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ENHANCEMENT OF DNA IMMUNIZATION AGAINST HEPATITIS B FOLLOWING COEXPRESSSION OF COSTIMULATORY MