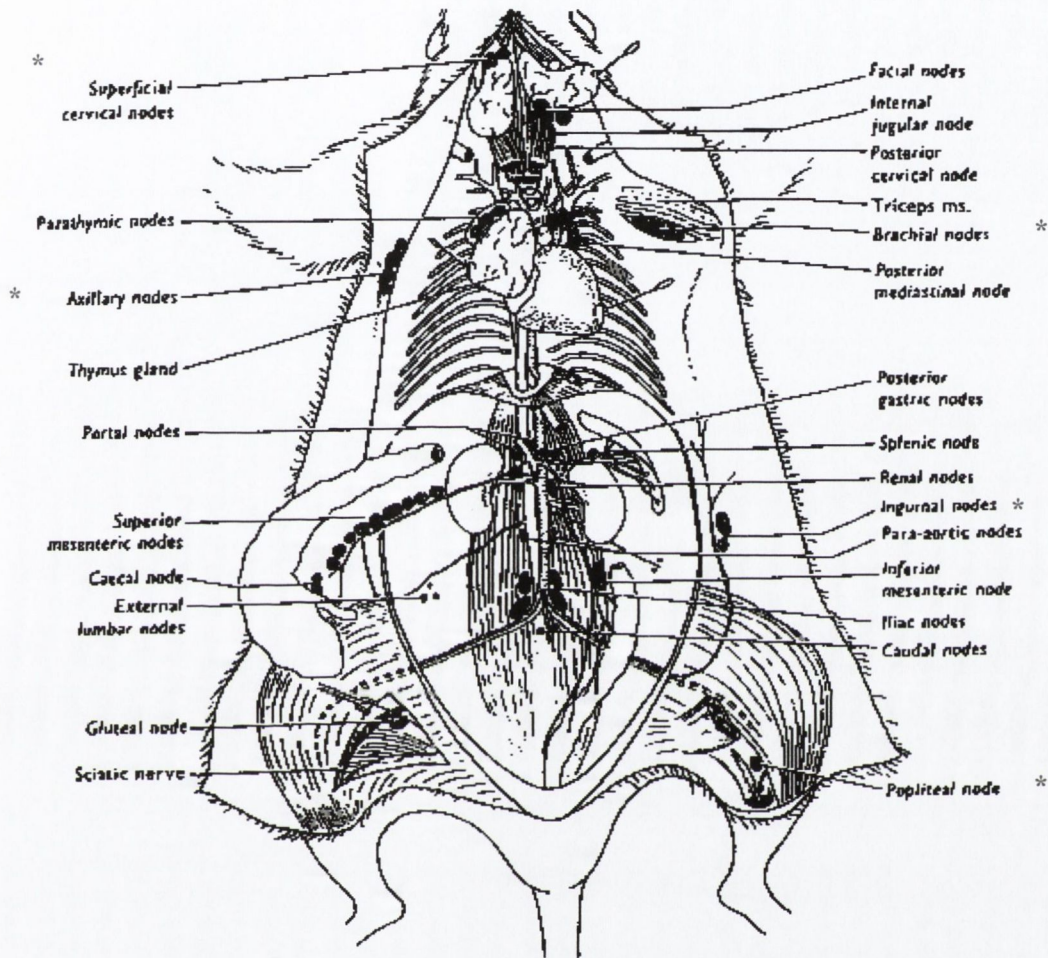


Figure 2.5. Lymphatic drainage system in the rat.

Lymph nodes of the rat are arranged in a similar manner to those found in the laboratory mouse. Cervical, axillary, brachial, inguinal and popliteal nodes (*) are assessed in the present study. Taken from Tilney *et al*, (1971).

glass fiber paper with an automatic cell harvester. The filters were then dried under an infra-red lamp and sealed in plastic sample bags with non-aqueous scintillation fluid, BetaScint, and [^3H]-thymidine incorporation assessed using a Beta-plate counter (Wallac). Results were expressed as mean counts per minute (cpm) of [^3H]-thymidine incorporation for triplicate cultures.

The concentrations of IL-4, IL-5 and IFN- γ were measured by enzyme linked immunosorbent assay (ELISA) using the antibodies described in table 2.1. Cytokine specific capture antibodies (50 μl /well at 1 $\mu\text{g}/\text{ml}$ in PBS) were added to 96-well microtitre plates (Nunc) and incubated overnight at 4°C. The plates were washed 4-5 times with wash solution (PBS/0.05% Tween 20) and then incubated with 200 μl /well of blocking solution (PBS supplemented with 10% w/v of dried milk (marvel®)) at room temperature for 2h to block non-specific binding sites. Following washing, plates were incubated overnight at 4°C with 50 μl /well of the test supernatant or the corresponding cytokine standard. The plates were then washed and incubated with 50 μl /well of a biotinylated anti-cytokine antibody and 1 $\mu\text{g}/\text{ml}$ in PBS at room temperature for 1h. After washing, the plates were incubated for 20 minutes at room temperature with 50 μl /well of extravidin alkaline phosphatase (Sigma Chemical Co. Ltd, Poole, Dorset, UK) at 1/2500 dilution in PBS. Finally, after washing, the plates were incubated with 100 μl /well of p-nitrophenyl phosphate (pNPP) substrate (Sigma) at 1mg/ml in 1M diethanolamine buffer pH 9.8. The O.D. value of test samples and cytokine standards were measured at 405nm using a microtiter plate reader (Titertex) and cytokine concentrations for test samples determined after reference to a standard curve prepared from recombinant cytokines of known concentration and potency. The limits of the assays were 0.5 ng/ml for IFN- γ and 20 pg/ml for IL-4 and IL-5.

2.5.2 DETERMINATION OF SERUM AND PLASMA IgG TITRES

Serum samples were prepared from peripheral blood removed from the thoracic cavity following cardiac puncture of euthanized mice. Alternatively, smaller quantities of plasma were obtained by tail bleeds from anaesthetized mice using syringes containing 10 μ l 0.1 M EDTA to prevent blood clotting during the bleed. For the collection of serum, the blood was allowed to clot for 1 hour at room temperature and then placed at 4°C for 2-4 hours. Following centrifugation at 15,000 g for 5 min in a bench top microfuge at room temperature, serum was pipetted into fresh tubes and stored at -20°C. The levels of antigen-specific IgG antibodies in the sera of naïve or immunized mice was determined by ELISA. Gp120 (1 μ g/ml in PBS) or influenza virus (stock @ 12,800 HAU, 1/160 dilution in carbonate buffer (one capsule of calcium carbonate diluted in 100 ml ddH₂O)), was added to 96-well immunoassay plates and incubated overnight at 4°C. Excess antigen was washed with wash buffer (PBS/0.05% Tween 20) and non-specific binding sites were blocked by incubating the plates with PBS supplemented with 10% w/v of dried milk for two hours. Sera were serially diluted in PBS on the washed plates to determine the endpoint titres. In general, an initial dilution of 1/50 was used for serum from mice that received one or two immunizations. The plates with the serum samples were incubated overnight at 4°C or for 2h at room temperature. After washing, plates were incubated for 1h at room temperature with 50 μ l/well of an alkaline phosphatase conjugated rat anti-mouse IgG monoclonal antibody (1/10,000 dilution in PBS/2%FCS) or with antibodies specific for IgG1 (1/4000), IgG2a (1/500), IgG2b (1/2000) and IgG3 (1/2000) (Table 2.1). The plates were then washed and incubated with 100 μ l/well of p-nitrophenyl phosphate in diethanolamine buffer (see section 2.1.3). The absorbance was measured at 405nm. Results are expressed as log₁₀ endpoint antibody titres. OD_{405nm} values were determined by extrapolation to the control, naïve serum and readings taken at two standard deviations above the control. Control wells in which the antigen, serum sample or detection antibodies were omitted gave negligible absorbances in the range 0.1-0.2.

2.5.3 DETERMINATION OF LUNG IgA ANTIBODY TITRES

Lungs were removed following the collection of blood and homogenized in 1 ml of ice cold sterile complete RPMI containing 1 µg/ml of PMSF (protease inhibitor to prevent protein degradation during homogenization). The cellular debris was removed by centrifugation at 15,000 g for 10 minutes and the supernatant fluid was collected and stored at -20°C. Lung IgA titres were determined by ELISA using the procedure described for IgG analysis, however the initial dilution used for lung homogenates was 1/4. Bound IgA was detected using sheep anti-mouse IgA (1/5000 dilution in PBS, 50 µl/well), and an alkaline phosphatase conjugated donkey anti-sheep antibody (1/2000 dilution in PBS, 50 µl/well).

2.6 GENERATION OF BONE MARROW DERIVED DENDRITIC CELLS (BMDC)

The method described by Lutz *et al*, (1999) was used to generate BMDC from BALB/c (H-2^d) mice. Mice were euthanized and the tibia and femur were dissected under sterile conditions. The muscle tissue was removed from the bone with a sterile scissors. The bone marrow was washed out into a petri dish containing sterile complete RPMI. Cell aggregates were broken up using a 19-gauge needle and were transferred to a sterile tube. The cell suspension was pelleted by centrifugation at 1200 rpm for 5 minutes at 4°C. The pellet was resuspended in complete RPMI and the cell viability assessed. The cells were seeded at 1×10^6 per tissue culture flask in 9 ml fresh RPMI and 1 ml GM-CSF (20 ng/ml, Pharmingen). Three days later 9 ml of fresh RPMI and 1 ml GM-CSF were added to each flask. On day six the cells were pelleted at 1200 rpm for 5 minutes at 4°C and resuspended in fresh RPMI and GM-CSF as before. This was repeated on day nine and finally on day 10 the viability of the BMDC was finally assessed and the cells were now ready for use.

2.6.1 IN VITRO ANALYSIS OF BMDC MATURATION, CYTOKINE AND CHEMOKINE INDUCTION

BMDC (1×10^6 /ml) were stimulated *in vitro* with various concentrations of the relative antigens, mitogens (positive control) or medium alone (negative control). Following 24 hour of stimulation, the cells were gently removed from the base of the 24 well tissues culture Nunc plates. The cell suspension was washed twice with sterile RPMI and resuspended in ice-cold PBA buffer, and cell viability count was

performed. DC maturation was assessed by changes in surface marker expression determined by FACs analysis (see section 2.6.6). Plates were set up in duplicate so that supernatants could be removed after various time points and stored at -20°C , to be used later to assess the concentrations of cytokines and chemokines.

2.6.2 THE DETECTION OF IL-12 BY IMMUNOASSAY

IL-12 was detected by immunoassay using commercially available antibodies (Genzyme) specific for the IL-12 p40 subunit. These antibodies recognise IL-12 p40 either as a monomer, heterodimer, or as part of the p70 heterodimer. 96-well microtiter plates were coated with $50\mu\text{l}/\text{well}$ of a capture anti-IL-12 monoclonal antibody (as described in Table 2.1) at a concentration of $2\mu\text{g}/\text{ml}$ in PBS and incubated overnight at 4°C . Following washing non-specific binding sites were blocked at room temperature for 2hr using $200\mu\text{l}/\text{well}$ of PBS with 10% w/v dried milk. After washing $50\mu\text{l}$ of the test sample or diluted IL-12 standards in the range of 0-5000pg/ml were added and incubated overnight at 4°C . The plates were then washed and incubated with the detecting antibody (rat anti-mouse IL-12) at a concentration of $2\mu\text{g}/\text{ml}$ in PBS ($50\mu\text{l}/\text{well}$) for 1hr at room temperature. An alkaline phosphatase conjugated mouse anti-rat IgG2a (PharMingen) antibody was then used to detect the second anti-IL-12 antibody. Plates were incubated with $50\mu\text{l}/\text{well}$ of a 1/1000 dilution of the alkaline phosphatase antibody for 2hr at room temperature. Finally, plates were washed and incubated with $100\mu\text{l}/\text{well}$ of pNPP substrate at $1\text{mg}/\text{ml}$ prepared in diethanolamine buffer (pH 9.8). OD values were measured at 405nm and IL-12 concentrations were determined from a standard curve prepared using recombinant murine IL-12 of known concentration.

2.6.3 IL-10 IMMUNOASSAY

The concentrations of IL-10 were determined by immunoassay using the antibodies described in Table 2.1. IL-10 specific capture antibody ($50\mu\text{l}/\text{well}$ in PBS at $2\mu\text{g}/\text{ml}$) was added to 96-well microtitre plates and incubated overnight at 4°C . The plates were washed 5 times with PBS/0.05% Tween 20 and then incubated with $300\mu\text{l}/\text{well}$ of blocking solution (1%BSA and 5% sucrose in PBS) for 2 hours at room temperature, to block non-specific binding sites. Following washing, plates were incubated overnight with $50\mu\text{l}/\text{well}$ of the test supernatant (with $50\mu\text{l}/\text{well}$ of 10%

BSA) and 100µl/well of the IL-10 cytokine standard in the range of 0-2000 pg/ml. The plates were then washed again and incubated with 50µl/well of a biotinylated detection antibody at 200ng/ml in 1% BSA, at room temperature for 2 hours. After washing the plates were incubated in the dark for 20 mins at room temperature with 100µl/well streptavidin-HRP (horseradish peroxidase conjugated) (Sigma chemical Co. Ltd., Poole, Dorset, UK), at a 1/1000 dilution in TBS as recommended by the manufacturer. Finally, after washing, the plates were incubated with 100µl/well of tetramethylbenzidine (TMB) substrate (Sigma). The plates were allowed to develop for approximately 20mins, and then the reaction was stopped with the addition of 50µl/well of 1M H₂SO₄. The O. D. value of test samples and cytokine standards were measured at 450nm using a microtiter plate reader (SpectraMax, Gemini) and cytokine concentrations for test samples determined after reference to a standard curve prepared from recombinant cytokines of known concentration and potency. The limit of the assay was 31 pg/ml.

2.6.4 IL-18 IMMUNOASSAY

The concentrations of IL-18 were determined using an enzyme-linked immunosorbent assay (ELISA), using the antibodies described in Table 2.1. IL-18 specific capture antibody (50µl/well at 1µg/ml in PBS) was added to 96-well microtitre plates and incubated overnight at 4°C. The plates were washed 5 times with PBS/0.05% Tween 20 and then incubated with 200µl/well of blocking solution (1%BSA, 5% sucrose, and 0.05% NaN₃ in PBS) for 2 hours at room temperature, to block non-specific binding sites. Following washing, plates were incubated overnight with 50µl/well of the test supernatant and the appropriate cytokine standard. The plates were then washed again and incubated with 50µl/well of a biotinylated detection antibody at 200ng/ml in TBS pH 7.3, at room temperature for 2 hours. After washing the plates were incubated with for 30mins at room temperature with 100µl/well streptavidin-HRP at a 1/1000 dilution in TBS as recommended by the manufacturer. Finally, after washing, the plates were incubated with 100µl/well of TMB substrate. The plates were allowed to develop for approximately 20mins, and then the reaction was stopped with the addition of 1M H₂SO₄. The O. D. value of test samples and cytokine standards were measured at 450nm using a microtiter plate reader (SpectraMax, Gemini) and cytokine concentrations for test samples determined after reference to a

standard curve prepared from recombinant cytokines of known concentration and potency. The limit of the assay was 31 pg/ml.

2.6.5 CHEMOKINE DETECTION BY IMMUNOASSAY

The chemokines, MIP-1 α , MIP-1 β , MIP-2 and RANTES were detected by immunoassay using commercially available antibodies (R&D) described in Table 2.1. The various chemokine specific capture antibodies (100 μ l/well at 1 μ g/ml in PBS) were added to 96-well immunoassay plates and incubated overnight at 4°C. The plates were washed 5 times with PBS/0.05% Tween 20 and then 200 μ l/well of blocking solution (1% BSA in PBS) was incubated for 2 hours at room temperature, to block non-specific binding sites. After washing 50 μ l of the test sample or diluted chemokine standards in the range of 0-5000pg/ml were added and incubated overnight at 4°C. The plates were then washed and incubated with the detecting antibody at a concentration of 0.2 μ g/ml in TBS (100 μ l/well) for 1hr at 37°C. After washing the plates were incubated for 15 mins at 37°C with 100 μ l/well streptavidin (horseradish peroxidase conjugated) (Sigma chemical Co. Ltd., Poole, Dorset, UK), at a 1/1000 dilution in PBS/Tween/1%BSA. Finally, after washing, the plates were incubated with 100 μ l/well of TMB substrate (Sigma). The plates were allowed to develop for approximately 20mins at room temperature, and then the reaction was stopped with the addition of 50 μ l 1M H₂SO₄. The O. D. value of test samples and cytokine standards were measured at 450nm using a microtiter plate reader (SpectraMax, Gemini) and cytokine concentrations for test samples determined after reference to a standard curve prepared from recombinant cytokines of known concentration and potency. The limit of the assays was 31 pg/ml.

2.6.6 FLOW CYTOMETRY ANALYSIS

Cell surface marker expression was assessed by flow cytometry using a FACScan (Becton-Dickenson, San Jose, CA). The FACScan was calibrated using the Autocomp software in conjunction with commercially prepared fluorescent beads (Calibrate beads, Becton-Dickenson). Direct and indirect staining methods were used to assess cell surface marker expression. The cell preparations, control and stimulated, were washed and resuspended in FACS tubes (Falcon) containing sterile PBA. The cells were then blocked with 2% NMS for 30 minutes on ice to prevent any non-specific binding. Specific cell marker expression was assessed using fluorescent conjugated antibodies specific for the particular cell type, e.g. FITC-conjugated CD11c for dendritic cells. In the case of indirect labeling the biotin-conjugated antibody specific for the cell type to be examined was added prior to addition of the fluorescent antibody. The cells were incubated for 30 minutes in the dark at 4°C followed by three washes in sterile PBA. Stained cells were then fixed with a 4% formaldehyde solution and flow cytometry analysis was performed using the FACScan. Unstained cells were used as a control to determine the level of autofluorescence and cells stained with and irrelevant antibody of matched isotype was used to assess any non-specific binding of the antibody. The results were analyzed using CELLQuest software.

2.7 STATISTICS

Statistical analysis of data was performed using the computer based mathematical package PrismII. Statistical significance of difference was assessed using the Student's t-test, as indicated in the text and figure legend.

TABLE 2.1 THE ORIGIN AND SPECIFICITIES OF ANTIBODIES

Antibody Clone	Specificity	Conjugate	Supplier
RA-6A2	mouse IFN- γ	-	PharMingen, San Diego, USA
XMG1.2	" IFN- γ	biotin	"
11-B-11	" IL-4	-	National Institute of Health, USA
BVD6-24G2	" IL-4	biotin	PharMingen, San Diego, USA
TRFK5	" IL-5	-	"
TRFK4	" IL-5	biotin	"
C17.8	" IL-12	-	"
C15.6	" IL-12	-	"
9A5	" IL-12	-	"
R19-15	" IgG2a	AKP	"
51817.111	" IL-18	-	R&D Systems, UK
-	" IL-18	biotin	"
-	" IL-10	-	"
-	" IL-10	biotin	"
-	" MIP-1 β	-	"
-	" MIP-1 β	biotin	"
-	" MIP-2	-	"
-	" MIP-2	biotin	"
-	" RANTES	-	"
-	" RANTES	biotin	"
A85-1	" IgG1	AKP	PharMingen, San Diego, USA
R19-15	" IgG2a	AKP	"
R12-3	" IgG2b	AKP	"
R40-82	" IgG3	AKP	"
16-10A1	" CD80	PE	"
GL1	" CD86	FITC	"

HM40-3	"	CD40	FITC	"
HL3	"	CD11c	FITC	"
M1/70	"	CD11b	PE	"
HM40-3	"	CD40	FITC	"
G235-2356	Hamster IgG		FITC	"
-	Mouse CCR5		PE	"

TABLE 2.2 RECOMBINANT CYTOKINES

Cytokine	Source
Mouse IFN- γ	NIBSC, Herts, UK
" IL-2	"
" IL-4	"
" IL-5	PharMingen, San Diego, USA
" IL-12	Stanley Wolf, Genetics Institute Inc., Mass., USA
" IL-18	R&D Systems, UK
" IL-10	"
" MIP-1 β	"
" MIP-2	"
" RANTES	"

CHAPTER 3

**COMPARISON OF IMMUNE RESPONSES INDUCED
FOLLOWING IMMUNIZATION WITH GP120 PROTEIN OR
GP120 ENCODING PLASMID DNA**

3.1 INTRODUCTION

Viruses that undergo antigenic variation, such as HIV and influenza, pose a considerable obstacle to the development of effective vaccines against these pathogens. Although successful, attenuated vaccines are associated with inherent risks of reversion, whereas recombinant protein vaccines demonstrate poor immunogenicity. An alternative approach aimed at mimicking the live attenuated vaccines, without the risks, is the use of naked DNA coding for genes from pathogenic organisms. In the early 1990's scientists discovered that intramuscular injection of plasmid DNA encoding reporter genes could be internalized and expressed by muscle cells (Tang *et al.*, 1992). DNA vaccines have been shown to induce both antigen-specific cellular and humoral immune responses. Their stability at extreme temperatures, rapid construction and the ability to stimulate potent immune responses has made them an attractive alternative to conventional killed, attenuated or subunit vaccines.

The immune response induced following DNA vaccination is influenced by numerous factors including plasmid backbone, nature of encoded antigen, internal immunostimulatory CpG motifs, dose, route of administration and immunization schedule. A wide range of routes for DNA delivery have been evaluated such as intramuscular (i.m.), intravenous (i.v.), intradermal (i.d.), subcutaneous (s.c.), intraperitoneal (i.p.) (Fynan *et al.*, 1993), (Bohm *et al.*, 1998), non-invasive skin-targeted plasmid vaccination (Shi *et al.*, 1999), topical ocular administration (Daheshia *et al.*, 1998), intranasal (i.n.) (Klavinskis *et al.*, 1997), (Ban *et al.*, 1997) and gene gun (Vahlsing *et al.*, 1994), (Tuting *et al.*, 1998). I.m. and gene gun methods of DNA delivery have been extensively studied. I.m. immunization with a plasmid encoding HA from influenza virus generates a potent Th1 response and protection following respiratory challenge (Johnson *et al.*, 2000). In contrast, gene gun immunization employing submicrogram quantities of gold or tungsten coated plasmid DNA, allows the plasmid to gain access to the cytosol of the cell where the coating detaches and allows the intracellular positioning of the plasmid DNA (Gregoriadis, 1998). This method of immunization results in the generation of predominant IgG1 antibodies, suggesting that it may promote induction of Th2 biased immune responses. However, despite the success of i.m. immunization at generating both cellular and humoral systemic immune responses, administration of the DNA vaccine by this route does not consistently enhance local IgA and hence may not be so

effective in protection against pathogen entry at the mucosal surfaces (Bagarezzi, 1999). Nevertheless, conventional polio and pertussis vaccines have been shown to induce protection at mucosal surfaces following i.m. immunization. IgA may not be essential for protective immunity against all infectious pathogens that infect via mucosal surfaces. However, this limitation of DNA vaccination at generating effective mucosal immunity is a major concern when designing effective vaccines against viruses such as HIV which enter the host primarily through mucosal surfaces. A recent study has demonstrated that i.m. administration of a DNA vaccine induced antigen-specific CD4⁺ T cells in the Peyer's patches and lymph nodes, tissues associated with the common mucosal system. Therefore, considerable interest has been generated in the targeting of DNA vaccines to various mucosal surfaces (respiratory, digestive, genitourinary tracts and ocular).

The correlates of protection against many pathogens remain undefined, nevertheless it is possible that protective immunity may require both cell mediated and humoral responses. Although DNA vaccines have demonstrated induction of strong immune responses, they can be further improved through prime/boost immunization schedules or formulation in delivery vehicles such as liposomes. Commonly used booster regimens include recombinant proteins or poxvirus. Boosting with recombinant protein has been shown to enhance antibody production following priming with plasmid DNA (Barnett *et al.*, 1997). The reversal of the plasmid prime/protein boost schedule abrogates any enhancement in immune responses. The mechanism by which this prime/boost regimen enhances immune responses remains unclear.

Liposomes have been shown to act as effective delivery systems for plasmid DNA, by facilitating efficient uptake by APC and presentation of the encoded antigen. They protect plasmid DNA from degradation by the host enzymatic environment and introduce structural versatility to further improve transfection efficiency (Gregoriadis, 1998). Liposomes composed of various lipid and cholesterol ratios can be formulated with antigens to increase the effectiveness of both parenterally and mucosally delivered protein vaccines (Templeton *et al.*, 1997), (Gao *et al.*, 1997). Formulation with liposomes has been shown to enhance immune responses with plasmid DNA following i.m., i.p. and i.n. administration, but not following s.c. or i.d. routes (Ishii *et al.*, 1997). Enhanced immunogenicity of i.m. delivered plasmid DNA formulated with cationic liposomes resulted in a significant increase in antibody titres and long lasting

HIV-1 specific CTL activity (Ishii *et al.*, 1997). Some liposome formulations have shown stability in acidic solutions, bile and pancreatin solutions, which demonstrate their suitability as oral vaccine delivery vehicles (Han *et al.*, 1997). Recently, it was demonstrated that i.n. immunization with liposome-formulated *Yersinia pestis* vaccine enhanced mucosal immune responses (Baca-Estrada *et al.*, 2000). In addition to liposomes, other delivery modes have been used successfully for DNA vaccines. Microparticles such as poly (lactic-co-glycolic acid) (PLGA) or polylactic acid (PLA) have been shown to prevent plasmid DNA degradation upon entry into the cell, facilitate uptake by APC and lead to production of both systemic and mucosal antibody responses (Jones *et al.*, 1997).

In the present study, gp120 recombinant protein absorbed to alum and plasmid DNA encoding gp120 (gp120pDNA) were compared for the ability to induce both cellular and humoral immune responses, which may benefit the design and development of an effective HIV vaccine. Furthermore, this study examined a prime/boost immunization schedule and the influence of two major routes of immunization on the immune response following DNA immunization. An effective viral vaccine may require dominant type-1 responses and the induction of local mucosal immunity. Therefore this study attempts to enhance the immune response generated by gp120pDNA by administering the plasmid with cationic liposomes (DMRIE-c) that aid in protecting the plasmid from degradation by the host enzymes and exhibit adjuvant properties. Finally, as mucosal immunity is an important consideration in HIV vaccine design, this study investigates induction of a mucosal immune responses following i.n. immunization with gp120pDNA and potential improvement by co-delivering DMRIE-c.

3.2 RESULTS

3.2.1 COMPARISON OF T CELL RESPONSES INDUCED FOLLOWING INTRAMUSCULAR IMMUNIZATION WITH GP120 PROTEIN ADSORBED TO ALUM OR PLASMID DNA ENCODING GP120

One approach to vaccination against HIV is to utilize the surface envelope glycoprotein gp120. The focus of these initial studies was to directly compare the immune responses generated following immunization of BALB/c mice with gp120 protein adsorbed to alum (gp120-alum) with gp120 encoded by plasmid DNA (gp120pDNA). Two weeks after a single i.m. immunization with 5µg of gp120-alum, spleen cells from immunized mice secreted significant amounts of IL-5 and relatively low levels of IFN-γ (<10ng/ml) following *in vitro* restimulation with various concentrations of gp120 antigen (Fig 3.1). To allow for variability in the PMA/aCD3 response it may be important to include a corrective index to compensate between test groups. The Th2 response generated after a single immunization was maintained following a booster dose, as indicated by high IL-5 concentrations and undetectable levels of IFN-γ (Fig 3.2). In contrast, i.m. immunization with 50µg of gp120pDNA generated a mixed Th1/Th2 immune response. High concentrations of IL-5 and modest levels of IFN-γ were produced by spleen cells from mice immunized with a single dose of gp120pDNA (Fig 3.1). Following a booster immunization the levels of IFN-γ ($p<0.01$) were greatly enhanced compared to those observed with gp120-alum, whereas IL-5 levels remained the same as that observed after a single dose (Fig 3.2).

3.2.2 SERUM ANTIBODY RESPONSES FOLLOWING IMMUNIZATION WITH GP120-ALUM OR GP120pDNA

Analysis of serum antibody response demonstrates that significant levels of anti-gp120 IgG, in the range of 3-4 log₁₀, were generated following immunization with either gp120-alum or gp120pDNA. IgG titres induced following a single immunization with gp120pDNA were marginally greater than those generated with gp120-alum (Fig 3.3A). However, following a booster at week 4, the IgG responses induced with gp120-alum were enhanced, but titres for gp120pDNA-immunized mice remained the same (Fig 3.4A). The IgG subclasses were assessed and it was found that IgG1 is dominant following immunization with gp120pDNA or gp120-alum. The IgG1:IgG2a ratio is high after a primary dose of gp120pDNA or gp120-alum, but

IgG2a increased after a booster dose with gp120-alum, but remained the same for gp120pDNA (Fig 3.3B and 3.4B).

3.2.3 THE INFLUENCE OF A PRIME/BOOST IMMUNIZATION SCHEDULE ON SYSTEMIC T CELL RESPONSES

As a type-1 T cell response may be important for protective anti-viral immunity, it is considered a key element in viral vaccine design. A prime/boost immunization schedule was examined for its potential to induce a stronger Th1 type response with gp120pDNA. BALB/c mice were primed with either gp120-alum or gp120pDNA and boosted at four weeks with either plasmid DNA (DNA) or protein (DNA/DNA, Protein/Protein, DNA/Protein, Protein/DNA). Two immunizations with gp120pDNA or soluble gp120 generated a mixed and polarized Th2 immune response respectively (Fig 3.5.). However, immunization with gp120pDNA followed by a booster dose of gp120 protein resulted in a 2-fold increase in the level of IFN- γ production ($p < 0.05$) and a concurrent dramatic reduction in IL-5 production by antigen-stimulated spleen cells from immunized mice ($p < 0.05$) compared to DNA/DNA immunization. Immunization with gp120-alum followed by a booster with gp120pDNA, did not induce significant levels of IFN- γ producing spleen cells, whereas IL-5 production was marginally enhanced compared to that following protein-protein immunization (Fig 3.5).

The influence of the prime/boost immunization schedule on cytokine production from the lymph node T cells was also assessed, as the various lymph nodes are likely to be sites of T cell activation. A polarized Th2 immune response was detected in the lymph nodes draining the site of i.m. immunization (axillary, brachial and popliteal nodes) with gp120-alum or gp120pDNA immunization. Gp120 stimulated lymph node cells secreted significant IL-5, but undetectable IFN- γ ; the concentrations of IL-5 were particularly high following immunization with gp120pDNA. However, IL-5 production was not as high in lymph node cells from mice primed with DNA and boosted with protein. Interestingly the reverse, protein priming and DNA boosting, resulted in similar levels of IL-5 as generated with two doses of gp120pDNA in the popliteal lymph node (Fig 3.6).

3.2.4 DNA PRIMING FOLLOWED BY A PROTEIN BOOST ENHANCES SERUM IGG

The prime/boost immunization schedule enhanced both Th1 type responses in the spleen following i.m. immunization with DNA/Protein and Th2 responses following Protein/DNA immunization. Therefore the influence of prime/boost immunization schedule on the humoral response was examined. A comparison of serum IgG titres revealed that the prime/boost schedule did not greatly affect the induction of antibody responses. Two immunizations with gp120pDNA or gp120 protein generated similar IgG titres, although protein followed by a DNA boost resulted in the lowest IgG levels. Interestingly, DNA followed by a protein boost induced the highest antibody titre ($4.2 \log_{10}$), which was almost a log increase over that observed with other combinations ($p < 0.05$ compared to DNA/DNA and $p < 0.01$ compared to Protein/DNA) (Fig 3.7).

3.2.5 IGG SUBCLASS INDUCED FOLLOWING DIFFERENT PRIME/BOOST IMMUNIZATION SCHEDULES

The IgG subclass induced was affected by the prime/boost schedule employed. DNA/DNA generated gp120-specific antibodies predominantly of the IgG1 subclass. Protein/Protein also induced a highly polarized IgG1 phenotype, which correlates with that observed with protein-alum formulations. The Protein/DNA immunization schedule also selectively induced predominant, IgG1 but the overall titres were lower than that observed with DNA or protein alone. Compared to other immunizing schedules, immunizing mice with DNA followed by protein enhanced the IgG2a titres, with only moderate increases in IgG1; this correlates with the T cell responses observed in the spleen (Fig 3.8).

3.2.6 CO-ADMINISTRATION OF PLASMID DNA AND DMRIE-C ENHANCES SYSTEMIC T CELL RESPONSES TO PARENTERALLY BUT NOT NASALLY ADMINISTERED GP120PDNA

As many viruses gain entry to their host through mucosal surfaces, generation of a immune responses at these surfaces may be important aspect of HIV vaccine design. In this study, systemic and local immune responses were assessed following either i.m. or i.n. immunization with gp120pDNA administered with cationic DMRIE-c. Assessment of gp120-specific spleen cell cytokine production demonstrated that i.n. administration of gp120pDNA was poorly immunogenic and this was not enhanced

by the codelivery of gp120pDNA with DMRIE-c. However, spleen cells from mice immunized by the i.m. route induced a mixed Th1/Th2 type profile, indicated by significant IL-5 production and low-level IFN- γ (Fig 3.9). Co-administration of cationic DMRIE-c did not alter the level of IL-5 secreted from spleen cells, but did enhance IFN- γ production by almost 2-fold ($p<0.05$) (Fig 3.9A).

3.2.7 ANTIBODY RESPONSES FOLLOWING PARENTERAL OR MUCOSAL IMMUNIZATION WITH GP120PDNA AND DMRIE-C

Humoral responses were assessed following immunization with gp120pDNA by the i.m. and i.n. routes. Serum anti-gp120 antibodies were only produced following i.m. immunization. Administration of gp120pDNA alone generated high titres when assessed 4 and 6 weeks after immunization. However, co-administration of DMRIE-c appeared to adversely affect the total IgG titres induced after a single dose, but not after booster immunization ($p<0.01$) (Fig 3.10A). Since the nasal route of immunization is associated with induction of secretory IgA responses, IgA in lung homogenates was examined from mice immunized by both the i.n. and i.m. routes of immunization. High titres of gp120-specific IgA were detected following i.n. immunization, and this was not enhanced when formulated with DMRIE-c ($p<0.01$). In comparison with the i.n. route, i.m. immunization generated lower titres of gp120-specific IgA and these titres were not enhanced by the addition of DMRIE-c to the vaccine composition (Fig 3.10B).

Analysis of serum IgG subclasses revealed that i.m. immunization with gp120pDNA generated anti-gp120 antibodies predominantly of the IgG1 subclass and this was not altered by the addition of DMRIE-c. Gp120pDNA induced IgG2a and IgG2b in a ratio of 1:1, however IgG2b significantly increased ($p<0.05$) when DMRIE-c were included in the vaccine formulation (Fig 3.11).

3.3 DISCUSSION

Immunization with plasmid DNA encoding microbial antigens can induce immune responses against antigens or infectious agents (Fynan *et al.*, 1993) and is being considered as an alternative strategy to conventional killed attenuated or subunit vaccines. Certain viruses, that are highly variable due to antigenic drift, can evade virus neutralizing antibody responses and therefore T cell responses against conserved regions of the virus may be essential in cross-reactive immunity. Furthermore, Th1 cells are required for the induction of murine IgG2a antibody responses, considered to be protective in many viral infections. Therefore, the present study evaluated the ability of a plasmid DNA encoding the HIV gp120 envelope surface glycoprotein, to induce distinct type-1 and type-2 T cells. Intramuscular immunizations with gp120 protein absorbed to alum induced a polarized Th2 response, whereas gp120pDNA induced a mixed Th1/Th2 type response. Induction of gp120-specific immune responses were influenced by the route of administration, prime/boost immunization schedules and co-administration of DMRIE-c.

These studies demonstrate that BALB/c mice i.m. immunized with gp120pDNA, generate a mixed Th1/Th2 type response in the spleen and a polarized Th2 type response in the draining lymph nodes. In contrast, i.m. immunization of recombinant gp120 protein absorbed to alum generated IL-5 producing spleen cells, which is consistent with the potent Th2 response previously reported with protein absorbed to alum (Moore *et al.*, 1995), (Conway *et al.*, 2000). The difference in the immune responses induced following gp120 protein or gp120pDNA immunization may be explained by the fact that DNA vaccination not only relies on the expression of the foreign gene of interest, but also on the adjuvant properties of bacterial DNA containing immunostimulatory sequences, such as CpG motifs (Sato *et al.*, 1996), (Yi *et al.*, 1996). These motifs are known to activate IL-12 production, which enhances the induction of Th1 cells (Bauer *et al.*, 1999), (Walker *et al.*, 1999). CpG motifs can also induce chemokine and IFN- γ secretion leading to upregulation of MHC class II on muscle cells, which can efficiently present to CD4⁺ T cells *in vitro* (Stan *et al.*, 2001).

In the present study, it was demonstrated that distinct antigen-specific T cell responses could be induced in both spleen and lymph nodes following i.m. immunization with gp120pDNA. Many DNA vaccines have been shown to induce polarized Th1 type responses (Johnson *et al.*, 2000), furthermore it has been

suggested that low antigen doses favor Th1 responses (Hernandez-Pando *et al.*, 1997). In this study, mice were inoculated at four muscular sites, which may result in a higher proportion of cells transfected with the plasmid, effectively increasing the dose of antigen available to the APC allowing induction of a Th2 type response with gp120pDNA. As initial T cell activation is reported to occur in the draining lymph nodes (Akbari *et al.*, 1999), (Casares *et al.*, 1997), cells directly transfected following plasmid DNA injection may migrate to the nodes, where the continuous expression of antigen would favor CD4⁺ Th2 induction. Furthermore, it has been shown that injected plasmid DNA can be transported away from the site of injection to initiate immune responses at distal sites (Klinman *et al.*, 1998) and antigen circulating in the blood can be processed for antigen presentation in the spleen (Wang *et al.*, 1993). It may be possible that following injection some plasmid DNA may enter the circulation, bound to APC and reach the spleen; the CpG motifs in the plasmid backbone may bind to TLR9 and stimulate IL-12 production by DCs leading to the induction of Th1 cells from naïve precursor T cells (Hemmi *et al.*, 2000).

The mechanism of generating immune responses following DNA vaccination has yet to be fully elucidated. However, the induction of cellular immune responses requires antigen processed by APC and presented to T cells as an MHC: antigen complex. There are a number of mechanisms by which T cell activation may be mediated following DNA vaccination; these include direct priming by somatic cells, direct transfection of professional APC or cross-priming of APC by transfected somatic cells. The role of muscle and DC in initiating immune responses, have received considerable attention. Skeletal muscle can directly take up plasmid DNA where it can persist for at least 30 days (Danko and Wolff, 1994) and secrete the encoded protein (Wolff *et al.*, 1992). Therefore cross-priming can occur where muscle cells act as antigen reservoirs for APC (Corr *et al.*, 1999), (Aihara and Miyazaki, 1998). Furthermore, injection into muscle results in local inflammation that can recruit APC (Tighe *et al.*, 1998). Studies have reported that DCs play a key role in initiating T helper cell responses following DNA vaccination (Akbari *et al.*, 1999), (Casares *et al.*, 1997). DCs containing plasmid DNA can be detected in draining lymph nodes and spleen of mice following i.d. immunization (Bouloc *et al.*, 1999), (Akbari *et al.*, 1999). It is possible that muscle cells are directly transfected with gp120pDNA and cross-prime DC that circulate to the spleen and lymph nodes to prime T cells. Alternatively, plasmid DNA may directly transfect DC recruited to the

site of injection, which could lead the encoded antigen and CpG, influencing the maturation of the DC that triggers the final T cell subtype induction.

In this study, a prime/boost immunization schedule was examined as a means of modulating the immune response that can be generated with gp120pDNA. Compared to homologous DNA/DNA immunization, DNA priming followed by a protein boost, resulted in enhancement of a type-1 immune response with increased IFN- γ and significantly reduced IL-5 production. Similar studies have revealed the dominant induction of IFN- γ producing CD4⁺ T cells in a tuberculosis DNA vaccine prime and exogenous protein boost (Tanghe *et al.*, 2001). MHC class I restricted CD8⁺ T cells, mediate protective immune responses against malaria and are strongly implicated in HIV protection. A specific DNA prime/MVA boost strategy was sufficient to generate complete protection in C57Bl/6 mice, that are highly susceptible to malaria sporozite challenge, by inducing high levels of IFN- γ producing CD8⁺ T cells. However, the effect was eliminated with the reverse order of administration (Schneider *et al.*, 1998). Therefore, this immunization method may be a successful means of generating type-1 responses against viral pathogens.

Assessment of antibody responses revealed a significant increase in anti-gp120 IgG following the DNA prime/protein boost immunization schedule compared to any other combination. Haddad *et al.*, (1999) provide evidence that this regimen induced high antibody titres and durable anamnestic responses. Induction of anti-malarial IgG1 antibody subclass was also reported using the same immunization protocol. In this study, examination of the IgG subclasses demonstrated a shift from predominant IgG1 induction with gp120 protein immunization to a more IgG1, IgG2a profile following priming with gp120pDNA and a booster dose of gp120 protein. Other studies have shown that IgG1 subclass is predominant generated following protein immunization, whereas DNA or DNA prime/protein boost induced predominant IgG2a antibodies (Tanghe *et al.*, 2001), (Haddad *et al.*, 1999). In addition to boosting with recombinant protein, attenuated viruses have been used effectively for boosting i.n. following immunization with plasmid DNA. Boosting with recombinant vaccinia virus coding for human papillomavirus (HPV) (Chen *et al.*, 2000) or HIV-1 *env* antigens (Gherardi *et al.*, 2000) enhanced IFN- γ secreting CD8⁺ T cell responses. Similarly, immunization with a plasmid encoding five structural and non-structural proteins (capsid/E1/E2/NS2/NS3) from Hepatitis C Virus (HCV) can

be improved with a canarypox virus boost encoding the same HCV genes (Pancholi *et al.*, 2000).

The mechanism by which DNA priming and protein boosting generates high antibody titres and Th1 responses is as yet undefined. Many studies examining DNA immunization have shown that they induce predominant Th1 type responses. Boosting with gp120 recombinant protein may expand the pre-existing gp120-specific type 1 T cell population (Cuisinier *et al.*, 1999). It has been suggested that the success of the DNA/ Modified Vaccinia Ankara (MVA) immunization regimen reflects the ability of the plasmid to prime sufficient CD8⁺ T cells to allow the MVA boost to focus on the viral epitopes in a display of immunodominance (Gilbert *et al.*, 1999), (Hanke and McMichael, 1999). Therefore, APCs or myocytes transfected during immunization may selectively prime type-1 T cells following the DNA priming and additional exposure to gp120 recombinant protein enhances this population of memory T cells. Interestingly, the route of booster immunization also influences the strength of the immune response following DNA priming. The intravenous route has been shown to induce significantly better responses compared to i.d. and i.m. immunization routes (Schneider *et al.*, 1998).

Immunization by mucosal routes has several advantages over conventional parenteral vaccination. They eliminate needle usage, reduce the risk of contamination and potentially increase vaccine coverage. Furthermore, initiation of an efficient mucosal immune response may be critical for vaccines targeted against pathogens that enter the host via mucosal surfaces. Studies have shown that mucosal immunization with DNA vaccines results in dissemination of plasmid DNA via the bloodstream and therefore may lead to the induction of both mucosal and systemic cellular immune responses (Chun *et al.*, 1999). The results of this study demonstrate a lack of systemic T cell and serum anti-gp120 IgG antibody induction following i.n. immunization of BALB/c mice with gp120pDNA, however significant lung IgA titres (2 log₁₀) were detected in these animals. These findings are consistent with other reports demonstrating the induction of IgA following both i.n and i.m. immunization of pDNA encoding *env* and *rev* genes of HIV (Sasaki *et al.*, 1998). Furthermore, IgA responses were detected in lung and vaginal washes following i.n. instillation with a DNA vaccine encoding HIV antigens (Asakura *et al.*, 1999). These studies suggest that i.n. administration of naked gp120pDNA can induce limited mucosal immunity. In contrast, the i.m route of gp120pDNA delivery generated mixed Th1/Th2 type

responses in the spleen, moderate lung IgA titres and significant serum IgG antibody production. To further define the role of IgA in this system, the type of IgA (sIgA or serum IgA) could have been determined by ELISA. It is known that secretory IgA is predominantly located at mucosal surfaces, whereas serum IgA triggers inflammatory responses following Fc α R1 myeloid IgA receptor.

Although naked DNA can induce immune responses at mucosal surfaces, the immunogenicity does not appear to equal that observed by the i.m. route. Consequently, novel injection methods and formulations have been used to enhance the efficacy of DNA vaccines delivered by mucosal routes. It has been reported that co-administering liposomes with either antigen or plasmid DNA can enhance immune responses. However the effect is lost if injected in separate liposomes vehicles, indicating the liposomes effect is only observed when both antigen and plasmid target the same APC (Gursel *et al.*, 1999). Nasal administration of *Streptococcus mutants* antigen formulated with liposomes was shown to enhance local secretory IgA in human volunteers (Childers *et al.*, 1999). Similarly, formulation into cationic liposome complexes for i.n. administration of a plasmid encoding the firefly luciferase gene induced enhanced local humoral responses, CTL and proliferate T cell responses in the spleen and iliac lymph nodes (Klavinskis *et al.*, 1997).

Liposomes have been reported to protect plasmid DNA and improve systemic T cell responses following i.m. immunization. The present study showed that i.m. administration of gp120pDNA with cationic DMRIE-c moderately enhanced gp120-specific IFN- γ from spleen cells and serum anti-gp120 IgG antibody production. Interestingly, the addition of DMRIE-c to gp120pDNA adversely affected the induction of anti-gp120 IgA antibodies in the lung. This observation is consistent with other studies demonstrating a reduction in IgA titres following immunization with whole measles virus vaccine with liposomes. Serum IgG titres were moderately enhanced, but IgA titres were not improved (de Haan *et al.*, 1995). One possible explanation is that the nasal surface is covered with a mucous layer and may inhibit movement and uptake of the liposomes complexes to the mucosal inductive areas of the nose (Harokopakis *et al.*, 1998). Alternatively, the liposome complex may be cleared by ciliary action of the nasal hairs (Heritage *et al.*, 1998). However, the present study demonstrated that gp120pDNA/DMRIE-c induced anti-gp120 IgG in the serum after i.n. immunization. Therefore, it is possible that the liposomes may somehow

block epithelia cell transfection, which primarily mediate mucosal induction, but still allow entry to the circulation to facilitate serum IgG induction.

This study has shown that a plasmid DNA encoding the HIV surface glycoprotein gp120 is capable of inducing a mixed Th1/Th2 type of response in comparison to a polarized Th2 profile observed following immunization with gp120-alum. These findings are at variance with the general observations that i.m. immunization with DNA vaccines predominantly generates a type-1 response, as was observed in this lab with a plasmid encoding the hemagglutinin (HA) gene from influenza virus (Johnson *et al.*, 2000). Therefore this study examined the influence of route, immunization schedule and delivery vehicles on the immune response induced with gp120pDNA. Evidence was found for enhanced IFN- γ producing spleen cells and IgG2a subclass induction using a DNA prime/protein boost immunization schedule. Injection of plasmid DNA into the enzymatic environment of the host can result in degradation of the DNA and could ultimately reduce the effectiveness of the vaccine. However, co-administration of gp120pDNA and DMRIE-c induced a moderate increase in both IFN- γ and IgG titres. However reduction in mucosal IgA following i.n. immunization was observed when plasmid DNA was co-administered with DMRIE-c. These findings reveal that modification of the DNA vaccines formulation or immunization schedule can generate immune responses that may be useful against viral pathogens.

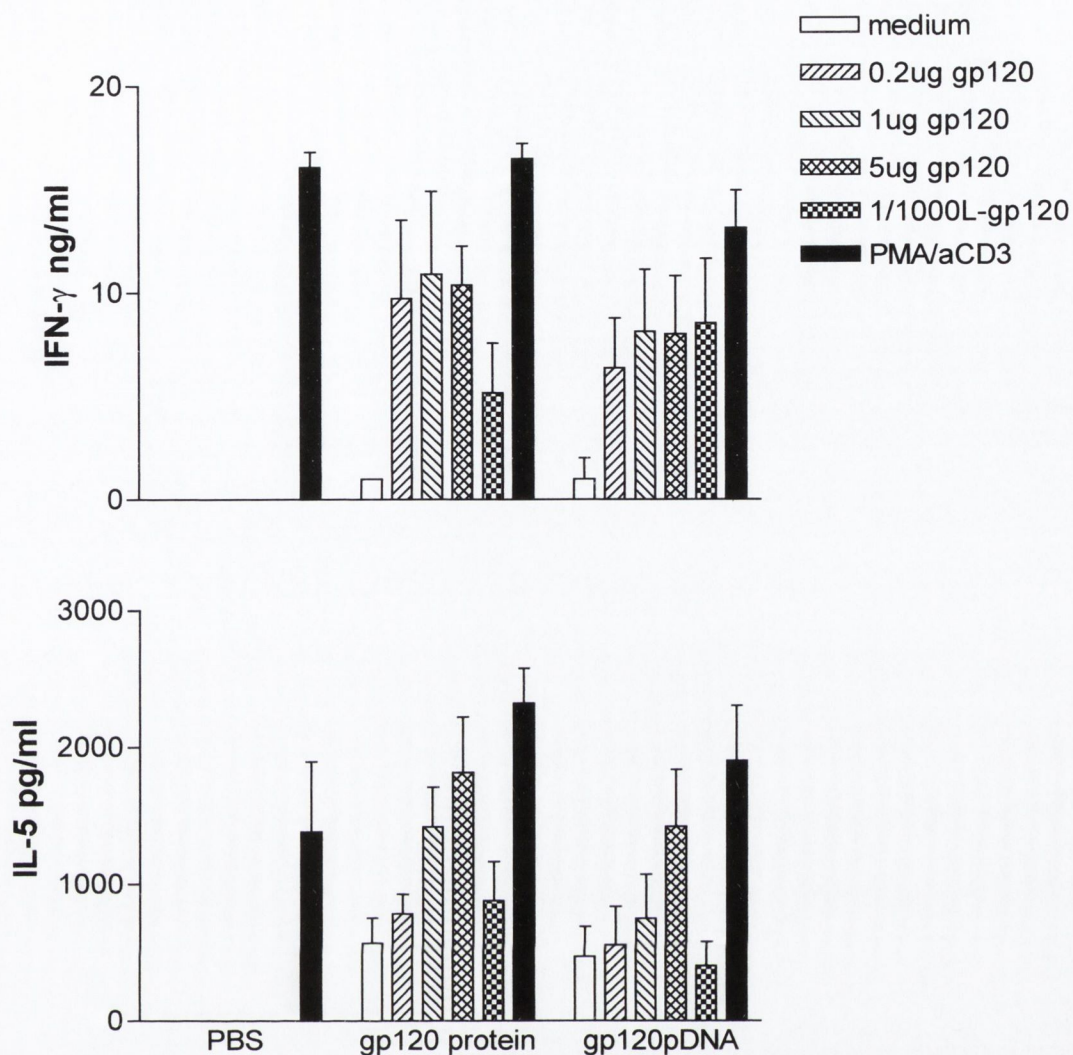


Figure 3.1. Intramuscular immunization with a single dose of gp120 absorbed to alum or gp120pDNA induces a mixed systemic Th1/Th2 response in mice. BALB/c mice were immunized with either 5 μ g of gp120 protein absorbed to alum or 50 μ g of gp120pDNA. At two weeks spleen cells were stimulated *in vitro* with gp120 antigen (0.2, 1, 5 μ g) or 1/1000 dilution of gp120 coupled latex beads (1/1000 L-gp120). Medium or PMA and anti-CD3 acted as negative and positive controls respectively. Supernatants were removed after 72 hours and cytokine concentrations were determined by specific immunoassay. Results from a single experiment expressed as the mean $\pm$ standard error (S.E.) for four mice per group.

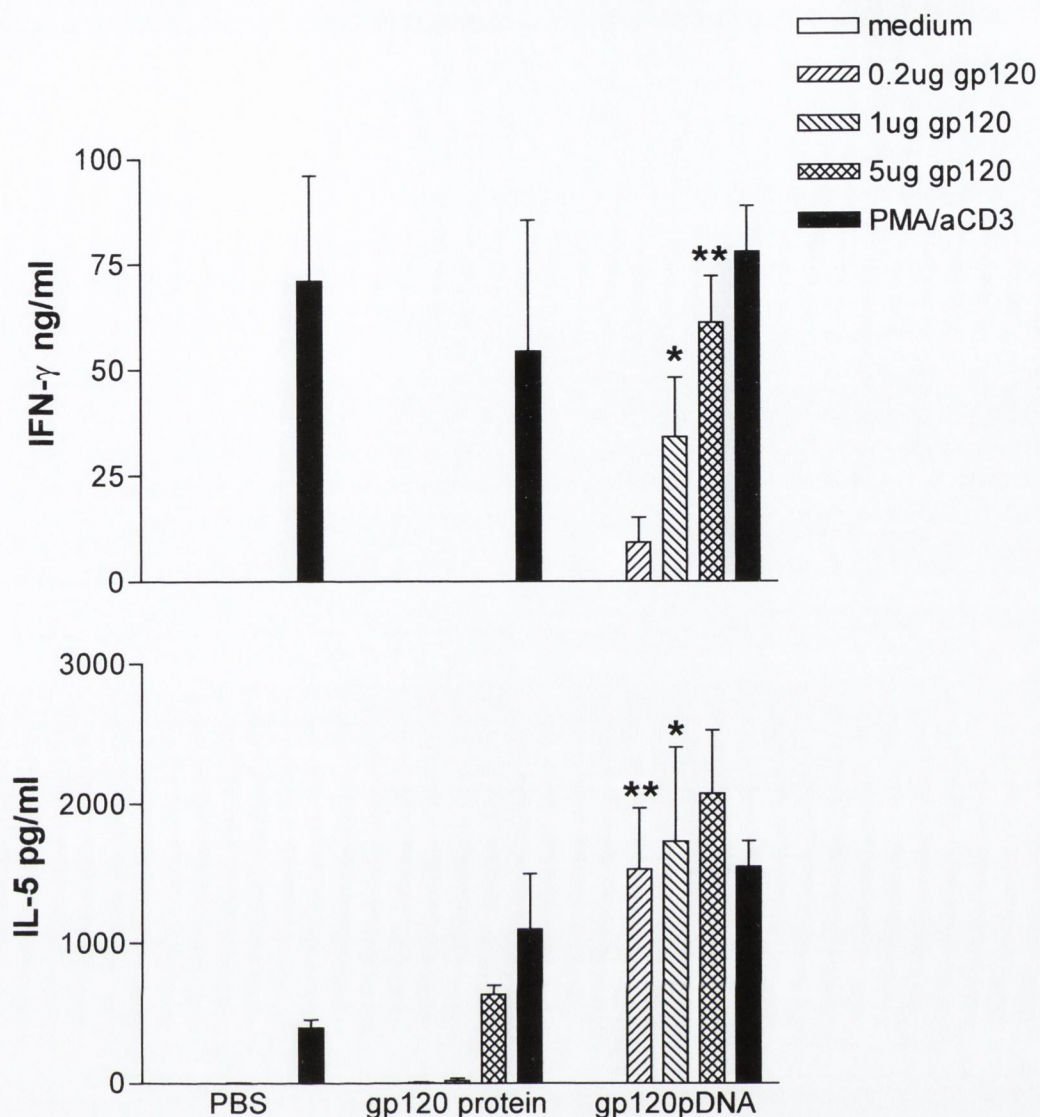


Figure 3.2. Persistence of systemic T cell responses following booster i.m. immunization with gp120 absorbed to alum or gp120pDNA. BALB/c mice were immunized at week 0 and boosted at week four. Two weeks later spleen cells were stimulated *in vitro* as described in the legend to figure 3.1. Cytokine concentrations from 72 hour supernatants were determined by specific IFN- γ and IL-5 immunoassays. Results from a single experiment expressed as the mean $\pm$ S.E. for four mice per group. * $p < 0.05$, ** $p < 0.01$, gp120-alum versus gp120pDNA, determined by Student's t-test.

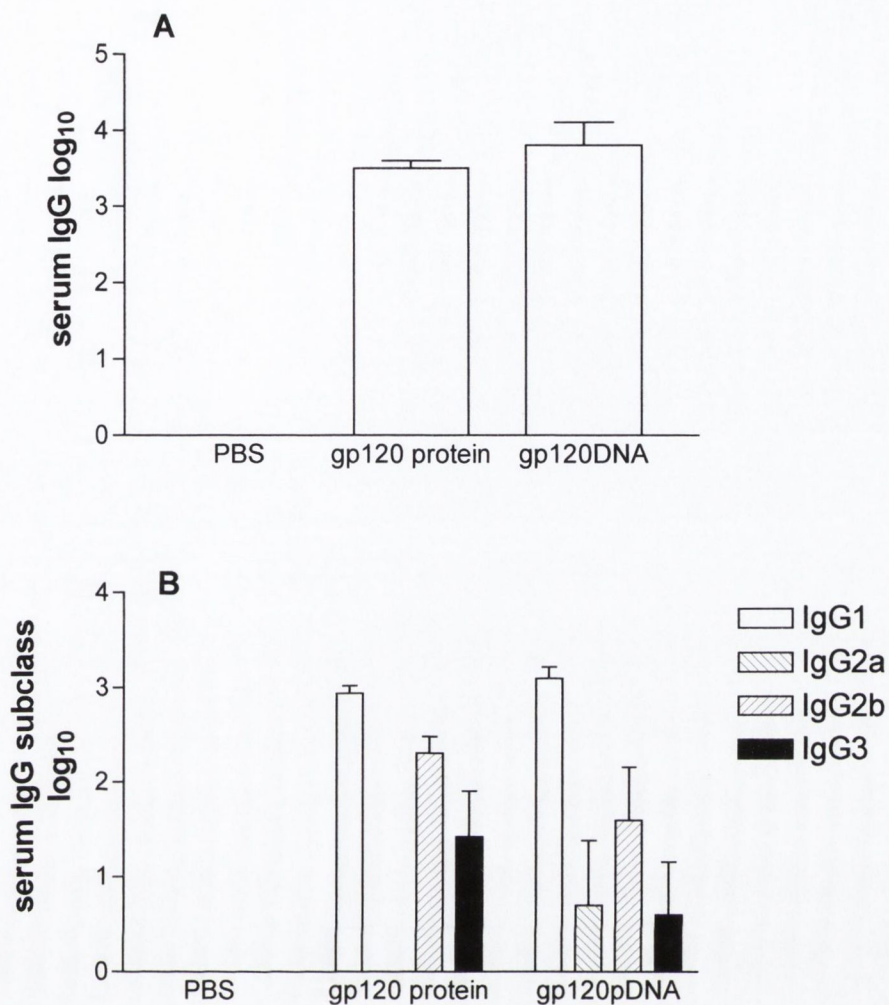


Figure 3.3. Antibody responses following a single intramuscular immunization with gp120 protein absorbed to alum or gp120pDNA. Mice were immunized as described in the legend to figure 3.1. Gp120-specific serum IgG titres were determined by ELISA. **(A)** total anti-gp120 IgG endpoint titres, **(B)** anti-gp120 IgG subclasses. Results are determined from endpoint titres and expressed as mean $\pm$ SE values for four mice in each group.

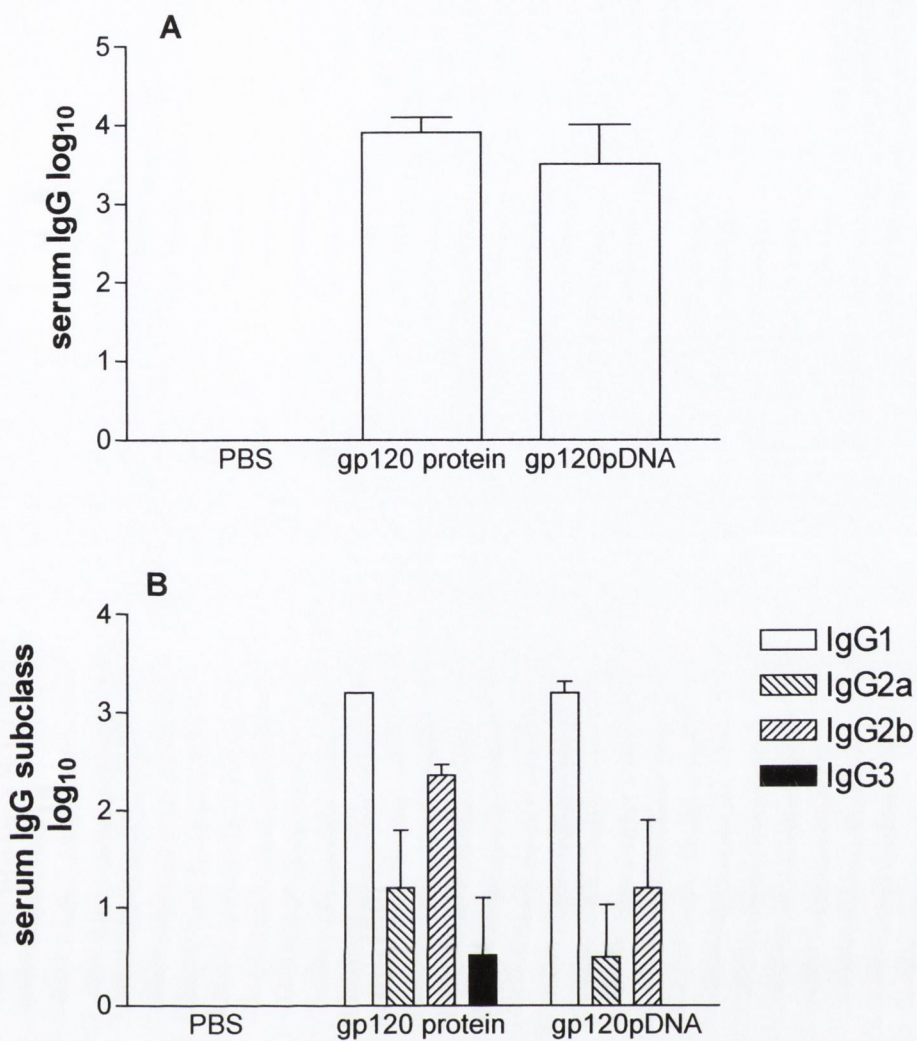
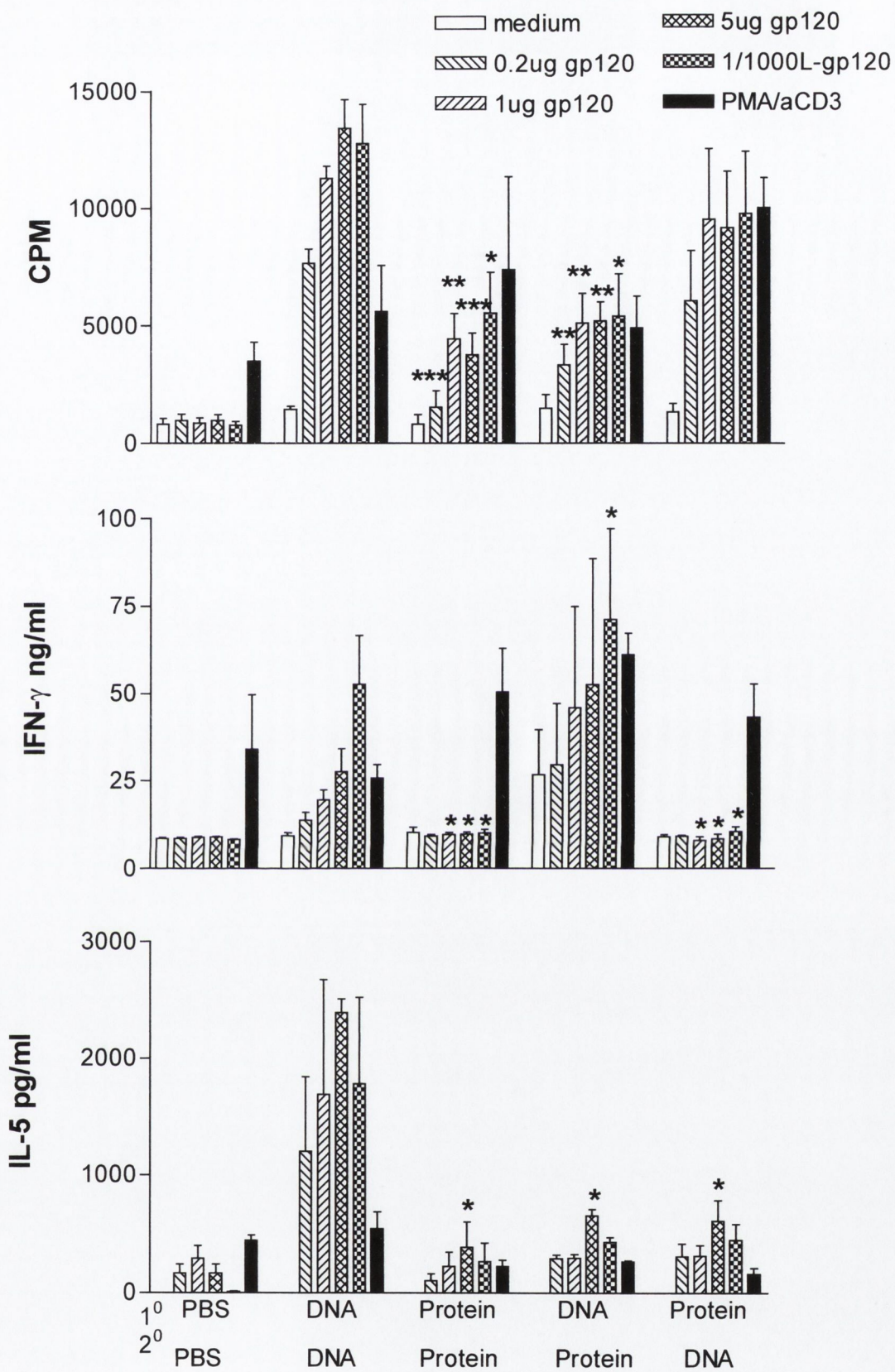


Figure 3.4. Antibody responses following two intramuscular immunizations with gp120 protein absorbed to alum or gp120pDNA. Mice were immunized as described in legend to figure 3.2. Gp120-specific serum IgG titres were determined by ELISA. **(A)** total anti-gp120 IgG endpoint titres, **(B)** anti-gp120 IgG subclasses. Results are determined from endpoint titres and expressed as mean $\pm$ SE endpoint titres for four mice in each group.

Figure 3.5. Effect of prime/boost immunization schedule on systemic T cell responses. Mice recieved a primary immunization with either 5 µg of gp120 protein absorbed to alum or 50 µg gp120pDNA and were boosted at week four as described above. At week six the mice were sacrificed and spleen cell cytokine production assessed as described in the legend to figure 3.1. Proliferation was assessed at 4 days by 3H-thymidine incorporation. Results from a single experiment expressed as the mean $\pm$ S.E. for four mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, DNA/DNA versus protein/protein, DNA/protein and protein/DNA by Student's t-test.



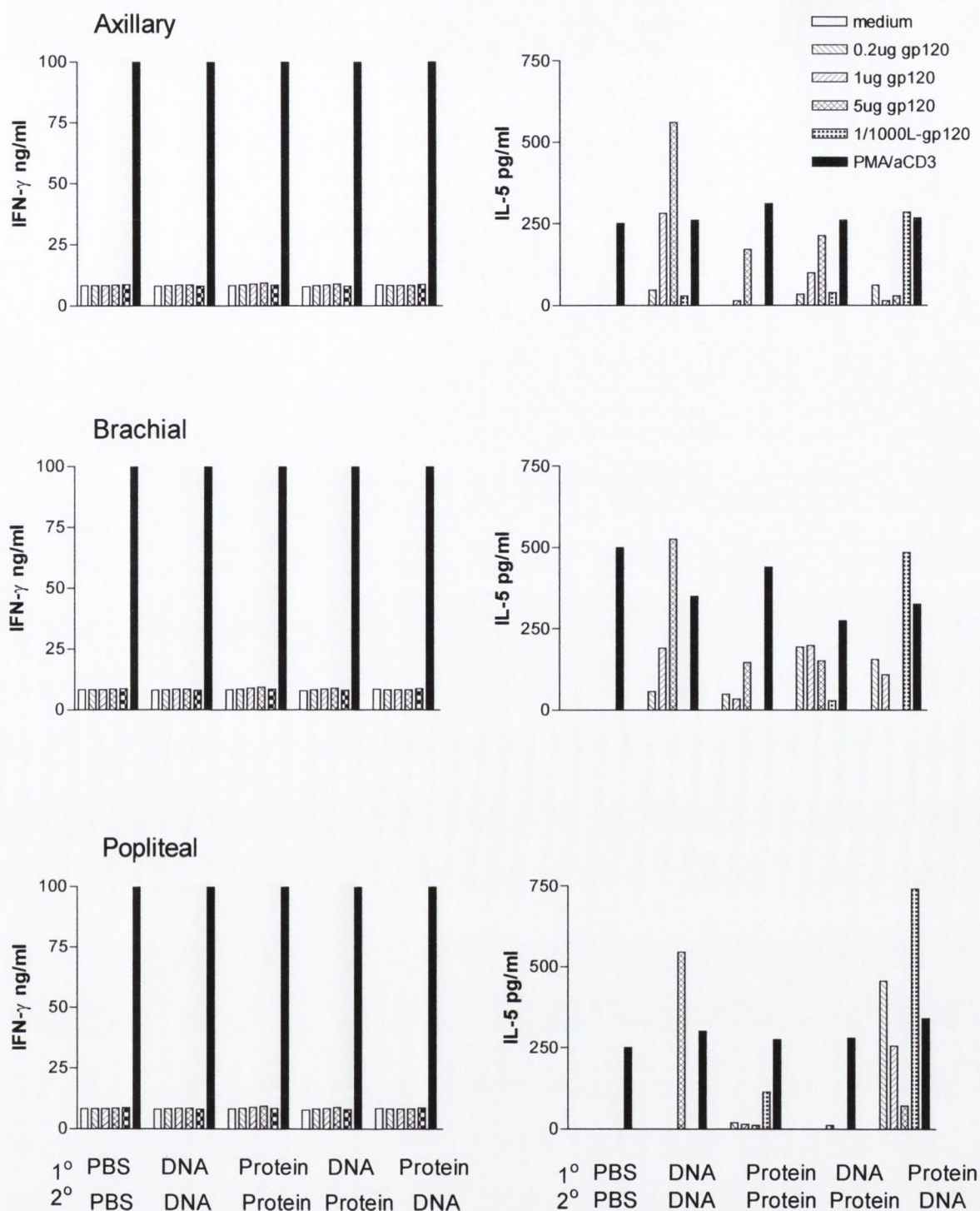


Figure 3.6. Effect of prime/boost immunization schedule on lymph node T cell responses. Mice were immunized as described in the legend to figure 3.5. At week six mice were sacrificed and T cell proliferation and cytokine production from axillary, brachial and popliteal lymph node cells was assessed by specific immunoassays as described in the legend to figure 3.5. Results represent responses from pooled lymph nodes for four mice per group.

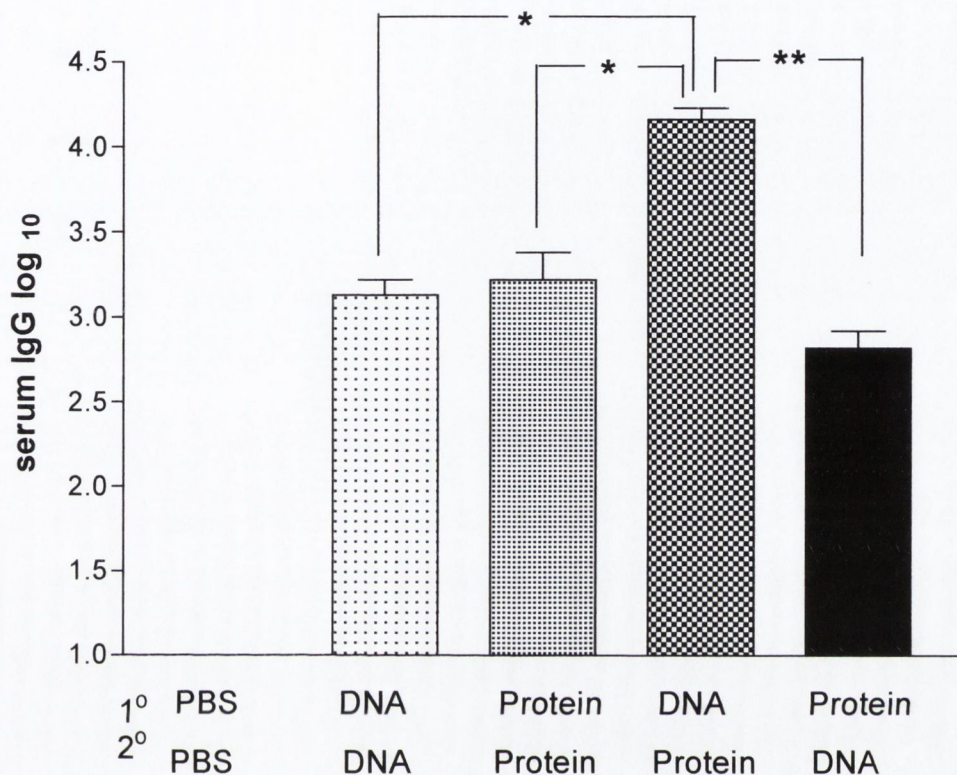


Figure 3.7. DNA prime and protein boost enhances anti-gp120 antibody responses. Mice were immunized as described in figure legend 3.5. Gp120-specific serum IgG titres were determined by ELISA. Results are mean $\pm$ SE endpoint titres for four mice in each group. * $p < 0.05$ and ** $p < 0.01$, DNA/protein versus DNA/DNA, protein/protein and protein/DNA by Student's t-test.

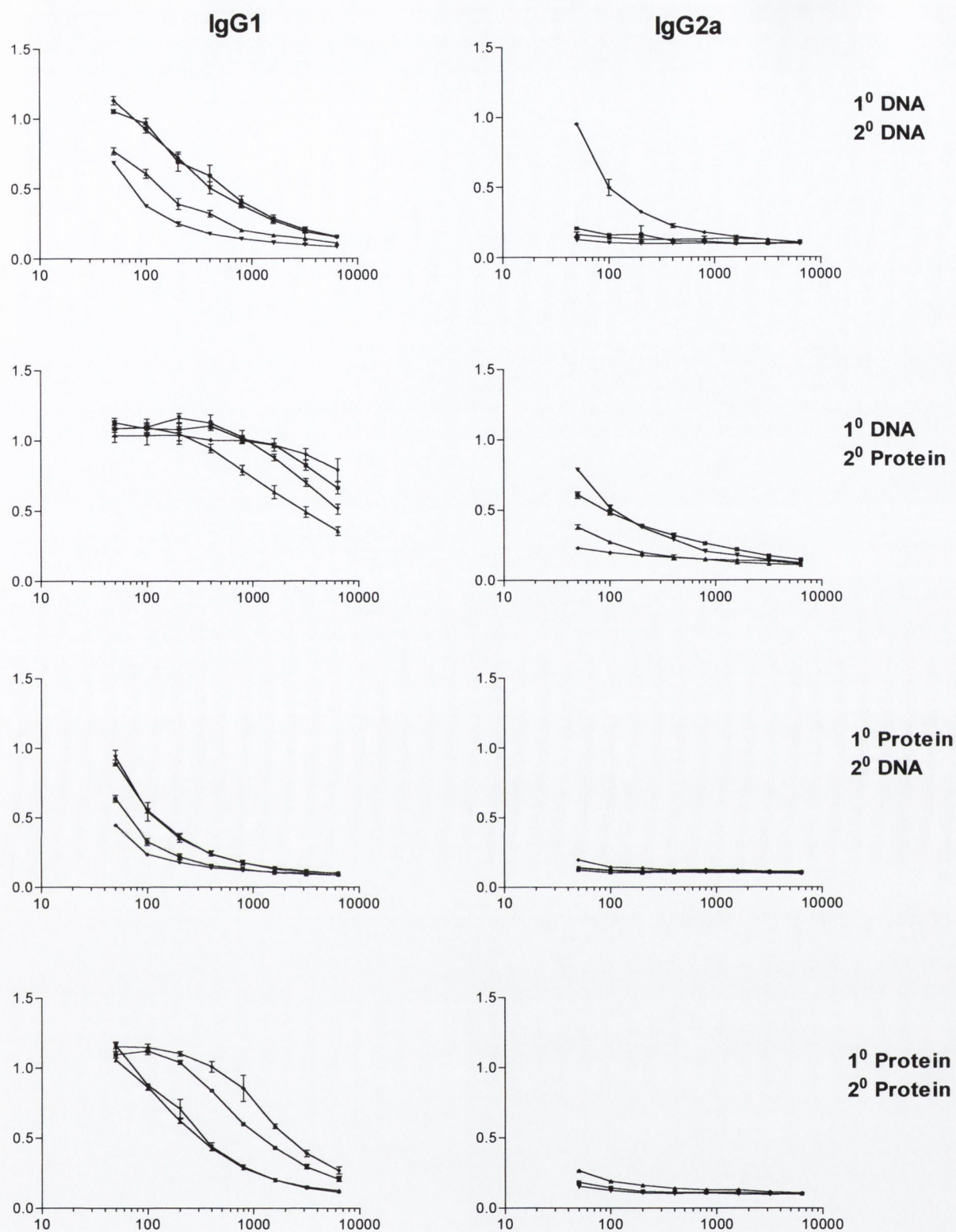


Figure 3.8. Anti-HIV gp120 IgG subclass induction following DNA prime and protein boost regime. Mice were immunized as described in the legend to figure 3.5. Gp120-specific serum IgG subclass titres were determined by ELISA. Each curve represents responses for individual mice (n=4).

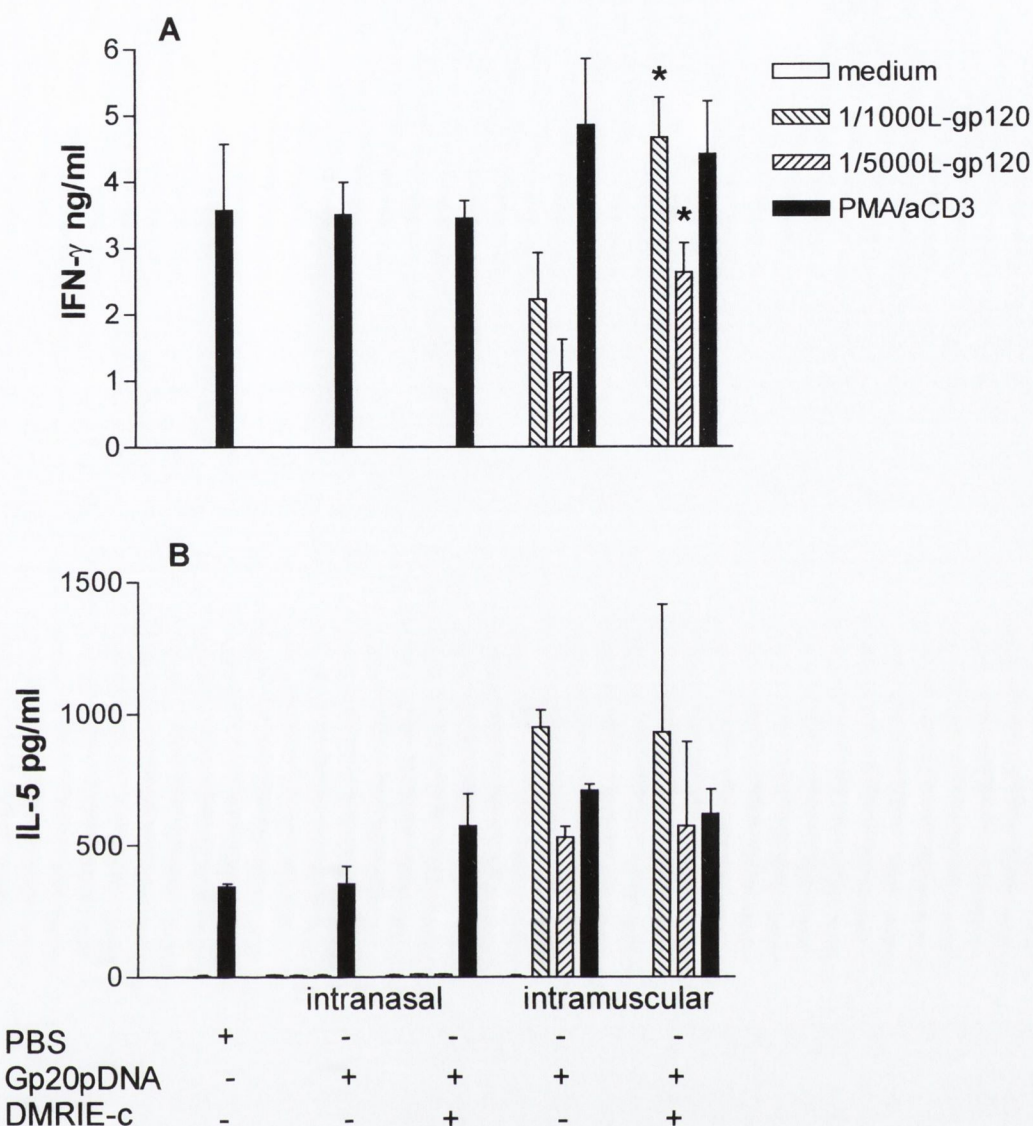


Figure 3.9. DMRIE-c enhance systemic T cell responses to parenterally but not nasally delivered gp120pDNA. Mice were immunized twice, at weeks 0 and 4, either i.m. or i.n. with PBS alone or 50 μ g gp120pDNA $\pm$ 10 μ g DMRIE-c. Supernatants were removed after 72 hours and the concentrations of **(A)** IFN- γ and **(B)** IL-5 were detected by specific immunoassay. Results from a single experiment expressed as the mean $\pm$ S.E. for four mice per group. * $p < 0.05$, gp120pDNA alone versus gp120pDNA + DMRIE-c determined by Student's t-test.

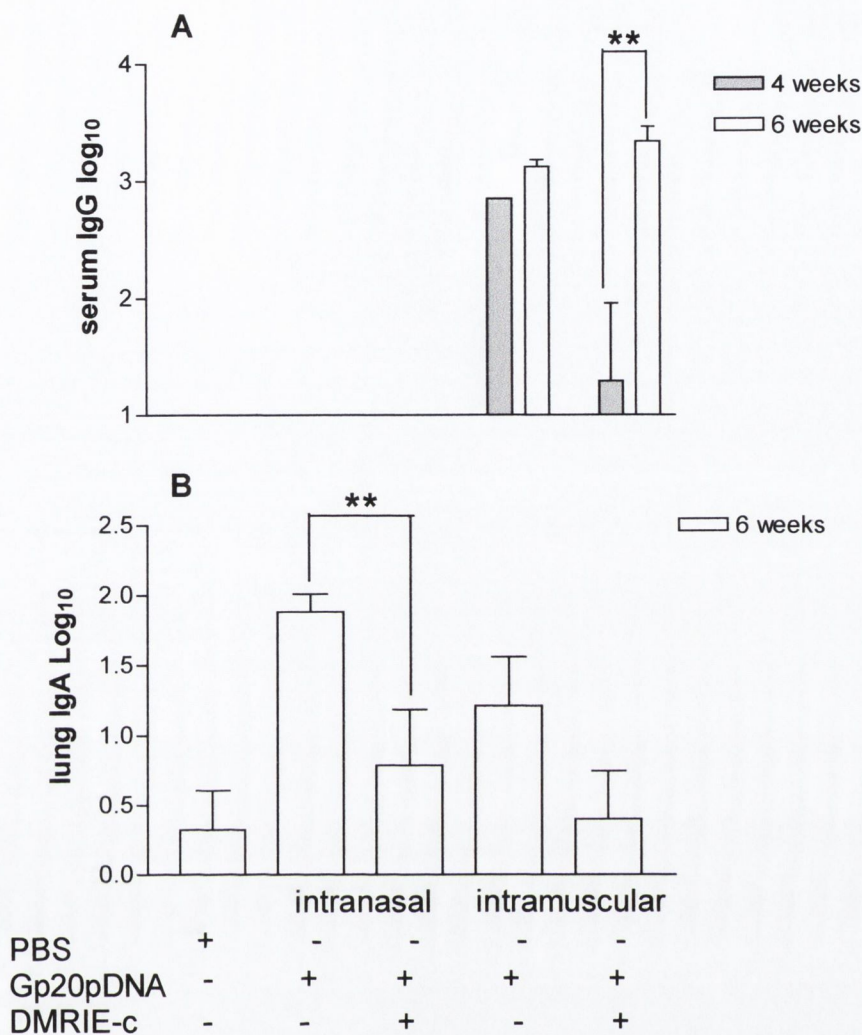


Figure 3.10. DMRIE-c reduces gp120-specific IgA titres but does not alter IgG antibody responses. Mice were immunized as described in the legend to figure 3.9. Gp120-specific serum IgG titres (**A**) and lung anti-gp120 specific IgA (**B**) were determined by immunoassay. Results are mean $\pm$ SE endpoint titres for four mice in each group. ** $p < 0.01$, gp120pDNA alone versus gp120pDNA + DMRIE-c, determined by Student's t-test.

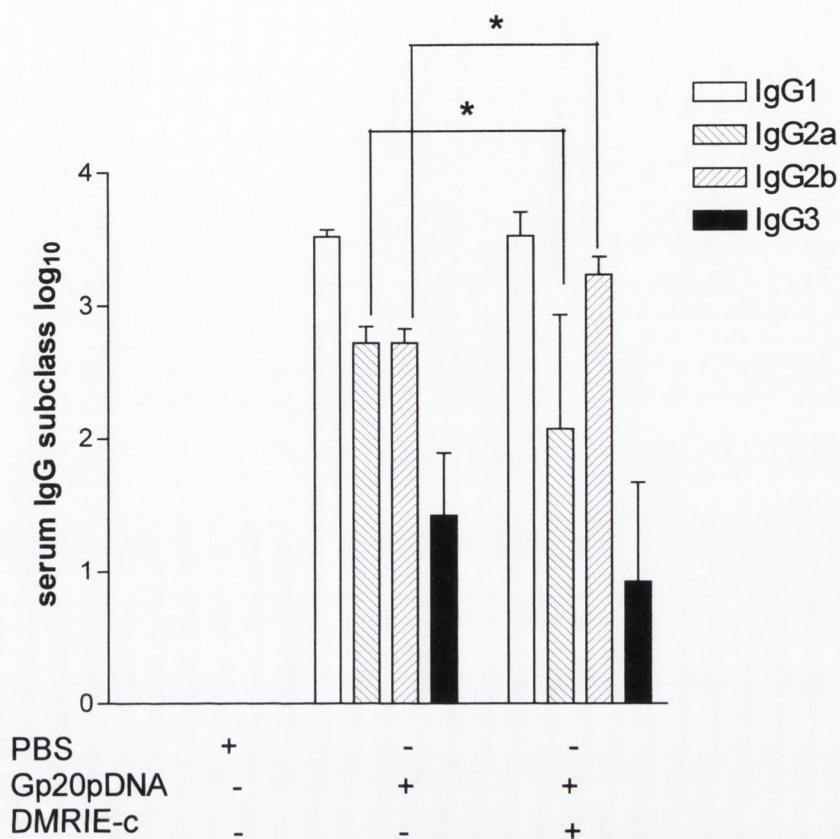


Figure 3.11. Effect of DMRIE-c on induction of specific IgG subclasses. Mice were immunized as described in the legend to figure 3.9. Gp120-specific serum IgG subclass titres were determined by immunoassay. Results are mean $\pm$ SE endpoint titres for four mice in each group. * $p < 0.05$, gp120pDNA alone versus gp120pDNA + DMRIE-c, determined by Student's t-test.

CHAPTER 4

APPROACHES TO ENHANCE TYPE-1 RESPONSES INDUCED WITH GP120PDNA

4.1 INTRODUCTION

Once an intracellular pathogen, such as HIV, has breached the host's first line of defense, the immune system plays a crucial role in the containment of the pathogen. However, a lack of potent neutralizing antibodies against envelope glycoproteins, infection together with depletion of the CD4⁺ T helper cell population and evasion of CD8⁺ CTL responses by rapid genetic changes, permits HIV to avoid immune control (Mascola and Nabel, 2001). Immune dysregulation between Th1 and Th2 immune responses contributes to the pathogenesis of HIV and progression to AIDS in HIV⁺ individuals due to a progressive reduction in dominant type-1 cytokine production (IL-12 and IFN- γ) and concomitant increase in type-2 cytokines (IL-4, IL-5, IL-6 and IL-10) (Clerici and Shearer, 1994). The generation and maintenance of effective immune responses against viral infections is dependant on both virus-specific CD4⁺ T helper cells and CD8⁺ CTL responses (Altfeld and Rosenberg, 2000). It has been suggested that IL-12 cytokine based therapy may selectively augment type-1 responses; administration of IL-12 to HIV patients significantly increased their CD4⁺ T cell counts (Clerici and Shearer, 1994).

In the previous chapter, it was demonstrated that i.m. immunization with a plasmid coding for HIV gp120 induced HIV-specific cells in the spleen capable of producing both Th1 and Th2 type cytokines. However, evidence from the literature suggests that CD4⁺ Th1 and CD8⁺ CTL may be critical for protection against HIV. Cytokine and co-stimulatory molecules play a crucial role in directing T cell responses and have been co-administered with DNA vaccines to improve immune responses against encoded foreign antigens. Co-stimulatory molecules, such as B7, and regulatory cytokines, such as IL-12, can alter the direction of T cell subset differentiation and improve the immunogenicity of DNA constructs. The heterodimeric cytokine IL-12 (composed of p35 and p40 subunits) is central to the development of a Th1 responses and plasmid coding for IL-12, IFN- γ and GM-CSF have been shown to enhance IFN- γ , IgG2a and proliferation responses with a concurrent reduction in IgG1 subclass titres (Gurunathan *et al.*, 2000).

Immune responses to DNA vaccines will be ineffective if the plasmid DNA is degraded by the host's enzymatic and acidic environment. Vaccine delivery systems such as PLG, microspheres, chitosan, liposomes, or the use of live attenuated vectors have been assessed for the delivery of DNA vaccines (Gurunathan *et al.*, 2000).

Injection of plasmid DNA formulated into delivery systems, such as liposomes can both protect the antigen from extracellular enzymatic degradation and facilitate enhanced uptake of plasmid DNA by macrophages and DCs.

The rationale behind this study was to evaluate the possibility of modulating the immune response induced following vaccination with gp120pDNA towards a more potent type-1 profile, known to be important in anti-viral immunity. The first approach was to characterize the optimal dose of gp120pDNA that would generate the strongest immune responses in mice. The potential of a plasmid coding for the IL-12 gene was evaluated for its ability to enhance the induction of gp120-specific responses following i.m. immunization with gp120pDNA. The optimal ratio of gp120pDNA and DMRIE-c combination, that can enhance the response to gp120pDNA vaccines, was established. Finally a formulation composed of gp120pDNA with both IL-12pDNA and DMRIE-c was administered to mice in an attempt to further enhance the gp120-specific immune type-1 response.

4.2 RESULTS

4.2.1 CO-ADMINISTRATION OF PLASMID CODING FOR IL-12 CYTOKINE ENHANCES TYPE-1 T CELL RESPONSES TO Gp120pDNA

The results reported in chapter 3 established that gp120pDNA administered via the i.m. route induced a mixed Th1/Th2 type response. Co-administration of plasmids coding for cytokine genes can modulate the differentiation of naïve T cells to the Th1 or Th2 subtype. In the present study, the immune profile induced by gp120pDNA was examined following co-administration of a plasmid DNA coding for the heterodimeric cytokine, IL-12 (IL-12pDNA).

Female BALB/c mice were immunized i.m. with 50 µg of either gp120pDNA or IL-12pDNA or a combination of the two plasmids with a final dose of 100 µg of plasmid DNA. Analysis of the systemic T cell responses revealed that the addition of the IL-12pDNA to the vaccine formulation could modulate the immune response generated by gp120pDNA alone. Examination of the cytokine responses demonstrated that spleens from gp120pDNA immunized mice produced both IL-5 and IFN-γ in response to specific antigen stimulation *in vitro* (Fig 4.1). Co-administration of IL-12pDNA enhanced IFN-γ production ($p<0.05$) and reduced the production of gp120-specific IL-5 ($p<0.05$). Spleen cells from mice immunized with PBS or IL-12pDNA did not produce any significant cytokine in response to antigen stimulation *in vitro*. However, the cells from these mice were functional as they could produce cytokines in response to the polyclonal activator PMA and anti-CD3 (Fig 4.1).

The cytokine profile generated in the draining lymph nodes (axillary, brachial and cervical) was also examined. It was shown in chapter 3 that immunization with gp120pDNA does not induce gp120-specific IFN-γ secretion in local draining lymph node cells. The data is only presented for gp120-specific IL-5 data as no IFN-γ was detected in antigen-stimulated lymph node cells from mice immunized with gp120pDNA. The results demonstrated that co-administration of IL-12pDNA reduced the IL-5 response to gp120pDNA (Fig 4.2). Lymph node cells from mice immunized with gp120pDNA produced IL-5 in response to gp120pDNA, with strongest responses observed in the brachial and cervical nodes. In contrast, co-administration of IL-12pDNA with gp120pDNA adversely affected IL-5 production from lymph node cells (Fig 4.2) and this correlates with the findings in the spleen (Fig 4.1)

4.2.2. CO-ADMINISTRATION OF IL-12pDNA WITH Gp120pDNA REDUCES SERUM IGG BUT ENHANCES IGG2A ANTIBODIES.

Co-administration of IL-12pDNA with gp120pDNA enhances gp120-specific Th1 type responses in the spleen and reduced IL-5 production in both spleen and draining lymph node cells. The influence of co-delivery of IL-12pDNA and gp120pDNA on serum IgG production was analyzed and revealed a moderate reduction ($p < 0.05$) in serum anti-gp120 IgG titres (Fig 4.3A). The pattern was observed after both single and booster immunizations. However both groups of mice immunized with or without IL-12pDNA induced higher titres of anti-gp120 IgG antibodies ($\sim 0.5 \log_{10}$) following a booster immunization (Fig 4.3A). Examination of the subclass induced revealed a significant increase in the IgG2a subclass ($p < 0.05$) following co-administration of IL-12pDNA and gp120pDNA (Fig 4.3B). Immunization with gp120pDNA alone induced IgG1: IgG2a in a ratio of 3.4: 2.6 that was shifted toward a balanced IgG subclass profile by the addition of IL-12pDNA (Fig 4.3B).

4.2.3. SYSTEMIC T CELL RESPONSES ARE DEPENDANT ON THE DOSE OF PLASMID DNA AND CAN BE ALTERED BY CO-ADMINISTRATION OF DMRIE-C

The optimal dose of plasmid DNA that would induce the strongest immune response was determined by immunizing BALB/c mice with a range of doses of gp120pDNA (0.05, 0.5, 5, 50 μg). Increasing the dose of gp120pDNA administered enhanced the concentration of cytokine production by spleen cells from immunized mice (Fig 4.4). IL-5 production by spleen cells from immunized mice increased with increasing immunizing dose of plasmid. Only the high doses of gp120pDNA (5 and 50 μg) induced detectable gp120-specific IFN- γ , whereas lower doses (0.05 and 0.5 μg) induced dominant Th2 type responses with IL-5 and no detectable IFN- γ (Fig 4.4). Therefore, high doses (5 and 50 μg) generated a mixed Th1/Th2 type response, whereas a polarized Th2 type response was induced following immunization with the two lower doses of gp120pDNA (0.5 and 0.05 μg) (Fig 4.4B).

In contrast to the dose dependant pattern observed with gp120pDNA administration alone, the addition of cationic liposomes revealed a different pattern. The combination of gp120pDNA and DMRIE-c induced gp120-specific IL-5 and IFN- γ cytokine producing spleen cells in mice immunized with the high doses (5 and 50 μg) of gp120pDNA (Fig 4.4B). However, addition of DMRIE-c abrogated the IL-5

production ($p < 0.05$) previously observed at the two lower doses (0.5 and 0.05 μg) of gp120pDNA.

4.2.4. INFLUENCE OF DOSE AND FORMULATION WITH DMRIE-C ON CYTOKINE RESPONSES IN LYMPH NODES CELLS

Polarization towards a dominant Th2 type response was observed in the spleen by reducing the dose of gp120pDNA administered. Analysis of cytokine profiles generated in the draining lymph nodes (axillary, brachial, popliteal, cervical and inguinal) following i.m. immunization with gp120pDNA generated potent Th2 type responses indicated by the sole production of IL-5 (Fig 4.5). The lowest dose of gp120pDNA (0.05 μg) resulted in low to detectable concentrations of IL-5 that increased with increasing the dose of gp120pDNA. Axillary, brachial and cervical lymph node cells induce more consistent IL-5 production compared with the popliteal and inguinal nodes.

Co-administration with DMRIE-c enhances gp120-specific IL-5 production in response to 5 μg gp120pDNA (Fig 4.5C). However, co-administration of DMRIE-c with other doses of gp120pDNA resulted in an overall reduction in the ability of gp120pDNA to induce IL-5 producing cells in the lymph nodes (Fig 4.5). IL-5 production could not be detected in antigen-stimulation lymph node cells from mice immunized with the lowest doses of gp120pDNA (0.05 and 0.5 μg) administered with DMRIE-c (Fig 4.5A and 4.5B). The higher doses of gp120pDNA (5 and 50 μg) produced gp120-specific IL-5 producing lymph node cells, however low-levels of IL-5 were generated following immunization with 50 μg gp120pDNA and DMRIE-c.

4.2.5 SERUM IgG RESPONSES FOLLOWING GP120pDNA ADMINISTRATION ARE SIGNIFICANTLY ENHANCED WITH DMRIE-C

In the previous chapter, DMRIE-c was shown to moderately enhance gp120-specific IFN- γ and IgG responses but adversely affected IgA production in mice following i.m. immunization with gp120pDNA. Here, the potential adjuvant and protective influence of DMRIE-c was examined over a range of doses of gp120pDNA. BALB/c mice were immunized with gp120pDNA (0.05, 0.5, 5, 50 μ g) $\pm$ 10 μ g DMRIE-c. To ensure all variables are assessed it would be valuable to investigate the 5:1 ratio of plasmid DNA and liposomes at each dilution, however it is impractical to measure 0.01 μ g DMRIE-c. Anti-HIV gp120 serum IgG responses were assessed two weeks post immunization and revealed a dose dependent effect. The 50 μ g dose of gp120pDNA induced ~100-fold stronger gp120-specific IgG responses than the 0.05 μ g dose. However, co-administration of gp120pDNA with 10 μ g DMRIE-c enhanced serum IgG titres. Even the lowest 0.05 μ g dose of gp120pDNA ($p < 0.001$), which generated a serum IgG titre of $\sim 2.5 \log_{10}$, which was almost 2 logs greater than that observed with gp120pDNA administered alone (Fig 4.6).

4.2.6 NON-CODING PLASMID DNA ENHANCES SYSTEMIC IMMUNE RESPONSES

It has been suggested that the plasmid backbone, in particular the CpG content, may influence the induction of Th1 responses with DNA vaccines. This study examined the influence of the plasmid type on gp120-specific IFN- γ production in response to gp120pDNA. BALB/c mice were immunized i.m. with 50 μ g gp120pDNA and 50 μ g of empty non-coding plasmid, JA4304 (parent plasmid of gp120pDNA), IL-12pDNA or PI.19 (parent plasmid of IL-12pDNA). Immunization with gp120pDNA generated gp120-specific cells in the spleen that produced IL-5 and IFN- γ producing spleen cells when stimulated *in vitro* with gp120 antigen (Fig 4.7). Addition of 50 μ g IL-12pDNA to gp120pDNA enhanced the production of cytokines, with spleen cell IFN- γ production increasing over that from mice immunized with gp120pDNA alone. However, co-administration of the empty parent plasmids, JA4304 and PI.19, also enhanced T cell responses to gp120pDNA. Inclusion of JA4304 with gp120pDNA resulted in a moderate increase in IL-5 production ($p < 0.05$) from spleens cells but no enhancement in antigen-specific IFN- γ production (Fig 4.7). Furthermore, co-administration of PI.19 resulted in a 4-fold increase in IL-5 cytokine production

($p < 0.01$) with a similar IFN- γ responses to those observed with gp120pDNA alone (Fig 4.7).

This study also examined the influence of non-coding plasmid on the gp120 specific responses in the lymph nodes following i.m. immunization with gp120pDNA. It was demonstrated in chapter 3 that mice immunized i.m. with gp120pDNA generated a consistent Th2 type response in the axillary, brachial and cervical lymph nodes. The data is only presented for gp120-specific IL-5 data as no IFN- γ was detected in antigen-stimulated lymph node cells from immunized mice. Co-administration of non-coding parent plasmids, JA4034 and PI.19, with gp120pDNA enhanced the gp120-specific type-2 responses especially in the brachial lymph node (Fig 4.8). Addition of IL-12pDNA to gp120pDNA reduced IL-5 producing lymph node cells. Therefore it appears that non-coding parent plasmids may possess a Th2 inducing capacity that is abrogated following the insertion of the IL-12 cytokine gene (Fig 4.8).

4.2.7 CO-ADMINISTRATION OF GP120pDNA AND IL-12pDNA WITH DMRIE-C ENHANCES GP120-SPECIFIC TYPE-1 T CELL RESPONSES

It was established in chapter 3 that systemic T cell responses induced with gp120pDNA can be polarized to the type-1 subtype with IL-12pDNA or DMRIE-c. Therefore, it was decided to examine the combined effect of IL-12pDNA and DMRIE-c into one formulation, in an attempt to further enhance the gp120-specific Th1 type response.

BALB/c mice were immunized i.m. with 50 μ g of gp120pDNA and IL-12pDNA with or without 10 μ g DMRIE-c. Spleens from immunized mice were isolated at 2 weeks and assessed for gp120-specific cytokine production. In this assay the concentrations of IFN- γ detected by immunoassay were low and background concentrations of IL-5 were high. However, co-administration of IL-12pDNA or PI.19 significantly ($p < 0.05$) improved the production of gp120-specific IFN- γ from spleen cells, compared to that induced by gp120pDNA alone. Addition of IL-12pDNA, but not PI.19, to gp120pDNA reduced the concentrations of IL-5 production by antigen-stimulated spleen cells. The inclusion of DMRIE-c further reduced gp120-specific IL-5 production ($p < 0.05$) to almost background levels and a concurrent increase in the concentrations of gp120-specific IFN- γ ($p < 0.05$) (Fig 4.9).

It was demonstrated in Fig. 4.9 that co-administration of IL-12pDNA and DMRIE-c with gp120pDNA improved gp120-specific Th1 type responses in the spleen. Therefore, this formulation was examined for its capacity to modulate responses in lymph nodes. Following a single i.m. immunization with all three vaccine components (gp120pDNA, IL-12pDNA and DMRIE-c), gp120-specific IL-5 concentrations detected in the axillary, brachial and cervical nodes were significantly lower than in mice immunized with gp120pDNA alone (Fig 4.10). Antigen-specific IFN- γ production was undetectable.

4.3 DISCUSSION

The gp120, but not gp160, envelope protein from HIV has been reported to generate protective responses in chimpanzees (Berman *et al.*, 1990). This study investigated the ability of gp120pDNA to induce responses that may fulfill some of the criteria for the development of a prophylactic vaccine for HIV. The significant findings of this study are that unlike other DNA vaccines, plasmid DNA coding for gp120 generates Th2 or Th1/Th2 type responses in mice. Furthermore, co-administration of IL-12pDNA and DMRIE-c cationic liposomes was shown to enhance gp120-specific type-1 and to reduce type-2 responses.

The strength and type of immune response induced with DNA immunization depends not only on the route of administration but also on the quantity of injected plasmid DNA. To determine the effect of dose, BALB/c mice were immunized with a range of doses of gp120pDNA. The strength of the T cell and antibody responses increased as the dose of gp120pDNA was increased from 0.005 μ g to 50 μ g. This dose dependant effect has been observed in other studies; increasing the dose of HApDNA (from 0.1 μ g to 100 μ g) administered to BALB/c mice resulted in increased HA-specific IFN- γ production from spleen cells of immunized mice (Johnson *et al.*, 2000). Interestingly, at the lower doses of gp120pDNA there was an absence of detectable IFN- γ , which resulted in a polarized Th2 type response. The polarization may be due to the low concentration of plasmid DNA and ultimately lower quantities of immunostimulatory CpG motifs reaching the target APC. Therefore, the resulting type-2 response may be largely influenced by the persistent antigen expression of the encoded gp120 protein, which induces polarized Th2 responses (Mellado *et al.*, 1998).

There is evidence to suggest that a switch from Th1 to Th2 is associated with the progression to AIDS in infected individuals. Interestingly, vaccination of seronegative volunteers with HIV-1 immunogens, gp120 and the V3 loop peptide, induced predominantly IFN- γ secreting cells, with an increase in IL-4 and antibody production following multiple immunizations (Evans *et al.*, 1998). Switching of dominant immunoregulatory cytokines towards type-2 following HIV immunization may lead to immune deterioration as a result of priming the immune system in a direction that favors HIV survival. However, apoptosis of peripheral blood monocytes in HIV⁺ infected individuals is promoted by type-2 cytokines and prevented by type-1

cytokines (Clerici and Shearer, 1994). Candidate HIV vaccines have been shown to be effective at inducing neutralizing antibodies but fail to protect against infection in non-human primate models, therefore cellular responses, in particular virus-specific CD4⁺ Th cells and CD8⁺ CTL response (Altfeld and Rosenberg, 2000) may play a critical role in preventing infection with HIV (Cease and Berzofsky, 1994).

The studies in chapter 3 demonstrated that i.m. immunization with gp120pDNA induced a mixed Th1/Th2 type response in the spleen and polarized Th2 type response in lymph nodes. Immunization strategies that induce Th2 type responses may be ineffective against HIV infection and may contribute to the progression to AIDS. However, enhancement of type-1 cytokine production, in particular IL-12, would preferentially activate Th1 differentiation from naive precursor T cells. IL-12 may reduce IL-10 production induced by gp120 and prevent production of Th2 cells that may contribute to HIV persistence (Taoufik *et al.*, 1997). Therefore, vaccines composed of plasmids coding for cytokine genes, such as IL-12 that can enhance the induction of Th1 type responses, could be useful in HIV vaccine design. Vaccines composed of IL-12 or IFN- γ can be used to improve Th1 type responses (Kim *et al.*, 2001), whereas IL-4/5/10 cytokine genes that direct Th2 responses can be incorporated into extracellular bacterial vaccine designs. Co-administration of a hepatitis B virus (HBV) DNA vaccine with IL-12 or IFN- γ cytokine plasmids enhanced antigen-specific Th1 responses, whereas IL-2 or GM-CSF plasmid co-administration was shown to enhance HepBAG-specific Th2 responses (Chow *et al.*, 1998).

Co-administration of the IL-12 cytokine gene was chosen for this study due to the pivotal role in activation of Th1 cells and ability to induce IFN- γ secretion from a range of immune cells, including T cells. Addition of IL-12pDNA to gp120pDNA enhanced the induction of IFN- γ secreting cells with a concomitant decrease in IL-5 production. This may potentially be a direct result of an increase in the quantity of plasmid administered. This variable can be controlled by including the empty backbone for the IL-12pDNA to determine the direct influence on the response with the IL-12 cytokine. Nevertheless, these findings are consistent with a study that examined the adjuvant role of IL-12pDNA in a murine herpes infection model which demonstrated that co-delivery of IL-12pDNA with a herpes simplex virus type-2 (HSV-2) DNA vaccine significantly enhanced IL-2, IFN- γ , RANTES and MIP-1 α

production, but inhibited antigen-specific antibody and Th2 cytokine production (Sin *et al.*, 1999). These findings also correlate with the observations in this study, whereby IL-12pDNA co-administration drives antigen-specific type-1 responses. Moreover, following challenge with simian HIV (SHIV), viremia was controlled and anemastic responses were enhanced, in non-human primates immunized with condon-modified DNA plasmids and augmented with DNA plasmid coding for IL-12-Ig (Mascola and Nabel, 2001).

Co-administration of IL-12pDNA enhanced IFN- γ production but the empty parent plasmid, PI.19, also enhanced IFN- γ production following co-administration with gp120pDNA. Therefore, the enhancement effect of IL-12pDNA may be partially due to additional immunostimulatory sequences in the overall dose of plasmid DNA administered. Others have reported similar findings but dismissed the notion that CpG motifs contributed to the increase in IFN- γ production. Studies investigating IL-12 as cytokine therapy for tumor regression showed that delivery of a plasmid coding for IL-12 in the reverse order or additional CpG-ODN could not effectively reduce metastasis formation in a similar manner to IL-12pDNA. This indicated that the effect was a direct result of the IL-12 cytokine (Schultz *et al.*, 2000). The current co-immunization study revealed a slight reduction in serum anti-gp120 IgG antibody titres, but enhanced murine IgG2a associated with type-1 responses. These finding are consistent with studies demonstrating addition of anti-IL-12 or anti-IFN- γ can enhance antibody production (Toda *et al.*, 1997). Barouch *et al.*, (1998) demonstrated the importance of timing the administration of additional plasmids. A distrionic DNA vaccine encoding HIV-1 gp120_{IIB} and IL-2 or IL-4 plasmids induced lower gp120-specific antibody responses than HIV-1 gp120 plasmid DNA alone. In contrast, administering IL-2 or IL-4 plasmids separately, and after the initial priming with the gp120 plasmid, augmented immune responses.

Cationic liposomes that can act as antigen delivery systems and exhibit adjuvant activity represent an attractive alternative to other delivery systems for DNA vaccines. Incorporation of plasmid DNA into cationic lipids DMRIE/DOPE can increase the duration of antigen expression (Barnfield *et al.*, 1997). A prolonged HIV-specific immune response may be necessary as humans may encounter the virus months or years after vaccination. It has been demonstrated that immunization with liposome/plasmid DNA combination resulted in a HIV specific CTL response that

could be detected 4 months following immunization (Ishii *et al.*, 1997). Similarly, encapsulation of HIV gp120 recombinant protein into biodegradable PLG microparticles induced long lasting CD8⁺ CTL and Th1 immune responses in mice (Moore *et al.*, 1995). Furthermore, mice injected with CpG-ODN and OVA protein, encapsulated in sterically stabilized liposomes, generated a 2-fold increase in the duration of protective immune responses against pathogenic challenge and significantly enhanced both antigen-specific IFN- γ and IgG antibody titres (Gursel *et al.*, 2001).

The present study has shown that liposomes can enhance Th1 type responses following parenteral immunization with gp120pDNA. Intramuscular administration of liposomes complexes is feasible, as muscle cells in a post-mitotic state of maturation, can facilitate prolonged expression of encoded antigen, which has been demonstrated in transfected cultured murine muscle cells with cationic lipid: DNA complexes (Dodds *et al.*, 1998). The formulation of liposomes is reported to influence induction of immune responses, as cellular and molecular barriers can result in reduced cell transfection efficiency. Escape of homogeneous DNA/lipid complex from endosomal aggregates and dissociation from lipid complexes are potentially limiting steps in the transfer of DNA from the cell surface to the nucleus (Zabner *et al.*, 1995). In this study gp120pDNA and DMRIE-c were simply mixed by continuous rotation to generate a heterogeneous population of gp120pDNA and DMRIE-c. However, these complexes were capable of inducing responses in both the spleen and draining lymph nodes, indicating that gp120pDNA was sufficiently protected to allow induction of improved Th1 type responses. DMRIE-c which contain the neutral lipid, cholesterol, have been shown to increase the stability of liposomes and aid in preventing disruption of plasmid DNA: liposomes complexes following systemic DNA delivery (Templeton *et al.*, 1997). Intravenous delivery of cholesterol with plasmid DNA encoding luciferase in cationic liposome multilamellar vesicles increased luciferase gene expression by 1,740-fold compared to that observed in the absence of cholesterol (Liu *et al.*, 1997).

Inclusion of DMRIE-c with gp120pDNA adversely affected the induction of gp120-specific IL-5 production detected in draining lymph node cells, but did moderately increase IFN- γ production from spleen cells of immunized mice. Similarly, i.m. injection of a HIV DNA vaccine combined with liposomes (DC-chol

and DOPE) in a ratio of 17:3, increased IFN- γ production from lymphoid cells (Toda *et al.*, 1997). It appears that improvement in type-1 induction following immunization with gp120DNA may result from prolonged circulation of intact plasmid DNA protected by the liposomes. Further evidence of adjuvant activity of liposomes was demonstrated in the present study in mice following co-administration of gp120pDNA, IL-12pDNA and DMRIE-c; this formulation induced a significant increase in gp120-specific IFN- γ and marked reduction in antigen-specific IL-5 secretion from both spleen and lymph nodes. These findings correlate with a study showing that i.n. administration of *env* gp160 and *rev* coding HIV DNA vaccine combined with liposomes and IL-12pDNA induced strong HIV-specific CTL responses. Taken together these studies demonstrate the effectiveness of combining the IL-12pDNA and liposomes into one vaccine composition (Okada *et al.*, 1997).

Specific IgA and IgG antibodies were detected in BALB/c mice immunized i.n. or i.m. with 50 μ g plasmid DNA formulated with DMRIE/DOPE (Gao *et al.*, 1997). The present study demonstrates that co-administration of DMRIE-c significantly enhanced anti-gp120 IgG antibodies when combined with gp120pDNA, suggesting that liposomes may enhance APC uptake of plasmid DNA. This may occur indirectly through T cell help mediated by IFN- γ promoting B cells secretion of IgG2a. Similarly, i.m. immunization of BALB/c mice with cationic liposomes and 5 μ g plasmid DNA encoding HIV antigens induced antibodies against V3 peptide antigen which were significantly enhanced compared to titres achieved with plasmid DNA administered alone (Ishii *et al.*, 1997). Furthermore, mannan-coated liposomes administered i.m or i.n. with a HIV DNA vaccine, encoding *env* and *rev* genes, were shown to significantly enhance both fecal IgA and serum IgG antibody titres. IgG2a responses were also enhanced suggesting that Th1 responses were also augmented by co-administered liposomes (Toda *et al.*, 1997). The improvement of immune responses with co-administered liposomes may be a result of increased cell transfection efficiency leading to higher concentrations of plasmid DNA reaching the nucleus of the cells and ultimately increased expression of the encoded antigen.

Synthetic ODN containing immunostimulatory CpG motifs have been shown to act as adjuvants for induction of systemic Th1 responses (Klinman *et al.*, 1999) following parenteral (Putney and Burke, 1998) and mucosal administration of protein antigens (McCluskie *et al.*, 2000). However, LT was found to be a more potent Th1

adjuvant than CpG-ODN for mucosal immunization (Olszewska *et al.*, 2000). In the present study, co-administration of non-coding plasmids with gp120pDNA enhanced spleen and lymph node cell responses. Addition of the JA4304 (parent plasmid for gp120pDNA) moderately enhanced gp120-specific Th1 responses. Interestingly, inclusion of PI.19 (parent plasmid for IL-12pDNA) further improved these responses and this may be explained by the differences in plasmid composition, for example the content of CpG motifs that may shift help towards a type-1 response. It is noteworthy that PI.19 enhanced gp120-specific IL-5 production from local lymph nodes, indicating that some component of this vector can also contribute to Th2 induction. Others have shown that CpG-ODN, used as an oral adjuvant with hepatitis B surface antigen or tetanus toxoid, could enhance both Th1 and Th2 type responses (McCluskie *et al.*, 2000). Another explanation may be that the presence of IL-12 could suppress the IL-5 response, whereas the empty PI.19 plasmid with the absence of IL-12, can facilitate the induction of a strong type-2 response.

The design of successful vaccines against HIV is likely to be dependant on the identification of formulations that can induce and sustain potent HIV-specific cellular immune responses. This study demonstrates that the immunogenicity of a DNA vaccine encoding a surface envelope protein from HIV-1, especially for the induction of type-1 responses, can be enhanced by co-administering a plasmid coding for the Th1 activating cytokine, IL-12. Therefore, it may be possible to design custom-made HIV DNA vaccines capable of generating protective immunity against HIV infection.

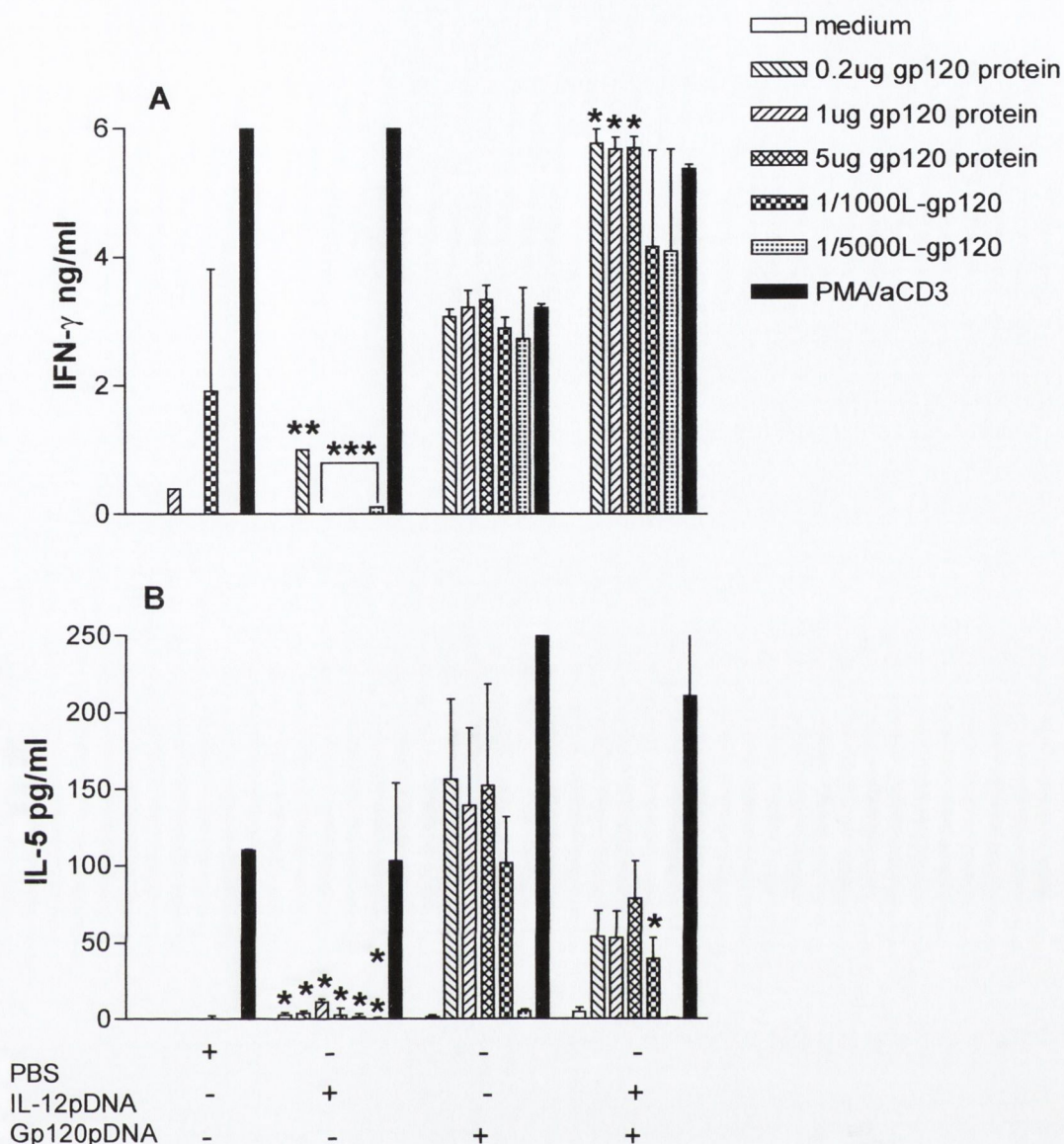


Figure 4.1. Modulation of spleen cell cytokine production following co-administration of gp120pDNA and IL-12pDNA. Mice were i.m. immunized twice at week 0 and boosted at week 4 with 50 μ g gp120pDNA $\pm$ 50 μ g IL-12pDNA. At six weeks spleen cells were stimulated *in vitro* with gp120 antigen (0.2, 1, 5 μ g) or 1/1000, 1/5000 dilution of gp120 coupled latex beads (1/1000, 1/5000L-gp120). Medium or PMA and anti-CD3 acted as negative and positive controls respectively. Supernatants were removed after 72 hours and the concentrations of (A) IFN- γ and (B) IL-5 were determined by specific immunoassay. The graph represents the results from a single experiment and are expressed as the mean of four mice per group $\pm$ S.E. * p<0.05, ** p<0.01, *** p<0.001, gp120pDNA alone versus IL-12pDNA or gp120pDNA + IL-12pDNA, determined by Student's t-test.

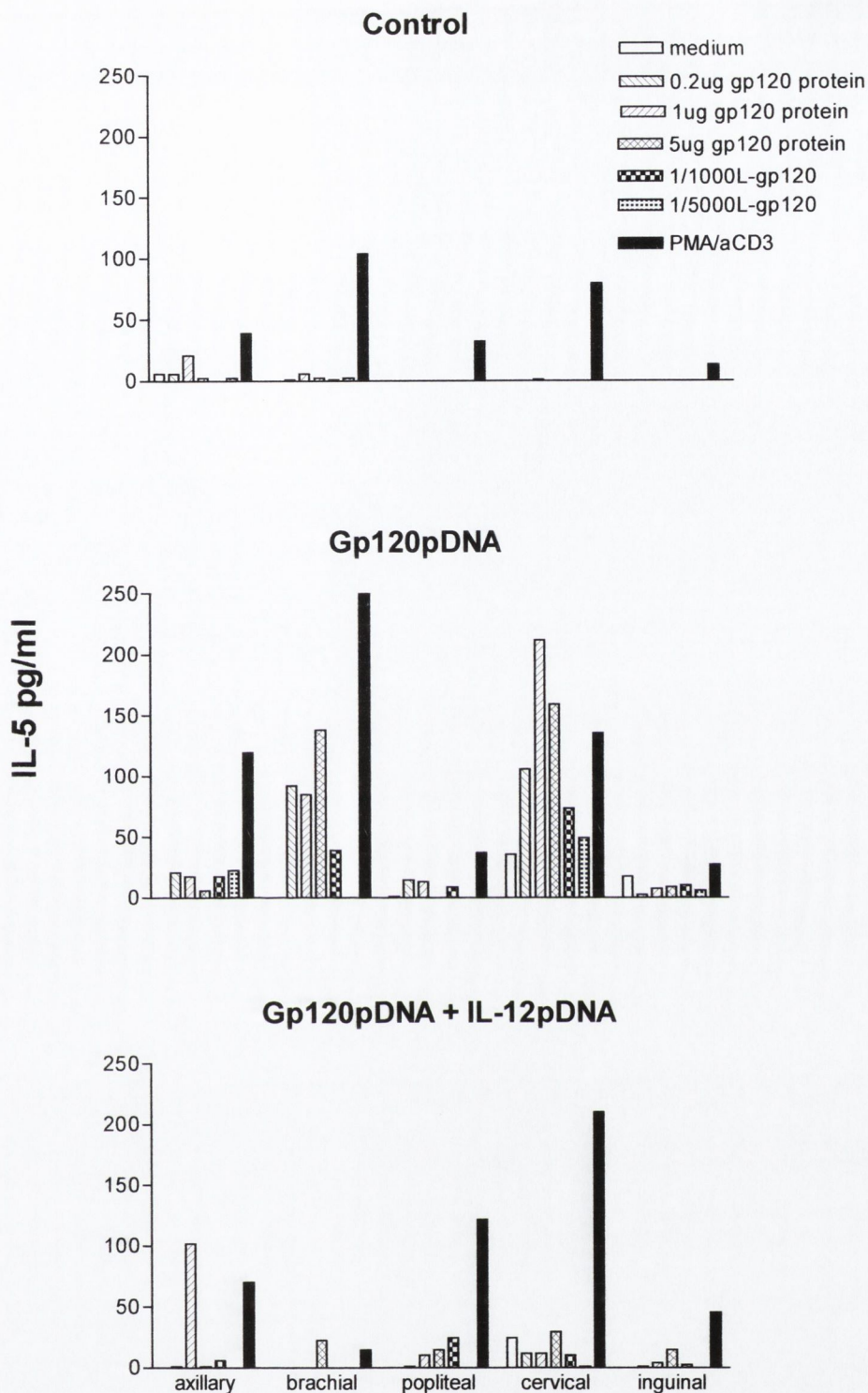


Figure 4.2. Gp120pDNA induces a polarized Th2 type response in the lymph nodes which is reduced by co-administration with IL-12pDNA. Mice were immunized as described in the legend to figure 4.1. The draining lymph nodes were pooled and restimulated *in vitro* as described in the legend to figure 4.1. Three days later the gp120-specific IL-5 concentrations in supernatants were determined by specific immunoassay. The graphs represent responses of pooled lymph node cells from one experiment.

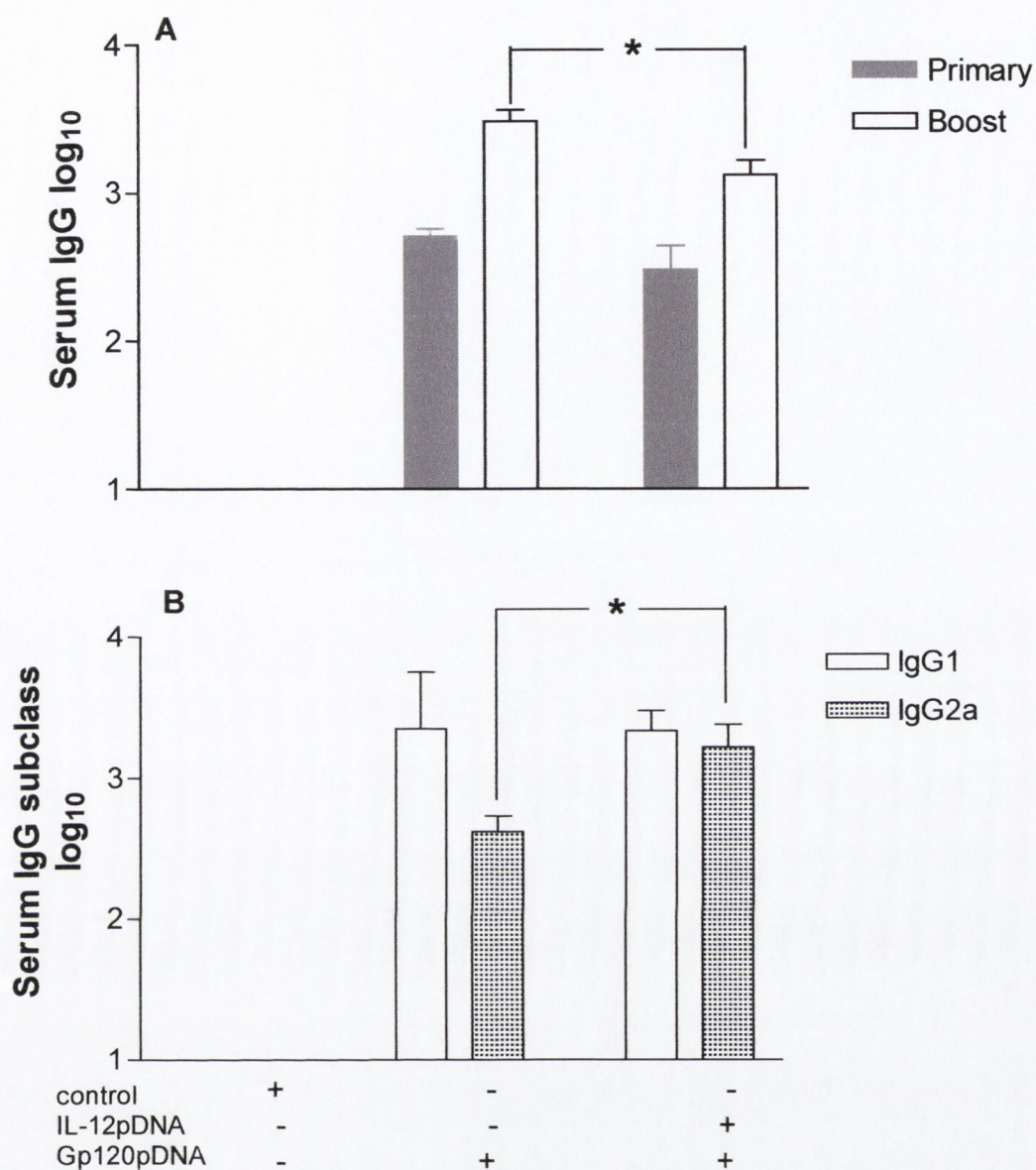
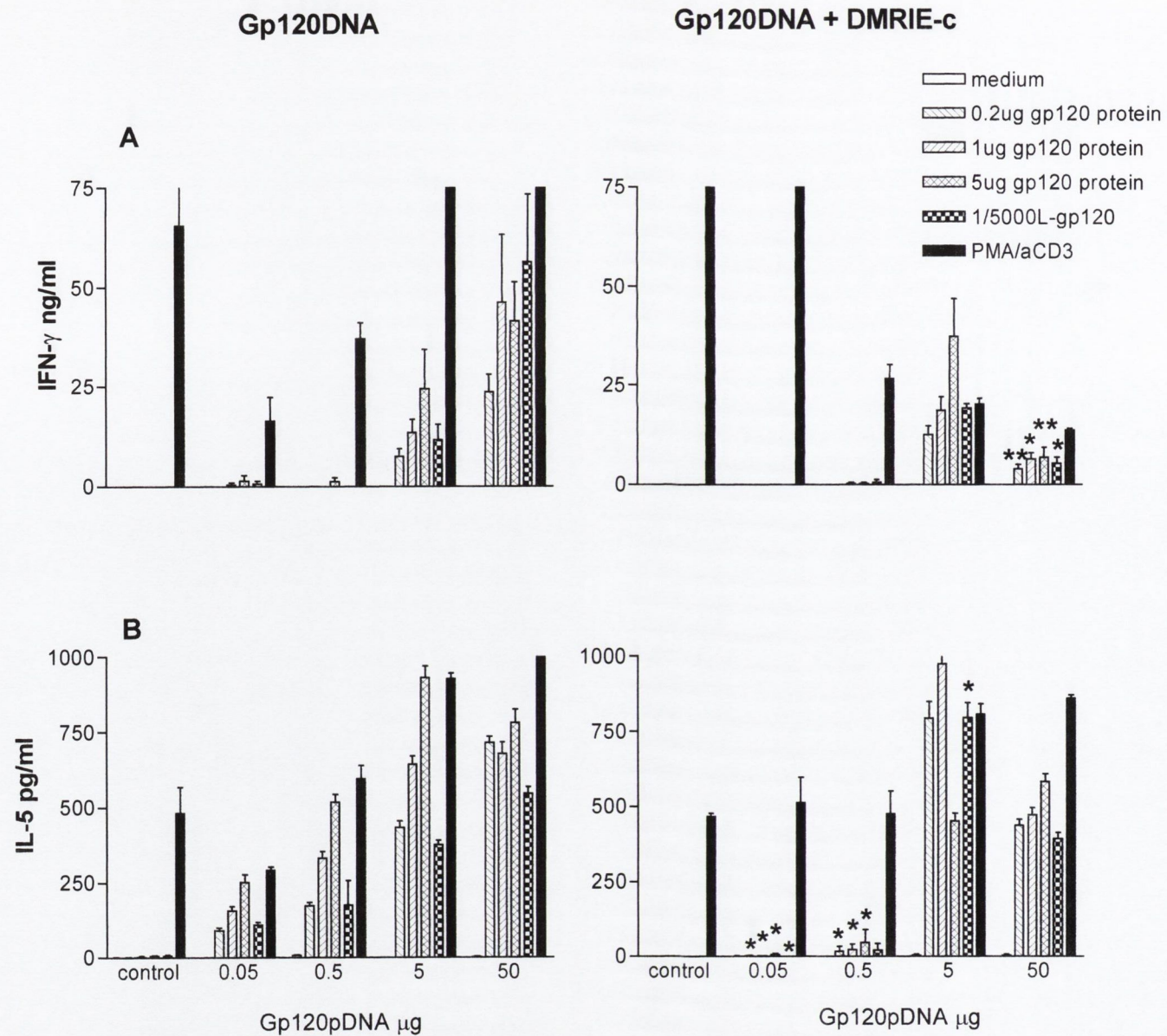


Figure 4.3. Co-administration of IL-12pDNA enhances IgG2a antibodies. Mice were immunized as described in the legend to figure 4.1. Gp120-specific serum IgG titres were determined by ELISA. **(A)** total anti-gp120 IgG endpoint titres, **(B)** anti-gp120 IgG specific subclasses. Results represent mean titres $\pm$ SE for four mice in each group. * $p < 0.05$, gp120pDNA alone versus gp120pDNA + IL-12pDNA determined by Student's t-test.

Figure 4.4. Effect of DNA dose and DMRIE-c on T cell responses in the spleen. Mice were immunized twice i.m. with gp120pDNA (0.05, 0.5, 5 or 50 μ g) $\pm$ 10 μ g DMRIE-c. At week six the spleens were isolated and restimulated *in vitro* as described in the legend to figure 4.1. Supernatants were removed after 72 hours of culture and assessed for cytokine concentrations. **(A)** IFN- γ and **(B)** IL-5 were determined by specific immunoassay. The graph represents the results from a single experiment and are expressed as the mean of four mice per group $\pm$ S.E. * $p < 0.05$, ** $p < 0.01$, gp120pDNA alone versus gp120pDNA + DMRIE-c determined by Student's t-test.



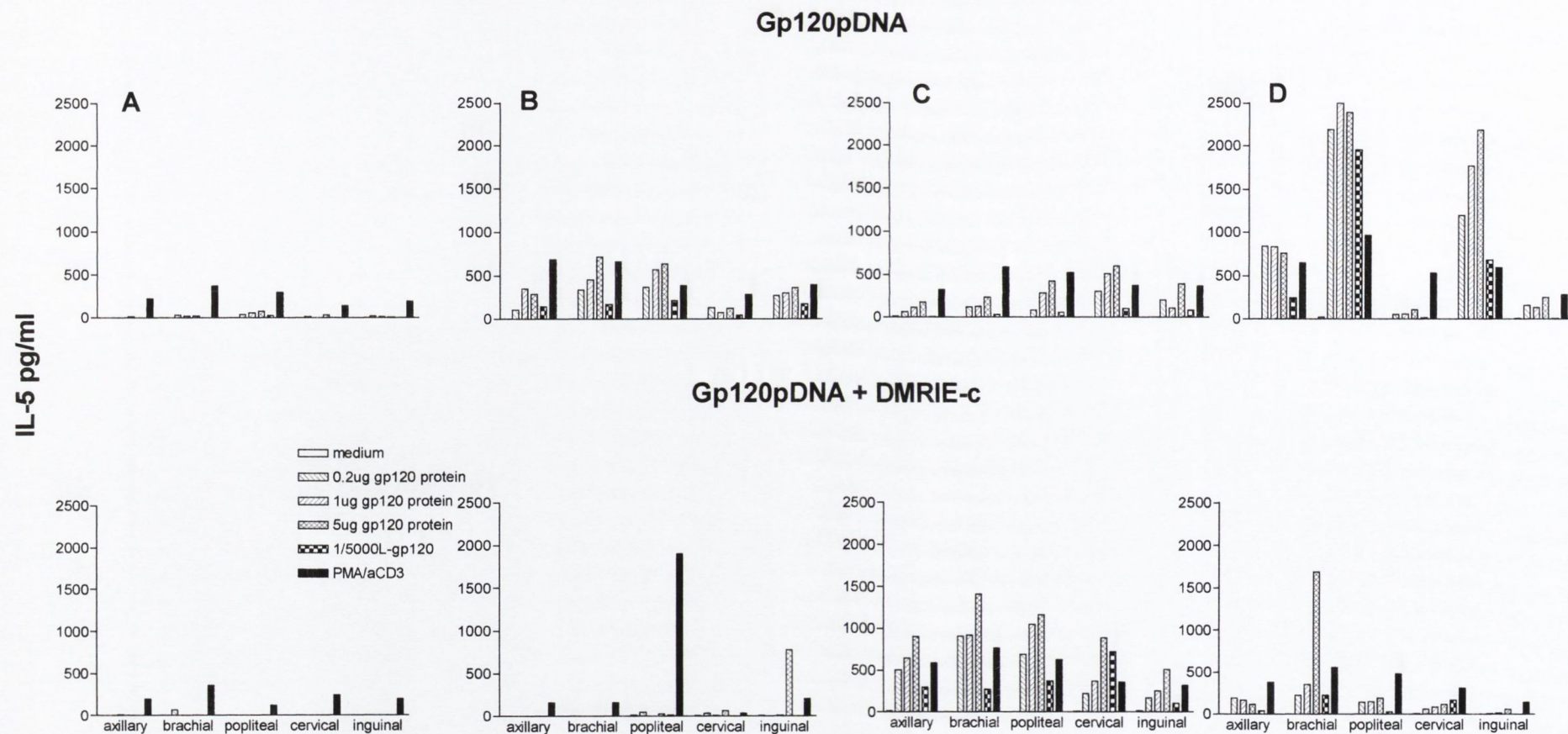


Figure 4.5. Effect of DNA dose and liposome formulation on T cell responses in the draining lymph nodes Mice were immunized as described in the legend to figure 4.4. Mice were immunized with (A) 0.05 μ g, (B) 0.5 μ g, (C) 5 μ g and (D) 50 μ g gp120pDNA. IL-5 production in supernatant of antigen-stimulated lymph node cells was determined by specific immunoassay. The graph represents the responses from pooled lymph node cells from a single experiment.

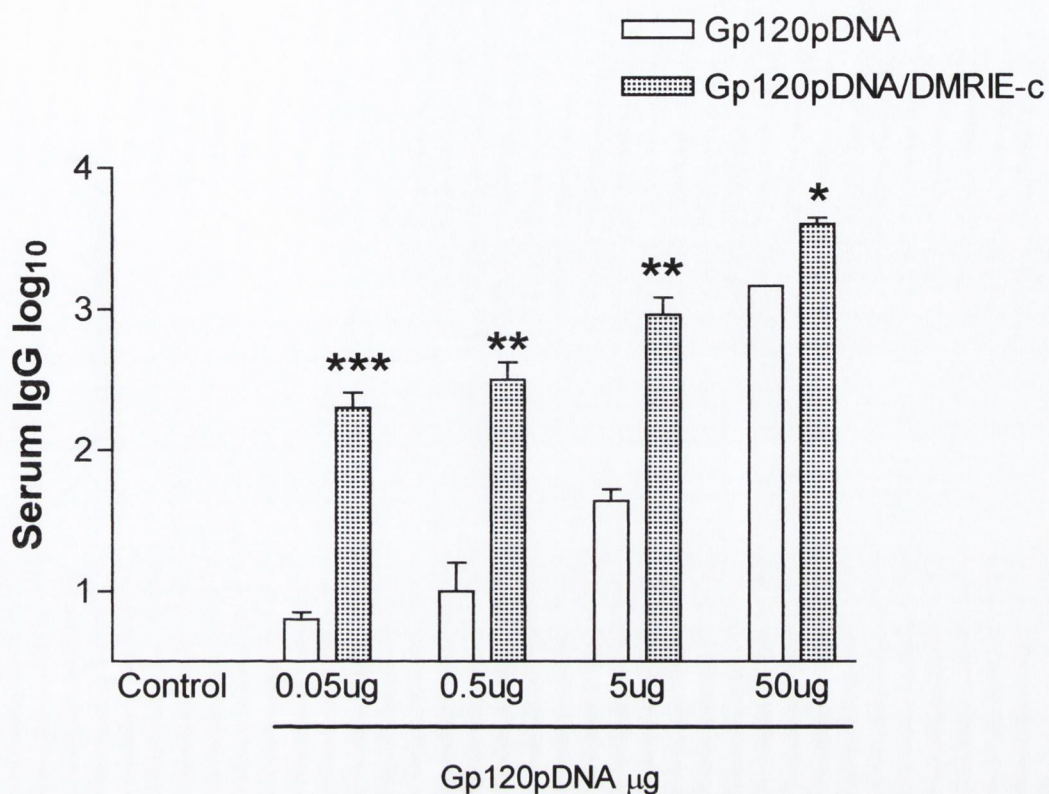


Figure 4.6. Antibody responses are enhanced by increasing dose of plasmid DNA and by co-administration of DMRIE-c. Mice were immunized as described in the legend to figure 4.5. Gp120-specific serum IgG titres were determined by ELISA. Results are mean endpoint titres $\pm$ SE for four mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, gp120pDNA alone versus gp120pDNA + DMRIE-c determined by Student's t-test.

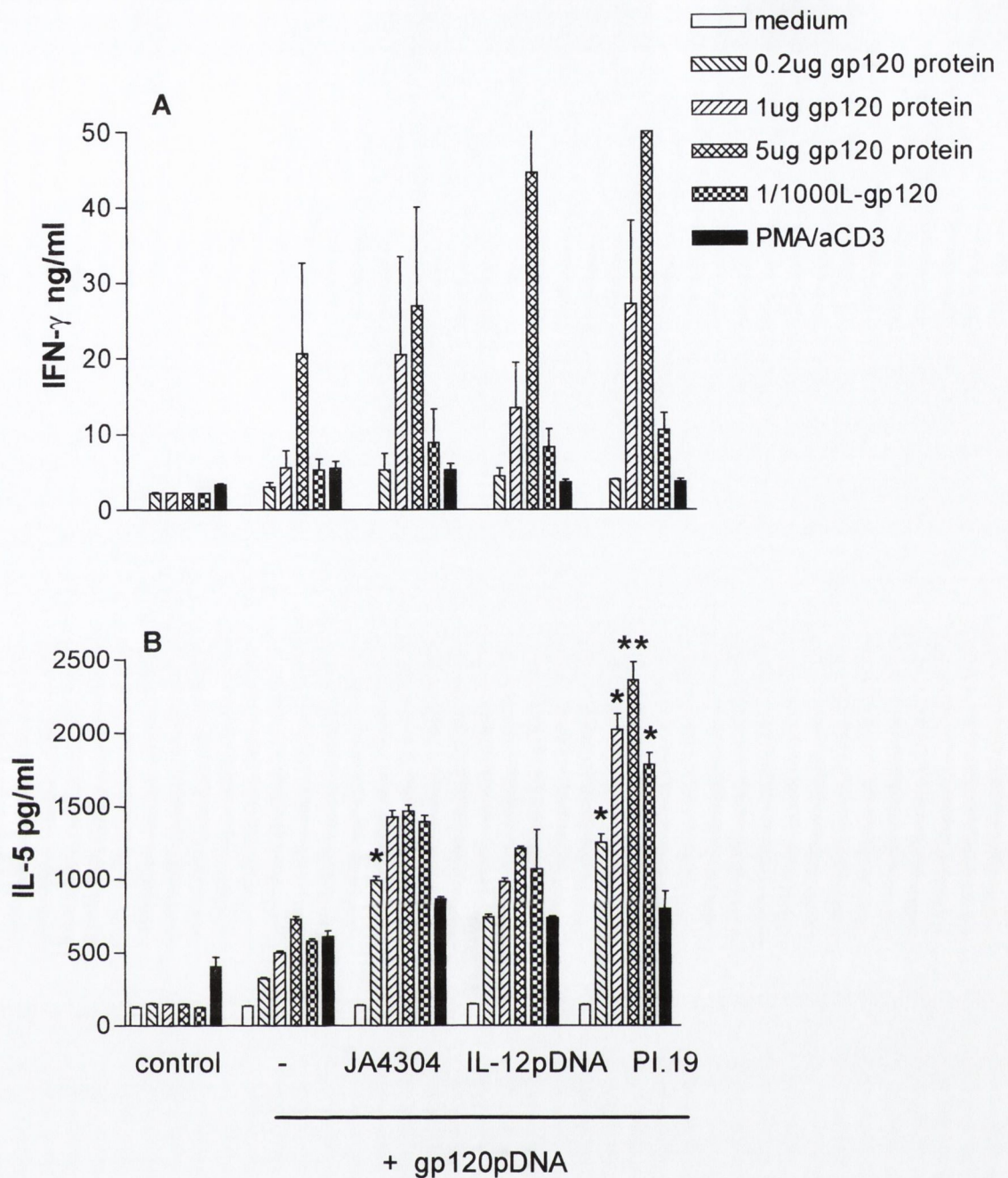
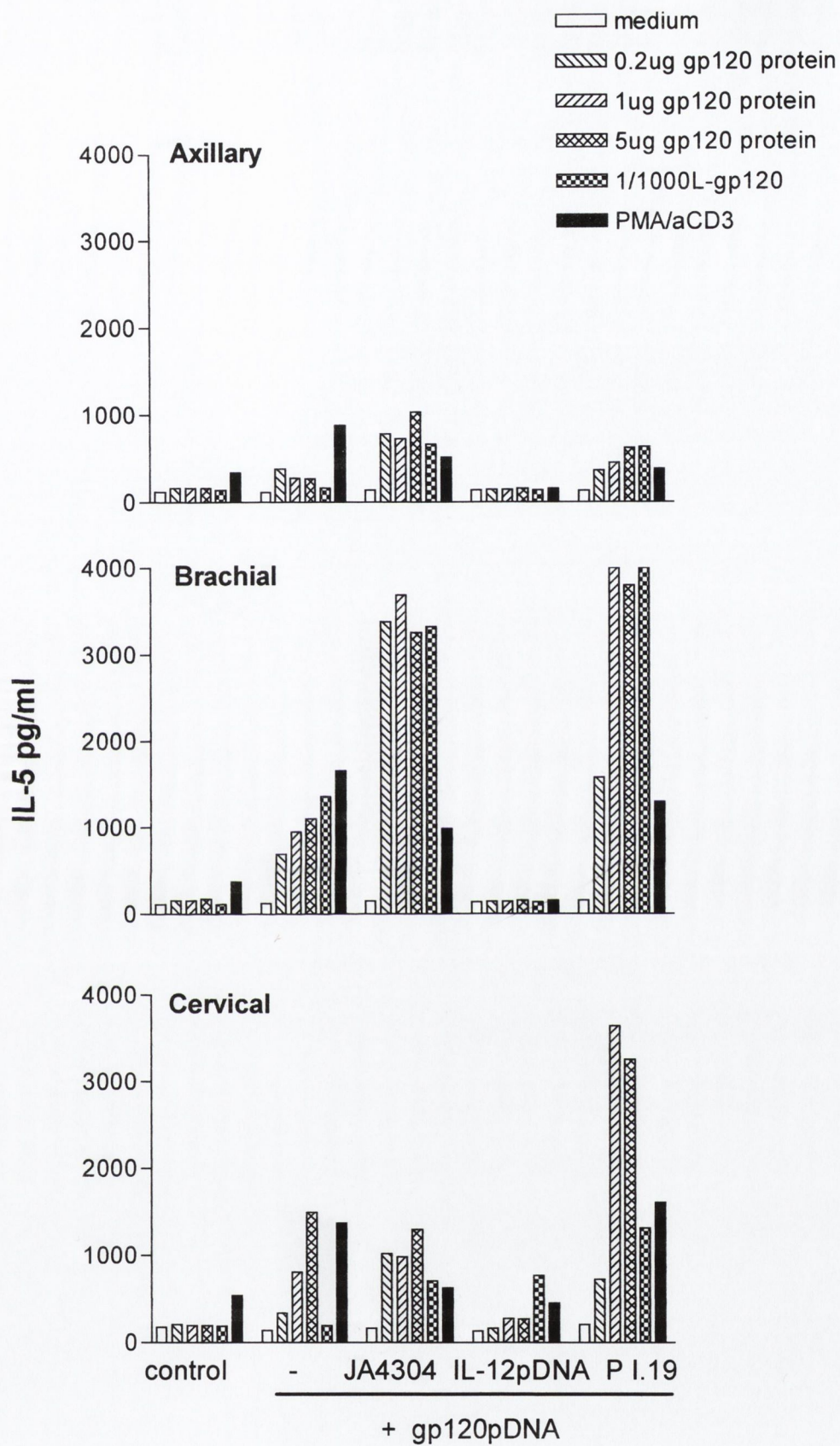


Figure 4.7. Influence of plasmid encoding an antigen or a non-coding plasmid on spleen cytokine production. Mice were immunized twice i.m with 50 μ g gp120pDNA and 50 μ g of either JA4304 (parent plasmid of gp120pDNA), IL-12pDNA or PI.19 (parent plasmid of IL-12pDNA). **(A)** IFN- γ and **(B)** IL-5 cytokine concentrations were determined by specific immunoassay. The graph represents the results from a single experiment and are expressed as the mean of four mice per group $\pm$ S.E. * p<0.05, ** p<0.01, gp120pDNA alone versus gp120pDNA + JA4034, IL-12pDNA or PI.19, determined by Student's t-test.

Figure 4.8. Co-administration of non-coding plasmid influences induction of a Th2 type response in the lymph nodes. Mice were immunized twice i.m as described in the legend to figure 4.7. Gp120-specific IL-5 cytokine concentrations in supernatants in antigen-stimulated lymph node cells were determined by specific immunoassay. The graphs represent responses of pooled lymph node cells from one experiment.



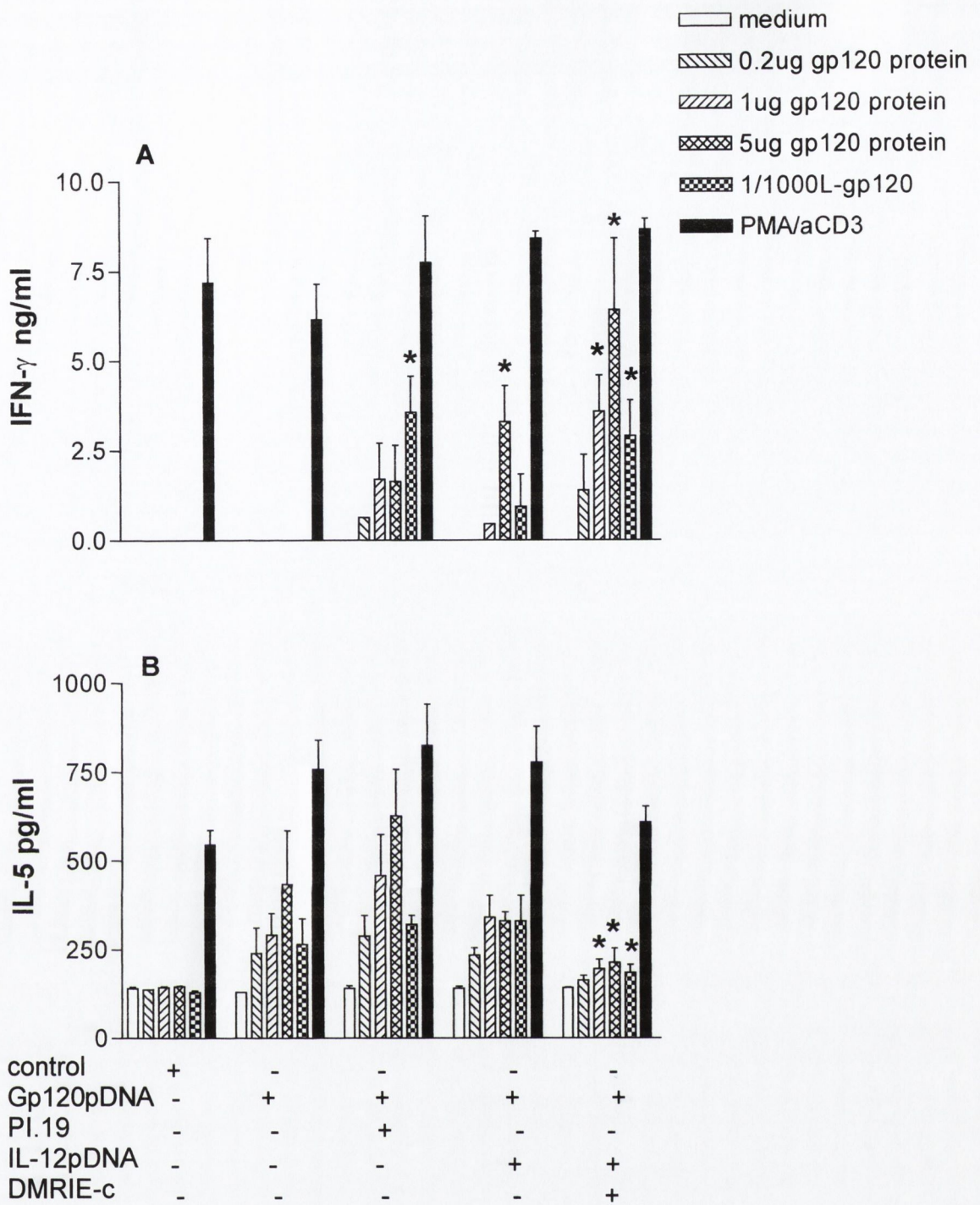
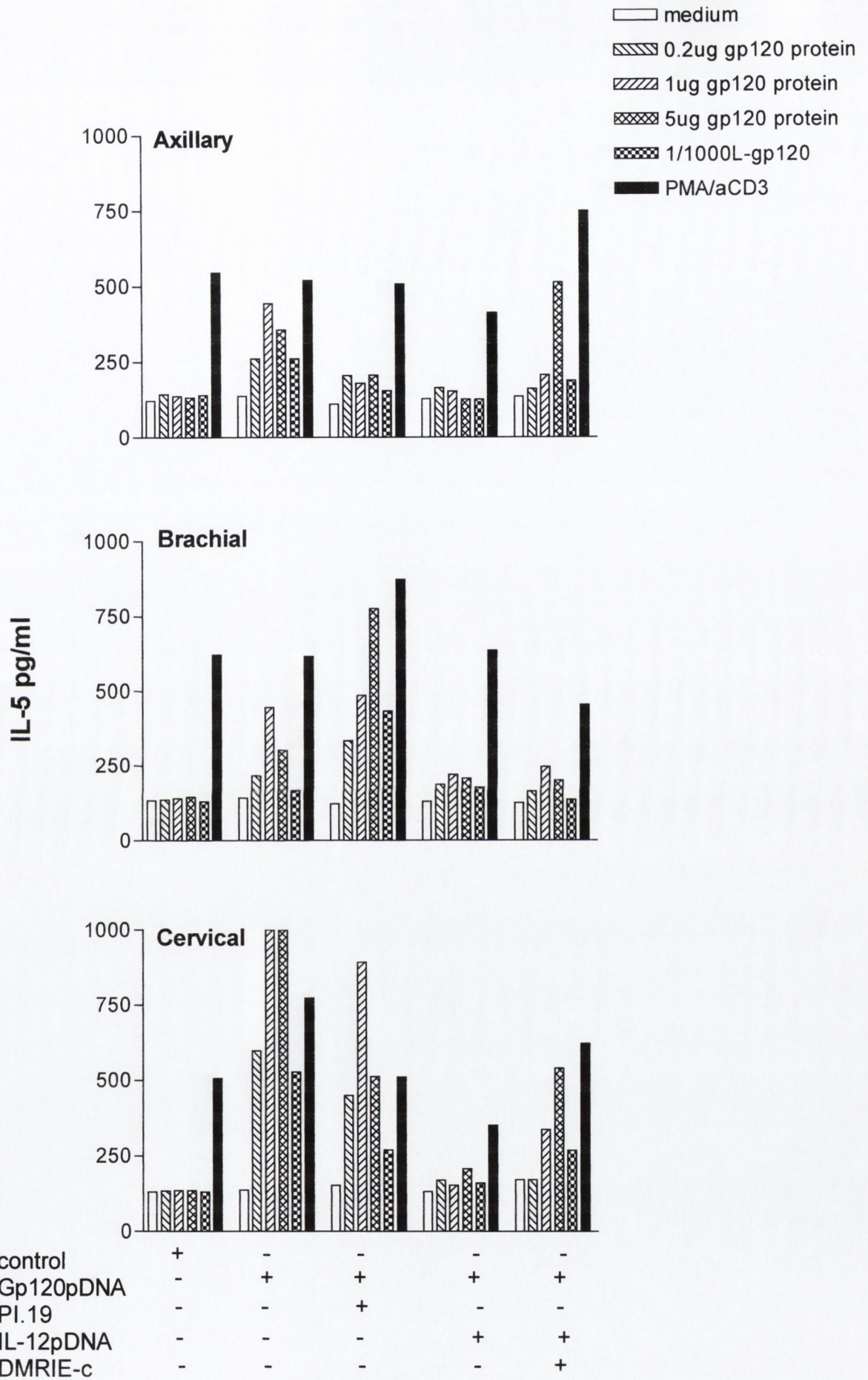


Figure 4.9. The Th1 type response generated with gp120pDNA can enhanced be by co-administration with IL-12pDNA and DMRIE-c. Mice were immunized twice i.m with 50 μ g gp120pDNA $\pm$ 50 μ g IL-12pDNA, 50 μ g PI.19 or 10 μ g DMRIE-c. At week six spleen cells were isolated and restimulated *in vitro* as described in the legend to figure 4.1. **(A)** IFN- γ and **(B)** IL-5 were determined by specific immunoassay. The graph represents the results from a single experiment and are expressed as the mean of four mice per group $\pm$ S.E. * $p < 0.05$, gp120pDNA alone versus gp120pDNA + PI.19 or IL-12pDNA $\pm$ DMRIE-c, determined by Student's t-test.

Figure 4.10. The Th2 type response in the lymph nodes induced with gp120pDNA is reduced by co-administration with IL-12pDNA and DMRIE-c. Mice were immunized as described in the legend to figure 4.9. The axillary, brachial and cervical node cells were analysed for production of IL-5 as described in the legend to figure 4.1. Gp120-specific IL-5 concentrations were determined by specific immunoassay. The graph represents the responses of pooled lymph node cells from a single experiment.



CHAPTER 5

INFLUENCE OF ENCODED ANTIGEN AND PLASMID ON INDUCTION OF IMMUNE RESPONSES

5.1 INTRODUCTION

The immune response generated following DNA immunization is influenced by a number of factors including the nature of the encoded antigen (Feltquate, 1998), (Haddad *et al.*, 1997), (Cardosa, 1998) and the immunostimulatory sequences of the bacterial plasmid backbone (Herve *et al.*, 2001). The type and magnitude of the immune response depends on whether an antigen is secreted, membrane bound, or retained within the cytosol of the cell. Antigen-specific IgG subclass induction is influenced by antigen type; secreted antigen predominantly generates the murine IgG1 subclass and cytosolic antigen generates a mixed IgG1/IgG2a response (Haddad *et al.*, 1998).

Bacterial plasmid DNA contains short immunostimulatory sequences (ISS), which consist of two 5' purines and two 3' pyrimidines on either side of an unmethylated CpG motif (Sato *et al.*, 1996). Bacterial DNA contains a higher frequency of CpG motifs compared to mammalian DNA and these CpG motifs play a prominent role in enhancing the immunogenicity of plasmid DNA vaccines. Methylation of CpG motifs causes a significant reduction in the immunogenicity of a DNA vaccine (Klinman *et al.*, 1997). CpG motifs present in bacterial plasmid DNA have been shown to activate macrophages and DCs to secrete a variety of cytokines and chemokines and up-regulate expression of surface markers associated with DC maturation (Sparwasser and Lipford, 2000). Furthermore, synthetic oligonucleotides containing CpG motifs (CpG-ODN) can stimulate APC secretion of IL-6, IL-12, TNF- α and IFN- γ and enhance B cell proliferation and antibody production (Pisetsky, 1997).

One of the great advantages of DNA vaccine technology is the potential to generate multivalent vaccines, whereby several plasmids coding for foreign antigens can be combined in a single vaccine formulation. Braun *et al.*, (1998) examined compatibility of combination vaccines and found that immune responses induced by individual plasmids were not compromised following co-administration of four individual plasmids encoding distinct viral glycoproteins from bovine herpesvirus-1, bovine parainfluenzavirus-3 and human influenza virus. A similar adjuvant effect of the plasmid and lack of antigenic competition was observed following co-immunization of four plasmids coding for malarial antigens; antigen-specific antibody

titres of the poorly immunogenic plasmid were enhanced and this was explained by the presence of additional plasmids containing ISS (Grifantini *et al.*, 1998).

The mechanism by which DNA vaccines induce immune responses following injection remains to be fully defined. It is clear that Th1/Th2 differentiation is influenced by the cytokine milieu during initiation of T cell priming (O'Garra, 1998). Following encounter with foreign pathogens or antigens, a controlled set of signals is provided to the innate immune system, primarily through DCs, which influences the differentiation and polarization of T cell subsets (Jankovic *et al.*, 2001), (Kalinski *et al.*, 1999). DCs process and present foreign antigen to T cells and therefore play a key role in the induction of immune responses. Furthermore, priming of immune responses following immunization with plasmid DNA appears to be possible with relatively few transfected DCs (Casares *et al.*, 1997), (Condon *et al.*, 1996), (Porgador *et al.*, 1998).

The present study directly compared the immune responses induced by two distinct DNA vaccines using plasmids coding for the gp120 HIV surface glycoprotein (gp120pDNA) and the HA glycoprotein of influenza virus (HApDNA). The studies examined how the nature of the encoded antigen and plasmid backbone influences the immune response induction following DNA immunization. Furthermore, the potential of antigenic competition during multi-plasmid vaccination was examined by co-administration of gp120pDNA and HApDNA in single vaccine formulation. APCs and the cytokine milieu present at the site of immunization play key roles in determining T cell polarization. Therefore, the effect of plasmid DNA on maturation of BMDCs was assessed. Finally, the study used mice deficient in key immunoregulatory cytokines to examine whether the absence of key regulatory cytokines can functionally skew the development of Th1 and Th2 type responses following immunization with plasmid DNA vaccines.

5.2 RESULTS

5.2.1 DISTINCT IMMUNE RESPONSE INDUCTION FOLLOWING IMMUNIZATION WITH GP120pDNA OR HApDNA

This research group has previously demonstrated that i.m. immunization of BALB/c mice with HApDNA induced a potent Th1 type response, detectable in the spleen (Johnson *et al.*, 2000). Results from chapter 3 have shown that gp120pDNA induced a mixed Th1/Th2 type response in the spleen. This study has directly compared the induction of type-1 and type-2 responses in the lymph nodes following immunization with HApDNA or gp120pDNA. Two weeks after a single i.m. immunization with 50µg of HApDNA or 50µg of gp120pDNA, cytokine production from cervical lymph node cells of immunized mice were assessed following restimulation with gp120 antigen (1µg) or influenza virus (150HAU) *in vitro* (Fig 5.1). Cervical lymph node cells from mice immunized with gp120pDNA produced gp120-specific IL-5 and undetectable levels of IFN-γ. In contrast cervical lymph node cells from HApDNA immunized mice produced HA-specific IFN-γ production and with low concentrations of HA-specific IL-5 from cervical lymph node cells. The polarized type-2 responses induced with gp120pDNA and a type-1 response with HApDNA correlates with immune profiles previously reported in the spleen (chapters 3 and 4).

To further investigate the different immune response induced with gp120pDNA and HApDNA, antigen-specific serum IgG subclasses were examined. The ratio of IgG1:IgG2a generated with gp120pDNA (secreted protein) was strongly biased towards IgG1 ($p < 0.001$), whereas HApDNA (transmembrane protein) elicited stronger IgG2a subclass titres ($p < 0.001$) (Fig 5.2). These IgG subclasses are consistent with the observation that gp120pDNA induces a biased type-2 response in contrast to HApDNA, which induced a biased type-1 response.

5.2.2 IMMUNOGENICITY OF A COMBINATION DNA VACCINE

Mutli-component vaccine development is possible using DNA vaccine technology. A number of foreign antigens can be encoded by the same plasmid or by individual plasmids combined in one vaccine formulation. To investigate the reciprocal influences of a Th1-inducing plasmid or a Th2-inducing plasmid, this study examined the immune responses to a combination of gp120pDNA and HApDNA. BALB/c mice were immunized i.m. with 50µg of gp120pDNA or HApDNA individually or in combination resulting in a final dose of 100µg plasmid DNA. Spleen cells from gp120pDNA immunized mice predominantly produced gp120-specific IL-5, whereas HApDNA immunization generated HA-specific IFN-γ producing spleen cells (Fig 5.3). Antigenic competition and CpG saturation (Grifantini *et al.*, 1998) are elements of concern with regard to combination vaccines. When compared with gp120pDNA alone, administration of a combination vaccine composed of both plasmids significantly enhanced gp120-specific IFN-γ ($p<0.05$). Furthermore, an increase in HA-specific IL-5 producing spleen cells and concurrent decrease in HA-specific IFN-γ ($p<0.05$) was observed in spleen cells from mice immunized with the combined formulation (Fig 5.3).

5.2.3 DISTINCT CYTOKINE PRODUCTION FROM SPLEEN CELLS IMMUNIZED WITH GP120 ANTIGEN ENCODED BY DIFFERENT PLASMIDS

The type of immune response induced following immunization with plasmid DNA is influenced by at least two distinct entities of the DNA vaccine, the bacterial plasmid backbone and the nature of the encoded antigen. The design of an effective viral vaccine requires a strong type-1 response mediated primarily by IL-12 which enhances IFN-γ production. It was demonstrated in chapter 3 that gp120pDNA induces a mixed Th1/Th2 type response. Therefore the present study investigated the effect of encoding the gp120 protein in a different bacterial plasmid backbone. The gp120 protein was excised from gp120DNA by cleaving two restriction sites and then inserted into a PI.18 bacterial plasmid backbone to create a novel plasmid, PI.18sfigp120. Thus, gp120pDNA and PI.18sfigp120 encoded the same antigen but differed in plasmid backbones. Gp120pDNA has a combined total of 239 internal CpG motifs whereas PI.18 has 89, but it is important to note that not all these may be immunostimulatory. Mice were immunized i.m. with 50µg of gp120pDNA or

PI.18sfigp120. As previously demonstrated, spleen cells from mice immunized with gp120pDNA produced both gp120-specific IFN- γ and IL-5 (Fig 5.4). However, immunization with the gp120 in the PI.18 plasmid backbone resulted in a 4-fold increase in gp120-specific IFN- γ producing spleen cells and a 2-fold reduction in gp120-specific IL-5 concentrations (Fig 5.4). Assessment of anti-gp120 serum IgG subclasses revealed that both plasmids induced IgG1 and IgG2a subclasses. However gp120pDNA induced significantly higher titres ($p < 0.05$) of the IgG1 subclass (Fig 5.5).

5.2.4 MATURATION OF BMDC BY HAPDNA AND GP120PDNA

DCs activate T cells through CD40-CD40L interactions and secretion of immunoregulatory cytokines and chemokines. Immature DC express surface markers at low levels that become highly expressed following stimulation by conserved products of pathogens. DC maturation is associated with the up-regulation of certain surface markers, such as CD40, MHC class II, B7.1, B7.2 and down-regulation of CCR5. Bone marrow cells cultured *in vitro* with GM-CSF lead to the generation of immature myeloid BMDC (Lutz *et al.*, 1999), (Pulendran *et al.*, 1999). Expansion of the DC population alone can be verified using specific DCs markers such as CD11c/b, DEC-205 and CD8 α . CpG motifs trigger DC maturation and secretion of cytokines and chemokines *in vitro* (Sparwasser *et al.*, 1998), (Stan *et al.*, 2001).

In the present study, the influence of plasmid DNA on the maturation of BMDC *in vitro* was investigated to give an insight into how these molecules may behave *in vivo* following DNA immunization. BMDC were incubated for 24 hours with 10 μ g gp120pDNA or HApDNA and subsequently assessed for markers of maturation (Fig 5.6). BMDC cultured in medium alone or LPS/IFN- γ were used as the negative control or positive controls respectively. LPS/IFN- γ enhanced CD40, MHC class II, B7.1, B7.2 and reduced CCR5 surface marker expression. The effect of plasmid DNA was not as significant, however both HApDNA and gp120pDNA did induce marginal increases in certain surface markers examined with a small decrease in CCR5 indicative of DC maturation events. B7.1 expression was increased to a greater extent following incubation with HApDNA when compared with gp120pDNA. Furthermore, CD40 was upregulated to a greater extent following stimulation with HApDNA.

Cytokine and chemokine production by BMDC was also assessed following incubation with 2 or 10 μ g doses of gp120pDNA or HApDNA for 4 or 24 hours (Fig 5.7). BMDC produced low concentrations of MIP-1 β in response to stimulation with gp120pDNA. MIP-1 β is a β -chemokine known for its function in blocking and down modulating CCR5 which leads to reduced HIV infectivity. However BMDC incubated over a 24 hour period with the higher 10 μ g dose of HApDNA induced significant MIP-1 β concentrations. Interestingly, both plasmids induced MIP-2 with the highest concentration induced following stimulation with the 10 μ g dose of HApDNA. HIV-1 and recombinant gp120 protein, have been previously shown to induce IL-10 secretion from human monocytes and macrophages (Borghi *et al.*, 1995). BMDC incubated for 24 hours with gp120pDNA induced both IL-10 and IL-12 secretion. Interestingly, HApDNA stimulated BMDC to secrete high concentrations of IL-12 after 4 hours and this increased 2-fold when incubated with the 10 μ g dose. In comparison with gp120pDNA, HApDNA did not stimulate the same levels of IL-10 secretion from BMDC (Fig 5.7).

5.2.5 ROLE OF REGULATORY CYTOKINES IN IMMUNE RESPONSES INDUCTION FOLLOWING IMMUNIZATION WITH PLASMID DNA

The role of regulatory cytokines in skewing antigen-specific type-1 and type-2 responses following DNA immunization was investigated using mice deficient in IL-4, IL-10 and IL-12 genes. As reported in chapter 3, immunization of BALB/c mice with gp120pDNA induced a mixed gp120-specific Th1/Th2 type response. However, spleen cells from IL-12^{-/-} mice immunized with the same construct produced significantly less gp120-specific IFN- γ ($p < 0.001$) and significantly greater IL-5 ($p < 0.001$) suggesting that IL-12 does have a role in promoting the type-1 arm of the immune response with gp120pDNA (Fig 5.8A). Compared to the parent strain C57BL/6, spleen cells from IL-10^{-/-} and IL-4^{-/-} mice immunized with gp120pDNA produced significantly higher concentrations of antigen-specific IFN- γ ($p < 0.01$) and lower concentrations of IL-5 ($p < 0.001$) (Fig 5.8A). The immune response induced with gp120pDNA in IL-10^{-/-} mice was almost completely polarized to type-1, suggesting that IL-10 plays a major role in the induction of type-2 responses with gp120pDNA in normal mice.

Immunization of BALB/c and C57BL/6 mice with 50µg of HApDNA induced polarized type-1 responses, indicated by predominant HA-specific IFN-γ production and no detectable IL-5 from antigen-stimulated spleen cells (Fig 5.8B). Spleen cells from IL-12^{-/-} immunized mice produced significantly higher concentrations of HA-specific IL-5 ($p<0.01$) compared to the BALB/c parent strain. As expected, in the absence of type-2 cytokines, IL-4 and IL-10, HA-specific IFN-γ production from spleen cells was dramatically enhanced ($p<0.01$) compared to the C57BL/6 parent strain (Fig 5.8B).

Examination of antigen-specific cytokine production from local draining lymph nodes from immunized mice showed a similar pattern. It was shown in chapter 3 that a polarized Th2 type response was generated in the lymph nodes of mice following immunization with gp120pDNA. The Th2 type response from the cervical lymph node cells was not adversely affected by the absence of IL-4, IL-10 or IL-12 (Fig 5.9A). However, significantly enhanced gp120-specific IFN-γ production ($p<0.01$) was detected in antigen-stimulated cervical lymph nodes cells from immunized IL-10^{-/-} mice compared to the C57BL/6 parent strain (Fig 5.9A). Antigen-stimulated popliteal lymph nodes cell also produced significantly more gp120-specific IFN-γ ($p<0.01$) and IL-5 ($p<0.01$) from IL-10^{-/-} immunized mice compared to C57BL/6 (Fig 5.10A). In the absence of IL-4 and IL-10, lymph node cells from HApDNA immunized mice secreted significantly more IFN-γ ($p<0.001$) (Fig 5.9B and 5.10B). Likewise HA-specific IL-5 production ($p<0.01$) was significantly higher in popliteal lymph node cells from IL-12^{-/-} mice compared with the BALB/c parent strain (Fig 5.10B).

Analysis of antigen-specific antibody production revealed that mice immunized with both gp120pDNA and HApDNA produced high antigen-specific IgG titres in all strains of mice (Fig 5.11A). IL-4^{-/-} mice immunized with either plasmid produced lower antigen-specific serum IgG titres compared to the C57BL/6 parent strain. Examination of IgG subclass induction revealed that the IgG2a subclass was adversely affected by the absence of IL-12 cytokine (Fig 5.11B). BALB/c mice immunized with either gp120pDNA or HApDNA produced high antigen-specific IgG2a titres, which were reduced in the IL-12^{-/-} mice. In contrast, the IgG1 subclass was reduced in the absence of IL-4 and IL-10 cytokines. C57BL/6 mice immunized with gp120pDNA produced a mixed IgG1: IgG2a response. However, the IgG1 titre

was reduced in the absence of IL-10 and almost completely abrogated in the absence of IL-4 ($p<0.05$) (Fig 5.11B). In contrast, C57BL/6 mice immunized with HApDNA produced a predominant IgG2a response, which was marginally affected by the absence of either IL-4 or IL-10.

5.3 DISCUSSION

The present study demonstrates that both the nature of the encoded antigen and bacterial plasmid backbone can influence the activation of DCs, which subsequently impacts on the type of response induced following DNA immunization. Immunoregulatory cytokines, IL-4, IL-10 and IL-12 were found to play a role in directing the type of response induced with gp120pDNA and HApDNA. Co-administration of two DNA vaccines encoding HIV and influenza proteins enhanced the response induced by the individual plasmids. Therefore, the composition of each plasmid requires careful consideration and evaluation during the design and development of multi-plasmid vaccines.

The type of response generated following immunization with plasmid DNA can be influenced by a number of factors, including the dose, which can alter the magnitude of the response (Johnson *et al.*, 2000). The route of administration, with the same plasmid can generate distinct responses in different sites, such as skin, muscle or following administration by gene-gun (Feltquate *et al.*, 1997). The expression level exhibited by distinct plasmids may in some cases explain the differences in the type of response. To exclude this possibility it may be necessary to ensure that the plasmids to be compared should exhibit similar expression levels of the encoded protein to exclude this variable. Alternatively, knowledge of these expression levels may allow compensation for the differences in the final response. Two prominent aspects of DNA vaccination that influence the immune response are the nature of the encoded antigen and composition of the bacterial plasmid backbone. This study has compared directly the immune response induced in mice using plasmids coding for secreted (gp120) and membrane bound (HA) antigens. This research group has previously demonstrated that i.m. immunization of BALB/c mice with HApDNA induces a potent Th1 type response detectable in the spleen (Johnson *et al.*, 2000), which is consistent with other studies (Feltquate *et al.*, 1997). The present study confirmed that HApDNA, encoding a non-secreted antigen, generated a polarized type-1 response detectable in the spleen and lymph nodes. In contrast, i.m. immunization with gp120pDNA generated a mixed Th1/Th2 type response in the spleen and a polarized Th2 type response in the lymph nodes of BALB/c mice. Subcutaneous immunization with gp120 protein has previously been shown to prime Th2 cells in local draining lymph nodes (Mellado *et al.*, 1998). Other studies demonstrated that secreted antigen induced IL-4, whereas cell-associated antigen

induced IFN- γ secreting cells in the spleen, which underlines the influence that antigen type can have on T cell responses in both the spleen and draining lymph nodes (Lewis *et al.*, 1999). Plasmid DNA coding for either membrane anchored, secreted or intracellular protein versions of glycoprotein D from BHV-1 induced different serum IgG subclasses and primed distinct T cell subsets in the lymph nodes. Interestingly, the plasmid coding for an intracellular protein induced IFN- γ secreting cells (Lewis *et al.*, 1999), and this correlates the results obtained in this study, which showed the induction of a predominant Th1 type response with HApDNA.

The differences in gp120-specific cytokine secretion detected in the spleen and lymph nodes of mice immunized with gp120pDNA may result from transfection of distinct DC subsets. However, the same DC subset may also prime different T cell populations. It was previously demonstrated that CD8 α - lineage of DC generate both Th2 and Th1 responses in murine Peyer's patches and spleen respectively (Pulendran *et al.*, 2001). Gp120 protein induces IL-10 secretion from monocytes (Taoufik *et al.*, 1997), (Borghi *et al.*, 1995) and therefore it may be possible to activate Th2 differentiation with a DC subset already capable of directing Th1 differentiation through suppression of IL-12 secretion by IL-10 from CD8 α DC (Pulendran *et al.*, 2001).

The cellular localization of expressed antigens can influence the bias of the humoral response (Feltquate *et al.*, 1997), (Lewis *et al.*, 1997). Secreted antigen predominantly induces the IgG1 subclass, whereas cytosolic antigens induce dominant IgG2a subclasses (Haddad *et al.*, 1998). In addition, secreted forms of antigen were found to enhance antibody production compared to cytoplasmic or membrane bound antigens (Boyle *et al.*, 1997), (Prange and Werr, 1999). In the present study examination of IgG subclass demonstrated that dominant IgG1 or IgG2a subclasses were generated following immunization with gp120pDNA or HApDNA respectively. These observations are consistent with those reported by Feltquate *et al.*, (1997) who also found predominant IgG2a with a plasmid encoding the HA protein from influenza virus. However, BALB/c mice immunized with plasmid encoding HA using particle-mediated DNA transfer generated polarized IgG1 responses (Chen *et al.*, 1999) indicating that the route of immunization also plays a key role in the final immune profile.

Induction of immune responses following DNA vaccination are also largely affected by the bacterial plasmid backbone (Herve *et al.*, 2001) as they contain CpG motifs known to activate DC priming of type-1 T cells (Klinman *et al.*, 1997). Two distinct plasmid backbones, both expressing gp120 antigen, were generated in order to examine the influence of the plasmid backbone on the immune response induced. Interestingly, predominantly IgG1 subclass was induced with the encoded gp120 secreted antigen, regardless of the plasmid backbone. However, immunization with gp120pDNA generated significantly higher IgG1 subclass titres compared to those observed with PI.18sfigp120. Analysis of the systemic T cell responses following immunization with gp120 protein encoded by the PI.18 plasmid revealed switching to a dominant gp120-specific Th1 type profile in the spleen. This suggests that the plasmid backbone influences the direction of immune response induced following DNA immunization.

Development of multi-plasmid vaccines requires careful assessment of potential antigenic competition. Co-administration of gp120pDNA and HApDNA enhanced gp120-specific IFN- γ and reduced IL-5 HA-specific production from spleen cells of immunized mice, compared to responses induced by individual plasmids. A HA-specific IFN- γ response was induced following gp120pDNA immunization. This may be due to contamination of gp120pDNA in the preparation of the HApDNA vaccine and could be verified by PCR analysis to establish the exact content of the vaccine preparation. Cardoso *et al.*, (1998) demonstrated that HA and NP coding plasmids induced distinct IgG subclasses when individually injected by gene gun. Following co-administration the individual responses were switched to dominant IgG1, indicating that the presence of additional plasmids can influence the antibody subclass.

To date studies on multivalent vaccines have solely examined the effect on antibody responses. The present study demonstrated that co-administration of gp120pDNA and HApDNA enhanced gp120-specific IFN- γ production. Antigenic competition between the two plasmids may lead to reduced antigenic expression (Braun *et al.*, 1998). In part this may facilitate the Th1-inducing plasmid backbone to exert a greater influence and improve IFN- γ production. Grifantini *et al.*, (1998) has shown that the response generated by a poorly immunogenic plasmid could be enhanced in the presence of additional plasmids. Higher doses of plasmid DNA or co-

administration of multiple DNA vaccines may lead to an enhanced immune response due to the synergistic effect of the immunostimulatory CpG motifs in each plasmid DNA (Klinman *et al.*, 2000).

It is well established that bacterial DNA and immunostimulatory CpG motifs bind DCs through TLR9 and trigger IL-12 production, which facilitates Th1 differentiation and IFN- γ production (Sparwasser *et al.*, 1998), (Hemmi *et al.*, 2000). Therefore, the immunogenicity of DNA vaccines is dependant on adjuvant properties displayed by CpG motifs in the plasmid backbone, which can be eliminated by methylation (Klinman *et al.*, 1997). Furthermore, maturation of DCs can be stimulated following treatment with CpG or plasmid DNA (Klinman *et al.*, 2000). This was demonstrated in studies where immunostimulatory ODN containing CpG motifs, but not control GpC motifs, were shown to up-regulate expression of MHC class II, CD40, CD86 and limited increase in CD80 on DC (Behboudi *et al.*, 2000), (Sparwasser *et al.*, 1998). In addition, i.m. immunization of a plasmid DNA encoding HIV gp160 was shown to directly transfect macrophages and DC, leading to up-regulation of CD80 and CD86 on macrophage, but only CD86 expression on DC (Chattergoon *et al.*, 1998). A plasmid DNA encoding a non-secreted protein also resulted in both CD80 and CD86 up-regulation on DC (Akbari *et al.*, 1999). Following injection of plasmid DNA via muscle or skin, DC can present antigen to T cells and therefore it is appropriate to examine the effect of antigen encoding plasmid DNA containing CpG motifs on DC (Casares *et al.*, 1997). This study demonstrated that plasmid DNA encoding antigen can trigger BMDC maturation, with weak but detectable up-regulation of MHC class II, CD40, CD80, CD86 with a concurrent down-regulation in CCR5 expression. The non-secreted HA coding plasmid resulted in a marginally greater enhancement of CD80 expression compared to that invoked by the plasmid coding for the secreted gp120 protein. This lack of CD80 surface expression on DC by gp120pDNA may enhance the preferential activation of Th2 type cells (Sparwasser *et al.*, 1998), (Jankovic *et al.*, 2001) as it has been suggested that B7.2 and CD28 interaction is more important for Th2 than Th1 cells (Lenschow *et al.*, 1995), (Bottomly and Constant, 1997).

The cytokine milieu that prevails at the time of DC activation has significant effects on the outcome of induction of T cell response (Reid *et al.*, 2000). Th1/Th2 differentiation can be controlled by distinct DC subsets. Myeloid DC1 produce IL-4 and lymphoid DC2 produce IL-12, providing distinct effects on Th1 and Th2

differentiation respectfully (Rissoan *et al.*, 1999). In this study, BMDC were stimulated with GM-CSF to expand the myeloid DC subset that reportedly secrete high concentrations of IL-4 and IL-10 and produce lower levels of IL-12 compared to the lymphoid DC subset (Pulendran *et al.*, 1999), (Sousa *et al.*, 1999). The concentrations of IL-12 produced by myeloid DC are sufficient to induce T cells to secrete significant IFN- γ , however the Th1 response can be suppressed by a Th2 cytokine environment (Pulendran *et al.*, 1999). Gp120pDNA stimulated BMDC to secrete IL-10 that may act to suppress IL-12 induced Th1 polarization. HApDNA stimulated low concentrations of IL-10 and this may be insufficient to override the predominant IL-12 production from the BMDC and result in priming Th1 type response. Taking all of this evidence together it appears that the priming of distinct types of responses is influenced by factors affecting DC activation.

Initial antigenic encounter and the cytokine environment can dictate the generation of DC1 and DC2 subsets, that play a key role in CD4⁺ Th1 and Th2 cells differentiation respectively (Kourilsky and Truffa-Bachi, 2001), (Sato *et al.*, 1999), (Vieira *et al.*, 2000). Therefore, the absence of key cytokines can functionally skew the development of Th1 and Th2 subtypes. IL-12, IFN- γ and IL-18 are the major cytokines involved in differentiation of naïve CD4⁺ T cells into the Th1 subset (Seder *et al.*, 1993), whereas the development of CD4⁺ Th2 subset is primarily regulated by IL-4 (Kopf *et al.*, 1993). Furthermore, studies in IL-10^{-/-} mice reveal enhanced IL-12 secretion from DC subsets (Huang *et al.*, 2001). This demonstrates a negative feedback mechanism between IL-12 and IL-10 that regulate Th differentiation (Jeannin *et al.*, 1996). The present study demonstrated the importance of IL-4, IL-10 and IL-12 following DNA immunization in directing the type of antigen-specific T-cell response. IL-12-dependent IFN- γ production is necessary for Th1 development (Bancroft *et al.*, 1997), (Mountford *et al.*, 1999). Therefore, IL-12^{-/-} mice have a reduced capacity for IFN- γ production and effectively skewed the gp120-specific responses toward a Th2 type response. In contrast, the absence of IL-10 significantly improved type-1 induction. Studies with anti-IL-10 neutralizing antibodies have reported similar enhancement in IFN- γ responses (Huang *et al.*, 1999). Furthermore, IL-4^{-/-} deficient mice displayed little or no increase in IL-5 levels. This may be partially explained by observations that optimal induction of Th2 cells by DC2 requires IL-10 (Maldonado-Lopez and Moser, 2001), (Ria *et al.*, 1998). In addition,

IL-10 has been implicated in regulating both IL-12-driven type-1 and IL-4-driven type-2 responses in mice (Hoffmann *et al.*, 2000). This demonstrates the importance of both IL-4 and IL-10 in regulating T cell differentiation following DNA vaccination.

It has been widely reported that T cell derived cytokines influence antibody production by B cells (Finkleman *et al.*, 1990). Th1 cells promote subclass switching through IFN- γ , a potent inducer of IgG2a antibody secretion from B cells and inhibitor of Th2 associated IgG1. In contrast, IL-4 secreted by murine Th2 cells promotes IgG1 production, but suppresses IgG2a and IgG2b. Therefore, mice deficient in cytokines that mediate T cell differentiation can also indirectly influence B cell subclass switching. IL-12^{-/-} mice have reduced IFN- γ production therefore removing the trigger for B cell subclass switch to IgG2a and these mice developed enhanced IgG1 production. Gp120pDNA immunization generated a mixed IgG1:IgG2a antibody response. IL-4 deficiency resulted in reduced IgG1 subclass secretion and unchanged IgG2a levels. Similarly, HApDNA generated a polarized IgG2a subclass responses, which was reduced in IL-4^{-/-} mice, however the overall IgG titre was reduced in both IL-4^{-/-} and IL-10^{-/-} mice compared to the C57BL/6 parent strain following HApDNA immunization. Taken together these findings confirm the importance of IL-12, IL-10 and IL-4 as crucial components in T cell differentiation and subsequent B cell IgG subclass induction.

In conclusion this study provides evidence of the influence of both the nature of the encoded antigen and bacterial plasmid backbone components of DNA vaccines on the priming of distinct types of immune responses. However, the cytokine milieu plays an important role in directing the development of distinct DC subsets following initial antigenic stimulation and subsequent T cell differentiation. Influences that impact on the final specific immune response have direct implications on the future development of single or multi-plasmid DNA vaccine design.

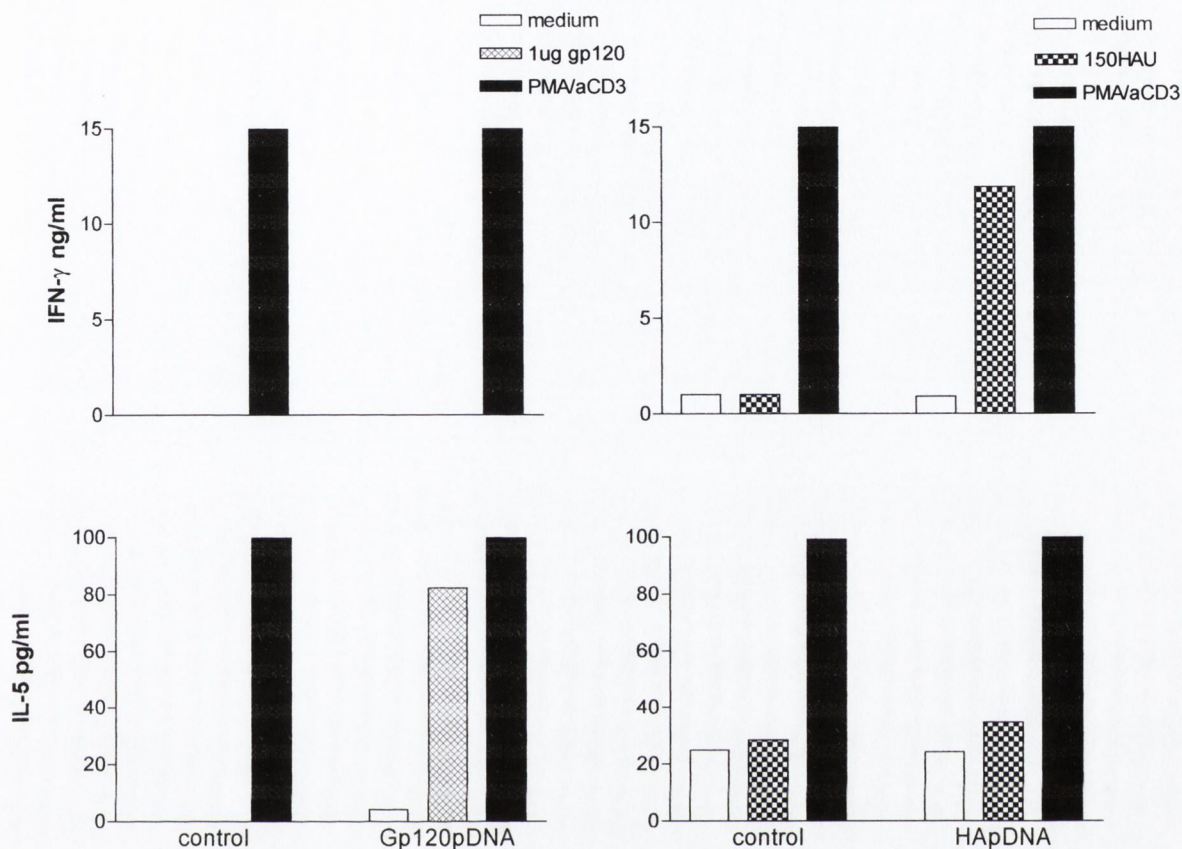


Figure 5.1. Distinct immune response induction in cervical lymph nodes following immunization with gp120pDNA or HApDNA. BALB/c mice were immunized with either 50 μ g of Gp120pDNA or 50 μ g of HApDNA. At two weeks cervical lymph node cells were stimulated *in vitro* with 1 μ g of gp120 antigen or 150HAU of influenza virus. Medium or PMA and anti-CD3 acted as negative and positive controls respectively. Supernatants were removed after 72 hours and cytokine concentrations were determined by specific immunoassay. Results from a single experiment represent responses from pooled cervical lymph node cells for four mice per group.

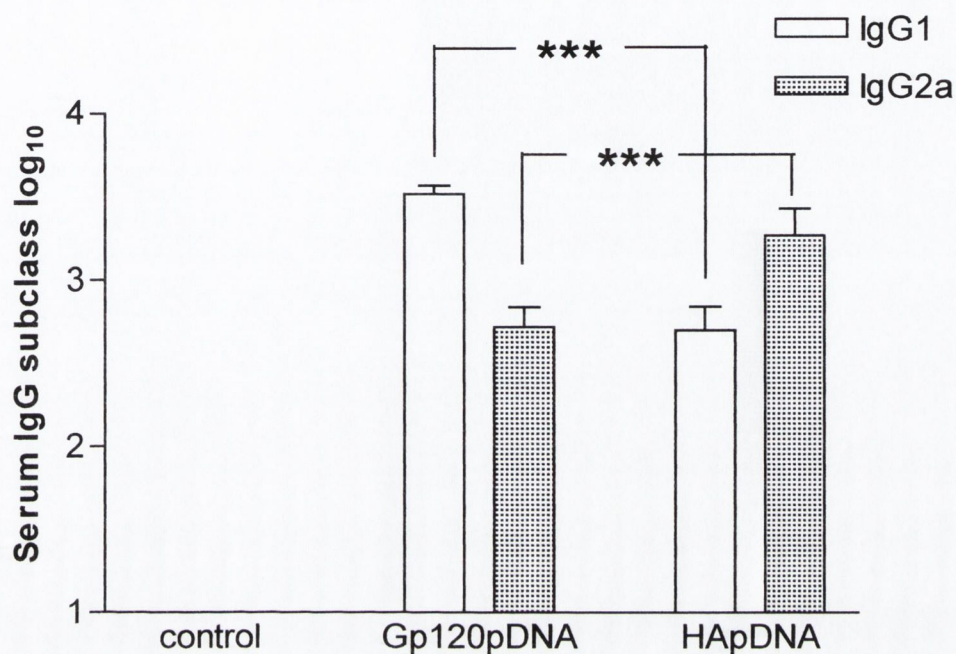


Figure 5.2. Gp120pDNA and HApDNA induction of distinct specific IgG subclasses. Mice were immunized as described in the legend to figure 5.1. Gp120- and HA-specific serum IgG subclasses were determined by ELISA. Results are mean $\pm$ SE endpoint titres for four mice in each group. *** $p < 0.001$, gp120pDNA versus HApDNA determined by Student's t-test.

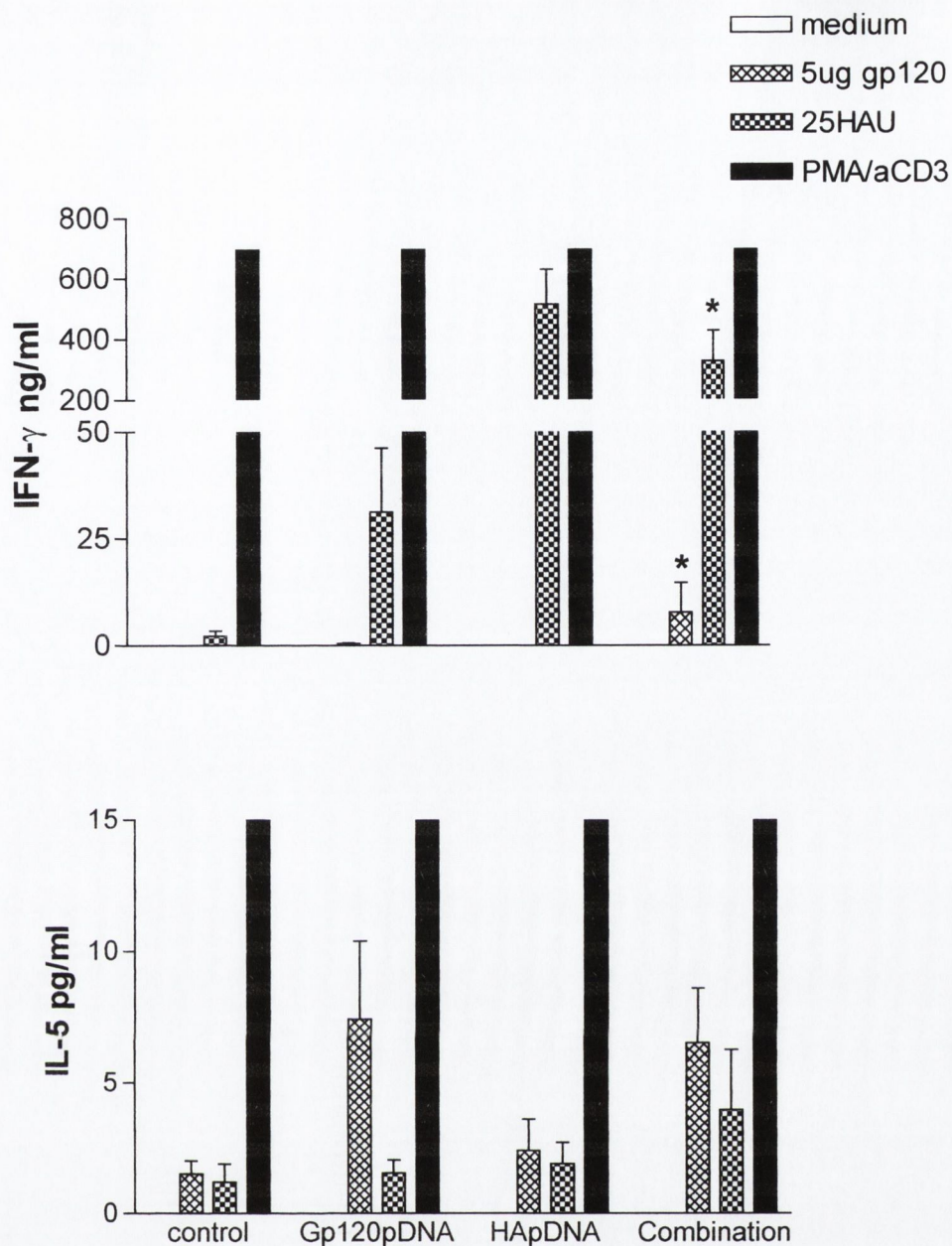


Figure 5.3. Immunization with a combination vaccine composed of both gp120pDNA and HApDNA does not adversely affect immune responses to individual plasmids. BALB/c mice were i.m. immunized with 50 μ g of gp120pDNA, 50 μ g of HApDNA or a combination 50 μ g gp120pDNA + 50 μ g of HApDNA. Two weeks later spleen cells were stimulated *in vitro* with 5 μ g of gp120 antigen or 25HAU of influenza virus. Cytokine concentrations from 72 hour supernatants were determined by specific IFN- γ and IL-5 immunoassays. Results from a single experiment expressed as the mean $\pm$ S.E. for four mice per group. * $p < 0.05$, plasmids alone versus combination determined by Student's t-test.

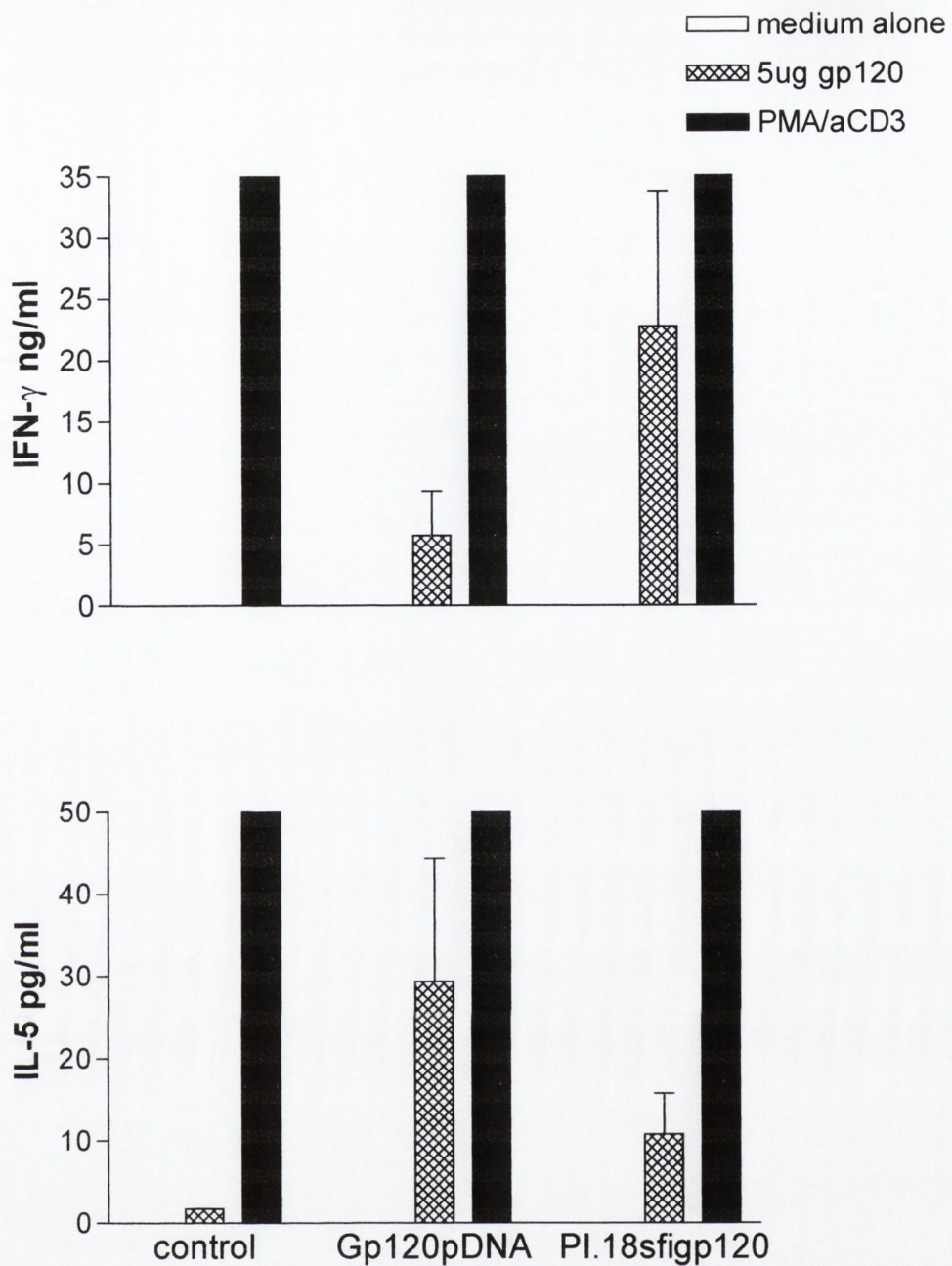


Figure 5.4. Gp120 antigen encoded by different plasmids induced distinct cytokine production from spleen of immunized mice. BALB/c mice were immunized with either 50 μ g of Gp120pDNA or PI.18sfigp120. At two weeks spleen cells were stimulated *in vitro* with 5 μ g of gp120 antigen. Medium or PMA and anti-CD3 acted as negative and positive controls respectively. Supernatants were removed after 72 hours and IFN- γ and IL-5 cytokine concentrations were determined by specific immunoassay. Results from a single experiment expressed as the mean $\pm$ S.E. for four mice per group.

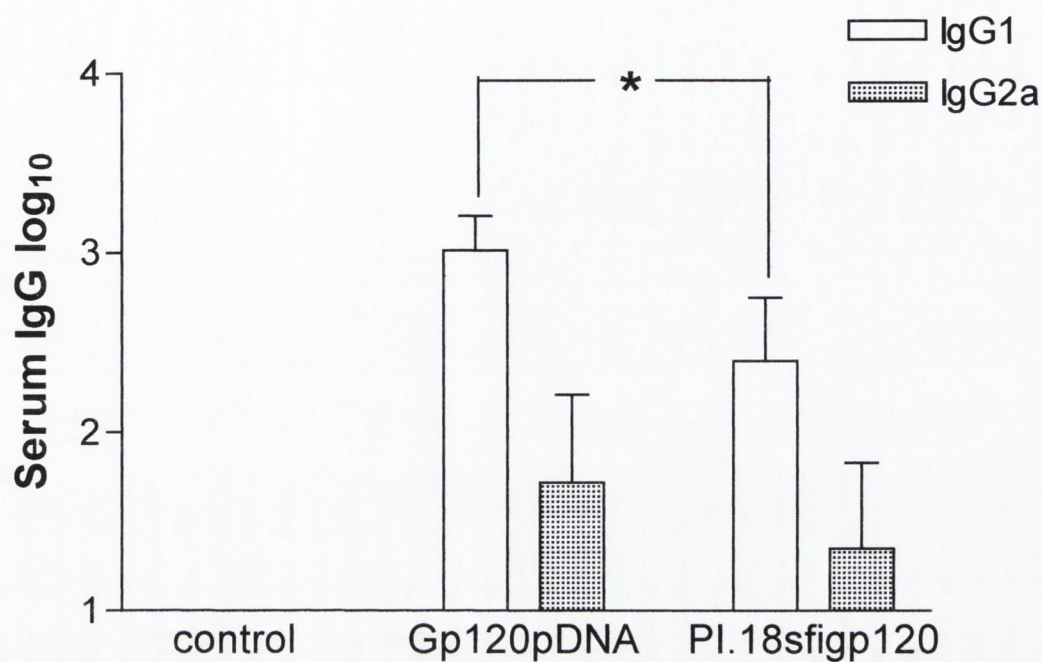


Figure 5.5. IgG subclass induction by gp120 antigen encoded by different plasmids. Mice were immunized as described in the legend to figure 5.4. Gp120 and HA specific serum IgG subclass titres were determined by ELISA. Results are mean endpoint titres $\pm$ SE for four mice in each group. * $p < 0.05$, gp120pDNA versus PI.18sfigp120 determined by Student's t-test.

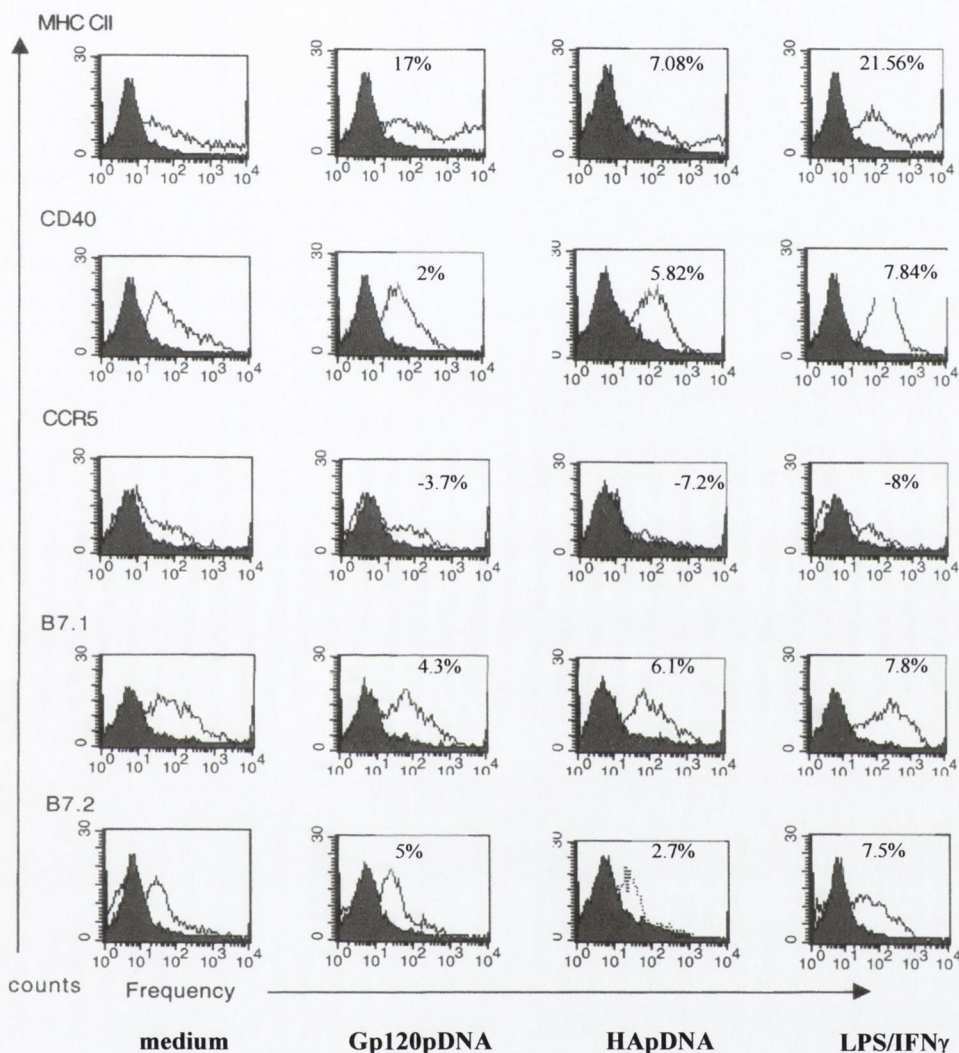


Figure 5.6. BMDC maturation induced by plasmid DNA. BMDC were stimulated *in vitro* with 10 μ g gp120pDNA or HApDNA. BMDC cultured in medium alone or with 1 μ g LPS and 10ng IFN- γ acted as negative and positive controls respectively. After 24 hours CD11c positive cells were washed and labeled with antibodies specific for MHC class II, CD40, CCR5, B7.1 and B7.2 (line). Isotype matched control antibodies were also used to assess non-specific binding (solid). % refers to staining to untreated BMDC.

Figure 5.7. Plasmid DNA can induce chemokine and cytokine production from BMDC. BMDC were stimulated *in vitro* for 24 hours with 2 and 10 µg of gp120pDNA or HApDNA. BMDC cultured in medium alone acted as a negative control, whereas BMDC stimulated with LPS & IFN-γ represent the positive control. Supernatants removed after 4 or 24 hours were assessed for concentrations of MIP-1β, MIP-2, IL-10 and IL-12 by specific immunoassays.

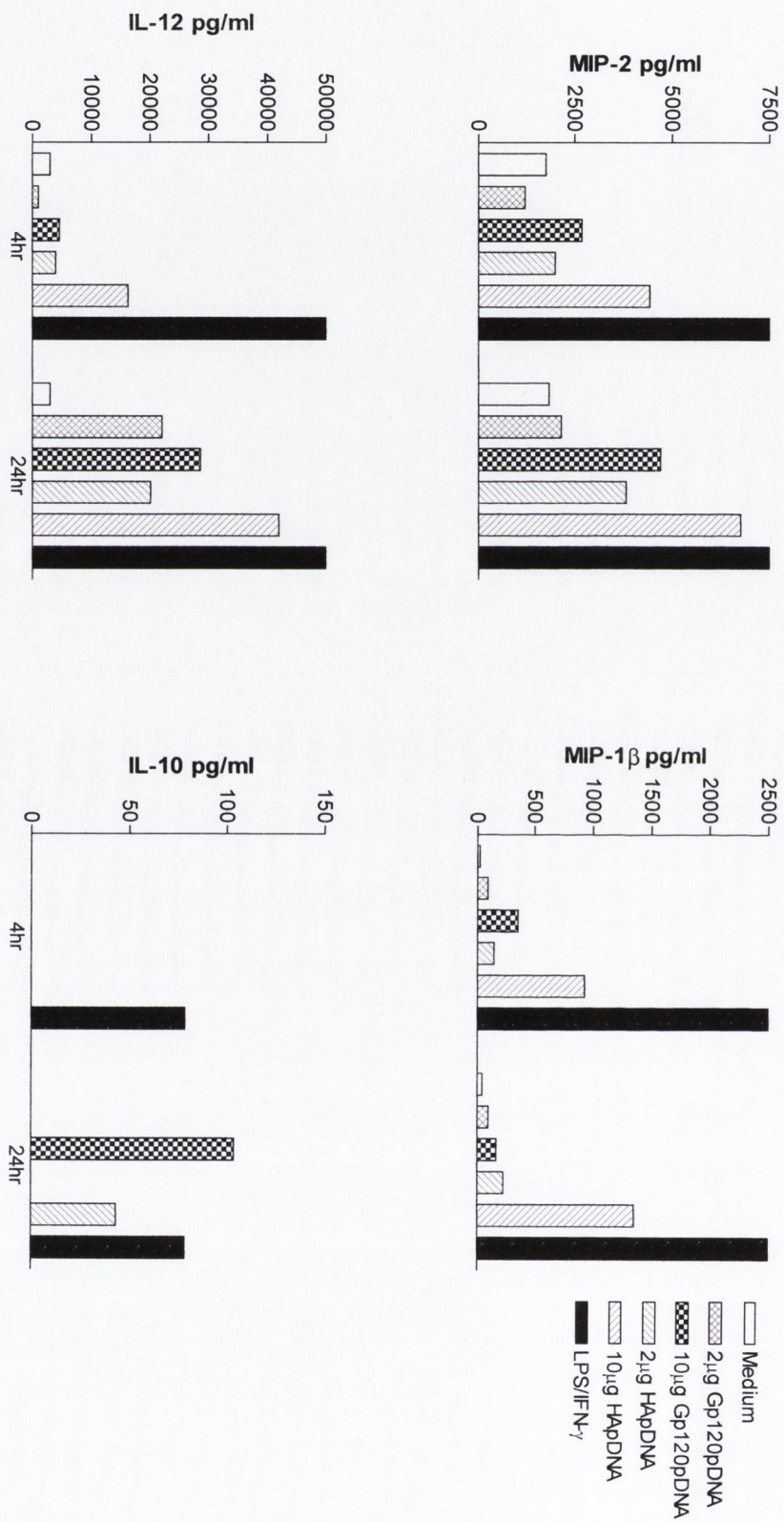
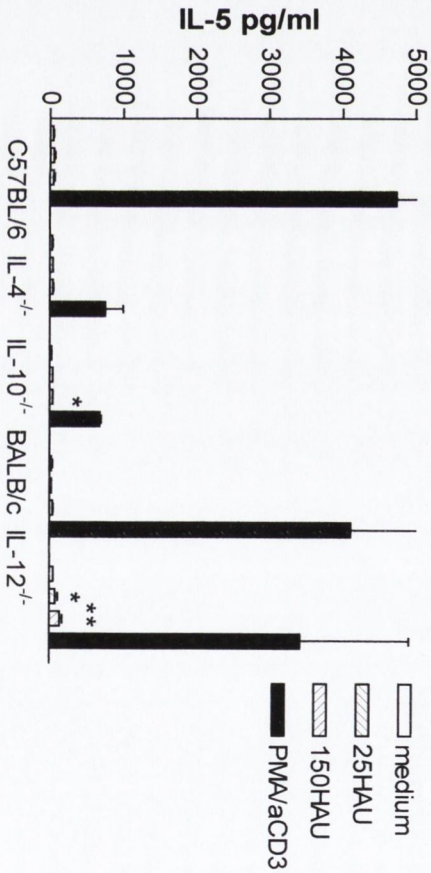
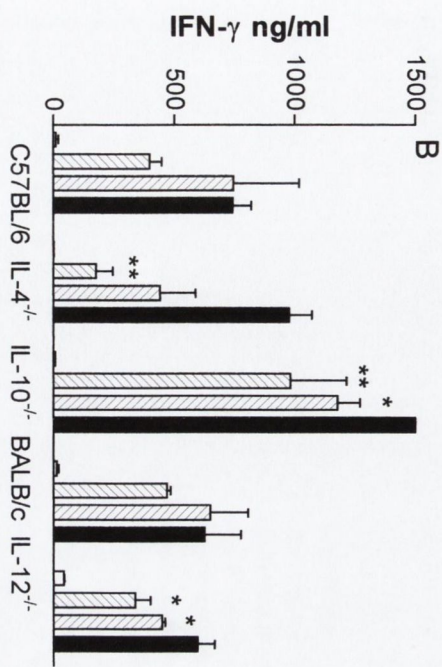
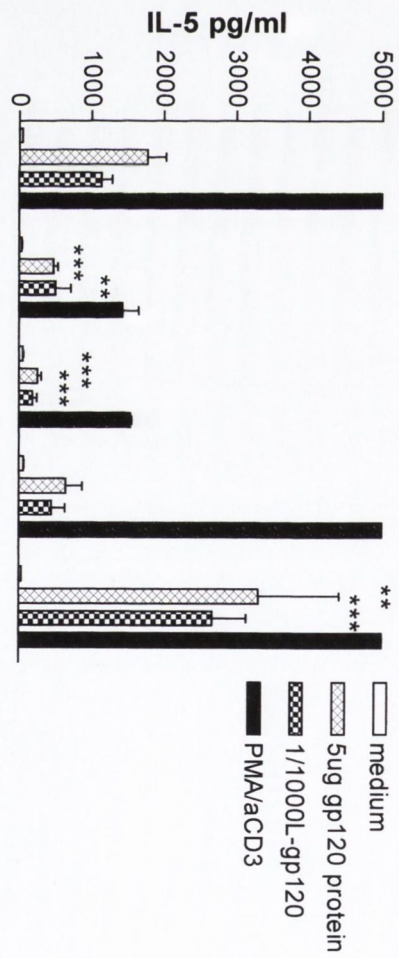
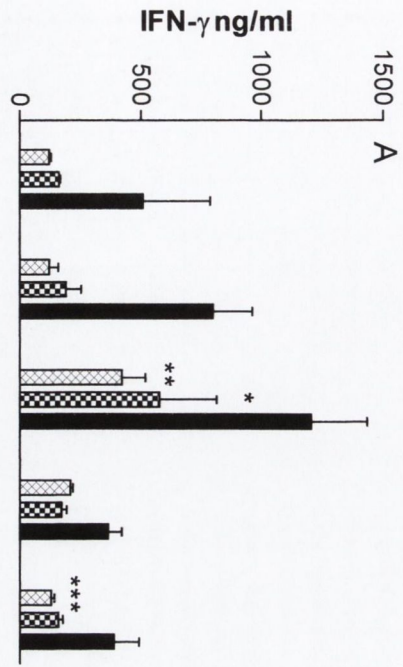


Figure 5.8. IL-10 and IL-12 primarily mediate induction of type-1 and type-2 responses in the spleen following DNA immunization. C57BL/6, IL-4^{-/-}, IL-10^{-/-}, BALB/c and IL-12^{-/-} mice were immunized with either 50 µg of Gp120pDNA or 50 µg of HApDNA at week 0 and boosted at week six. Two weeks after the booster immunization spleen cells from gp120pDNA (A) immunized mice were stimulated *in vitro* with 5 µg of gp120 antigen or 1/1000 dilution of gp120 coupled latex beads (1/1000 L-gp120), whereas spleen cells from HApDNA (B) immunized mice were stimulated with 25 or 150HAU of influenza virus. Medium or PMA and anti-CD3 acted as negative and positive controls respectively. Supernatants were removed after 72 hours and cytokine concentrations were determined by specific immunoassay. Results from a single experiment expressed as the mean ± S.E. for four mice per group. *p<0.05, ** p<0.01, *** p<0.001, C57BL/6 versus IL-4^{-/-} and IL-10^{-/-} and BALB/c versus IL-12^{-/-}, determined by Students t-test.



□ medium
 ▨ 5ug gp120 protein
 ▩ 1/1000L-gp120
 ■ PMA/aCD3

□ medium
 ▨ 25HAU
 ▩ 150HAU
 ■ PMA/aCD3

Figure 5.9. IL-10 and IL-12 primarily mediate induction of type-1 and type-2 responses in the cervical lymph node following DNA immunization. C57BL/6, IL-4^{-/-}, IL-10^{-/-}, BALB/c and IL-12^{-/-} mice were immunized as described in the legend to figure 5.8. Two weeks after the booster immunization cervical lymph node cells from gp120pDNA (A) or HApDNA (B) immunized mice were stimulated *in vitro* as described in the legend to figure 5.8. Medium or PMA and anti-CD3 acted as negative and positive controls respectively. Supernatants were removed after 72 hours and cytokine concentrations were determined by specific immunoassay. Results from a single experiment expressed as responses from pooled cervical lymph node cells for four mice per group. *p<0.05, ** p<0.01, *** p<0.001, C57BL/6 versus IL-4^{-/-} and IL-10^{-/-} and BALB/c versus IL-12^{-/-}, determined by Students t-test.

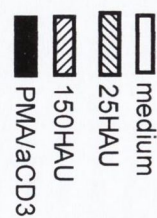
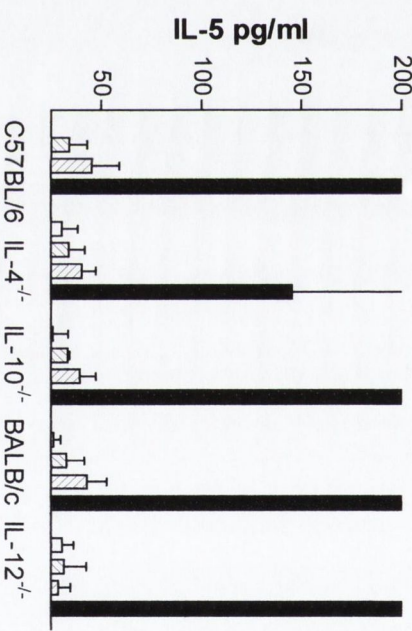
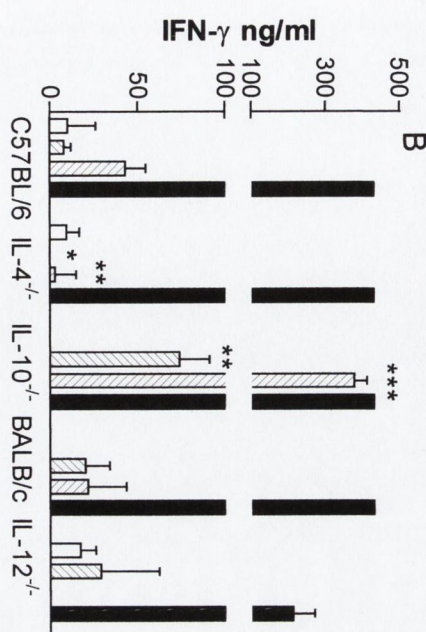
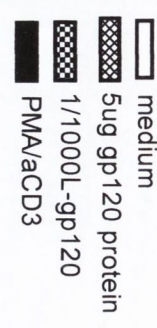
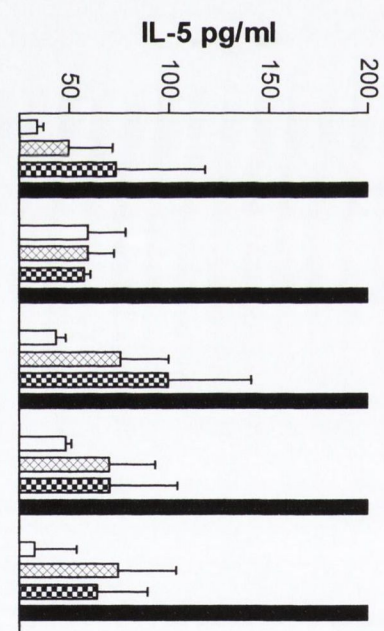
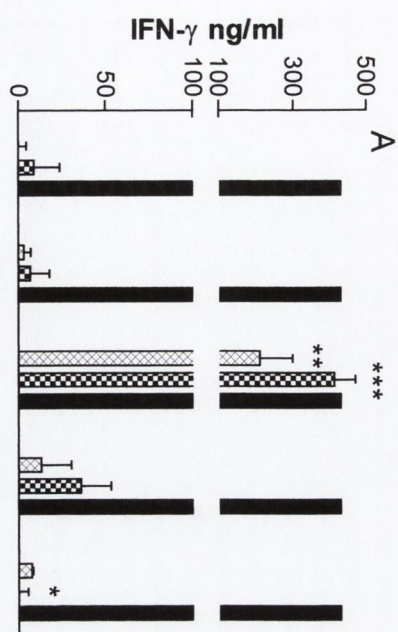
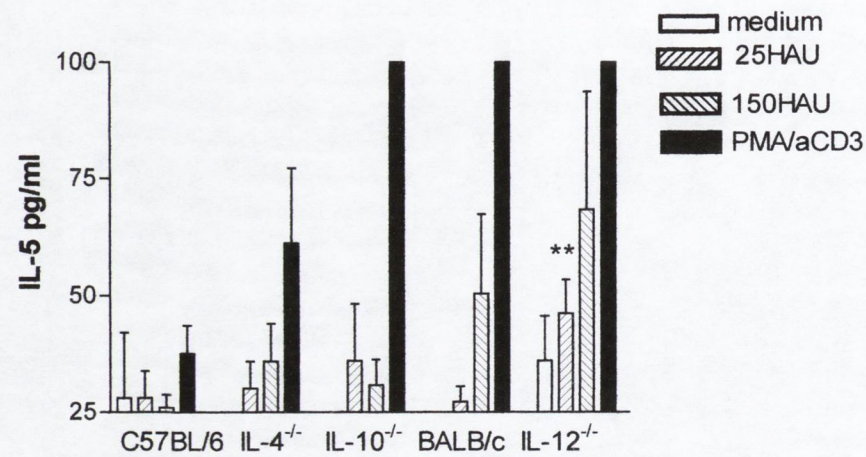
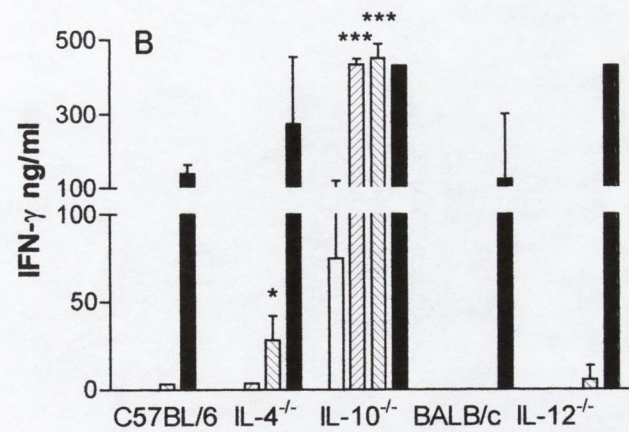
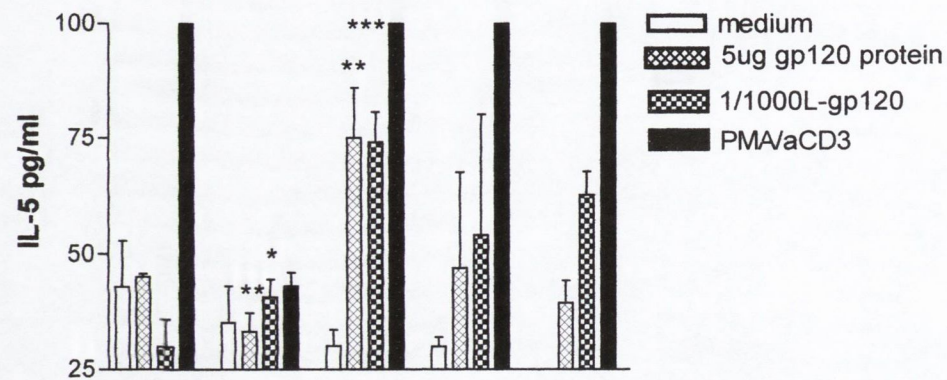
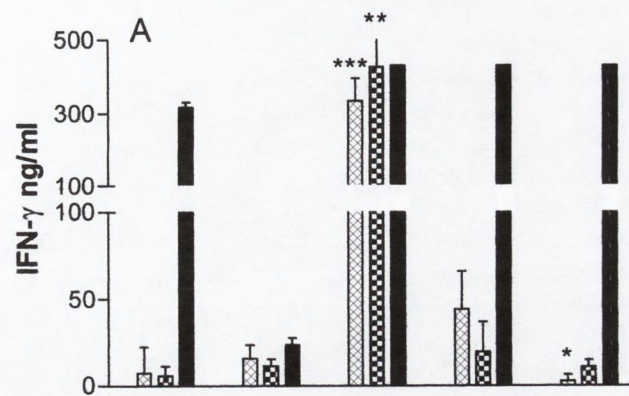


Figure 5.10. IL-10 and IL-12 primarily mediate induction of type-1 and type-2 responses in the popliteal lymph node following DNA immunization. C57BL/6, IL-4^{-/-}, IL-10^{-/-}, BALB/c and IL-12^{-/-} mice were immunized as described in the legend to figure 5.8. Two weeks after the booster immunization popliteal lymph node cells from gp120pDNA (A) or HApDNA (B) immunized mice were stimulated *in vitro* as described in the legend to figure 5.8. Medium or PMA and anti-CD3 acted as negative and positive controls respectively. Supernatants were removed after 72 hours and cytokine concentrations were determined by specific immunoassay. Results from a single experiment expressed as responses from pooled popliteal lymph node cells for four mice per group. *p<0.05, ** p<0.01, *** p<0.001, C57BL/6 versus IL-4^{-/-} and IL-10^{-/-} and BALB/c versus IL-12^{-/-}, determined by Students t-test.



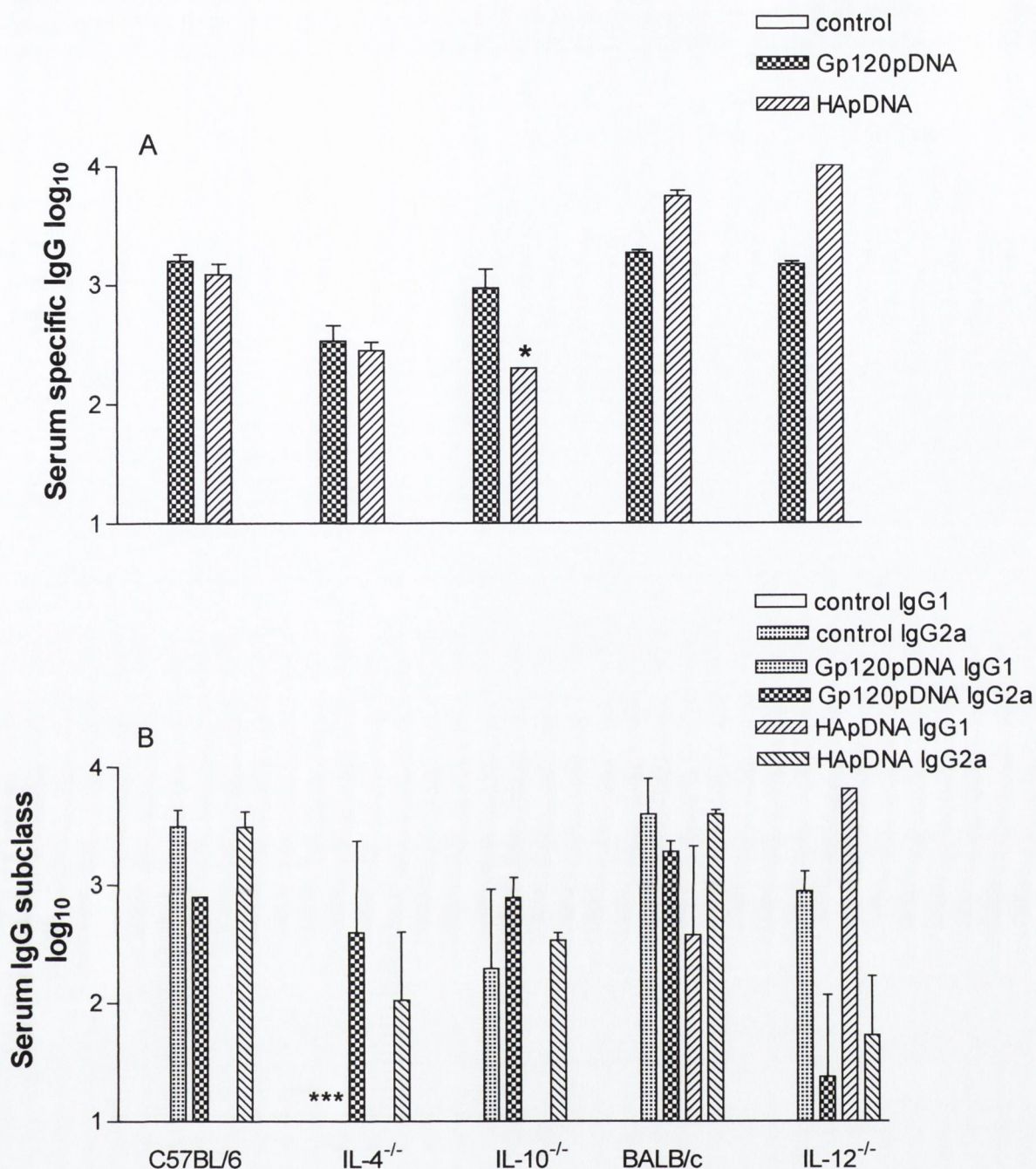


Figure 5.11. DNA vaccine induction of antibodies in mice deficient in key immunoregulatory cytokines. Mice were immunized as described in the legend to figure 5.8. Two weeks after the booster immunization serum from gp120pDNA or HApDNA immunized mice was assessed for **(A)** total anti-gp120 and anti-HA IgG endpoint titres, **(B)** anti-gp120 and anti-HA IgG subclasses. Results are determined from endpoint titres and expressed as mean $\pm$ SE values for four mice in each group. * $p < 0.05$, C57BL/6 versus IL-4^{-/-} and IL-10^{-/-} and BALB/c versus IL-12^{-/-}, determined by Student's t-test.

CHAPTER 6

REGULATION OF DC FUNCTION BY GP120DNA

6.1 INTRODUCTION

Viruses have evolved evasion mechanisms to counteract the selective pressure imposed by the immune system in its recognition and destruction of the pathogen. To this effect, many aspects of the immune system have been targeted by viruses, including abrogation of cell surface marker expression, required to initiate effective immune responses, initiation of apoptotic signaling pathways to facilitate viral replication, modulation of cytokine and chemokine molecules involved in regulating immune responses and manipulation of complement and Fc receptors during humoral responses (Tortorella *et al.*, 2000). Investigating these interactions and understanding the mechanisms involved will allow a more rational and directed approach to vaccine design.

It has been suggested that CD4⁺ T cells play a role in anti-HIV immunity, as they regulate antibody production and CD8⁺ effector functions (Altfeld and Rosenberg, 2000), (Pitcher *et al.*, 1999). However, priming of naïve T cells is dependant on interactions with peptide-MHC complexes on APC and co-stimulation signals provided by DCs. It has been reported that viruses, such as measles (Marie *et al.*, 2001) and influenza (Bender *et al.*, 1997/98), can interfere with DC maturation and functions. HIV may have developed mechanisms to impair the host immune system by targeting DC maturation events and subsequent chemokine and cytokine production, necessary for homing and priming of effective T cell responses. Extensive investigation has focused on the influence of the gp120 surface envelope glycoprotein of HIV on immune effector functions mediated by lymphocytes and monocytes. Most important in the context of vaccination is the ability of gp120 to induce IL-10 (Borghi *et al.*, 1995), (Taoufik *et al.*, 1997), which influences DC activation and priming of immune responses. It has been reported that IL-10 triggers functional decoy receptors (FDR) in the chemokine system (D'Amico *et al.*, 2000). IL-10 prevents upregulation of chemokine receptors associated with DC maturation. These receptors remain uncoupled but still function as molecular scavengers by recognizing specific ligands with high affinity, but are structurally incapable of triggering a signal cascade. This mechanism represents a major strategy for viruses to regulate chemokine and cytokine functions *in vivo*. A study by D'Amico *et al.*, (2000) demonstrates that IL-10 can inhibit LPS-induced DC maturation associated with downmodulation of chemokine receptors expression (CCR1, CCR2 and CCR5) and production of chemokines from DCs.

Although the correlates of immunity to HIV have yet to be fully established, it has been suggested that a type-1 T cell response may be required for protection against HIV infection (Clerici *et al.*, 1992). Depletion of CD4⁺ type-1 cells can disrupt immune homeostasis and the subsequent increase in type-2 T cells has been proposed to account for progression to AIDS in HIV infected individuals (Clerici and Shearer, 1993), (Evans *et al.*, 1998). Therefore, a vaccine that can induce type-1 T cell responses, mediated by cytokines such as IL-12, IL-18 and IFN- γ , may contribute to the control of an intracellular pathogen such as HIV. The results in chapter 4 demonstrate that gp120pDNA generates a mixed Th1/Th2 type response, modulated toward a type-1 profile by co-administration of an IL-12 coding plasmid DNA. The present chapter investigates the mechanism whereby gp120 induces T cell responses skewed to the type-2 subtype. The role of DC-derived IL-10 was assessed, including examination of BMDC maturation by surface marker expression and suppression of IL-12 and chemokine production. Understanding the mechanism of T cell induction with gp120pDNA should help in future HIV vaccine development.

6.2 RESULTS

6.2.1 Gp120pDNA INDUCES IL-10 PRODUCTION AND ANTI-IL-10 CAN ENHANCE IL-12 PRODUCTION BY BMDC.

To assess the production of the regulatory cytokines, IL-10 and IL-12, 1×10^6 BMDC were incubated *in vitro* for 24 hours with gp120pDNA (2 and $10 \mu\text{g}$). Supernatants removed at 4 and 24 hours were examined for the presence of cytokines. BMDC secreted high levels of IL-10 after 24 hours stimulation with gp120pDNA; the concentration was highly significant compared with untreated cells ($p < 0.001$) and similar to that induced with $1 \mu\text{g}$ LPS (Fig 6.1). However, kinetics of production was slower than that observed with LPS, which produced significant IL-10 after 4 hours and this may represent time required for synthesis of the gp120 protein by the BMDC. IL-12 production was analyzed by incubating BMDC with a range of doses of gp120pDNA and PI.18sfigp120, (0.08, 0.4, 2, $10 \mu\text{g}$) or $1 \mu\text{g}$ CpG-ODN as a positive control. PI.18sfigp120 encoded an identical version of gp120 to that encoded by gp120pDNA. However, the plasmid backbones were distinct. This allowed examination of the effect of the plasmid on DC cytokine production. Increasing doses of gp120pDNA and PI.18sfigp120 or CpG-ODN stimulated increased amounts of IL-12 production from BMDC (Fig 6.2A). CpG-ODN stimulated higher levels of IL-12 production compared with those induced with either gp120 encoding plasmid. Addition of neutralizing anti-IL-10 antibody significantly enhanced both gp120pDNA ($p < 0.001$) and PI-18sfigp120 ($p < 0.01$) induction of IL-12 from BMDC (Fig 6.2B). This effect was not observed when anti-IL-10 was added to BMDC and stimulated with CpG-ODN.

6.2.2 Gp120pDNA INHIBITS LPS INDUCED IL-12 PRODUCTION BY BMDC AND MACROPHAGES

Since gp120pDNA stimulated IL-10 production and anti-IL-10 antibodies enhanced gp120-induced IL-12 production, the effect of gp120 was assessed on LPS-stimulated IL-12 production. BMDC and J774 macrophage cells (1×10^6 cell/ml) were stimulated *in vitro* with a range of concentrations of recombinant gp120 protein or gp120pDNA (0.1, 1, $10 \mu\text{g}$) alone or with LPS/IFN- γ ($1 \mu\text{g}/10\text{ng}$). Increasing doses of gp120pDNA, stimulated high concentrations of IL-12 secretion from BMDC and lower levels from J774 macrophages (Fig 6.3). Recombinant gp120 protein failed to induce detectable

concentrations of IL-12 from either BMDC or macrophages. LPS/IFN- γ alone stimulated IL-12 production from both BMDC and macrophages. Addition of gp120 protein inhibited LPS/IFN- γ -induced IL-12 production from BMDC and this reduction was significant ($p < 0.05$) at the highest dose of gp120 protein tested (Fig 6.3A). In contrast, gp120pDNA had little effect on LPS/IFN- γ -induced IL-12 production by BMDC (Fig 6.3), but did inhibit LPS/IFN- γ -induced IL-12 production by macrophages.

6.2.3 GP120pDNA INDUCTION OF CHEMOKINE SECRETION FROM APC CAN BE ENHANCED BY BLOCKING IL-10 PRODUCTION

To assess the effect of gp120-induced IL-10 on chemokine production, J774 macrophage and DC lines were stimulated *in vitro* for 24 hours at 1×10^6 cell/well with either 10 μ g of gp120pDNA or 1 μ g CpG-ODN, LPS or medium alone acted as positive and negative controls respectively. Supernatant assessed after 24 hours showed that gp120pDNA stimulated secretion of macrophage inhibitory protein (MIP)-1 α , MIP-1 β and MIP-2 but not regulated on activation normal T-cell expressed (RANTES) from both macrophage and DC lines (Fig 6.4). The concentration of the inflammatory chemokine, MIP-1 α , secreted by the DC line was significantly higher than that produced by the macrophage cells. CpG-ODN and LPS stimulated both cell lines to secrete high concentrations of all four chemokines examined. Cells cultured in medium alone showed low-level constitutive release of MIP-1 β and MIP-2 from DC and MIP-1 β from macrophages.

Bone marrow cells cultured *in vitro* with GM-CSF lead to the generation of immature myeloid DC (Lutz *et al.*, 1999), (Pulendran *et al.*, 1999). Studies have described the effects of CpG motifs on both the maturation and production of cytokines from DCs *in vitro* (Sparwasser *et al.*, 1998) and chemokines from myocytes (Stan *et al.*, 2001). This study examined chemokine production from BMDC stimulated with gp120pDNA. Immature BMDC were incubated with 10 μ g gp120pDNA in the presence or absence of LPS/IFN- γ and/or anti-IL-10 antibody. Analysis of chemokine production demonstrated that gp120pDNA stimulated BMDC production of MIP-2 but not MIP-1 β after 24 hours. Addition of anti-IL-10 antibody or LPS/IFN- γ dramatically enhanced secretion of both MIP-1 β ($p < 0.001$) and MIP-2 ($p < 0.001$) from gp120pDNA activated BMDC (Fig 6.5).

6.2.4 GP120pDNA MATURATION OF BMDC IS ENHANCED BY ANTI-IL-10.

These studies have demonstrated that gp120pDNA induces IL-10 production and suppresses IL-12 secretion from BMDC in an IL-10 dependant mechanism. It has been reported that IL-10 can also inhibit DC maturation (D'Amico *et al.*, 2000) and adversely affect DC priming of T cell subtypes (Corinti *et al.*, 2001). The present study investigated whether gp120 induction of IL-10 would impair the ability of gp120pDNA to effectively mature BMDC and if blocking IL-10 would reverse this effect. Immature BMDC were incubated with 10µg gp120pDNA in the presence or absence of LPS/IFN-γ and/or anti-IL-10 antibody and tested by flow cytometric analysis for cell surface expression of B7.1, B7.2, MHC class II, CD40 and CCR5 (see section 2.6.6). BMDC incubated with gp120pDNA induced BMDC maturation as indicated by up-regulation of B7.1, B7.2, MHC class II and CD40 and marginal reduction in CCR5 surface expression (Fig 6.6). Addition of anti-IL-10 antibody significantly improved the maturation of BMDC by gp120pDNA. Expression of B7.1, MHC class II and CD40 were markedly improved whereas the level of B7.2 expression remained almost the same. Blocking IL-10 inhibition of BMDC maturation events also correlated with a significant reduction in CCR5 surface expression.

6.3 DISCUSSION

The anti-inflammatory cytokine, IL-10, has an important regulatory role in inflammatory cytokine and chemokine responses (D'Amico *et al.*, 2000), (Corinti *et al.*, 2001). HIV, through gp120 induction of IL-10, may have evolved a strategy to subvert immune responses by modulation of DC maturation and cytokine/chemokine production. IL-10 may function to prevent upregulation of chemokine receptors associated with DC maturation. These uncoupled receptors still act as a chemokine sink, but are unable to signal and trigger induction of immune responses. The results of this study demonstrate that immune responses generated with gp120pDNA are influenced by gp120 induced IL-10.

J774 macrophages or BMDC incubated with gp120pDNA produced low levels of IL-10 within 24 hours of stimulation. Furthermore, gp120pDNA stimulated macrophages and BMDC were shown to secrete low and high levels of IL-12 respectively. It has previously been demonstrated that gp120 protein can induce IL-10 from human monocytes (Borghi *et al.*, 1995) and inhibit monocyte IL-12 production (Taoufik *et al.*, 1997). Stimulation of DCs or macrophages, with a combination of LPS and IFN- γ , constitutes a powerful inflammatory stimulus that can induce IL-12 production (McGuirk and Mills, 2000), (Takenaka *et al.*, 1997). This study demonstrated that treatment of macrophages or BMDC with gp120pDNA significantly reduced LPS/IFN- γ -induced IL-12 production by macrophages. However, LPS-stimulated production of IL-12 by BMDC was only moderately reduced in the presence of gp120pDNA. These findings may suggest that the DCs were activated through TLR9 expressed on the surface through which CpG motifs, present in gp120pDNA, preferentially bind and trigger signal activation leading to IL-12 production (Hemmi *et al.*, 2000), (Warren *et al.*, 2000). It is possible that CpG-ODN may result in different levels of activation in DCs and macrophages.

In studies using IL-10-deficient mice, Huang *et al.*, (2001) have demonstrated that IL-10 regulates IL-12 production by DC. Furthermore, a recent study has confirmed that Th1 and Th2 priming is dependent on CD8 α^+ DC-derived IL-12 and IFN- γ and CD8 α^- DC- derived IL-10 (Maldonado-Lopez *et al.*, 2001). These findings have implications for developing vaccines against viral pathogens such as HIV where IL-10 may ultimately cause immune dysregulation.

In vitro studies demonstrated that antibodies to IL-10 enhanced IL-12 production by gp120pDNA stimulated BMDC. This effect was not exclusive to

gp120pDNA as PI.18sfigp120 plasmid, encoding an identical version of gp120, also stimulated BMDC production of IL-12 and was enhanced by the addition of antibodies to IL-10. Similar studies have shown that DCs treated with antibodies to IL-10 have increased ability to prime Th1 cells (Corinti *et al.*, 2001). These findings suggest gp120 protein encoded by gp120pDNA may suppress IL-12 production through the stimulation of IL-10.

Soluble chemokine molecules are produced by a number of immune cells, including leukocytes. Inflammatory chemokines primarily attract leukocytes, whereas lymphoid chemokines maintain trafficking of leukocytes during an immune response (Sallusto *et al.*, 2000). Sallusto *et al.*, (1999) have reported that DC stimulated *in vitro* with LPS, TNF- α or CD40L to rapidly secrete high-levels of MIP-1 α and MIP-1 β and sustain production of RANTES and monocyte chemotactic protein-1 (MCP-1). Macrophages stimulated with LPS, TNF- α or CD40L also induced a similar pattern of chemokine production. It has been demonstrated that macrophage-derived DC direct the polarization of naïve T cells to Th1 cells through β -chemokine induction of IFN- γ (Zou *et al.*, 2000). In the present study, BMDC stimulated with LPS or CpG-ODN, secreted MIP-1 α , MIP-1 β , MIP-2 and RANTES, whereas gp120pDNA stimulated only low concentrations of MIP-1 α , MIP-1 β and MIP-2. Examination of whole blood from individuals pretreated with IL-10 prior to intravenous injection with LPS demonstrated that IL-10 blocked the release of MIP-1 α , MIP-1 β and MIP-2 chemokines (Olszyna *et al.*, 2000). Furthermore, using IL-10-deficient mice, Shanley *et al.*, (2000) has shown that IL-10 is as a major regulator of neutrophil chemokine production in an LPS-induced acute lung inflammation model.

This study has shown that BMDC incubated with gp120pDNA produce IL-10 and low levels of inflammatory chemokines. Furthermore, it has been reported that Th1 responses are associated with MIP-1 β and RANTES and are downregulated by IL-10 (Sanjiv *et al.*, 2001). Blocking the effect of IL-10, through anti-IL-10 antibodies, was found to enhance chemokine release from BMDC stimulated with gp120pDNA. Similarly, neutralization of IL-10 in a bacterial pneumonia model resulted in increased MIP-1 α and MIP-2 production, which are required for effective bacterial clearance (Standiford *et al.*, 1996). IL-10 has been shown to block NK- κ B activation, which is necessary for a functional chemokine-signaling pathway (Schottelius *et al.*, 1999). Moreover, BMDC stimulated with IL-10 were shown to

bind MCP-1 from the site of inflammation and were subsequently unable to undergo migration (D'Amico *et al.*, 2000).

Immature DCs express CCR1, CCR2 and CCR5, receptors for inflammatory chemokines that mediate migration to the site of inflammation. Migration of mature DCs to lymph nodes is mediated primarily through upregulation of CCR7 surface expression (Sallusto *et al.*, 2000). De Smedt *et al.* (1997) have demonstrated that IL-10 affects DC maturation events and skews subsequent T cell priming in the favor of Th2 subset, by inhibiting IL-12 production. This study demonstrates that addition of gp120pDNA to immature BMDC lead to moderate enhancement of surface markers associated with DC maturation, B7.1, B7.2, MHC class II and CD40 and marginal reduction in CCR5 expression. Recent studies have reported that IL-10 blocks LPS-stimulated maturation of DC and can be abrogated by the addition of anti-IL-10-neutralizing antibodies (Corinti *et al.*, 2001). Addition of anti-IL-10 antibodies to gp120pDNA stimulated BMDC enhanced surface maker expression associated with DC maturation. This suggests a role of IL-10 induction by gp120 protein in the regulation of BMDC maturation.

Many viral evasion mechanisms have been characterized, such as the use of vMIP-2 encoded by kaposi's sarcoma herpes virus (HHV8) to block Th1 chemokine receptors and preferential binding to CCR8 (also reported to participate in HIV infection) to initiate type 2 responses (Sallusto *et al.*, 2000). The present study provides evidence to support the role of IL-10 in blocking chemokine receptors. Uncoupled chemokine receptors are unable to signal and therefore indirectly regulate DC maturation through inhibiting IL-12 and chemokine production required for effective type-1 T cell immunity. The ability of gp120 protein to adversely affect DC maturation, chemokine and cytokine production has important implications for the design of a prophylactic DNA vaccine against HIV.

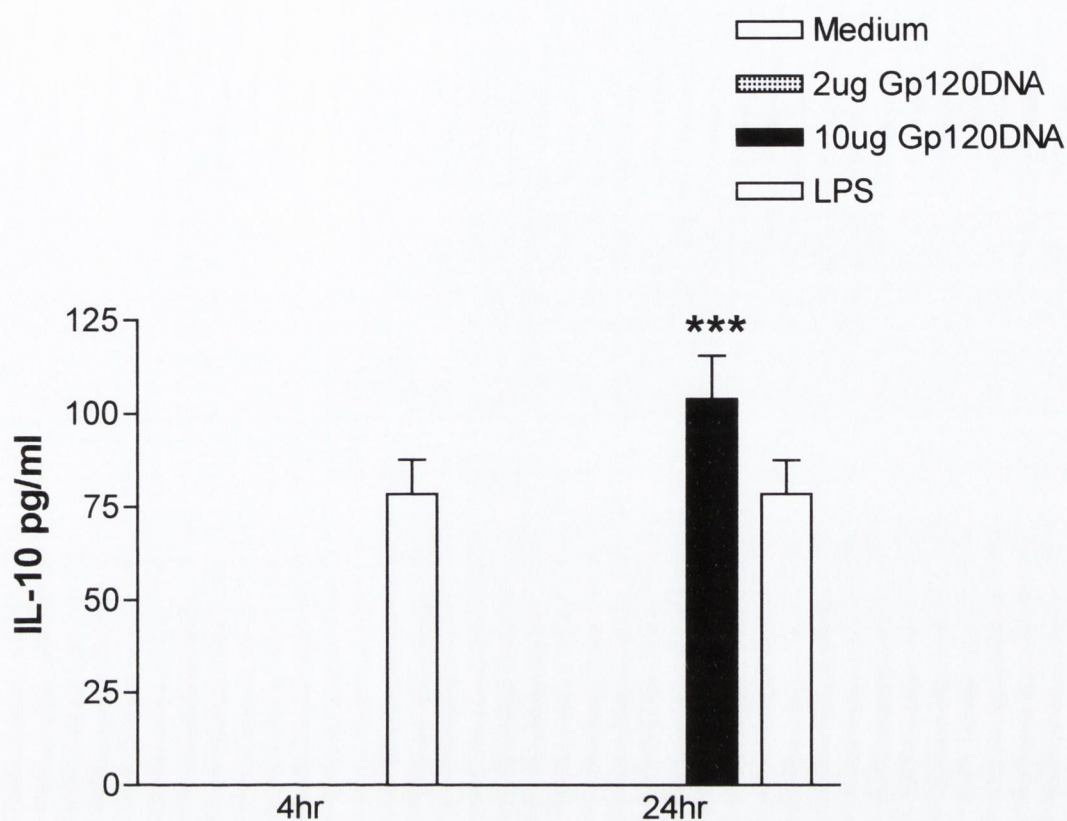


Figure 6.1. Gp120pDNA stimulates BMDC to produce IL-10.

BMDC were incubated *in vitro* with medium alone, gp120pDNA (2 or 10 μ g) or 1 μ g LPS. Supernatants were removed after 4 and 24 hours and the concentration of IL-10 was determined by specific immunoassay. *** $p < 0.001$, gp120pDNA versus cells alone determined by Student's t-test.

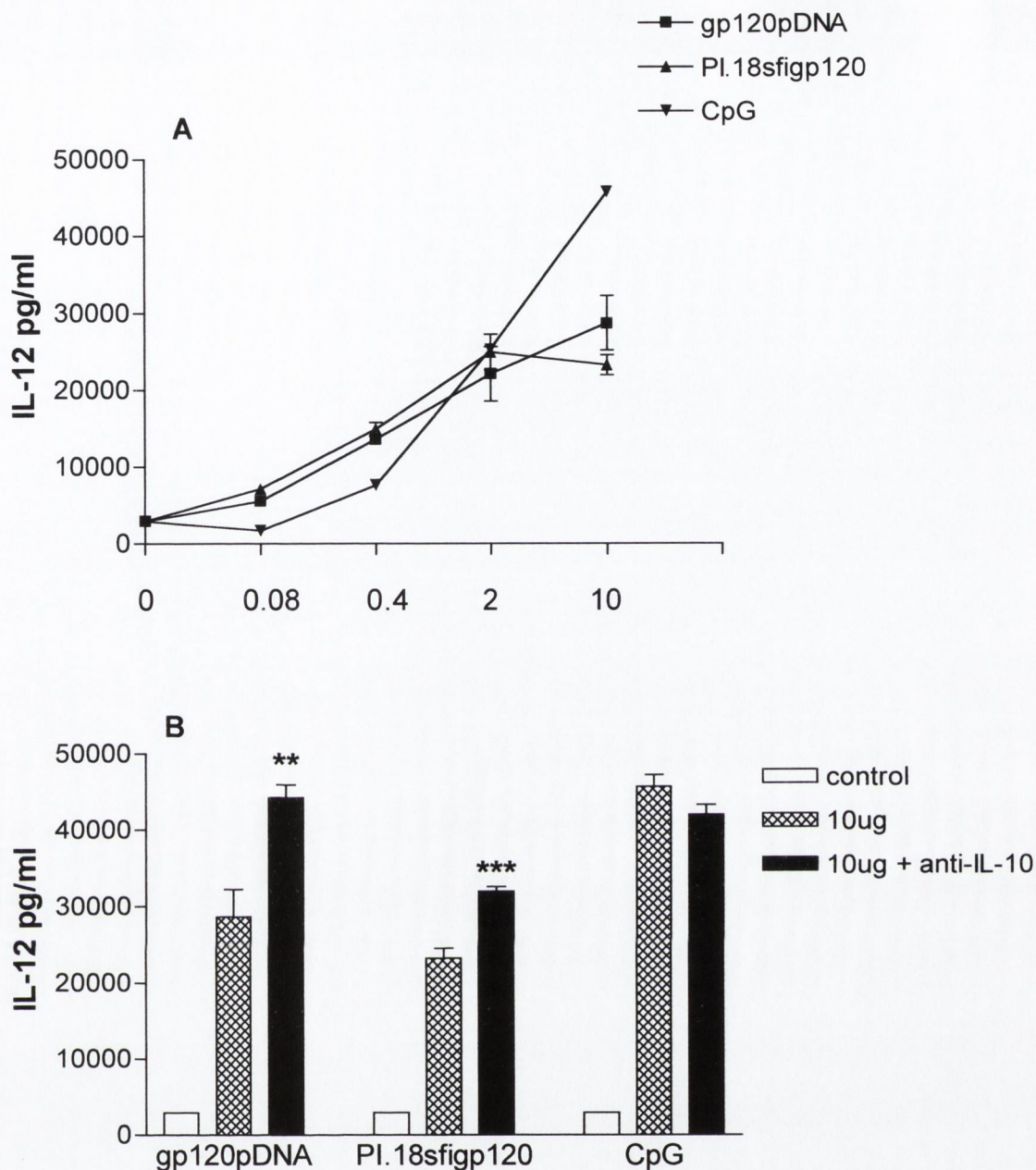


Figure 6.2. Gp120pDNA stimulates BMDC to secrete IL-12 which can be further enhanced by anti-IL-10. (A) BMDC were stimulated *in vitro* for 24 hours with gp120pDNA or PI.18sfigp120 (0.08, 0.4, 2, 10 μg/ml) or 1 μg CpG. (B) BMDC stimulated with gp120pDNA and PI.18sfigp120 (0.08, 0.4, 2, 10 μg/ml) with or without 10ng anti-IL-10 antibody or 1 μg CpG. Supernatants were removed after 24 hours and the concentration of IL-12p40 detected by specific immunoassay. ** $p < 0.01$, *** $p < 0.001$, plasmids alone versus plasmids with anti-IL-10 determined by Student's t-test.

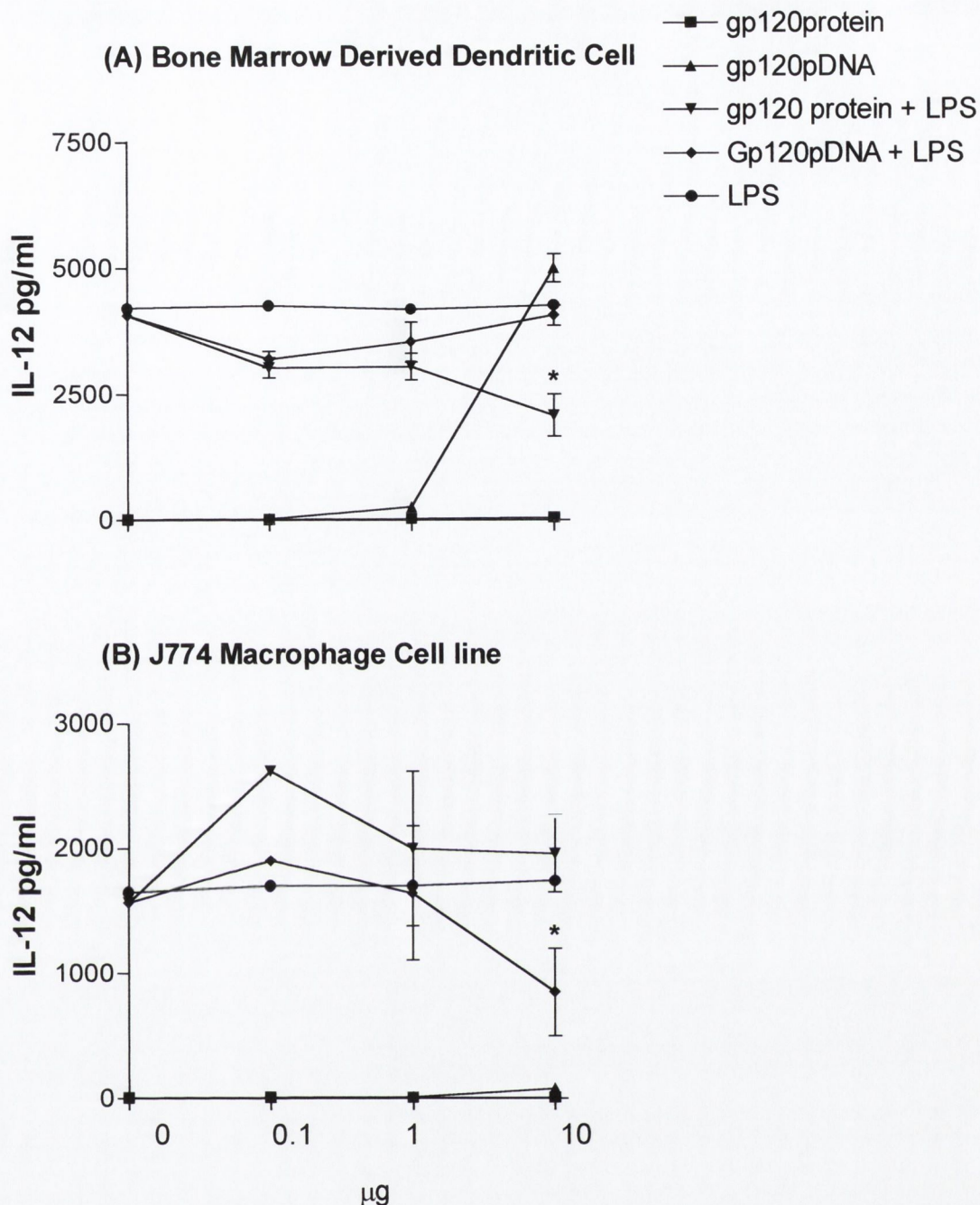


Figure 6.3. Gp120pDNA inhibits LPS induced IL-12 production from macrophages and BMDC. BMDC (A) and J774 macrophages (B) (1×10^6 cell/ml) were stimulated *in vitro* for 24 hours with gp120 protein, gp120pDNA (0.1, 1, 10 μ g) alone or LPS (1 μ g) and IFN- γ (10ng). Supernatants were removed after 24 hours and the IL-12p40 concentrations were determined by specific immunoassay. * $p < 0.05$, LPS alone versus 10 μ g gp120pDNA alone or gp120pDNA + LPS, determined by Student's t-test.

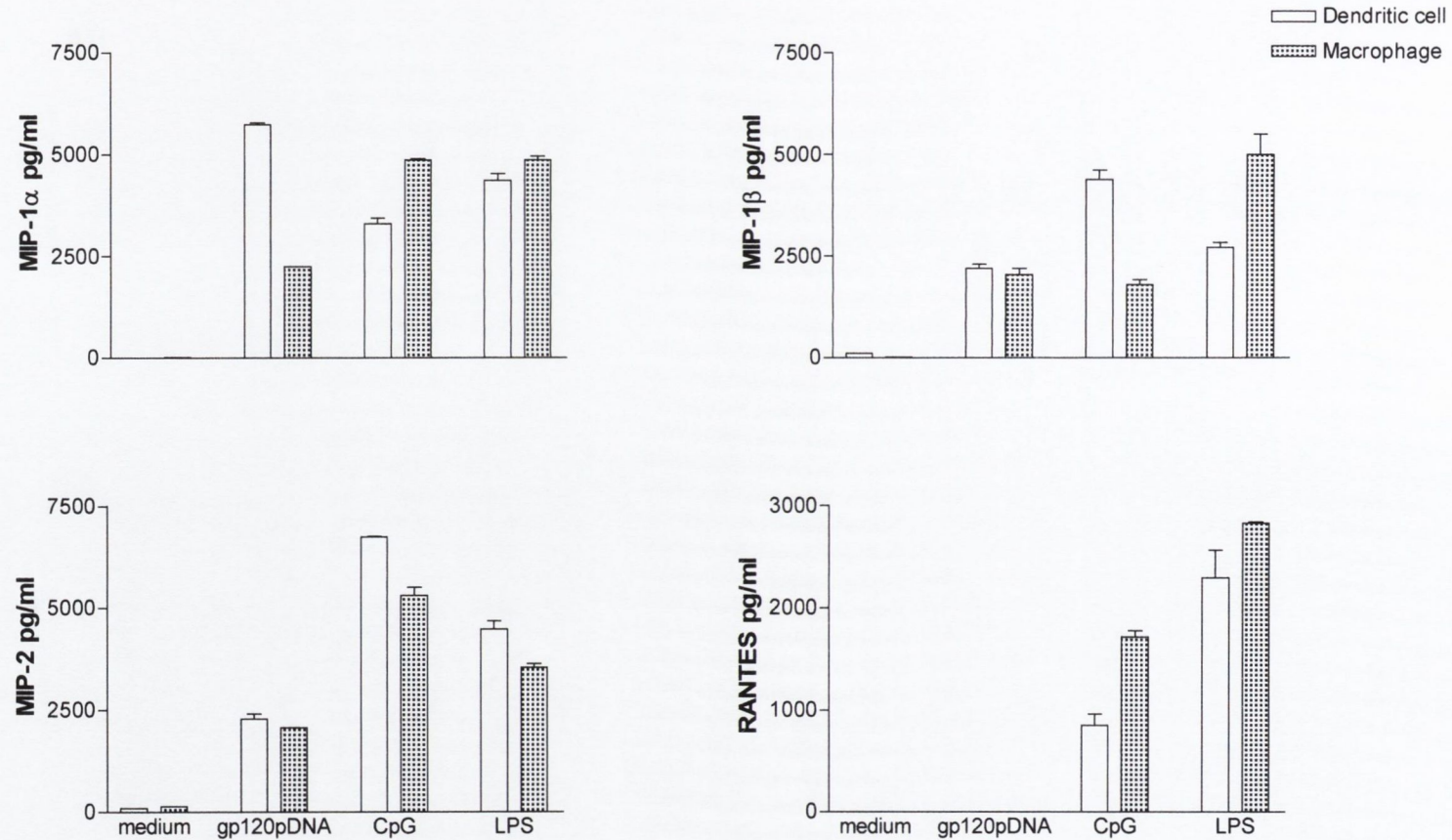


Figure 6.4. Gp120pDNA stimulates secretion of chemokines, MIP-1 α , Mip-1 β and MIP-2 but not RANTES, from dendritic and macrophage cell lines. TSDC and J774 macrophage cell lines were stimulated *in vitro* for 24 hours with 10 μ g Gp120pDNA. Cells cultured in medium alone acted as a negative control, whereas those stimulated with 1 μ g CpG or LPS acted as the positive controls. Supernatants were removed after 24 hours and the chemokine concentrations determined by specific immunoassays.

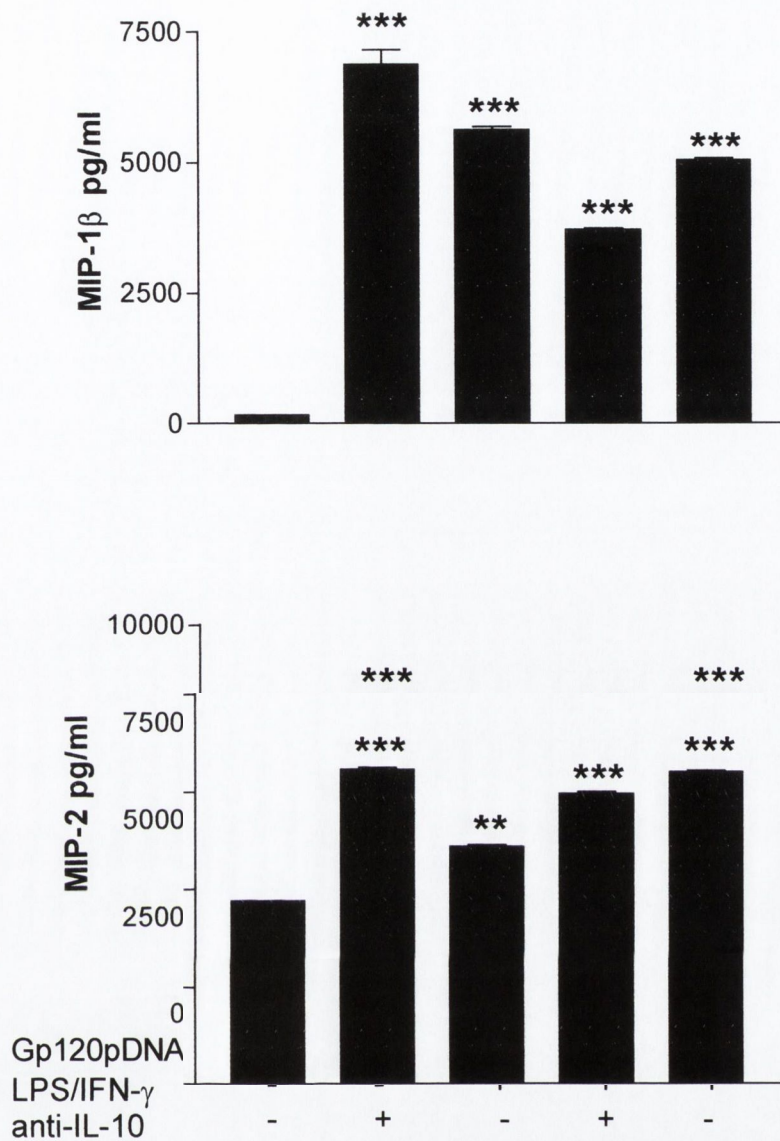


Figure 6.5. Blocking IL-10 can enhance secretion of MIP-1 β and MIP-2 chemokines from BMDC stimulated with gp120pDNA . BMDC were stimulated *in vitro* with 10 μ g gp120pDNA $\pm$ 1 μ g/10ng LPS/IFN γ or 10ng anti-IL-10 antibody. Supernatants were removed after 24 hours and the concentrations of MIP-1 β and MIP-2 were determined by specific immunoassay. ** p<0.01, *** p<0.001, gp120pDNA alone versus other gp120pDNA $\pm$ LPS/IFN- γ $\pm$ anti-IL-10, determined by Student's t-test.

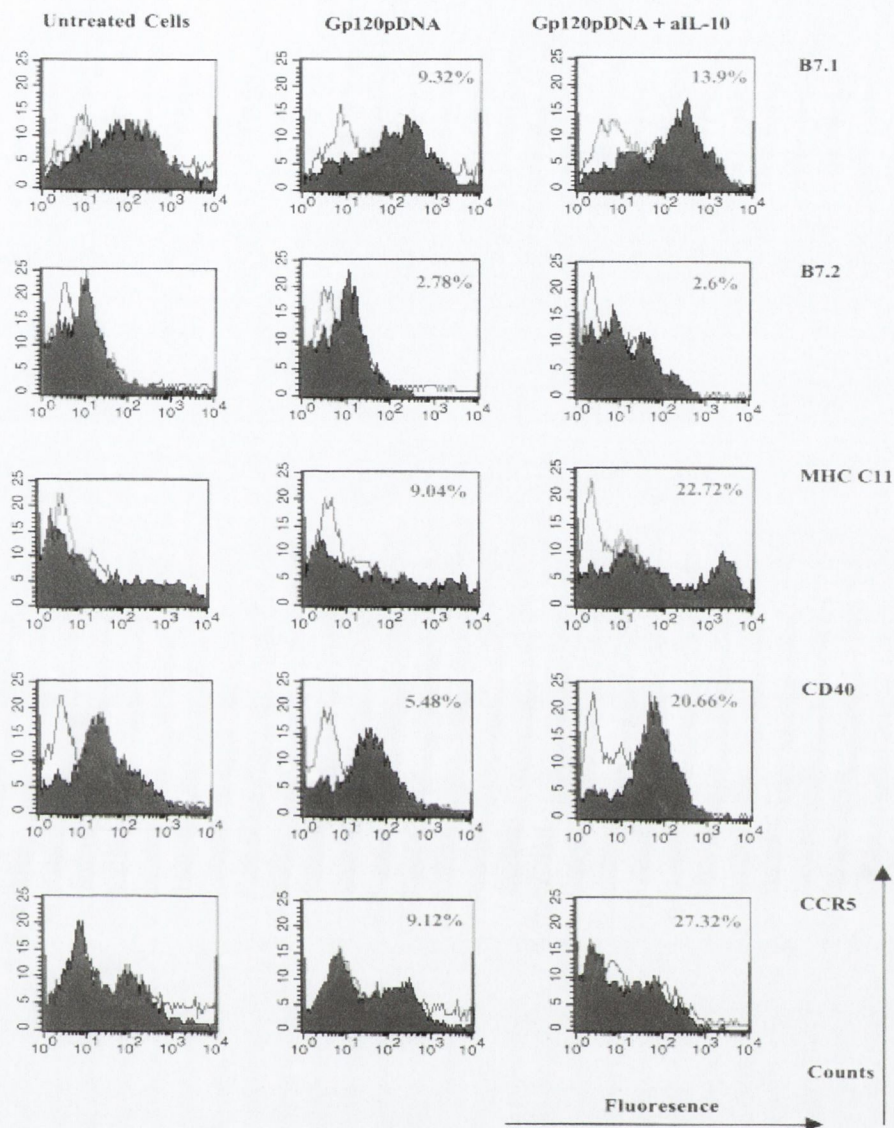


Figure 6.6. Anti-IL-10 enhances maturation of BMDC by gp120pDNA. BMDC were stimulated *in vitro* with 10 μ g gp120pDNA $\pm$ 10ng anti-IL-10 antibody. BMDC cultured in medium alone acted as a negative control. MHC class II, CD40, CCR5, B7.1 and B7.2 surface marker staining (solid) was preformed on CD11c-gated BMDC as described in section 2.6.6. Isotype matched control antibodies were also used to assess non-specific binding (line). % refers to staining compared to untreated BMDC.

CHAPTER 7

ROLE OF DC IN THE INDUCTION OF A TYPE-1 RESPONSE FOLLOWING IMMUNIZATION WITH HAPDNA

7.1 INTRODUCTION

Immunization with DNA vaccines has been shown to induce cell-mediated (Shiver *et al.*, 1997), (Roy *et al.*, 2000) and humoral immune responses (Okuda *et al.*, 1995), (Deck *et al.*, 1997) and protection against challenge with infectious organisms (Johnson *et al.*, 2000), (Webster *et al.*, 1994), (Polack *et al.*, 2000). However, the exact mechanisms involved in the induction of these immune responses remains to be defined.

Antigen processing and presentation following DNA vaccination may occur by at least three possible mechanisms: direct priming of somatic cells (e.g. muscle or skin cells), direct transfection of professional APC (e.g. DC) or cross-priming. Following i.m. immunization with plasmid DNA, muscle cells are predominantly transfected and produce protein through expression of the antigen encoded in the DNA vaccine (Wolff *et al.*, 1990). It has been shown that somatic cells, including muscle cells, can transfer antigen to professional APCs (Ulmer *et al.*, 1996), (Corr *et al.*, 1999), (Akbari *et al.*, 1999), (Fu *et al.*, 1997). Therefore, muscle cells represent non-professional APC that play a role in protein expression and generating antigen for cross-priming (Fu *et al.*, 1997), (Gurunathan *et al.*, 2000). Nevertheless, local inflammation may recruit professional APC following DNA immunization (Tighe *et al.*, 1998). A number of studies have suggested that direct transfection of professional APC may occur following DNA vaccination (Akbari *et al.*, 1999), (Casares *et al.*, 1997). For example, injection into the skin may result in direct transfection of specialized DCs (langerhans cells), that play a key role in the induction of immune responses (Torres *et al.*, 1997).

Several investigations have demonstrated rapid induction of specific immune responses following immunization with plasmid DNA. It has been reported that within 5 minutes of i.m. immunization of mice with a fluorescence-labeled plasmid DNA, muscle cells near the site of injection had taken up the plasmid DNA and expressed the transgene (Dupuis *et al.*, 2000). Furthermore, this research group has demonstrated that a Th1-type response can persist for at least three months following immunization with a plasmid coding for the HA protein expressed by A/Sichuan/87 influenza virus (HApDNA) (Johnson *et al.*, 2000). Similar investigations have shown that several months after i.m. immunization, plasmid DNA could still be detected in the muscle (Wolff *et al.*, 1992), (Danko and Wolff, 1994). Transfected muscle cells may act as antigen reservoirs by continuous expression of the plasmid-encoded

antigen and be responsible for induction and maintenance of the responses following DNA immunization. It has been demonstrated that in the absence of exogenous antigen, memory T cells are generated and persist for at least ten months following immunization with plasmid DNA (Akbari *et al.*, 1999). This memory response was maintained by a small number of transfected DCs that further activated the DC population resulting in repeated T cell activation (Akbari *et al.*, 1999). Therefore, DNA immunization in the absence of further antigenic stimulation can induce specific memory CD4⁺ T cells.

Immunization with DNA vaccines can rapidly induce specific immune responses and these responses have been shown to persist for prolonged periods. To understand the mechanism of action involved in induction of immune responses, the early events following immunization require attention. Previous studies in this laboratory have demonstrated that i.n. infection with A/Sichuan/87 influenza virus and immunization with HApDNA generated type-1 responses and protected against influenza challenge (Johnson *et al.*, 2000). This correlated with the established role of Th1 cells in generating protective immunity against influenza virus infection (Scherle *et al.*, 1992). The present study examined the mechanism of T cell induction following immunization with HApDNA. Secondary lymphoid organs, such as the spleen and lymph nodes, provide conditions necessary for efficient antigen processing as a result of their location, cell composition and ability to provide both cytokine and co-stimulatory signals required for activation of lymphocytes. Therefore, the presence of APCs in cervical lymph node cells from mice immunized i.m. with HApDNA were assessed for the ability to present antigen to a HA-specific Th1-type T cell clone *in vitro*. APCs present in spleen and popliteal lymph node cells were also examined from mice immunized subcutaneously via the footpad. Furthermore, to determine whether DCs were the APCs in the lymphoid organs responsible for induction of immune responses, BMDC were generated and incubated with HApDNA. The capacity of the activated BMDC to present HA antigen to the specific Th1-type T cell clone was examined *in vitro*.

7.2 RESULTS

7.2.1 TYPE-1 CYTOKINE PRODUCTION BY LYMPH NODE AND SPLEEN CELLS FROM MICE IMMUNIZED WITH HApDNA.

It is possible that the induction of Th1 cells following immunization with HApDNA (chapter 5) may have been facilitated through the stimulation of regulatory cytokines from innate cells within draining lymph nodes. To assess if cervical lymph nodes from mice immunized i.m. with HApDNA could produce cytokines associated with type-1 T cell responses (IFN- γ , IL-12 and IL-18), pooled cervical lymph nodes were isolated from mice 4 and 24 hours following immunization. Protein quantities were equalized using a Bio-rad protein assay and the concentrations of type-1 cytokines were assessed by specific immunoassays. Cervical lymph node cells from mice immunized with PBS were used as a negative control. Analysis of results revealed that IFN- γ , IL-12 and IL-18 were produced as early as 4 hours and were maintained for at least 24 hours in mice immunized with 50 μ g HApDNA (Fig 7.1). The levels of IL-12 decreased significantly 24 hours after immunization. As expected, mice immunized with PBS did not produce type-1 cytokines from the cervical lymph node cells.

To assess whether spleen and popliteal lymph nodes from mice s.c. immunized with HApDNA could produce regulator cytokines that enhance the induction of Th1 cells (IL-12 and IL-18), spleens and popliteal lymph nodes were isolated at 4 and 24 hours following subcutaneous/footpad immunization with 50 μ g HApDNA. Protein quantities were equalized in the samples after performing a bio-rad protein assay. Concentrations of IL-12 and IL-18 were then determined by specific immunoassays. Cells from mice immunized with PBS were used as a negative control. The results showed that IL-12 and IL-18 were produced in the lymph node and spleen after 4 hours, and were maintained for at least 24 hours in mice immunized with HApDNA (Fig 7.2). The concentrations of cytokines secreted by popliteal lymph node cells were significantly lower than those produced by spleen cells from HApDNA immunized mice (Fig 7.2). Spleen or popliteal lymph node cells from mice immunized with PBS did not produce detectable cytokines.

7.2.2 ANTIGEN-PRIMED APC IN THE SECONDARY LYMPHOID ORGANS OF MICE IMMUNIZED WITH HApDNA.

To expand these observations, it was then determined whether cervical lymph nodes from mice i.m. immunized with HApDNA contained HApDNA transfected cells or had taken up HA protein from other transfected cells. Irradiated cervical lymph node cells from mice immunized 24 hours earlier with 50 μ g HApDNA were examined for their ability to present antigen to HA-specific T cell clones *in vitro*. Activation of the T cell clone was assessed by IL-12 production in the absence of virus. Irradiated cervical lymph node cells from mice immunized with PBS were used as a negative control. Co-cultured of Th1 clones with cervical lymph node cells from mice i.m. immunized with HApDNA without virus (APC+T), stimulated significantly higher concentrations of IL-12 secretion ($p<0.001$) compared to Th1 clones with lymph node cells from mice immunized with PBS alone (Fig 7.3). In the presence of A/Sichuan/87 influenza virus (APC+T+V), lymph node cells from naïve mice produced high concentrations of IL-12, which represented the positive control. In the absence of the Th1 clone (APC+V) the cervical lymph node cells secreted lower levels of IL-12 (Fig 7.3). Therefore, in the absence of exogenous antigen, mice immunized with HApDNA were capable of activating the Th1 clone. This demonstrates that APC from the cervical lymph nodes were transfected or had taken up antigen from other cells 24 hours after i.m. immunization.

To determine whether spleen and popliteal lymph nodes from mice s.c. immunized with HApDNA contained HApDNA transfected cells or had taken up HA protein from other transfected cells, spleen and popliteal lymph nodes were isolated after 24 hours and examined for the ability to present antigen to HA-specific T cell clones *in vitro*. Activation of the T cell clone was assessed by IFN- γ production from the Th1 clones in the absence of virus. Irradiated spleen and popliteal lymph node cells from mice immunized with PBS were used as a control. In the absence of exogenous antigen (APC+T), spleen cells from mice immunized s.c. with 50 μ g HApDNA stimulated significantly higher concentrations of IFN- γ ($p<0.001$) and IL-12 ($p<0.01$) secretion from the Th1 clone compared to spleen cells from mice immunized with PBS alone (Fig 7.4A). Similarly, popliteal lymph node cells from mice immunized with HApDNA without virus stimulated higher concentrations of IFN- γ and IL-12 ($p<0.01$) production from the Th1 clone compared to popliteal lymph

node cells from PBS control mice immunized with PBS (Fig 7.4B). Following the addition of A/Sichuan/87 influenza virus to the culture (APC+T+V), spleen and popliteal lymph node cells from control mice stimulated the Th1 clone to secrete high concentrations of both IFN- γ and IL-12. These findings suggest that spleen and popliteal lymph node cells are capable of presenting antigen to the T cell clone in the absence of exogenous antigen. In the absence of the Th1 clone (APC+V), the spleen and popliteal lymph node cells secreted low levels of IFN- γ . However, modest levels of IL-12 were produced in response to stimulation with virus, but undetectable cytokines when incubated with medium only (Fig 7.4A and 7.4B). These findings demonstrate that in the absence of exogenous antigen, mice immunized with HApDNA were capable of activating the Th1 clone, which suggests that APC from both spleen and popliteal lymph nodes were either transfected or had taken up antigen from other cells 24 hours after subcutaneous immunization.

7.2.3 ANTIGEN-PRIMED BMDC FOLLOWING STIMULATION WITH PLASMID DNA.

To determine whether DCs could function as APCs for presenting plasmid derived HA to Th1 clones, BMDC were incubated with 10 μ g HApDNA overnight and irradiated BMDC were examined for the ability to present antigen to HA-specific T cell clones *in vitro*. Activation of the T cell clone was assessed by IFN- γ production from the Th1 clones in the absence of exogenous antigen in the form of A/Sichuan/87 influenza virus. Irradiated BMDC incubated with medium alone were used as a negative control (APC).

Significantly higher concentrations of IFN- γ ($p < 0.05$) were secreted by the Th1 clone in response to stimulation with HApDNA treated BMDC (APC+T) compared to untreated BMDC in the absence of A/Sichuan/87 influenza virus (Fig 7.5). Similarly Th1 clones stimulated by HApDNA treated BMDC (APC+T), resulted in the secretion of higher concentrations of IL-12 ($p < 0.05$) and IL-18 ($p < 0.05$) when compared with the untreated BMDC. This cytokine production may be attributed to the BMDC, as T cells do not produce IL-12 and IL-18. Interestingly preincubation of HApDNA enhanced the secretion of IL-12 and IL-18 from BMDC (APC+V) in response to A/Sichuan/87 influenza virus compared to BMDC without exogenous HA antigen. Following the addition of A/Sichuan/87 influenza virus to the culture, HApDNA treated BMDC and untreated BMDC stimulated the Th1 clone (APC+T+V) to secrete high concentrations of cytokines. These findings suggest that APCs were capable of presenting antigen to the T cell clone in the absence of exogenous antigen. Overall these findings suggest that BMDCs were capable of activating the Th1 clone in the absence of exogenous antigen.

7.3 DISCUSSION

The mechanism by which encoded antigen expressed by a plasmid DNA vaccine is processed and presented to the immune system to generate specific immune responses has yet to be fully defined. Subunit or inactivated vaccines contain significantly higher quantities of protein compared to the pg and ng protein concentrations expressed *in vivo* following DNA vaccination. Therefore, the type of APC transfected and/or adjuvant activity of immune enhancing CpG motifs present in bacterial DNA may account for the efficient antigen presentation and T cell activation following immunization with DNA vaccines. To investigate the mechanism of induction of immune responses following immunization with DNA vaccines, this study examined the ability of a plasmid DNA encoding the transmembrane HA protein expressed on the A/Sichuan/87 influenza virus (HApDNA) to transfect APCs and activate type-1 T cells using HA-specific Th1 cell clones. It was demonstrated that mice immunized i.m. and s.c. with HApDNA generated antigen-primed APCs in secondary lymphoid organs with the ability to activate a HA-specific Th1-type T cell clone *in vitro*. Furthermore, the role of DCs as the key professional APC involved in this mechanism was assessed.

Cervical lymph nodes from mice i.m. immunized with HApDNA were shown to contain high concentrations of type-1 cytokines, IFN- γ , IL-12 and IL-18. Differentiation towards a type-1 T cell subtype is predominantly mediated by IL-12 produced by DCs and/or macrophages (Gately *et al.*, 1998). The concentrations of IL-12 were found to be high after 4 hours and these levels decreased significantly by 24 hours following immunization. The immunostimulatory CpG sequences present in the plasmid DNA may be capable of rapidly activating type-1 cytokine production after i.m. immunization with HApDNA. This is consistent with the observation that CpG motifs are capable of acting as adjuvants for type-1 responses through maturation of DC1 and subsequent induction of IL-12 and inflammatory chemokine secretion, both necessary for priming of type-1 T cells (Behboudi *et al.*, 2000).

This study also assessed the ability of APC, present in cervical lymph nodes from mice i.m. immunized with HApDNA, to activate a HA-specific Th1-type T cell clone *in vitro*. It was previously demonstrated by this laboratory that CD4⁺ Th1 cells were induced during natural influenza infection; a H-2^d MHC class II-restricted HA-specific CD4⁺ Th1 clone was established from mice that had recovered from influenza virus infection (Johnson *et al.*, 2000). Further studies demonstrated that antigen-

primed APC, with the capacity to activate IFN- γ production by the HA-specific Th1 T cell clone, were present in the spleen as early as 1 hour post i.m. immunization with HApDNA, but these were undetectable after 24 hours (Conway, M., Johnson, P.J. and Mills, K.H.G. unpublished findings). In contrast, lymph nodes cells from i.m. immunized mice were unable to activate the Th1 T cell clone *in vitro*. This suggests that the main site of induction of immune responses following i.m. immunization with HApDNA may occur in the spleen rather than the local draining lymph nodes. However, the present study has shown that cervical lymph node cells from immunized mice were capable of activating IL-12 production. It is well documented that IL-12 is produced predominantly by both DCs and macrophages (Gately *et al.*, 1998). Studies have shown that DCs are transfected following plasmid DNA immunization and initiate T helper responses in the lymph nodes (Casares *et al.*, 1997), (Akbari *et al.*, 1999), (Porgador *et al.*, 1998). It is possible that transfected muscle cells may provide antigen for cross-priming DCs. These DCs may then localize to local lymph nodes, initiate a type-1 response, recruit more DCs, which then migrate to the spleen and further augment the response.

It has been reported by this research group that a type-1 response can persist for at least three months following immunization with the HApDNA (Johnson *et al.*, 2000). Other investigations have shown that several months after i.m. immunization, plasmid DNA could still be detected in the muscle (Wolff *et al.*, 1992), (Danko and Wolff, 1994). These findings suggest that transfected muscle cells may act as reservoirs for antigen by continuous expression of the encoded antigen and therefore may be partially responsible maintaining an immune response following DNA immunization. However, Corr *et al.* (1996) demonstrated that bone marrow derived APC and not muscle cells mediated immune response induction following DNA immunization. When bone-marrow expressing H-2^b or H-2^d MHC molecule were transferred to H-2^{bxd} mice, the CTL responses following DNA immunization were solely restricted to the MHC haplotype of the donors of the bone marrow.

As the skin contains a higher proportion of professional APC compared to muscles cells, the subcutaneous route of immunization was also assessed. This study demonstrated that spleen and popliteal lymph nodes from mice subcutaneously immunized via the footpad with HApDNA contained high concentrations of type-1 inducing cytokines, IL-12 and IL-18. Furthermore, irradiated spleen and popliteal lymph node cells contained HA-primed APCs that could activate the HA-specific Th1

clone to secrete the type-1 cytokine, IFN- γ , *in vitro*. These findings are consistent with studies that have demonstrated DCs containing plasmid DNA in draining lymph nodes and spleen of mice immunized with plasmids coding for hen egg lysozyme (Bouloc *et al.*, 1999) and the non secreted fifth element of complement protein (Akbari *et al.*, 1999). Furthermore, DCs isolated from draining lymph nodes of skin, from mice s.c. immunized with a plasmid DNA coding for A/PR/8/34 influenza peptide, were found to be transfected with the plasmid and presented encoded peptide to T cells (Casares *et al.*, 1997). Torres *et al.*, (1997) demonstrated that removal of muscle bundles after i.m. injection had no effect on the immune response, however skin biopsy following gene gun immunization abrogated the response completely, indicating that APC such as keratinocytes were directly transfected. Therefore, this suggests that the skin contains specialized DCs that play a key role in the induction of immune responses following DNA immunization.

The source of the type-1 inducing cytokines and identification of the antigen-primed APC responsible for activating the HA-specific Th1 clone is still unclear. However, evidence from *in vitro* studies suggests that DCs may be the primary APC involved in the induction of immune responses following plasmid DNA immunization. Immature DC generated *in vitro* from bone marrow were incubated in the presence of HApDNA, irradiated and found to activate production of the type-1 cytokines, IFN- γ , IL-12 and IL-18. Type-1 T cells secrete IFN- γ (Firestein *et al.*, 1989), whereas DCs and/or macrophages produce IL-12 (Macatonia *et al.*, 1995) and IL-18 (Wesa and Galy, 2001) which synergize and activate differentiation of type-1 T cells (McInnes *et al.*, 2000). It is possible that the activation of the T cell clone *in vitro*, by spleen cells from mice immunized with HApDNA, was due to the presence of HA-primed BMDC. Therefore, the predominant source of type-1 inducing cytokines, IL-12 and IL-18, may be attributed to activated DCs, possibly mediated by TLR9 signaling through CpG motifs present in the plasmid sequences (Hemmi *et al.*, 2000).

Although there are at least three mechanisms by which DNA vaccine immunization can induce specific immune responses, these studies have provided evidence that a combination of cross-priming by muscle cells and directly transfected DCs may contribute to the efficiency of antigen presentation and the generation of immune responses with DNA vaccines.

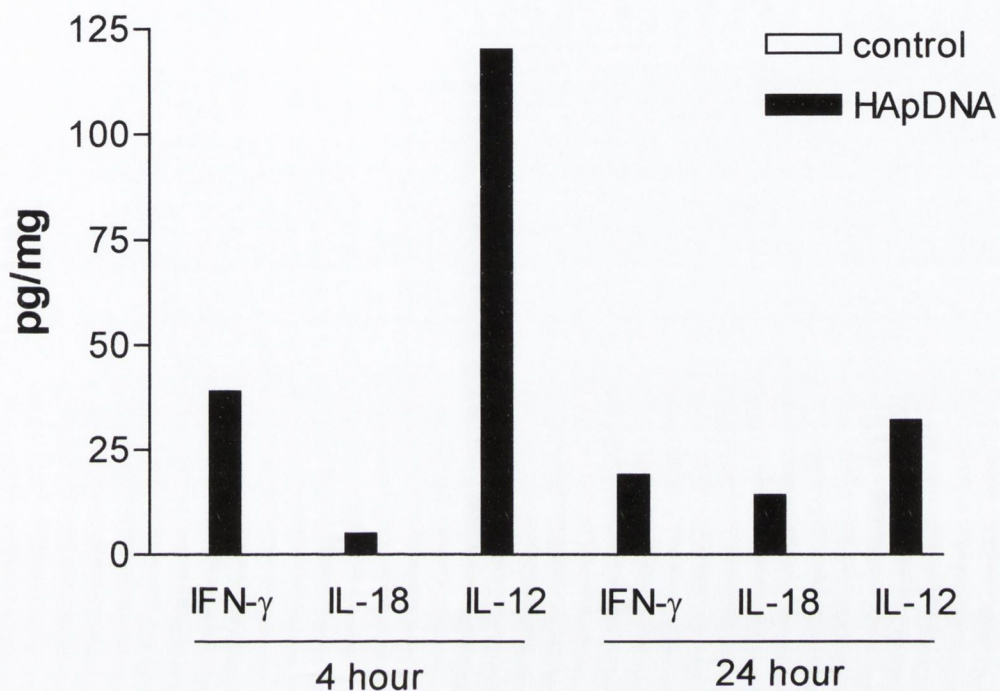


Figure 7.1. Intramuscular immunization with HApDNA induces cytokine production in the local cervical draining lymph node. BALB/c mice were immunized i.m with 50 μ g HApDNA or PBS. Cervical lymph nodes were isolated at 4 and 24 hours post immunization, homogenised and protein concentrations were normalized after performing a Bio-rad protein assay. Concentrations of IFN- γ , IL-18 and IL-12 were determined by specific immunoassay.

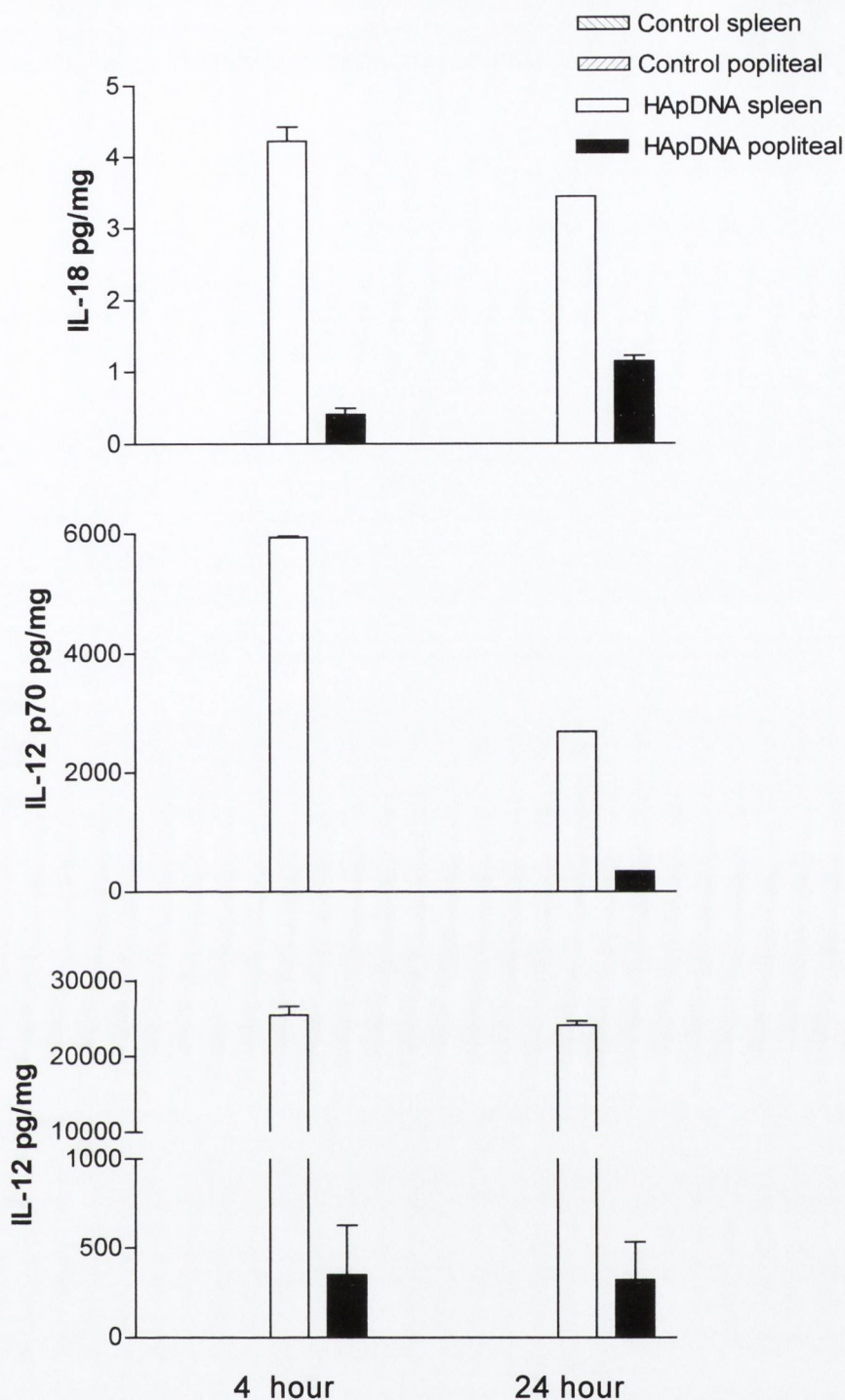


Figure 7.2. Subcutaneous immunization with HApDNA induces IL-12 and IL-18 production in the spleen and local popliteal draining lymph node. BALB/c mice were subcutaneously immunized into the footpad with 50 μ g HApDNA or PBS. Spleens and popliteal lymph nodes were isolated at 4 and 24 hours post immunization and homogenised. Following protein equalization, concentrations of IL-18, IL-12p70 and IL-12p40 were determined by specific immunoassay.

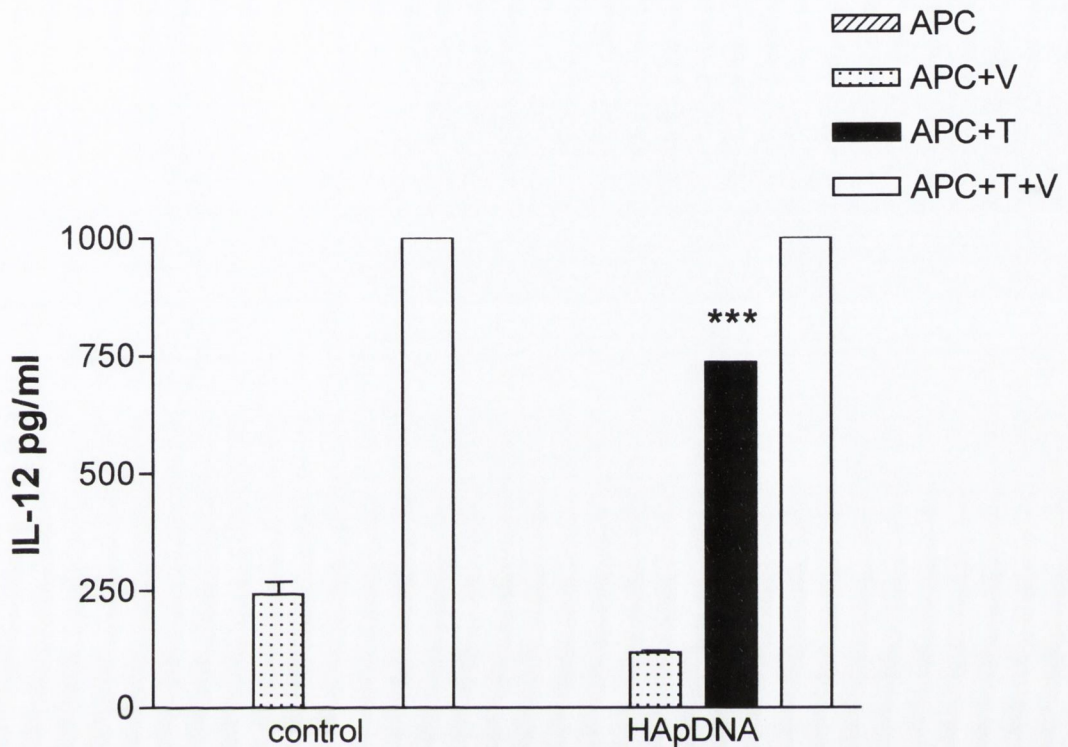


Figure 7.3. Activation of T cell clones by APC from cervical lymph nodes of mice immunized i.m. with HApDNA. Mice were immunized with 50 μ g HApDNA or PBS. Cervical lymph nodes were isolated after 24 hours and cultured alone (APC), with virus only (APC+ V), or with HA-specific T cell clones in the presence (APC+T+V) or absence of A/Sichuan/87 virus (APC+T). IL-12p40 production was assessed three days later by specific immunoassay. *** $p < 0.001$, HApDNA (APC+T) versus control (APC+T) determined by Student's t-test.

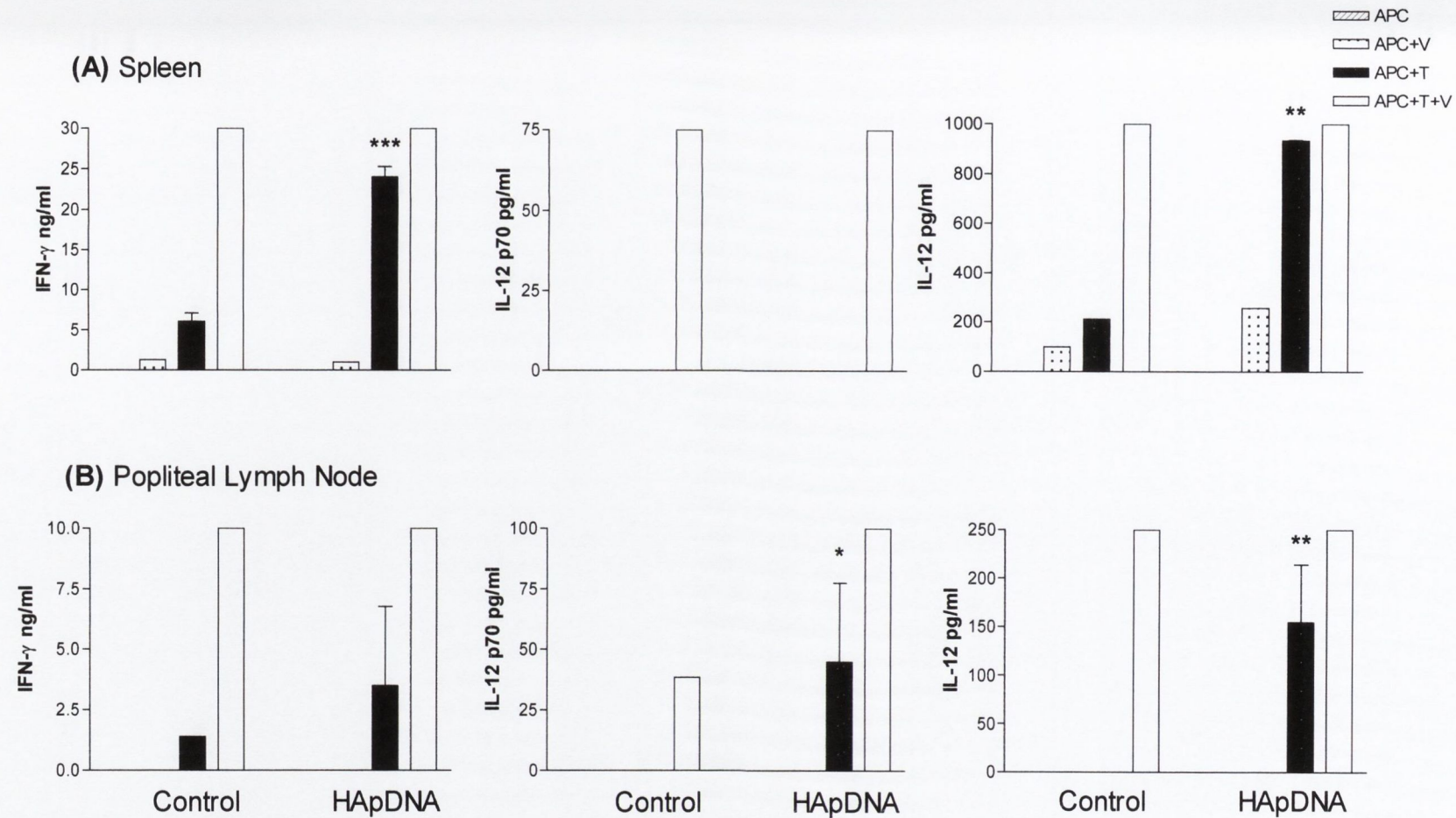


Figure 7.4. Activation of T cell clones by APC from spleen and local popliteal lymph node cells of mice subcutaneously immunized with HApDNA. Mice were immunized with 50 μ g HApDNA or PBS. Spleens and local popliteal lymph nodes were isolated after 24 hours, irradiated and cultured alone (APC), with virus only (APC+ V), or with HA-specific T cell clones in the presence (APC+T+V) or absence of A/Sichuan/87 virus (APC+T). IFN- γ and IL-12 production was assessed three days later by specific immunoassay. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, HApDNA (APC+T) versus control (APC+T) was determined by Student's t-test.

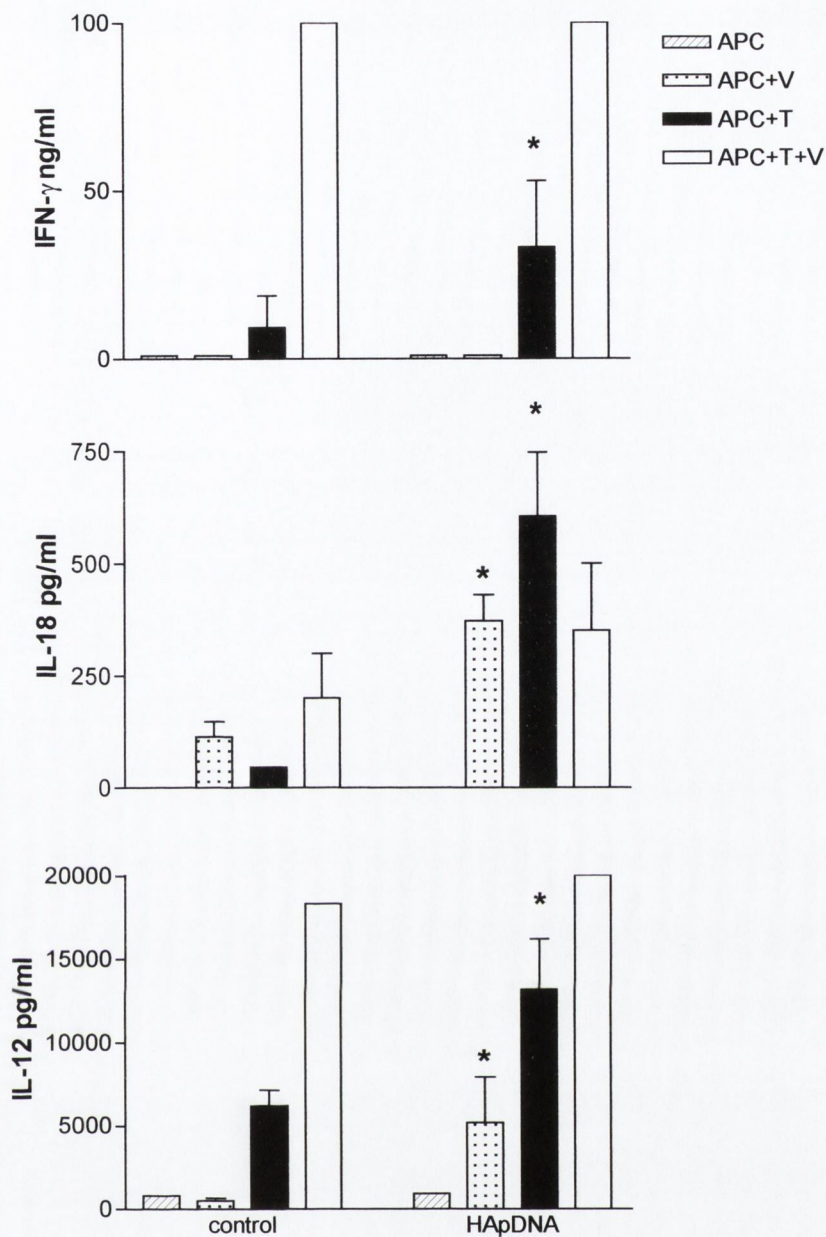


Figure 7.5. Activation of T cell clones by BMDC stimulated with HApDNA. BMDC were incubated with 10 μ g HApDNA or medium alone as a control. After 24 hours, 1 $\times$ 10⁴ BMDC were washed and cultured alone (APC), with virus (APC+ V), or with HA-specific T cell clones in the presence (APC+T+V) or absence of A/Sichuan/87 virus (APC+T). IFN- γ , IL-18 and IL-12p40 production was assessed three days later by specific immunoassay. * $p < 0.05$, HApDNA (ACP+T) versus control (APC+T) and HApDNA (ACP+V) versus control (APC+V) determined by Student's t-test.

CHAPTER 8

GENERAL DISCUSSION

8.1 GENERAL DISCUSSION

In the 21st century infectious diseases continue to represent the major cause of death in humans worldwide. Despite the discovery of penicillin in the 1940s by Alexander Fleming, which proved successful against many bacterial infections, antibiotic resistant strains of bacteria evolved, underlying the requirement to develop other means of controlling infectious microorganisms. Jenner and Pasteur, the pioneers of vaccination, exploited attenuated or killed microorganisms to generate protective immunity against several pathogens. By the 20th century this discovery had led to the control of many infectious diseases, including the eradication of smallpox (1980) and reduced incidence of diphtheria, polio, rabies, tuberculosis and pertussis (Makela, 2000). Vaccines have prevented more deaths and suffering than any other medical intervention. However, the emergence of new pathogens, such as HIV, has emphasized the requirement for ongoing development and improvement of current vaccination strategies.

Concerns regarding safety associated with killed and attenuated vaccines and poor immunogenicity of subunit vaccines provoked the development of safer immunogenic vaccines based on other technologies. Immunological and genomic innovations have allowed the identification of protective mechanisms against infectious pathogens permitting a more rational approach to vaccine development. The discovery of DNA vaccines has the potential to revolutionize vaccination and is based on the injection of plasmid DNA coding for the protective antigenic protein of the infectious microorganism (Wolff *et al.*, 1990), (Tang *et al.*, 1992), (Gurunathan *et al.*, 2000). Immunization with plasmid DNA encoding microbial antigens can induce immune responses against antigens from infectious pathogens (Fynan *et al.*, 1993), (Johnson *et al.*, 2000), (Huang *et al.*, 2000). In this study, factors that influence the induction of type-1 and type-2 responses following immunization with DNA vaccines were examined using the HIV gp120 surface envelope protein and the HA glycoprotein of influenza virus as model antigens.

It has been demonstrated that subunit vaccines based on recombinant proteins absorbed to alum can be poorly immunogenic, especially for cellular immune responses. In contrast, evidence from DNA vaccines suggests that plasmid DNA encoding antigen can induce potent cellular immune responses. A limited number of studies have directly compared DNA versus protein vaccines, therefore this study examined cellular and humoral immunity generated with gp120pDNA and the encoded gp120 protein. Continuous release of low dose antigen is thought to influence the differentiation of T cells towards a type-2 response (Guery *et al.*, 1996), (Rask *et al.*, 2000). The present study demonstrated that i.m. immunization with gp120 protein absorbed to alum generated a polarized Th2 response, whereas gp120pDNA induced a mixed Th1/Th2 type response. These findings are consistent with potent Th2 responses previously reported with protein antigens absorbed to alum (Moore *et al.*, 1995), (Conway *et al.*, 2000). The differences in the responses generated with gp120 protein and gp120pDNA may be explained by the adjuvant properties of bacterial DNA which contain immunostimulatory CpG motifs (Sato *et al.*, 1996), (Yi *et al.*, 1996) present in DNA vaccines which promote type-1 responses mediated by IL-12 secreted by CpG-activated APCs (Bauer *et al.*, 1999), (Walker *et al.*, 1999).

Compared to previous reports, the present study has focused on the potential of DNA vaccination rather than protein-alum vaccine strategies. Induction of immune responses following DNA vaccination can be influenced by a number of factors, including the composition of the bacterial plasmid backbone, nature of encoded antigen, frequency of CpG motifs, dose, route of administration and immunization schedule. These factors can also be exploited to enhance the immunogenicity of DNA vaccines. DNA vaccines have been promoted as vaccines against intracellular infections as they potentiate the development of Th1 cells. Although cell mediated and humoral immune responses have been extensively studied during HIV infection, the exact correlates of protection still require attention. However, there is some evidence to suggest that a type-1 response consisting of CTL CD8⁺ T cells (Schmitz *et al.*, 1999), (Parren *et al.*, 1999) and Th1 CD4⁺ T cells (Pitcher *et al.*, 1999) may play a role in protective immunity to HIV. This study examined approaches for enhancing type-1 responses to gp120pDNA.

Recombinant proteins have been used to boost immune responses primed with plasmid DNA. This prime/boost immunization schedule has been shown to enhance

antibody production, which can often be used with naked DNA (Barnett *et al.*, 1997). Therefore, this study analyzed a prime/boost immunization schedule for its potential to enhance responses with gp120pDNA. Priming with gp120pDNA followed by a gp120 protein boost augmented the type-1 response with enhanced gp120-specific IFN- γ and a concomitant reduction in IL-5. Furthermore, evaluation of antibody responses revealed an increase in both gp120-specific IgG1 and IgG2a titres. These results correspond to findings that protein immunization induces predominantly IgG1, whereas DNA or DNA prime/protein boost induces strong IgG2a responses (Tanghe *et al.*, 2001), (Haddad *et al.*, 1999). Although this immunization schedule appears to be highly effective for enhancing humoral and type-1 responses against the gp120 protein, the mechanism remains unclear. It is possible that the gp120 protein boost may selectively expand the pre-existing gp120-specific type-1 T cell population induced by priming with plasmid DNA (Cuisinier *et al.*, 1999). Comparable prime/boost immunization schedules have been used to improve the immunogenicity of HIV vaccine approaches in non-human primates; macaques primed with a HIV-1 DNA vaccine and boosted with recombinant fowlpox virus generated strong antigen-specific T helper and CTL responses (Kent *et al.*, 1998). Taken together these findings suggest that a DNA prime with a protein boost may represent an ideal approach for enhancing cell-mediated immunity to HIV-1 with DNA vaccines.

DNA vaccination also represents a novel means of introducing genes for immunomodulatory molecules that can modify and selectively augment distinct arms of the immune system (Kim *et al.*, 1998), (Gurunathan *et al.*, 2000). Co-administration of plasmids encoding genes for immunoregulatory cytokines can modulate the induction of Th1 or Th2 cells. This study demonstrated that co-administration of a plasmid DNA encoding the type-1 promoting cytokine, IL-12, with gp120pDNA enhanced antigen-specific IFN- γ production with a concurrent decrease in IL-5 production compared to immunization with gp120pDNA alone. These results are consistent with studies on herpes simplex virus type-2 (HSV-2) DNA vaccine where co-delivery of IL-12pDNA significantly augmented IFN- γ , type-1 chemokine secretion and inhibited antigen-specific antibody and Th2 cytokine production (Sin *et al.*, 1999). The current co-immunization study revealed a slight reduction in serum anti-gp120 IgG antibody titres, but enhanced murine IgG2a. These

findings are consistent with a report by Toda *et al.*, (1997) who demonstrated that addition of anti-IL-12 or anti-IFN- γ enhanced antibody production.

Liposomes are bilayered membrane vesicles that function as effective antigen delivery systems and possess certain immunoadjuvant qualities. They have been shown to protect DNA from degradation *in vivo* and possess structural versatility to improve cell transfection (Gregoriadis, 1998). The strength of immune response induced with DNA immunization is partially dependent on the quantity of injected plasmid DNA. However, it would be beneficial from a safety point of view, to administer lower quantities of plasmid and still achieve effective immune responses. Mice immunized with high doses of gp120pDNA induced a mixed Th1/Th2 type response. At lower doses gp120-specific IL-5 production was dominant, although this may be attributed to reduced CpG motifs frequencies as it has been suggested that persistent gp120 antigen expression may trigger induction of predominant type-2 responses (Mellado *et al.*, 1998). The results of this study demonstrate that gp120pDNA enhances IL-10 and suppresses IL-12 production *in vitro*.

Co-administration of DMRIE-c enhanced the response to lower doses of gp120pDNA which was comparable to those induced with higher doses of gp120pDNA alone. Ishii *et al.*, (1997) also demonstrated that low dose plasmid DNA encoding HIV antigens with cationic liposomes significantly enhance antibodies against the V3 peptide over that generated by administration with naked plasmid DNA. Consistent with these findings the present study showed that anti-gp120 IgG2a was also augmented in the presence of DMRIE-c. The improved immune responses with co-administered liposomes may be a result of increased cell transfection efficiency leading to higher concentrations of plasmid DNA reaching the nucleus of the cell and ultimately increased expression of the encoded antigen. Co-administration of IL-12pDNA with gp120pDNA and DMRIE-c, to generate a three-component vaccine, further enhanced gp120-specific IFN- γ and reduce IL-5 production, indicating an augmented type-1 response. Others have demonstrated significant HIV-specific CTL responses following i.n. administration of a similar vaccine combination; *env* gp160 and *rev* coding HIV DNA vaccine, liposomes and IL-12pDNA (Okada *et al.*, 1997). Taken together these studies show that IL-12pDNA can enhance type-1 responses against gp120pDNA, which can be further augmented by cationic liposomes.

Novel delivery systems and formulations are often required for mucosally delivered DNA vaccines, which pose many obstacles to induction of efficient immune responses. The present study attempted to enhance the immune response generated by gp120pDNA at mucosal surfaces by co-administering DMRIE-c. Nasal administration of gp120pDNA was poorly immunogenic and was not augmented by the co-delivery of DMRIE-c. Blockage by nasal mucous layers (Harokopakis *et al.*, 1998) or nasal hair (Heritage *et al.*, 1998) may have prevented initiation of immune responses. However, local and systemic antibodies were augmented following i.n. immunization of luciferase encoding plasmid when formulated into DMRIE/DOPE liposome complexes (Klavinskis *et al.*, 1999). Therefore, in certain systems, liposomes may facilitate increased antigen expression within mucosal inductive sites and local lymph nodes.

DNA vaccine technology has considerable potential for the development of mutli-component vaccines; several foreign antigens can be encoded by the same plasmid or by distinct plasmids combined into a single vaccine formulation. An important consideration is the effect that one vaccine may impose on the immune response generated by a co-administered vaccine. This study demonstrated that co-administration of gp120pDNA and HApDNA increased gp120-specific IFN- γ with a concomitant decline in HA-specific IL-5 production from spleen cells of immunized mice. Induction of type-1 response was not adversely affected by the presence of plasmids. The findings with this combination vaccine are consistent with those of Braun *et al.*, (1998) who reported that responses generated by individual plasmids were not adversely influenced following co-administration of four individual plasmids encoding discrete viral glycoproteins. Furthermore, poorly immunogenic plasmids can benefit from mutli-plasmid vaccine combinations and this can be attributed to additional plasmids exposure of the innate immune system to CpG or other immunogenic stimulatory sequences in bacterial DNA (Grifantini *et al.*, 1998).

The nature of the encoded antigen (Feltquate, 1998), (Haddad *et al.*, 1997), (Cardosa, 1998) and immunostimulatory CpG motifs present in the bacterial plasmid backbone (Sato *et al.*, 1996), (Herve *et al.*, 2001) can influence the immune response following immunization with DNA vaccines. Several studies that have investigated antigen type have focused on antibody induction and have reported that secreted antigen predominantly induces the murine IgG1 subclass, whereas cytosolic antigen generates a mixed IgG1/IgG2a response in mice (Haddad *et al.*, 1998). In the present

study, gp120pDNA induced dominant IgG1 antibodies, whereas HApDNA induced mainly IgG2a. However, the effect of antigen type on cell-mediated responses are less well established. This study showed that a plasmid coding for a secreted (gp120) induced a mixed Th1/Th2 type response, whereas the membrane bound (HA) antigen induced a polarized Th1 response. This may be partially explained by the nature of the antigen encoded and the subsequent mechanism of antigen presentation. The major contribution to the immunogenicity of DNA vaccines is immunostimulatory CpG motifs present in bacterial plasmid DNA (Sato *et al.*, 1996), (Klinman *et al.*, 1997). One problem with a direct comparison between gp120pDNA and HApDNA was the use of distinct expression plasmids. Therefore, gp120 was inserted into the expression plasmid for HApDNA. The plasmid backbone influenced the type of immune response with the plasmid PI.18 favoring the induction of type-1 T cell responses. However, IgG1 antibodies were predominantly induced by the encoded gp120 antigen regardless of the plasmid backbone. This study demonstrates that the encoded antigen can also influence the induction of immune responses. *In vitro* studies with DC demonstrate that gp120pDNA enhanced IL-10 production, whereas HApDNA did not. Furthermore, the strong influence of the expression plasmid on both antibody and T cell responses emphasizes the importance of selecting an appropriate plasmid backbone depending on the target pathogen.

CpG-ODN have been shown to possess adjuvant properties that promote the induction of systemic Th1 responses (Klinman *et al.*, 1999) following parenteral (Putney and Burke, 1998) or mucosal administration of protein antigens (McCluskie *et al.*, 2000). Co-administration of expression plasmids devoid of encoded antigens with gp120pDNA revealed moderate enhancement of gp120-specific Th1 type responses. Interestingly one expression plasmid, PI.19, also augmented gp120-specific IL-5 production from local lymph nodes. This suggests that an unidentified component of bacterial plasmids may also promote Th2 type responses. Another study has recently demonstrated comparable findings; administration of CpG-ODN as an oral adjuvant with hepatitis B surface antigen or tetanus toxoid enhanced both Th1 and Th2 type responses (McCluskie *et al.*, 2000).

Understanding the mechanism involved in the induction of immune responses is of paramount importance for rational vaccine design. Initiation of responses against pathogens is dependant on activating cells of the innate immune system including those involved in antigen presentation, which in turn direct the induction of T cells of the acquired immune response. Regulatory cytokines produced by innate cells play a major role in activation and suppression of the immune system following antigenic encounter by influencing DC1 and DC2 maturation and subsequent differentiation of T cells towards a specific T cell subtype (Kourilsky and Truffa-Bachi, 2001), (Gajewski, *et al.* 1991).

Elimination of intracellular pathogens in naïve or vaccinated individuals is often associated with Th1 type responses. As IL-12 favors the differentiation of T cells towards a type-1 phenotype (Seder *et al.*, 1993), (Bellinghausen *et al.*, 1999), (Gately *et al.*, 1998), and IL-4 favors Th2 development (Kopf *et al.*, 1993), these immunoregulatory cytokines are likely to play key roles in the development of immune responses following immunization with DNA vaccines. The induction of IL-12 production and development of type-1 responses are also influenced IL-10 (Jeannin *et al.*, 1996). It has been recently been shown that IL-10 promotes T regulatory cell induction (Mc Guirk *et al.*, manuscript in preparation). Studies in IL-10^{-/-} mice demonstrated that IL-10 plays a major role in controlling IL-12 production from DCs (Huang *et al.*, 2001). Mice deficient in key regulatory cytokines may indirectly influence B cell subclass induction (Finkleman *et al.*, 1990). This study used knockout mice deficient in key immunoregulatory cytokines, IL-12, IL-4 and IL-10, to provide evidence for their role in the induction of distinct T cell subtypes following immunization with both gp120pDNA and HApDNA. Wild type mice immunized with gp120pDNA produced a mixed Th1/Th2 response compared to a polarized Th1 response with HApDNA. However, IL-12^{-/-} mice immunized with both gp120pDNA and HApDNA had reduced IFN- γ production. These mice also developed weaker IgG2a and higher IgG1 antibody responses compared with wild-type mice. These results are in agreement with findings from other studies that demonstrated a shift from a polarized Th1 to Th2 response in certain systems where IL-12 was absent (Seder *et al.*, 1993). Likewise, IL-10 deficiency facilitated a significant improvement in type-1 IFN- γ production. Neutralization studies in other systems have also reported a role for IL-10 in regulating enhancement of IFN- γ

responses (Huang *et al.*, 1999). Production of the Th2 cytokine, IL-5, from gp120pDNA-immunized mice was more adversely affected by the absence of IL-10 than IL-4. This suggests that IL-10 may play a more crucial role in the induction of type-2 responses following immunization with gp120pDNA. The absence of IL-4 adversely affected the ability of B cells to produce the IgG1. Antibody production in IL-10^{-/-} mice was partially reduced, but did not exhibit the dramatic pattern observed in IL-4^{-/-} mice. Taking together these observations suggest that the balance between IL-12 and IL-10 during initiation of T cells responses is more crucial than the presence of IL-4. However IL-4 and IL-12 appear to play a role in mediating induction of IgG subclasses following DNA immunization. Therefore, the balance of immunoregulatory cytokines following immunization with plasmid DNA is important in directing the differentiation of T cells and antibody responses and appears to be independent of antigen-type.

As discussed above, the immune response induced by DNA immunization in mice is altered in the absence of key immunoregulatory cytokines. However, viruses have evolved evasion mechanisms to subvert the immune response (Marie *et al.*, 2001), (Bender *et al.*, 1997/98), by inducing cytokines such as IL-10 that can suppress protective Th1 responses. HIV may induce IL-10 to influence activation of immature DCs and subsequently disrupt induction of effective immune responses. Gp120 encoded by gp120pDNA was shown to stimulate DCs to secrete IL-10. Further examination revealed that gp120 protein encoded by distinct plasmid backbones stimulated IL-12, MIP-1 β and MIP-2 secretion and maturation of BMDC, which were significantly improved by blocking IL-10 with anti-IL-10 antibodies. Others have observed similar effects on IL-12 (Huang *et al.*, 2001), chemokines (D'Amico *et al.*, 2000) and DC maturation (Corinti *et al.*, 2001) by blocking IL-10. Filamentous haemagglutinin a major virulence factor of *Bordetella pertussis* has been shown to induce IL-10 production by DC and to suppress IL-12 and Th1 responses (McGuirk and Mills, 2000). Therefore, the ability of gp120 protein encoded by plasmid DNA, to modulate DC maturation, chemokine and cytokine production by gp120pDNA may reflect a role evolved by gp120 protein to subvert immune responses during HIV infection.

The influence of immunoregulatory cytokines has been established in this study using gp120pDNA and HApDNA, however the exact APC type and the mechanisms involved in the induction of T cell responses will require further study. It

has been proposed that antigen processing and presentation following DNA vaccination may occur by three possible mechanisms including both direct and indirect priming of APCs. Current opinion suggests that DCs represent the key APC involved in inducing immune responses following DNA immunization. Transfected muscle cells can express proteins encoded by the DNA vaccine and may also generate antigen for cross priming (Fu *et al.*, 1997), (Gurunathan *et al.*, 2000). DNA vaccines contain CpG motifs that function as adjuvants for type-1 responses; this is mediated through enhancement of DC1 maturation and subsequent secretion of IL-12 and inflammatory chemokines, which are required for differentiation of Th1 cells (Behboudi *et al.*, 2000). IL-12 is predominantly produced by both DCs and macrophages (Gately *et al.*, 1998) and DCs have been demonstrated as being the primary cell transfected following immunization with plasmid DNA, leading to initiation of T helper responses in the lymph nodes (Casares *et al.*, 1997), (Akbari *et al.*, 1999), (Porgador *et al.*, 1998). Therefore, BMDC were evaluated as the possible source of these immunoregulatory cytokines following immunization with HApDNA. The study demonstrated that mice immunized with HApDNA produced IL-12, IL-18 and IFN- γ in secondary lymphoid organs. Furthermore, BMDC stimulated *in vitro* with HApDNA activated the HA-specific Th1 clone to secrete type-1 cytokines. Collectively these findings point to a role for DCs as the principal source of regulatory cytokines that enhance Th1 responses with DNA vaccines. Secretion of these cytokines from activated BMDC in response to plasmid DNA probably result from TLR9 signaling through CpG motifs present in the plasmid backbone (Hemmi *et al.*, 2000). However, it remains unclear whether the DCs are directly transfected or if the muscle could act as a source of antigen for DCs. This area would require further investigation to establish the full role of muscle cells and DC in the initiation of immune responses.

In conclusion, this project has identified several factors that influence the immunogenicity of DNA vaccines. Understanding the dynamics of this technology will allow a more comprehensive appreciation of how these exciting vaccines can express their full potential.

CHAPTER 9

REFERENCES

- Aihara, H., and J. Miyazaki. 1998. Gene transfer into muscle by electroporation in vivo. *Nature Biotechnology* 16:867.
- Akbari, O., N. Panjwani, S. Garcia, R. Tascon, D. Lowrie, and B. Stockinger. 1999. DNA vaccination: transfection and activation of dendritic cells as key events for immunity. *J. Exp. Med.* 189:169.
- Akiba, H., Y. Miyahira, M. Atsuta, K. Takeda, C. Nohara, T. Futagawa, H. Matsuda, and K. Okumura. 2000. Critical contribution of OX40 ligand to helper cell type 2 differentiation in experimental leishmaniasis. *J. Exp. Med.* 191:375.
- Al-Mariri, A., A. Tibor, M. P., X. De Bolle, P. Michel, J. Godefroid, K. Walravens, and J. J. Letesson. 2001. Protection of BALB/c mice against *Brucella abortus* 544 challenge by vaccination with bacterioferritin or P39 recombinant proteins with CpG oligodeoxynucleotides as adjuvant. *Infect. Immun.* 69:4816.
- Almeida, A. J., and H. O. Alpar. 1996. Nasal Delivery of Vaccines. *Journal of Drug Targeting* 3:455.
- Altfeld, M., and E. S. Rosenberg. 2000. The role of CD4+ T helper cells in the cytotoxic T lymphocyte response to HIV-1. *Curr. Opin. Immun.* 12:375.
- Amerongen, H. M., R. Weltzin, C. M. Farnet, P. Michetti, W. A. Haseltine, and M. R. Neutra. 1991. Transepithelial transport of HIV-1 by intestinal M cells: A mechanism for transmission of AIDS. *J. Acquir. Immune Defic. Synd.* 4:760.
- Asakura, Y., P. Lundholm, A. Kjerrstrom, R. Benthin, E. Lucht, J. Fukushima, S. Schwartz, K. Okuda, B. Wahren, and J. Hinkula. 1999. DNA-plasmids of HIV-1 induce systemic and mucosal immune responses. *Biol. Chem.* 380:375.
- Ayash-Rashkovsky, M., Z. Weisman, S. Zlotnikov, E. Raz, Z. Bentwich, and G. Borkow. 2001. Induction of antigen-specific Th1-biased immune responses by plasmid DNA in schistosoma-infected mice with a preexistent dominant Th2 immune profile. *Biochem. Biophys. Res. Commun.* 282:1169.

Baca-Estrada, M. E., M. Foldvari, M. Snider, K. Harding, B. Kournikakis, L. Babiuk, and P. Griebel. 2000. Intranasal immunization with liposome-formulated *Yersinia pestis* vaccine enhances mucosal immune responses. *Vaccine* 18:2203.

Badley, A., J. McElhinny, P. Leibson, D. Lynch, M. Alderson, and C. Paya. 1994. Upregulation of Fas ligand expression by human immunodeficiency virus in human macrophages mediates apoptosis of uninfected T lymphocytes. *J. Virol.* 70:199.

Bagarezzi, M. L. e. a. 1999. Systemic and mucosal immunity is elicited after both intramuscular and intravaginal delivery of human immunodeficiency virus type 1 DNA plasmid vaccines to pregnant chimpanzees. *J. Infect. Dis.* 180:1351.

Ban, E. M., F. W. van Ginkel, J. W. Simecka, H. Kiyono, H. L. Robinson, and J. R. McGhee. 1997. Mucosal immunization with DNA encoding influenza hemagglutinin. *Vaccine* 15:811.

Bancroft, A. J., K. J. Else, J. P. Sypek, and R. K. Grencis. 1997. Interleukin-12 promotes a chronic intestinal nematode infection. *Eur. J. Immunol.* 27:866.

Barackman, J. D., G. Ott, and D. T. O'Hagan. 1999. Intranasal immunization of mice with influenza vaccine in combination with the adjuvant LT-R72 induces potent mucosal and serum immunity which is stronger than that with traditional intramuscular immunization. *Infect.Immun.* 67:4276.

Barnard, A., B. Mahon , J. Watkins, K. Redhead, and K. Mills. 1996. Th1/Th2 cell dichotomy in acquired immunity to *Bordetella pertussis*: variables in the *in vivo* priming and *in vitro* cytokine detection techniques affect the classification of T-cell subsets as Th1, Th2 or Th0. *Immunology* 87:372.

Barnett, S. W., S. Rajasekar, H. Legg, B. Doe, D. H. Fuller, J. R. Haynes, C. M. Walker, and K. S. Steimer. 1997. Vaccination with HIV-1 gp120 DNA induces immune responses that are boosted by a recombinant gp120 protein subunit. *Vaccine* 15:869.

Barnfield, C., L. Gao, S. Parker, R. Ward, and L. S. Klavinskis. 1997. Characterization of gene expression following intranasal immunization with nucleic acid. *Biochem. Soc. Trans.* 25:335S.

Barry, M. A., and S. A. Johnston. 1997. Biological features of genetic immunization. *Vaccine* 15:788.

Bauer, M., K. Heeg, H. Wagner, and G. B. Lipford. 1999. DNA activates human immune cells through a CpG sequence-dependent manner. *Immunology* 97:699.

Bauer, S., C. J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, and G. B. Lipford. 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc. Natl. Acad. Sci. U S A.* 98:9237.

Behboudi, S., D. Chao, P. Klenerman, and J. Austyn. 2000. The effects of DNA containing CpG motif on dendritic cells. *Immunology* 99:361.

Bellinghausen, I., U. Brand, A. H. Enk, J. Knop, and J. Saloga. 1999. Signals involved in the early TH1/TH2 polarization of an immune response depending on the type of antigen. *J. Allergy. Clin. Immunol.* 103:298.

Bendelac, A., and D. T. Fearon. 1997. Innate Immunity: Innate pathways that control acquired immunity. *Curr. Opin. Immun.* 9:1.

Bendelac, A., and D. T. Fearon. 2000. Innate Immunity: Receptors and effectors of innate immunity. *Curr. Opin. Immun.* 12:11.

Bender, A., M. Albert, A. Reddy, M. Feldman, B. Sauter, G. Kalpan, W. Hellman, and N. Bhardwaj. 1997/98. The distinctive features of influenza virus infection of dendritic cells. *Immunobiology* 198:552.

Berman, P. W., T. J. Gregory, L. Riddle, G. R. Nakamura, M. A. Champe, J. P. Porter, F. M. Wurm, R. D. Hershsberg, E. K. Cobb, and J. W. Eichberg. 1990.

- Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant gp120 but not gp160. *Nature* 345:622.
- Beutler, B. 2000. Tlr4: central component of the sole mammalian LPS sensor. *Curr. Opin. Immunol.* 12:20.
- Biron, C. A., and L. Brossay. 2001. NK cells and NKT cells in innate defense against viral infections. *Curr. Opin. Immunol.* 13:458.
- Blazevic, V., C. M. Trubey, and G. M. Shearer. 2000. Comparison of *in vitro* immunostimulatory potential of live and inactivated influenza viruses. *Human Immunology* 61:845.
- Bogdan, C., M. Rollinghoff, and A. Diefenbach. 2000. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr. Opin. Immunol.* 12:64.
- Bohle, B., B. Jahn-Schmid, D. Maurer, D. Kraft, and C. Ebner. 1999. Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN- γ production in cells from allergic individuals and inhibit IgE synthesis *in vitro*. *Eur. J. Immunol.* 29:2344.
- Bohm, W., T. Mertens, R. Schirmbeck, and J. Reimann. 1998. Routes of plasmid DNA vaccination that prime murine humoral and cellular immune responses. *Vaccine* 16:949.
- Borghi, P., L. Fantuzzi, B. Varano, S. Gessani, P. Puddu, L. Conti, M. R. Capobianchi, F. Amelio, and F. Belardelli. 1995. Induction of interleukin-10 by human immunodeficiency virus type 1 and its gp120 protein in human monocytes/macrophages. *J. Virol.* 69:1284.
- Borrow, R., M. Stapleton, S. Windsor, A. McKnight, and T. Andronova. 1992. The control of antibody isotype response to recombinant human immunodeficiency virus gp120 antigen by adjuvants. *J. Virol.* 68:6103.

- Bottomly, K., and S. L. Constant. 1997. Induction of the Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Ann. Rev. Immunol.* 15:297.
- Bouloc, A., P. Walker, J. C. Grivel, J. C. Vogel, and S. I. Katz. 1999. Immunization through dermal delivery of protein-encoding DNA: a role for migratory dendritic cells. *Eur. J. Immunol.* 29:446.
- Boyle, J. S., C. Koniaras, and A. M. Lew. 1997. Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization. *Int. Immunol.* 9:1897.
- Braun, R., L. A. Babiuk, and H. van Drunen Littel-van den. 1998. Compatibility of plasmids expressing different antigens in a single DNA vaccine formulation. *J. Gen. Virol.* 79:2965.
- Brenner, B., U. Koppenhoefer, A. Lepple-Wienhues, H. Grassme, C. Muller, C. Speer, and E. Gulbins. 1997. The CD40 ligand directly activates T-lymphocytes via tyrosine phosphorylation-dependent PKC activation. *Biochem. Biophys. Res. Commun.* 1997:11.
- Brewer, J., J. Richmond, and J. Alexander. 1994. The demonstration of an essential role for macrophages in the in vivo generation of IgG2a antibodies. *Clin. Exp. Immunol.* 97:164.
- Cairns, J. S., and N. Sarver. 1998. New viral vectors for HIV vaccine delivery. *AIDS Res. Hum. Retroviruses* 14:1501.
- Calarota, S., G. Bratt, S. Nordlund, J. Hinkula, A. C. Leandersson, E. Sandstrom, and B. Wahren. 1998. Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. *Lancet* 351:1320.
- Cardosa, M. J. 1998. Dengue vaccine design: issues and challenges. *Br. Med. Bull.* 54:395.

Casares, S., T. D. Brumeanu, A. Bot, and C. A. Bona. 1997. Protective immunity elicited by vaccination with DNA encoding for a B cell and a T cell epitope of the A/PR/8/34 influenza virus. *Viral Immunol.* 10:129.

Casares, S., K. Inaba, T. D. Brumeanu, R. M. Steinman, and C. A. Bona. 1997. Antigen presentation by dendritic cells after immunization with DNA encoding a major histocompatibility complex class II-restricted viral epitope. *J. Exp. Med.* 186:1481.

Cassell, D. J. 2001. Validity of the two-signal model for activation of CD28-deficient T lymphocytes: quantitative characterization of an alternative costimulatory function of dendritic cells. *Scand. J. Immunol.* 53:346.

Cease, K. B., and J. A. Berzofsky. 1994. TOWARD A VACCINE FOR AIDS: The Emergence of Immunobiology Based Vaccine Development. *Annu. Rev. Immunol.* 12:923.

Chambers, C. A. 2001. The expanding world of co-stimulation: the two signal model of revisited. *Trends Immunol.* 22:217.

Chattergoon, M. A., T. M. Robinson, J. D. Boyer, and D. B. Weiner. 1998. Specific Immune Induction Following DNA-Based Immunization Through In Vivo Transfection and Activation of Macrophages/Antigen-Presenting Cells. *J. Immunol.* 160:5707.

Chen, C., T. Wang, C. Hung, D. M. Pardoll, and T. Wu. 2000. Boosting with recombinant vaccinia increases HPV-16 E7-specific T cell precursor frequencies of HPV-16 E7-expressing DNA vaccines. *Vaccine* 18:2015.

Chen, Y., V. K. Kuchroo, J. Inobe, D. A. Hafler, and H. I. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265:1237.

Chen, Z., K. Matsuo, H. Asanuma, H. Takahashi, T. Iwasaki, Y. Suzuki, C. Aizawa, T. Kurata, and S. Tamura. 1999. Enhanced protection against a lethal influenza virus challenge by immunization with both hemagglutinin- and neuraminidase-expressing DNAs. *Vaccine* 17:653.

Chen, Z., Y. Sahashi, K. Matsuo, H. Asanuma, H. Takahashi, T. Iwasaki, Y. Suzuki, C. Aizawa, T. Kurata, and S. Tamura. 1998. Comparison of the ability of viral protein-expressing plasmid DNAs to protect against influenza. *Vaccine* 16:1544.

Childers, N. K., G. Tong, S. Mitchell, K. Kirk, M. Russell, and S. Michalek. 1999. A controlled clinical study of the effect of nasal immunization with a *Streptococcus mutans* antigen alone or incorporated into liposomes on the induction of immune responses. *Infect. Immun.* 67:618.

Choe, H., M. Farazan, Y. Sun, N. Sullivan, B. Rollins, D. Ponath, L. Wu, C. R. Mackey, G. Larosa, W. Newman, N. Gerard, C. Gerard, and J. Sodroski. 1996. The B-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 85:1135.

Chow, Y. H., B. L. Chiang, Y. L. Lee, W. K. Chi, W. C. Lin, Y. T. Chen, and M. H. Tao. 1998. Development of Th1 and Th2 Populations and the Nature of Immune Responses to Hepatitis B Virus DNA Vaccines Can Be Modulated by Codelivery of Various Cytokine Genes. *J. Immunol.* 160:1320.

Chu, W. M., X. Gong, Z. W. Li, K. Takabayashi, H. H. Ouyang, Y. Chen, A. Lois, D. J. Chen, G. C. Li, M. Karin, and E. Raz. 2000. DNA-PKcs is required for activation of innate immunity by immunostimulatory DNA. *Cell* 103:909.

Chun, S., M. Daheshia, S. Lee, S. K. Eo, and B. T. Rouse. 1999. Distribution Fate and Mechanism of Immune Modulation Following Mucosal Delivery of Plasmid DNA Encoding IL-10. *J. Immunol.* 163:2393.

Clark, E. A., and L. A. Ledbetter. 1994. How B and T cells talk to each other. *Nature* 367:425.

Clerici, M., J. V. Giorgi, C. C. Chou, V. K. Gudeman, J. A. Zack, P. Gupta, H. N. Ho, P. G. Nishanian, J. A. Berzofsky, and G. M. Shearer. 1992. Cell mediated immune responses to human immunodeficiency virus type 1 (HIV-1) in seronegative homosexuals with recent sexual exposure to HIV-1. *J. Infect. Dis.* 165:1012.

Clerici, M., and G. M. Shearer. 1993. A Th1-> Th2 switch is a critical step in the etiology of HIV infection. *Immunology Today* 14:107.

Clerici, M., and G. M. Shearer. 1994. The Th1-Th2 hypothesis of HIV infection: new insights. *Immunology Today* 15:575.

Clerici, M., T. A. Wynn, J. A. Berzofsky, S. A. Blatt, C. W. Hendrix, A. Sher, R. L. Coffman, and G. M. Shearer. 1994. Role of interleukin-10 in T helper cell dysfunction in asymptomatic individuals with the human immunodeficiency virus. *J. Clin. Invest.* 93:768.

Coffman, R. L., D. A. Leberman, and P. Rothman. 1993. Mechanism and Regulation of Immunoglobulin Isotype Switching. *Adv. Immunol.* 54:229.

Condon, C., S. C. Watkins, C. M. Celluzzi, K. Thompson, and L. D. J. Falo. 1996. DNA-based immunization by *in vivo* transfection of dendritic cells. *Nature Medicine* 2:1122.

Coney, L., B. Wang, K. E. Ugen, J. Boyer, D. McCallus, V. Srikantan, M. Agajanyan, C. J. Pachuk, K. Herold, M. Merva, L. Gilbert, K. Deng, K. Moelling, M. Newman, W. V. Williams, and D. B. Weiner. 1994. Facilitated DNA inoculation induces anti-HIV-1 immunity *in vivo*. *Vaccine* 12:1545.

Conway, M. A., L. Madrigal-Estebas, S. McClean, D. J. Brayden, and K. H. Mills. 2000. Protection against *Bordetella pertussis* infection following parenteral immunization with antigens entrapped in biodegradable particles: effect of formulation and route of immunization on induction of Th1 and Th2 responses. *Vaccine* 19.

- Corinti, S., C. Albanesi, A. la Sala, S. Pastore, and G. Girolomoni. 2001. Regulatory activity of autocrine IL-10 on dendritic cell functions. *J. Immunol.* 166:4312.
- Corr, M., D. J. Lee, D. A. Carson, and H. Tighe. 1996. Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J. Exp. Med.* 184:1555.
- Corr, M., A. van Damm, D. J. Lee, and H. Tighe. 1999. In Vivo Priming by DNA Injection Occurs Predominantly by Antigen Transfer. *J. Immunol.* 163:4721.
- Cuisinier, A. M., A. Meyer, B. Chatrenet, A. S. Verdier, and A. Aubert. 1999. Attempt to modify the immune response developed against FIV gp120 protein by preliminary FIV DNA injection. *Vaccine* 17:415.
- Curti, A., M. Ratta, S. Corinti, G. Girolomoni, F. Ricci, P. Tazzari, M. Siena, A. Grande, M. Fogli, S. Tura, and R. M. Lemoli. 2001. Interleukin-11 induces Th2 polarization of human CD4(+) T cells. *Blood* 97:2758.
- Cusi, M. G., R. Zurbriggen, M. Valassina, S. Bianchi, P. Durrer, P. E. Valensin, M. Donati, and R. Gluck. 2000. Intranasal immunization with mumps virus DNA vaccine delivered by influenza virosomes elicits mucosal and systemic immunity. *Virology* 277:111.
- Daheshia, M., N. Kuklin, E. Manickan, S. Chun, and B. T. Rouse. 1998. Immune induction and modulation by topical ocular administration of plasmid DNA encoding antigens and cytokines. *Vaccine* 16:1103.
- D'Amico, G., G. Frascaroli, G. Bianchi, P. Transidico, A. Doni, A. Vecchi, S. Sozzani, P. Allavena, and A. Mantovani. 2000. Uncoupling of inflammatory chemokine receptors by IL-10: generation of functional decoys. *Nat. Immunol.* 1:387.
- Daniel, M. D., F. Kirchoff, S. C. Czajak, P. K. Sehgal, and R. C. Desrosiers. 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the *nef* gene. *Science* 258:1938.

Danko, I., and J. A. Wolff. 1994. Direct gene transfer into muscle. *Vaccine* 12:1499.

Das, G., S. Sheridan, and C. A. J. Janeway. 2001. The source of early ifn-gamma that plays a role in th1 priming. *J. Immunol.* 167:2004.

Davis, H. L., R. Weeranta, T. J. Waldschmidt, L. Tygrett, J. Schorr, and A. M. Kreig. 1998. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant Hepatitis B surface antigen. *J. Immunol.* 160:870.

de Haan, A., J. F. C. Tomee, J. P. Huchshorn, and J. Wilschut. 1995. Liposomes as an immunoadjuvant system for stimulation of mucosal and systemic antibody responses against inactivated measles virus administered intranasally to mice. *Vaccine* 13:1320.

Deck, R. R., C. M. DeWitt, J. J. Donnelly, M. A. Liu, and J. B. Ulmer. 1997. Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. *Vaccine* 15:71.

DeKruuff, R. H., Y. Fang, and D. T. Umetsu. 1992. IL-4 synthesis by in vivo primed keyhole limpet hemocyanin-specific CD4+ T cells. Influence of antigen concentration and antigen-presenting cell type. *J. Immunol.* 149:3468.

Dietrich, G., A. Kolb-Maurer, S. Spreng, M. Scharl, W. Goebel, and I. Gentschev. 2001. Gram-positive and Gram-negative bacteria as carrier systems for DNA vaccines. *Vaccine* 19:2506.

Ding, L., P. S. Linsey, L. Y. Huang, R. N. Germain, and E. M. Shevach. 1993. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting up-regulation of B7 expression. *J. Immunol.* 151:1224.

Dodds, E., M. G. Dunkley, K. Naujoks, U. Michaelis, and G. Dickson. 1998. Lipofection of cultured mouse muscles cells: a direct comparison of Lipofectamine and DOSPER. *Gene Therapy* 5:542.

Doherty, T. M., and R. L. Coffman. 1999. Ability of macrophage subsets to transfer resistance to murine leishmania-dependent on IL-12 production. *Eur. J. Immunol.* 29:522.

Dong, C., A. E. Juedes, U. A. Temann, S. Shresta, J. P. Allison, N. H. Ruddle, and R. A. Flavell. 2001. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 409:97.

Donnelly, J. J., J. B. Ulmer, J. W. Shiver, and M. A. Lui. 1997. DNA Vaccines. *Ann. Rev. Immunol.* 15:617.

D'Orazio, T. J., and J. Y. Niederkorn. 1998. The nature of antigen in the eye has a profound effect on the cytokine milieu and resultant immune response. *Eur. J. Immunol.* 28:1544.

Dupuis, M., K. Denis-Mize, C. Woo, C. Goldbeck, M. J. Selby, M. Chen, G. R. Otten, J. B. Ulmer, J. J. Donnelly, G. Ott, and D. M. McDonald. 2000. Distribution of DNA vaccines determines their immunogenicity after intramuscular injection in mice. *J. Immunol.* 165:2850.

Elkins, K. L., T. R. Rhinehart-Jones, S. Stibitz, J. S. Conover, and D. M. Klinman. 1999. Bacterial DNA containing CpG motifs stimulates lymphocyte-dependent protection of mice against lethal infection with intracellular bacteria. *J. Immunol.* 162:2291.

Etchart, N., R. Buckland, M. A. Liu, T. F. Wild, and D. Kaiserlian. 1997. Class I-restricted CTL induction by mucosal immunization with naked DNA encoding measles virus haemagglutinin. *J. Gen. Virol.* 78:1577.

Evans, T. G., T. Fitzgerald, D. C. Gibbons, M. C. Keefer, H. Soucier, and T. A. V. E. Group. 1998. Th1/Th2 cytokine responses following HIV-1 immunization in seronegative volunteers. *Clin. Exp. Immunol.* 111:243.

- Fearon, D. T., and R. M. Locksley. 1996. The instructive role of the innate immunity in the acquired immune response. *Science* 272:50.
- Feltquate, D. M. 1998. DNA vaccines: vector design, delivery, and antigen presentation. *J. Cell. Biochem. Suppl.* 30-31:304.
- Feltquate, D. M., S. Heaney, R. G. Webster, and H. L. Robinson. 1997. Different T Helper Cell Types and Antibody Isotypes Generated by Saline and Gene Gun DNA Immunization. *J. Immunol.* 158:2278.
- Fennelly, G. J., S. A. Khan, M. A. Abadi, T. F. Wild, and B. R. Bloom. 1999. Mucosal DNA vaccine immunization against measles with a highly attenuated *Shigella flexneri* vector. *J. Immunol.* 162:1603.
- Ferreira, G. N., G. A. Monteiro, D. M. Prazeres, and J. M. Cabral. 2000. Downstream processing of plasmid DNA for gene therapy and DNA vaccine applications. *Trends Biotechnol.* 18:380.
- Finkelman, F. D., S. C. Morris, T. Orekhova, M. Mori, D. Donaldson, S. L. Reiner, N. L. Reilly, L. Schopf, and J. F. Urban. 2000. Stat6 regulation of *in vivo* IL-4 responses. *J. Immunol.* 164:2303.
- Finkleman, F., J. Holmes, I. M. Katona, J. F. Urban, M. P. Beckmann, L. S. Park, K. A. Schooley, R. L. Coffman, T. R. Mosmann, and W. E. Paul. 1990. Lymphokine control of *in vivo* immunoglobulin isotype selection. *Ann. Rev. Immunol.* 8:303.
- Firestein, G. S., W. D. Roeder, J. A. Laxer, K. S. Townsend, C. T. Weaver, J. T. Hom, J. Linton, B. E. Torbett, and A. L. Glasebrook. 1989. A new murine CD4⁺ T cell subset with an unrestricted cytokine profile. *J. Immunol.* 143:518.
- Fu, T. M., J. B. Ulmer, M. J. Caulfield, R. R. Deck, A. Friedman, S. Wang, X. Liu, J. J. Donnelly, and M. A. Liu. 1997. Priming of cytotoxic T lymphocytes by DNA vaccines: requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes. *Mol. Med.* 3:362.

- Fust, G., M. P. Dierich, and T. Hidvegi. 1995. Role of humoral factors in the progression of HIV disease. *Immunology Today* 16:167.
- Fynan, E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson. 1993. DNA vaccines: Protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. USA* 90:11478.
- Gajewski, T. F., M. Pinnas, T. Wong, and F. W. Fitch. 1991. Murine Th1 and Th2 clones proliferate optimally in response to distinct antigen presenting cell populations. *J. Immunol.* 146:1750.
- Gallo, R. C. 1999. Tat as one key to HIV-induced immune pathogenesis and Tat (correction of Pat) toxoid as an important component of a vaccine. *Proc. Natl. Acad. USA.* 96:8324.
- Gallucci, S., and P. Matzinger. 2001. Danger signals: SOS to the immune system. *Curr. Opin. Immunol.* 13:114.
- Gao, L., C. M. Barnfield, S. Parker, R. Ward, and L. S. Klavinskis. 1997. Mucosal delivery of nucleic acid elicits both cellular and humoral immunity. *Biochem. Soc. Trans.* 25:336S.
- Gately, M. K., L. M. Renzetti, J. Magram, A. S. Stern, L. Adorni, U. Gubler, and D. H. Presky. 1998. The interleukin-12/interleukin-12-receptor system: role in normal allergic pathologic immune responses. *Annu. Rev. Immunol.* 16:495.
- Gherardi, M. M., J. C. Ramirez, and M. Esteban. 2000. Interleukin-12 (IL-12) enhancement of the cellular immune response against human immunodeficiency virus type 1 env antigen in a DNA prime/vaccinia virus boost vaccine regimen is time and dose dependent: suppressive effects of IL-12 boost are mediated by nitric oxide. *J. Virol.* 74:6278.

Gilbert, S. C., J. Schneider, M. Plebanski, C. M. Hannan, T. J. Blanchard, G. L. Smith, and A. V. Hill. 1999. Ty virus-like particles, DNA vaccines and Modified Vaccinia Virus Ankara; comparisons and combinations. *Biol. Chem.* 380:299.

Girad, J. P., and T. A. Springer. 1995. High endothelial venules (HEVs): specialized endothelium for lymphocyte migration. *Immunol. Today.* 16:449.

Gollob, K. J., W. O. Dutra, and R. L. Coffman. 1996. Early message expression of interleukin-4 and interferon-gamma, but not of interleukin-2 and interleukin-10, reflects later polarization of primary CD4⁺ T cell cultures. *Eur. J. Immunol.* 26:1565.

Gregoriadis, G. 1998. Genetic Vaccines : Strategies for Optimization. *Pharmacuetical Research* 15:661.

Grewal, I. S., and R. A. Flavell. 1996. The role of CD40 ligand in costimulation and T-cell activation. *Immunol. Rev.* 153:85.

Grifantini, R., O. Finco, E. Bartolini, M. Draghi, G. Del Giudice, C. Knocken, A. Thomas, S. Abrignani, and G. Grandi. 1998. Multi-plasmid DNA vaccination avoids antigenic competition and enhances immunogenicity of a poorly immunogenic plasmid. *Eur. J. Immunol.* 28:1225.

Grohmann, U., M. L. Belladonna, C. Vacca, R. Bianchi, F. Fallarino, C. Orabona, M. C. Fioretti, and P. Puccetti. 2001. Positive Regulatory Role of IL-12 in Macrophages and Modulation by IFN- γ . *J. Immunol.* 167:221.

Groux, H., A. O'Garra, M. Bogler, M. Rouleau, S. Antonenka, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4⁺ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737.

Guery, J. C., F. Galbiati, S. Smiroldo, and L. Adorini. 1996. Selective development of T helper (Th)2 cells induced by continuous administration of low dose soluble proteins to normal and beta(2)-microglobulin-deficient BALB/c mice. *J Exp Med* 183:485.

Gursel, I., M. Gursel, K. J. Ishii, and D. M. Klinman. 2001. Sterically Stabilized Cationic Liposomes Improve the Uptake and Immunostimulatory Activity of CpG Oligonucleotides. *J. Immunol.* 167:3324.

Gursel, M., S. Tunca, M. Ozkan, G. Ozcengiz, and G. Alaeddinoglu. 1999. Immunoadjuvant action of plasmid DNA in liposomes. *Vaccine* 17:1376.

Gurunathan, S., K. R. Irvine, C.-Y. Wu, J. I. Cohen, E. Thomas, C. Prussin, N. P. Restifo, and R. A. Seder. 1998. CD40 ligand/trimer DNA enhances both humoral and cellular immune responses and induces protective immunity to infectious and tumor challenge. *J. Immunol.* 161:4563.

Gurunathan, S., D. M. Klinman, and R. A. Seder. 2000. DNA VACCINES: Immunology, Application, and Optimization. *Annu. Rev. Immunol.* 18:927.

Gurunathan, S., D. L. Sacks, D. R. Brown, S. L. Reiner, H. Charest, N. Glaichenhaus, and R. A. Seder. 1997. Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with *Leishmania major*. *J. Exp. Med.* 186:1137.

Gurunathan, S., L. Stobie, C. Prussin, D. L. Sacks, N. Glaichenhaus, D. J. Fowell, R. M. Locksley, J. T. Chang, C. Y. Wu, and R. A. Seder. 2000. Requirements for the maintenance of Th1 immunity in vivo following DNA vaccination: a potential immunoregulatory role for CD8⁺ T cells. *J. Immunol.* 165:915.

Gurunathan, S., C. Y. Wu, B. L. Freidag, and R. A. Seder. 2000. DNA vaccines: a key for inducing long-term cellular immunity. *Curr. Opin. Immunol.* 12:442.

Haddad, D., S. Liljeqvist, S. Stahl, I. Andersson, P. Perlmann, K. Berzins, and N. Ahlborg. 1997. Comparative study of DNA-based immunization vectors: effect of secretion signals on the antibody responses in mice. *FEMS Immunol. Med. Microbiol.* 18:193.

Haddad, D., S. Liljeqvist, S. Stahl, M. Hansson, P. Perlmann, N. Ahlborg, and K. Berzins. 1999. Characterization of antibody responses to a *Plasmodium falciparum* blood-stage antigen induced by a DNA prime/protein boost immunization protocol. *Scand. J. Immunol.* 49:506.

Haddad, D., S. Liljeqvist, S. Stahl, P. Perlmann, K. Berzins, and N. Ahlborg. 1998. Differential induction of immunoglobulin G subclasses by immunization with DNA vectors containing or lacking a signal sequence. *Immunol. Lett.* 61:201.

Hammond, S. A., R. C. Bollinger, P. E. Stanhope, T. C. Quinn, D. Schwartz, M. I. Clements, and R. F. Siliciano. 1992. Comparative clonal analysis of human immunodeficiency virus type 1 (HIV-1)-specific CD4⁺ and CD8⁺ cytolytic T lymphocytes isolated from seronegative humans immunized with candidate HIV-1 vaccines. *J. Exp. Med.* 176:1531.

Han, M., S. Watarai, K. Kobayashi, and T. Yasuda. 1997. Application of liposomes for development of oral vaccines: study of *in vitro* stability of liposomes and antibody responses after oral immunization. *J. Vet. Med.* 59:1109.

Hanke, T., and A. McMichael. 1999. Pre-clinical development of a multi-CTL epitope-based DNA prime MVA boost vaccine for AIDS. *Immunol. Lett.* 66:177.

Harding, C. V., and R. Song. 1994. Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class I MHC molecules. *J. Immunol.* 153:4925.

Harokopakis, E., G. Hajishengallis, and S. M. Michalek. 1998. Effectiveness of liposomes possessing surface-linked recombinant B subunit of cholera toxin as an oral antigen delivery system. *Infect. Immun.* 66:4299.

Hartshorn, K., K. Reid, M. White, J. Jensenius, S. Morris, A. Tauber, and E. Crouch. 1996. Neutrophil deactivation by influenza A viruses: mechanisms of protection after viral opsonization with collectins and hemagglutination-inhibiting antibodies. *Blood* 15:3450.

Hatada, E. N., D. Krappmann, and C. Scheidereit. 2000. NF- κ B and the innate immune response. *Curr. Opin. Immunol.* 12:52.

Hathcock, K. S., G. Laszlo, C. Pucillo, P. Linsley, and R. J. Hodes. 1994. Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression function. *J. Exp. Med.* 180:631.

Heinzel, F. P., D. S. Schoenhaut, R. M. Rerko, L. E. Rosser, and M. K. Gately. 1993. Recombinant interleukin 12 cures mice infected with *Leishmania major*. *J. Exp. Med.* 177:1505.

Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740.

Heritage, P. L., M. A. Brook, B. J. Underdown, and M. R. McDermott. 1998. Intranasal immunization with polymer-grafted microparticles activates the nasal associated lymphoid tissue and draining lymph nodes. *Immunology* 93:249.

Hernandez-Pando, R., L. Pavon, K. Arriaga, H. Orozco, V. Madrid-Marina, and G. A. W. Rook. 1997. Pathogenesis of tuberculosis in mice exposed to low and high doses of an environmental mycobacterial saprophyte before infection. *Infect. Immun* 65:3317.

Herve, M., L. Dupre, E. Ban, A. Schacht, A. Capron, and G. Riveau. 2001. Features of the antibody response attributable to plasmid backbone adjuvanticity after DNA immunization. *Vaccine* 19:4549.

Hinkula, J., P. Lundholm, and B. Wahren. 1997. Nucleic acid vaccination with HIV regulatory genes: a combination of HIV-1 genes in separate plasmids induces strong immune responses. *Vaccine* 15:874.

Hino, A., and H. Nariuchi. 1996. Negative feedback mechanism suppresses interleukin-12 production from antigen presenting cells interacting with T helper 2 cells. *Eur. J. Immunol.* 26:623.

Hocart, M. J., J. S. Mackenzie, and G. A. Stuart. 1989. The IgG subclass response to influenza hemagglutinin in the same mouse; effect of route of inoculation. *J. Gen. Virol.* 70:809.

Hoffmann, K. F., A. W. Cheever, and T. A. Wynn. 2000. IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. *J. Immunol.* 164:6406.

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

Huang, L., A. M. Krieg, N. Eller, and D. E. Scott. 1999. Induction and regulation of Th1-inducing cytokines by bacterial DNA, lipopolysaccharide, and heat-inactivated bacteria. *Infect. Immun.* 67:6257.

Huang, L. Y., C. Reis e Sousa, Y. Itoh, J. Inman, and D. E. Scott. 2001. IL-12 induction by a TH1-inducing adjuvant in vivo: dendritic cell subsets and regulation by IL-10. *J. Immunol.* 167:1423.

Huang, Y. H., J. C. Wu, M. H. Tao, W. J. Syu, S. C. Hsu, W. K. Chi, F. Y. Chang, and S. D. Lee. 2000. DNA-based immunization produces Th1 immune responses to hepatitis delta virus in a mouse model. *Hepatology* 32:104.

Hunter, C. A., and S. L. Reiner. 2000. Cytokines and T cells in host defense. *Curr. Opin. Immunol.* 12:413.

Igietseme, J. U., G. A. Ananaba, J. Bolier, S. Bowers, T. Moore, T. Belay, F. O. Eko, D. Lyn, and C. M. Black. 2000. Suppression of endogenous IL-10 gene expression in dendritic cells enhances antigen presentation for specific Th1 induction: potential for cellular vaccine development. *J. Immunol.* 164:4212.

Ihata, A., S. Watabe, S. Sasaki, A. Shirai, J. Fukushima, K. Hamajima, J. Inoue, and K. Okuda. 1999. Immunomodulatory effect of a plasmid expressing CD40 ligand on DNA vaccination against human immunodeficiency virus type-1. *Immunology* 98:436.

Ishii, N., J. Fukushima, T. Kaneko, E. Okada, K. Tani, S. I. Tanaka, K. Hamajima, K. Q. Xin, S. Kawamoto, W. Koff, K. Nishioka, T. Yasuda, and K. Okuda. 1997. Cationic liposomes are a strong adjuvant for a DNA vaccine of human immunodeficiency virus type 1. *AIDS Res. Hum. Retroviruses* 13:1421.

Iwasaki, A., B. J. Stiernholm, A. K. Chan, N. L. Berinstein, and B. H. Barber. 1997. Enhanced CTL responses mediated by plasmid DNA immunogens encoding costimulatory molecules and cytokines. *J. Immunol.* 158:4591.

Jankovic, D., M. C. Kullberg, N. Noben-Trauth, P. Casper, W. E. Paul, and A. Sher. 2000. Single cell analysis reveals that IL-4 receptor/Stat6 signalling is not required for the *in vivo* and *in vitro* development of CD4 (+) lymphocytes with a Th2 cytokine profile. *J. Immunol.* 164:3047.

Jankovic, D., Z. Lui, and W. C. Gause. 2001. Th1- and Th2-cell commitment during infectious disease: asymmetry in divergent pathways. *Trends Immunol.* 22:450.

Jeannin, P., Y. Delneste, M. Seveso, P. Life, and Y. L. Bonnefoy. 1996. IL-12 synergises with IL-2 and other stimuli in inducing IL-10 production by human T cells. *J. Immunol.* 156:3159.

Johnson, P. A., M. A. Conway, J. Daly, C. Nicolson, J. Robertson, and K. H. Mills. 2000. Plasmid DNA encoding influenza virus haemagglutinin induces Th1 cells and

- protection against respiratory infection despite its limited ability to generate antibody responses. *J. Gen. Virol.* 81 Pt 7:1737.
- Jones, D. H., S. Corris, S. McDonald, J. C. Clegg, and G. H. Farrar. 1997. Poly(DL-lactide-co-glycolide)-encapsulated plasmid DNA elicits systemic and mucosal antibody responses to encoded protein after oral administration. *Vaccine* 15:814.
- Justewicz, D. M., M. J. Morin, H. L. Robinson, and R. G. Webster. 1995. Antibody-forming cell response to virus challenge in mice immunized with DNA encoding the influenza virus hemagglutinin. *J. Virol.* 69:7712.
- Justewicz, D. M., and R. G. Webster. 1996. Long-term maintenance of B cell immunity to influenza virus hemagglutinin in mice following DNA-based immunization. *Virology* 224:10.
- Kaisho, T., and S. Akira. 2001. Dendritic-cell function in Toll-like receptor- and MyD88-knockout mice. *Trends Immunol.* 22:78.
- Kalinski, P., M. U. Hilkens, E. A. Wierenga, and M. L. Kapsenberg. 1999. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol. Today* 20:561.
- Kamath, A. T., C. G. Feng, M. Macdonald, H. Briscoe, and W. J. Britton. 1999. Differential protective efficacy of DNA vaccines expressing secreted proteins of *Mycobacterium tuberculosis*. *Infect. Immun.* 67:1702.
- Kaneko, H., I. Bednarek, A. Wierzbicki, I. Kiszka, M. Dmochowski, T. J. Wasik, Y. Kaneko, and D. Kozbor. 2000. Oral DNA vaccination promotes mucosal and systemic immune responses to HIV envelope glycoproteins. *Virology* 267:8.
- Kawabata, S., Y. Terao, T. Fujiwara, I. Nakagawa, and S. Hamada. 1999. Targeted salivary gland immunization with plasmid DNA elicits specific salivary immunoglobulin A and G antibodies and serum immunoglobulin G antibodies in mice. *Infect. Immun.* 67:5863.

Kawamura, T., K. Takeda, S. K. Mendiratta, H. Kawamura, L. Van Kaer, H. Yagita, and K. Okumura. 1998. Critical role of NK1+ T cells in IL-12-induced immune responses in mice. *J. Immunol.* 160:16.

Kent, S. J., A. Zhao, S. J. Best, J. D. Chandler, D. B. Boyle, and I. A. Ramshaw. 1998. Enhanced T-cell immunogenicity and protective efficacy of a human immunodeficiency virus type 1 vaccine regimen consisting of consecutive priming with DNA and boosting with recombinant fowlpox virus. *J. Virol.* 72:10180.

Kim, J. J., N. N. Trivedi, L. K. Nottingham, L. Morrison, A. Tsai, Y. Hu, S. Mahalingam, K. Dang, L. Ahn, N. K. Doyle, D. M. Wilson, M. A. Chattergoon, A. A. Chalian, J. D. Boyer, M. G. Agadjanyan, and D. B. Weiner. 1998. Modulation of amplitude and direction of *in vivo* immune responses by co-administration of cytokine gene expression cassettes with DNA immunogens. *Eur. J. Immunol.* 28:1089.

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

Klavinskis, L. S., C. Barnfield, L. Gao, and S. Parker. 1999. Intranasal immunization with plasmid DNA-lipid complexes elicits mucosal immunity in the female genital and rectal tracts. *J. Immunol.* 162:254.

Klavinskis, L. S., L. Gao, C. Barnfield, T. Lehner, and S. Parker. 1997. Mucosal immunization with DNA-liposome complexes. *Vaccine* 15:818.

Klein, M. 2001. Current progress in the development of human immunodeficiency virus vaccines: research and clinical trials. *Vaccine* 19:2210.

Klinman, D. M., K. M. Barnhart, and J. Conover. 1999. CpG motifs as immune adjuvants. *Vaccine* 17:19.

Klinman, D. M., K. J. Ishii, and D. Verthelyi. 2000. CpG DNA Augments the Immunogenicity of Plasmid DNA Vaccines. *Curr. Top. Microbiol. Immunol.* 247:131.

Klinman, D. M., J. M. G. Sechler, J. Conover, M. Gu, and A. S. Rosenberg. 1998. Contribution of Cells at the Site of DNA Vaccination to the Generation of Antigen-Specific Immunity and Memory. *J. Immunol.* 160:2388.

Klinman, D. M., G. Yamshchikov, and Y. Ishigatsubo. 1997. Contribution of CpG Motifs to the Immunogenicity of DNA Vaccines. *J. Immunol.* 158:3635.

Koch, F., U. Stanzl, P. Jennewein, K. Janke, C. Heufler, E. Kampgen, and N. Romani. 1996. High level IL-12 production by murine dendritic cells: upregulation of MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J. Exp. Med.* 184:741.

Komase, K., S. Tamura, K. Matsuo, K. Watanabe, N. Hattori, A. Odaka, Y. Suzuki, T. Kurata, and C. Aizawa. 1998. Mutants of *Escherichia coli* heat-labile enterotoxin as an adjuvant for nasal influenza vaccine. *Vaccine* 16:248.

Kopf, M., G. Le Gros, M. Bachmann, M. C. Lamers, H. Bluethmann, and G. Kohler. 1993. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* 362:245.

Kopp, E. B., and R. Medzhitov. 1999. The Toll-receptor family and control of innate immunity. *Curr. Opin. Immunol.* 11:13.

Kourilsky, P., and P. Truffa-Bachi. 2001. Cytokine fields and the polarization of the immune response. *Trends Immunol.* 22:502.

Kovarik, J., P. Bozzotti, L. Love-Homan, M. Pihlgren, H. L. Davis, P. H. Lambert, A. M. Krieg, and C. A. Siegrist. 1999. CpG oligodeoxynucleotides can circumvent the Th2 polarization of neonatal responses to vaccines but may fail to fully redirect Th2 responses established by neonatal priming. *J. Immunol.* 162:1611.

Krieg, A. M., T. Wu, R. Weeratna, S. M. Efler, L. Love-Homan, L. Yang, A. K. Yi, D. Short, and H. L. Davis. 1998. Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. *Proc. Natl. Acad. Sci. U S A.* 95:12631.

Kuwano, K., and S. Arai. 1996. Involvement of two distinct killing mechanisms in bystander target cells induced by a cytotoxic T lymphocyte clone. *Cell. Immunol.* 169:288.

Kwong, P. D., R. Wyatt, J. Robinson, R. W. Sweet, J. Sodroski, and W. A. Hendrickson. 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* 393:648.

Lanier, L. L., S. O'Fallon, C. Somoza, J. H. Philips, P. S. Linsey, K. Okumura, D. Ito, and M. Azuma. 1995. CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. *J. Immunol.* 154:97.

Leclerc, C., E. Deriaud, M. Rojas, and R. G. Whalen. 1997. The preferential induction of a Th1 immune response by DNA-based immunization is mediated by the immunostimulatory effect of plasmid DNA. *Cell. Immunol.* 179:97.

Lekutis, C., J. W. Shiver, M. A. Liu, and N. L. Letvin. 1997. HIV-1 env DNA vaccine administered to rhesus monkeys elicits MHC class II-restricted CD4⁺ T helper cells that secrete IFN-gamma and TNF-alpha. *J. Immunol.* 158:4471.

Lenschow, D. J., S. C. Ho, H. Scatter, G. Rhee, G. Gray, N. Nabavi, K. C. Herold, and J. A. Bluestone. 1995. Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in a non-obese diabetic mouse. *J. Exp. Med.* 181:1145.

Lewis, P. J., G. J. Cox, S. van Drunen Littel-van den Hurk, and L. A. Babiuk. 1997. Polynucleotide vaccines in animals: enhancing and modulating responses. *Vaccine* 15:861.

Lewis, P. J., S. van Drunen Littel-van den Hurk, and L. A. Babiuk. 1999. Altering the Cellular Location of an Antigen Expressed by a DNA-based Vaccine Modulates the Immune Response. *J. Virol.* 73:10214.

Li, S., C. Liu, A. Kilmov, K. Subbarao, M. L. Perdue, D. Mo, Y. Ji, L. Woods, S. Hietala, and M. Bryant. 1999. Recombinant influenza A virus vaccines for the pathogenic human A/HongKong/97 (H5N1) viruses. *J. Infect. Dis.* 179:1132.

Lipford, G. B., T. Sparwasser, M. Bauer, S. Zimmermann, E. S. Koch, K. Heeg, and H. Wagner. 1997. Immunostimulatory DNA: sequence-dependant production of potentially harmful or useful cytokines. *Eur. J. Immunol.* 27:3420.

Liu, M. A., W. McClements, J. B. Ulmer, J. Shiver, and J. Donnelly. 1997. Immunization of non-human primates with DNA vaccines. *Vaccine* 15:909.

Liu, Y., L. C. Mounkes, H. D. Liggitt, C. S. Brown, I. Solodin, T. D. Heath, and R. J. Debs. 1997. Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. *Nat. Biotech.* 15:167.

Liu, Y. J., H. Kanzler, V. Soumelis, and M. Gilliet. 2001. Dendritic cell lineage, plasticity and cross-regulation. *Nat. Immunol.* 2:585.

Lobo, C. A., R. Dhar, and N. Kumar. 1999. Immunization of mice with DNA-based Pfs25 elicits potent malaria transmission-blocking antibodies. *Infect. Immun.* 67:1688.

Lukacher, A. E., V. L. Braciale, and T. J. Braciale. 1984. *In vivo* effector function of influenza virus-specific cytotoxic T cell clones is highly specific. *J. Exp. Med.* 160:814.

Lundholm, P., Y. Asakura, J. Hinkula, E. Lucht, and B. Wahern. 1999. Induction of mucosal IgA by a novel jet delivery technique for HIV-1 DNA. *Vaccine* 17:2036.

Lutz, M., N. Kukutsch, A. Ogilvie, S. Rossner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Meth.* 223:77.

Macatonia, S. E., N. A. Hosken, M. Litton, P. Viera, C. S. Hsieh, J. A. Culpepper, G. Wysock, K. M. Murphy, and A. O'Garra. 1995. Dendritic cells produce IL-12 and direct the development of Th1 and Th2 CD4+ T cells. *J. Immunol.* 154:5071.

Macatonia, S. E., C. S. Hsieh, K. M. Murphy, and A. O'Garra. 1993. Dendritic cells and macrophages are required for Th1 development of T cells from alpha beta TCR transgenic mice: IL-12 substitution for non-APC to stimulate IFN-gamma production is IFN-gamma-dependent. *Int. Immunol.* 5:1119.

MacGregor, R. R., J. D. Boyer, K. E. Ugen *et al.* 1998. First Human Trial of a DNA-based Vaccine for Treatment of Human Immunodeficiency Virus Type 1 Infection: Safety and Host Response. *J. Infect. Dis.* 178:92.

Mahlknecht, U., and G. Herbein. 2001. Macrophages and T-cell apoptosis in HIV infection: a leading role for accessory cells? *Trends Immunol.* 22:256.

Makela, P. H. 2000. Vaccines, coming of age after 200 years. *FEMS Microbiol. Rev.* 24:9.

Maldonado-Lopez, R., C. Maliszewski, J. Urbain, and M. Moser. 2001. Cytokines regulate the capacity of CD α (+) and CD α (-) dendritic cells to prime Th1/Th2 cells in vivo. *J. Immunol.* 167:4345.

Maldonado-Lopez, R., and M. Moser. 2001. Dendritic cell subsets and the regulation of Th1/Th2 responses. *Semin. Immunol.* 13:275.

Malone, J. G., P. J. Bergland, P. Liljestrom, G. H. Rhodes, and R. W. Malone. 1997. Mucosal immune responses associated with polynucleotide vaccination. *Behring Inst. Mitt.* 63.

Marie, J. C., J. Kehren, M. C. Trescol-Biemont, A. Evlashev, H. Valentin, T. Walzer, R. Tedone, B. Loveland, J. F. Nicolas, C. Roubourdin-Combe, and B. Horvat. 2001. Mechanism of measles virus-induced suppression of inflammatory responses. *Immunity* 14:69.

Marx, P. A., R. W. Compans, A. Gettie, J. A. Staas, R. M. Gilley, M. J. Mulligan, G. V. Yamshchikov, D. Chen, and J. H. Eldridge. 1993. Protection against SIV transmission with microencapsulated vaccine. *Science* 260:1323.

Mascola, J. R., and G. J. Nabel. 2001. Vaccines for the prevention of HIV-1 disease. *Curr. Opin. Immunol.* 13:489.

Matthews, D. J., C. L. Emson, G. L. McKenzie, H. E. Jolin, J. M. Blackwell, and A. N. McKenzie. 2000. IL-13 is a susceptibility factor for *Leishmania major* infection. *J. Immunol.* 164:1458.

Mbawuike, I. N., S. Pacheco, C. L. Acuna, K. C. Switzer, Y. Zhang, and G. R. Harriman. 1999. Mucosal immunity to influenza without IgA: an IgA knockout mouse model. *J. Immunol.* 162:2530.

McCluskie, M. J., and H. L. Davis. 1999. Mucosal immunization with DNA vaccines. *Microbes and Infection* 1:685.

McCluskie, M. J., and H. L. Davis. 2001. Oral, intrarectal and intranasal immunizations using CpG and non-CpG oligonucleotides as adjuvants. *Vaccine* 19:413.

McCluskie, M. J., R. D. Weeratna, A. M. Krieg, and H. L. Davis. 2000. CpG DNA is an effective oral adjuvant to protein antigens in mice. *Vaccine* 19:950.

McElrath, M. J., L. Corey, D. Montefiori, M. Wolff, D. Schwartz, M. Keefer, R. Belshe, B. S. Graham, T. Matthews, P. Wright, G. Gorse, R. Dolin, P. Berman, D. Francis, A. M. Duliege, D. Bolognesi, D. Stablein, N. Ketter, and P. Fast. 2000. A phase II study of two HIV type 1 envelope vaccines, comparing their immunogenicity

- in populations at risk for acquiring HIV type 1 infection. AIDS Vaccine Evaluation Group. *AIDS Res. Hum. Retroviruses* 16:907.
- McGuirk, P., and K. H. G. Mills. 2000. Direct anti-inflammatory effect of a bacterial virulence factor: IL-10-dependent suppression of IL-12 production by filamentous hemagglutinin from *Bordetella pertussis*. *Eur. J. Immunol.* 30:415.
- McInnes, I. B., J. A. Gracie, B. P. Leung, X. Q. Wei, and F. Y. Liew. 2000. Interleukin 18: a pleiotropic participant in chronic inflammation. *Immunol. Today* 21:312.
- Medzhitov, R., and C. Janeway. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 91:295.
- Mellado, M., M. Llorente, J. M. Rodriguez-Frade, P. Lucas, C. Martinez-A, and G. del Real. 1998. HIV-1 envelope protein gp120 triggers a Th2 response in mice that shifts to Th1 in the presence of human growth hormone. *Vaccine* 15:1111.
- Moore, A., P. McGuirk, S. Adams, W. C. Jones, J. P. McGee, D. T. O'Hagan, and K. H. G. Mills. 1995. Immunization with a soluble recombinant HIV protein entrapped in biodegradable microparticles induces HIV-specific CD8⁺ cytotoxic T lymphocytes and CD4⁺ Th1 cells. *Vaccine* 13:1741.
- Mor, G., G. Yamshchikov, M. Sedegah, M. Takeno, R. Wang, R. A. Houghten, S. Hoffman, and D. M. Klinman. 1996. Induction of neonatal tolerance by plasmid DNA vaccination of mice. *J. Clin. Invest.* 98:2700.
- Morokata, T., J. Ishikama, and T. Yamada. 2000. Antigen dose defines T helper 1 and T helper 2 responses in the lungs of C57BL/6 and BALB/c mice independently of splenic responses. *Immunol. Lett.* 72:119.
- Mosmann, T. R., and R. L. Coffman. 1989. Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv. Immunol.* 46:111.

- Mosmann, T. R., and S. Sad. 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today* 17:138.
- Moss, R. B., J. Diveley, F. C. Jensen, E. Gouveia, and D. J. Carlo. 2001. Human immunodeficiency virus (HIV)-specific immune responses are generated with the simultaneous vaccination of a gp120-depleted, whole-killed HIV-1 immunogen with cytosine-phosphorothioate-guanine dinucleotide immunostimulatory sequences of DNA. *J. Hum. Virol.* 4:39.
- Mountford, A. P., P. S. Coulson, A. W. Cheever, A. Sher, R. A. Wilson, and T. A. Wynn. 1999. Interleukin-12 can directly induce T-helper 1 responses in interferon (IFN-gamma) receptor-deficient mice, but requires IFN-gamma signal to downregulate T-helper 2 responses. *Immunology* 97:588.
- Mueller, D. L., M. K. Jenkins, and R. H. Schwartz. 1989. Clonal expansion versus functional clonal inactivation: a costimulation signalling pathway determines the outcome of T cell antigen receptor activation. *Annu. Rev. Immunol.* 7:445.
- Murphy-Corb, M., L. N. Martin, B. Davison-Fairburn, R. C. Montelaro, M. Miller, M. West, S. Ohkawa, G. B. Baskin, J.-Y. Zhang, S. D. Putney, A. C. Allison, and D. A. Eppstein. 1989. A formalin-inactivated whole SIV vaccine confers protection in macaques. *Science* 246:1293.
- Nakamura, K., H. Okamura, M. Wada, K. Nagata, and T. Tamura. 1989. Endotoxin-induced serum factor that stimulates gamma interferon. *Infect. Immun.* 57:590.
- Nelson, P. J., and M. Krensky. 1998. Chemokines, lymphocytes and viruses: what goes around comes around. *Curr. Opin. Immunol.* 10:265.
- Nishikomori, R., R. O. Ehrhardt, and W. Strober. 2000. T helper type 2 cell differentiation occurs in the presence of interleukin 12 receptor beta 2 chain expression and signalling. *J. Exp. Med.* 191:847.

- Noben-Trauth, N., J. Hu-Li, and W. E. Paul. 2000. Conventional, naive CD4⁺ T cells provide an initial source of IL-4 during Th2 differentiation. *J. Immunol.* 165:3620.
- Noll, A., N. Bucheler, E. Bohn, R. Schirmbeck, J. Reimann, and I. B. Autenrieth. 1999. DNA immunization confers systemic, but not mucosal, protection against enteroinvasive bacteria. *Eur. J. Immunol.* 29:986.
- Norman, J. A., P. Hobart, M. Manthorpe, P. Felgner, and C. Wheeler. 1997. Development of improved vectors for DNA-based immunization and other gene therapy applications. *Vaccine* 15:801.
- Oehen, S., T. Junt, C. Lopez-Macias, and T. A. N. Kramps. 2000. Antiviral protection after DNA vaccination is short lived and not enhanced by CpG DNA. *Immunology* 99:163.
- O'Garra, A. 1998. Cytokines Induce the Development of Functionally Heterogeneous T Helper Cell Subsets. *Immunity* 8:275.
- O'Garra, A., and K. Murphy. 1994. Role of cytokines in determining T-lymphocyte function. *Curr. Opin. Immunol.* 6:458.
- O'Hagan, D. T., and R. Rappuoli. 2001. Novel delivery systems for intranasal immunization with inactivated influenza vaccine. *Drug Delivery Systems and Sciences* 1:41.
- Okada, E., S. Sasaki, N. Ishii, I. Aoki, T. Yasuda, K. Nishioka, J. Fukushima, J. Miyazaki, B. Wahren, and K. Okuda. 1997. Intranasal immunization of a DNA vaccine with IL-12- and granulocyte-macrophage colony-stimulating factor (GM-CSF)-expressing plasmids in liposomes induces strong mucosal and cell-mediated immune responses against HIV-1 antigens. *J. Immunol.* 159:3638.

Okuda, K., H. Bukawa, K. Hamajima, S. Kawamoto, K. Sekigawa, Y. Yamada, S. Tanaka, N. Ishi, I. Aoki, and M. Nakamura. 1995. Induction of potent humoral and cell-mediated immune responses following direct injection of DNA encoding the HIV type 1 env and rev gene products. *AIDS Res. Hum. Retroviruses* 11:933.

Olszewska, W., C. D. Partidos, and M. W. Steward. 2000. Antipeptide antibody responses following intranasal immunization: effectiveness of mucosal adjuvants. *Infect. Immun.* 68:4923.

Olszyna, D. P., D. Pajkrt, F. N. Lauw, v. D. S.J., and T. van Der Poll. 2000. Interleukin 10 inhibits the release of CC chemokines during human endotoxemia. *J. Infect. Dis.* 181:613.

Oosterwegel, M. A., D. A. Mandelbrot, S. D. Boyd, R. B. Lorsbach, D. Y. Jarrett, and J. Abbas. 1999. The role of CTLA-4 in regulating Th2 differentiation. *J. Immunol.* 163:2634.

Openshaw, P., E. E. Murphy, N. A. Hosken, V. Maino, K. Davis, K. Murphy, and A. O'Garra. 1995. Heterogenicity of intracellular cytokine synthesis at the single-cell level in polarized T helper1 and T helper 2 populations. *J. Exp. Med.* 182:1357.

Orson, F. M., B. M. Kinsey, P. J. Hua, B. S. Bhogal, C. L. Densmore, and M. A. Barry. 2000. Genetic immunization with lung-targeting macroaggregated polyethyleneimine-albumin conjugates elicits combined systemic and mucosal immune responses. *J. Immunol.* 164:6313.

Ouyang, W., M. Lohning, Z. Gao, M. Assenmacher, S. Ranganath, A. Radbruch, and K. M. Murphy. 2000. Stat6-independant GATA-3 autoactivation directs IL-4-independant Th2 development and commitment. *Immunity* 12:27.

Oxenius, A., M. M. Martinic, H. Hengartner, and P. Klenerman. 1999. CpG-containing oligonucleotides are efficient adjuvants for induction of protective antiviral immune responses with T-cell peptide vaccines. *J. Virol.* 73:4120.

Pacheco, S. E., R. A. Gibbs, A. Ansari-Lari, and P. Rogers. 2000. Intranasal immunization with HIV reverse transcriptase: effect of differential induction of helper T cell type 1 and 2 immunity. *AIDS Res. Hum. Retroviruses* 16:2009.

Pancholi, P., Q. Liu, N. Tricoche, P. Zhang, M. E. Perkus, and A. M. Prince. 2000. DNA prime-canarypox boost with polycistronic hepatitis C virus (HCV) genes generates potent immune responses to HCV structural and nonstructural proteins. *J. Infect. Dis.* 182:18.

Parish, C. R., and F. Y. Liew. 1972. Immune responses to chemically modified flagellin III. Enhanced cell mediated immunity during high and low zone antibody tolerance to flagellin. *J. Exp. Med.* 135:298.

Park, A. Y., B. D. Handowicz, and P. Scott. 2000. IL-12 is required to maintain a Th1 responses during *Leishmania major* infection. *J. Immunol.* 165:896.

Parker, D. C. 1993. T Cell-Dependent B Cell Activation. *Annu. Rev. Immunol.* 11:331.

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

Peng, X., A. Kasran, P. A. Warmerdam, M. de Boer, and J. L. Cueppens. 1996. Accessory signaling by CD40 for T cell activation: induction of Th1 cell cytokines and synergy with interleukin-12 for interferon-gamma production. *Eur. J. Immunol.* 26:1621.

Perk, B. 2001. A role for the proteasome in HIV infection. *Trends Immunol.* 22:12.

Perrie, Y., P. M. Frederik, and G. Gregoriadis. 2001. Liposome-mediated DNA vaccination: the effect of vesicle composition. *Vaccine* 19:3301.

- Pinto, L. A., S. Sharpe, D. I. Cohen, and G. M. Shearer. 1998. Alloantigen-stimulated anti-HIV activity. *Blood* 92:3346.
- Pisetsky, D. S. 1997. Immunostimulatory DNA: A clear and present danger? *Nat. Med.* 3:829.
- Pitcher, C. J., C. Quittner, D. M. Peterson, M. Connors, R. A. Koup, V. C. Maino, and L. J. Picker. 1999. HIV-1-specific CD4⁺ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. *Nat. Med.* 5:518.
- Polack, F. P., S. H. Lee, S. Permar, E. Manyara, H. G. Nousari, Y. Jeng, F. Mustafa, A. Valsamakis, R. J. Adams, H. L. Robinson, and D. E. Griffin. 2000. Successful DNA immunization against measles: neutralizing antibody against either the hemagglutinin or fusion glycoprotein protects rhesus macaques without evidence of atypical measles. *Nat. Med.* 6:776.
- Porgador, A., K. R. Irvine, A. Iwasaki, B. H. Barber, N. P. Restifo, and R. N. Germain. 1998. Predominant role for directly transfected dendritic cells in antigen presentation to CD8⁺ T cells after gene gun immunization. *J. Exp. Med.* 188:1075.
- Prange, R., and M. Werr. 1999. DNA-mediated immunization to hepatitis B virus envelope proteins: preS antigen secretion enhances the humoral response. *Vaccine* 17:617.
- Pulendran, B., J. Banchereau, E. Maraskovsky, and C. Maliszewski. 2001. Modulating the immune response with dendritic cells and their growth factors. *Trends Immunol.* 22:41.
- Pulendran, B., J. L. Smith, G. Caspary, K. Brasel, D. Pettit, E. Maraskovsky, and C. R. Maliszewski. 1999. Distinct dendritic cell subsets differentially regulate the class of immune response *in vivo*. *Proc. Natl. Acad. Sci. USA* 96:1036.

- Putney, S. D., and P. A. Burke. 1998. Improving protein therapeutics with sustained-release formulations. *Nat. Biotech.* 16:153.
- Rask, C., J. Holmgren, M. Fredriksson, M. Lindblad, I. Nordstrom, J. B. Sun, and A. Czerkinsky. 2000. Prolonged oral treatment with low doses of allergen conjugated to cholera toxin B subunit suppresses immunoglobulin E antibody responses in sensitized mice. *Clin. Exp. Allergy.* 30:1024.
- Redfield, R. R., D. C. Wright, W. D. James, T. S. Jones, C. Brown, and D. S. Burke. 1987. Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. *N. Engl. J. Med.* 316:673.
- Reid, S. D., P. Guiseppe, and L. Adorini. 2000. The control of T cell responses by dendritic cell subsets. *Curr. Opin. Immunol.* 12:114.
- Ria, F., G. Penna, and L. Adorini. 1998. Th1 cells induce and Th2 inhibit antigen-dependent IL-12 secretion by dendritic cells. *Eur. J. Immunol.* 28:2003.
- Rissoan, M.-C., V. Soumelis, N. Kadowaki, G. Grouard, F. Briere, R. d. Malefyt, and Y.-J. Liu. 1999. Reciprocal Control of T Helper Cell and Dendritic Cell Differentiation. *Science* 283:1183.
- Robinson, H. L. 1997. Nucleic acid vaccines: an overview. *Vaccine* 15:785.
- Rogers, P. R., and M. Croft. 1999. Peptide dose, affinity, and time of differentiation can contribute to the cytokine balance. *J. Immunol.* 163:1205.
- Roy, K., H. Q. Mao, S. K. Huang, and K. W. Leong. 1999. Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat. Med.* 5:387.

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

Ruedl, C., M. F. Bachmann, and M. Kopf. 2000. The antigen dose determines T helper subset development by regulation of CD40 ligand. *Eur. J. Immunol.* 30:2056.

Sagodira, S., S. Iochmann, M. N. Mevelec, I. Dimier-Poisson, and D. Bout. 1999. Nasal immunizations of mice with *Cryptosporidium parvum* DNA induces systemic and intestinal immune responses. *Parasite Immunol.* 21:507.

Sakai, K., A. Yokoyama, N. Kohno, and K. Hiwada. 1999. Effect of different sensitizing doses of antigen in a murine model of atopic asthma. *Clin. Exp. Immunol.* 118:9.

Sallusto, F., C. R. Mackey, and A. Lanzavecchia. 2000. The role of chemokine receptors in primary, effector and memory immune responses. *Ann. Rev. Immunol.* 18:593.

Sambhara, S., A. Kurich, R. Miranda, A. Tame, R. Arpino, O. James, U. Guinness, A. Kandil, B. Underdown, M. Klein, and D. Burt. 1998. Enhanced immune responses and resistance against infection in aged mice conferred by Flu-ISCOMs vaccine correlate with up-regulation of co-stimulatory molecule CD86. *Vaccine* 18:1698.

Sanjiv, A., L. Cyster, and J. G. Cyster. 2001. Chemokines as regulators of T cell differentiation. *Nat. Immunol.* 2:102.

Sasaki, S., K. Hamajima, J. Fukushima, A. Ihata, N. Ishii, I. Gorai, F. Hirahara, H. Mohri, and K. Okuda. 1998. Comparison of Intranasal and Intramuscular Immunization against Human Immunodeficiency Virus Type 1 with a DNA-Monophosphoryl Lipid A Adjuvant Vaccine. *Infect. Immun.* 66:823.

Sato, M., K. Iwakabe, S. Kimura, and T. Nishimura. 1999. Functional skewing of bone marrow-derived dendritic cells by Th1- or Th2-inducing cytokines. *Immunol. Lett.* 15:63.

Sato, Y., M. Roman, H. Tighe, D. Lee, M. Corr, M.-D. Nguyen, G. J. Silverman, M. Lotz, D. A. Carson, and E. Raz. 1996. Immunostimulatory DNA Sequences Necessary for Effective Intradermal Gene Immunization. *Science* 273:352.

Scherle, P. A., G. Palladino, and W. Gerhard. 1992. Mice can recover from pulmonary influenza virus infection in the absence of class I-restricted cytotoxic T cells. *J. Immunol.* 148:212.

Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasserville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, and e. al. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 283:857.

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

Schottelius, A. J., M. W. Mayo, R. B. Sartor, and A. S. J. Baldwin. 1999. Interleukin-10 signaling blocks inhibitor of kappa B kinase activity and nuclear factor kappaB DNA binding. *J. Biol. Chem.* 274:31868.

Schultz, J., L. Heinzerling, J. Pavlovic, and K. Moelling. 2000. Induction of long-lasting cytokine effect by injection of IL-12 encoding plasmid DNA. *Cancer Gene Therapy* 7:1557.

Sedegah, M., R. Hedstrom, P. Hobart, and S. L. Hoffman. 1994. Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. *Proc. Natl. Acad. Sci. USA.* 91:9866.

Seder, R. A., R. Gazzinelli, A. Sher, and W. E. Paul. 1993. Interleukin 12 acts directly on CD4⁺ T cells to enhance priming for IFN-gamma production and diminishes interleukin 4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA* 90:10188.

Shearer, G. M., L. A. Pinto, and M. Clerici. 1999. Alloimmunization for immune-based therapy and vaccine design against HIV/AIDS. *Immunol. Today* 20:66.

Shi, Z., D. T. Curiel, and D. Tang. 1999. DNA-based non-invasive vaccination onto the skin. *Vaccine* 17:2136.

Shiver, J. W., M. E. Davies, Y. Yasutomi, H. C. Perry, D. C. Freed, N. L. Letvin, and M. A. Liu. 1997. Anti-HIV env immunities elicited by nucleic acid vaccines. *Vaccine* 15:884.

Sin, J., J. J. Kim, C. Pachuk, C. Satishchandran, and D. B. Weiner. 2000. DNA vaccines encoding interleukin-8 and RANTES enhance antigen-specific Th1-type CD4(+) T-cell-mediated protective immunity against herpes simplex virus type 2 in vivo. *J. Virol.* 74:11173.

Sin, J. I., J. Kim, C. Patchuk, and D. B. Weiner. 2000. Interleukin 7 can enhance antigen-specific cytotoxic-T-lymphocyte and/or Th2-type immune responses in vivo. *Clin. Diagn. Lab. Immunol.* 7:751.

Sin, J. I., J. J. Kim, R. L. Arnold, K. E. Shroff, D. McCallus, C. Pachuk, S. P. McElhiney, M. W. Wolf, S. J. Pompa-de Bruin, T. J. Higgins, R. B. Ciccarelli, and D. B. Weiner. 1999. IL-12 gene as a DNA vaccine adjuvant in a herpes mouse model. IL-12 enhances Th1-type CD4⁺ T cell-mediated protective immunity against herpes simplex virus-2 challenge. *J. Immunol.* 162:2912.

Sin, J. I., J. J. Kim, J. D. Boyer, R. B. Ciccarelli, T. J. Higgins, and D. B. Weiner. 1999. In vivo modulation of vaccine-induced immune responses toward a Th1 phenotype increases potency and vaccine effectiveness in a herpes simplex virus type 2 mouse model. *J. Virol.* 73:501.

- Sizemore, D. R., A. A. Branstrom, and J. C. Sadoff. 1997. Attenuated bacteria as a DNA delivery vehicle for DNA-mediated immunization. *Vaccine* 15:804.
- Smeltz, R. B., J. Chen, J. Hu-Li, and E. M. Shevach. 2001. Regulation of interleukin (IL)-18 receptor alpha chain expression on T cells during T helper (Th)1/Th2 differentiation. Critical downregulation of IL-4. *J. Exp. Med.* 194:143.
- Smith, H. A. 1994. Regulatory considerations for nucleic acid vaccines. *Vaccine* 12:1515.
- Sousa, C. R., A. Sher, and P. Kaye. 1999. The role of dendritic cells in the induction and regulation of immunity to microbial infection. *Curr. Opin. Immunol.* 11:392.
- Sparwasser, T., E. S. Koch, R. M. Vabulas, K. Heeg, G. B. Lipford, J. W. Ellwart, and H. Wagner. 1998. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* 28:2045.
- Sparwasser, T., and G. B. Lipford. 2000. Consequences of Bacterial CpG DNA-Driven Activation of Antigen-Presenting Cells. *Curr. Top. Microbiol. Immunol.* 247:59.
- Spier, R. E. 1995. Conference Report: Nucleic Acid Vaccines. *Vaccine* 13:131.
- Stan, A. C., S. Casares, T. D. Brumeanu, D. M. Klinman, and C. A. Bona. 2001. CpG motifs of DNA vaccines induce the expression of chemokines and MHC class II molecules on myocytes. *Eur. J. Immunol.* 31:301.
- Standiford, T. J., S. L. Kunkel, M. J. Greenberger, L. L. Laichalk, and R. M. Strieter. 1996. Expression and regulation of chemokines in bacterial pneumonia. *J. Leukoc. Biol.* 59:24.
- Stott, E. J., P. A. Kitchin, M. Page, B. Flanagan, L. F. Taffs, W. L. Chan, K. H. G. Mills, P. Silvera, and A. Rodgers. 1991. Anti-cell antibody in macaques. *Nature* 353:393.

Strasser, J. E., R. L. Arnold, C. Pachuk, T. J. Higgins, and D. I. Bernstein. 2000. Herpes simplex virus DNA vaccine efficacy: effect of glycoprotein D plasmid constructs. *J. Infect. Dis.* 182:1304.

Svirshchevskaya, E. V., L. G. Alekseeva, T. M. Andronova, and V. P. Kurup. 2001. Do T helpers 1 and 2 recognize different class II MHC molecules? Effect on cellular immune responses to soluble allergen from *Aspergillus fumigatis* f2. *Clin. Immunol* 100:349.

Szabo, S. J., A. S. Dighe, U. Gubler, and K. M. Murphy. 1997. Regulation of the interleukin (IL)-12R subunit expression in T helper 1 (Th1) and Th2 cells. *J. Exp. Med.* 185:817.

Takasawa, N., N. Ishii, N. Higashimura, K. Murata, Y. Tanaka, M. Nakamura, T. Sasaki, and K. Sugamura. 2001. Expression of gp34 (OX40 ligand) and OX40 on human T cell clones. *Jpn. J. Cancer. Res.* 92:377.

Takeda, K., M. Kamanaka, T. Tanaka, T. Kishimoto, and S. Akira. 1996. Impaired IL-13-mediated functions of macrophages in STAT6-deficient mice. *J. Immunol.* 157:3220.

Takeda, K., T. Tanaka, W. Shi, M. Matsumoto, M. Minami, S. Kashiwamura, N. Nakan, T. Kishimoto, and S. Akira. 1996. Essential role of Stat6 in IL-4 signaling. *Nature* 380:627.

Takeda, K., H. Tsutsui, T. Yoshimoto, O. Adachi, N. Yoshida, T. Kishimoto, K. Okamur, and S. Akira. 1998. Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity* 8:383.

Takenaka, H., S. Maruo, N. Yamamoto, M. Wysocka, S. Ono, M. Kobayashi, H. Yagita, K. Okumura, T. Hamaoka, G. Trinchieri, and H. Fujiwara. 1997. Regulation of T cell-dependent and -independent IL-12 production by the three Th2-type cytokines IL-10, IL-6, and IL-4. *J. Leukoc. Biol.* 61:80.

- Takeshita, F., and D. M. Klinman. 2000. CpG ODN-mediated regulation of IL-12 p40 transcription. *Eur. J. Immunol.* 30:1967.
- Tang, D. L., M. Devit, and S. A. Johnston. 1992. Genetic immunization is a simple method for eliciting an immune response. *Nature* 356:152.
- Tang, F., and J. A. Hughes. 1998. Introduction of a disulphide bond into a cationic lipid enhances transgene expression of plasmid DNA. *Biochem. Biophys. Res. Commun.* 242:141.
- Tanghe, A., O. Denis, B. Lambrecht, V. Motte, T. van den Berg, and K. Huygen. 2000. Tuberculosis DNA vaccine encoding Ag85A is immunogenic and protective when administered by intramuscular needle injection but not by epidermal gene gun bombardment. *Infect. Immun.* 68:3854.
- Tanghe, A., S. D'Souza, V. Rosseels, O. Denis, T. H. Ottenhoff, W. Dalemans, C. Wheeler, and K. Huygen. 2001. Improved immunogenicity and protective efficacy of a tuberculosis DNA vaccine encoding Ag85 by protein boosting. *Infect. Immun.* 69:3041.
- Taoufik, Y., O. Lantz, C. Wallon, A. Charles, E. Dussaix, and J. F. Delfraissy. 1997. Human Immunodeficiency Virus gp120 Inhibits Interleukin-12 Secretion by Human Monocytes: An Indirect Interleukin-10-Mediated Effect. *Blood* 89:2842.
- Tau, G. Z., T. von der Weid, B. Lu, S. Cowan, M. Kvatyuk, A. Pernis, G. Cattoretti, N. S. Braunstein, R. L. Coffman, and P. B. Rothman. 2000. Interferon gamma signaling alters the function of T helper type 1 cells. *J. Exp. Med.* 192:977.
- Templeton, N. S., D. D. Lasic, P. M. Frederik, H. H. Strey, D. D. Roberts, and G. N. Pavlakis. 1997. Improved DNA:liposomes complexes for increased systemic delivery and gene expression. *Nat. Biotech.* 15:647.
- Tighe, H., M. Corr, M. Roman, and E. Raz. 1998. Gene vaccination: plasmid DNA is more than just a blueprint. *Immunol. Today* 19:89.

Toda, S., N. Ishii, E. Okada, K. I. Kusakabe, H. Arai, K. Hamajima, I. Gorai, K. Nishioka, and K. Okuda. 1997. HIV-1-specific cell-mediated immune responses induced by DNA vaccination were enhanced by mannan-coated liposomes and inhibited by anti-interferon-gamma antibody. *Immunology* 92:111.

Torres, C. A., A. Iwasaki, B. H. Barber, and H. L. Robinson. 1997. Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. *J. Immunol.* 158:4529.

Tortorella, D., B. E. Gewurz, M. H. Furman, D. J. Schust, and H. L. Ploegh. 2000. Viral subversion of the immune system. *Ann. Rev. Immunol.* 18:861.

Treanor, J. J., and R. F. Betts. 1998. Evaluation of live, cold-adapted influenza A and B virus vaccines in elderly and high-risk subjects. *Vaccine* 18:1756.

Tuting, T., W. J. Storkus, and L. D. Falo Jr. 1998. DNA Immunization Targeting the Skin: Molecular Control of Adaptive Immunity. *J. Invest. Dermatol.* 111:183.

Ugen, K. E., J. D. Boyer, B. Wang, M. Bagarazzi, A. Javadian, P. Frost, M. M. Merva, M. G. Agadjanyan, S. Nyland, W. V. Williams, L. Coney, R. Ciccarelli, and D. B. Weiner. 1997. Nucleic acid immunization of chimpanzees as a prophylactic/immunotherapeutic vaccination model for HIV-1: prelude to a clinical trial. *Vaccine* 15:927.

Ulmer, J. B., R. R. Deck, C. M. DeWitt, T. M. Fu, J. J. Donnelly, M. J. Caulfield, and M. A. Liu. 1997. Expression of a viral protein by muscle cells in vivo induces protective cell-mediated immunity. *Vaccine* 15:839.

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

Ulmer, J. B., J. C. Sadoff, and M. A. Liu. 1996. DNA vaccines. *Curr. Opin. Immunol.* 8:531.

Vahlsing, H. L., M. A. Yankauckas, M. Sawdey, S. H. Gromkowski, and M. Manthorpe. 1994. Immunization with plasmid DNA using a pneumatic gun. *J. Immunol. Meth.* 175:11.

Verthelyi, D., K. Ishii, M. Gursel, F. Takeshita, and D. Klinman. 2001. Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. *J. Immunol.* 166:2372.

Vieira, P. L., E. C. de Jong, E. A. Wierenga, M. L. Kapsenberg, and P. Kalinski. 2000. Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. *J. Immunol.* 164:4507.

Walker, P. S., T. Scharton-Kersten, A. M. Krieg, L. Love-Howman, E. D. Rowton, and J. C. Vogel. 1999. Immunostimulatory oligonucleotides promote protective immunity and provide systemic therapy for leishmanias via IL-12 and IFN-gamma-dependant mechanisms. *Proc. Natl. Acad. Sci. USA* 96:6970.

Wang, B., K. E. Ugen, V. Srikantan, M. G. Agadjanyan, K. Dang, Y. Refaeli, A. I. Sato, J. Boyer, W. V. Williams, and D. B. Weiner. 1993. Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* 90:4156.

Warren, T. L., S. K. Bhatia, A. M. Acosta, C. E. Dahle, T. L. Ratliff, A. M. Krieg, and G. J. Weiner. 2000. APC stimulated by CpG oligodeoxynucleotide enhance activation of MHC class I-restricted T cells. *J. Immunol.* 165:6244.

Webster, R. G., E. F. Fynan, J. C. Santoro, and H. Robinson. 1994. Protection of ferrets against influenza challenge with a DNA vaccine to the haemagglutinin. *Vaccine* 12:1495.

Weeratna, R., C. L. Brazolot Millan, A. M. Krieg, and H. L. Davis. 1998. Reduction of antigen expression from DNA vaccines by coadministered oligodeoxynucleotides. *Antisense Nucleic Acid Drug Dev.* 8:351.

Wesa, A. K., and A. Galy. 2001. Regulation of T cell cytokine production by dendritic cells generated in vitro from hematopoietic progenitor cells. *Cell Immunol.* 208:115.

Wolff, J. A., J. J. Ludtke, G. Acsadi, P. Williams, and A. Jani. 1992. Long term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum. Mol. Genet.* 1:363.

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

Wu, Y., X. Wang, K. L. Csencsits, A. Haddad, N. Walters, and D. W. Pascual. 2001. M cell-targeted DNA vaccination. *Proc. Natl. Acad. Sci.* 98:9318.

Xu, D., and F. Y. Liew. 1995. Protection against leishmaniasis by injection of DNA encoding a major surface glycoprotein, gp63, of *L. major*. *Immunology* 84:173.

Xu, Y., and F. C. Szoka Jr. 1996. Mechanism of DNA Release from Cationic Liposomes/DNA Complexes Used in Cell Transfection. *Biochemistry* 35:5616.

Yamamoto, M., J. L. Vancott, N. Okahashi, M. Marinaro, H. Kiyono, K. Fujihashi, R. J. Jackson, S. N. Chatfield, H. Bluethmann, and J. R. McGhee. 1996. The role of Th1 and Th2 cells for the mucosal IgG responses. *Ann. N.Y. Acad. Sci.* 778:64.

Yang, X., R. S. Gieni, T. R. Mosmann, and K. T. HayGlass. 1993. Chemically modified antigen preferentially elicits induction of Th1-like cytokine synthesis patterns in vivo. *J. Exp. Med.* 178:349.

Yi, A. K., D. M. Klinman, T. L. Martin, S. Matson, and A. M. Krieg. 1996. Rapid Immune Activation by CpG Motifs in Bacterial DNA. *J. Immunol.* 157:5394.

Yoshimoto, T., K. Takeda, T. Tanaka, K. Ohkusu, S. Kashiwamura, H. Okamura, and K. Nakanishi. 1998. IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells and synergism with IL-18 for IFN-gamma production. *J. Immunol.* 161:3400.

Zabner, J., A. J. Fasbender, T. Moninger, K. A. Poellinger, and M. J. Welsh. 1995. Cellular and Molecular Barriers to Gene Transfer by a Cationic Lipid. *J. Biol. Chem.* 270:18997.

Zhang, Y., R. Apilado, J. Coleman, S. Ben-Sasson, S. Tsang, J. Hu-Li, and W. E. Paul. 2001. Interferon gamma stabilizes the T helper cell type 1 phenotype. *J. Exp. Med.* 194:165.

Zhang, Y. Q., R. J. Joost van Neerven, S. W. Van Gool, L. Coorevits, M. de Boer, and Ceup. 1997. B7-CD28 interaction is a late acting co-stimulatory signal for human T cell responses. *Int. Immunol.* 9:1095.

Zou, W., J. Borvak, F. Marches, S. Wei, P. Galanaud, D. Emilie, and T. J. Curiel. 2000. Macrophage-derived dendritic cells have strong Th1-polarizing potential mediated by beta-chemokines rather than IL-12. *J. Immunol.* 165:4388.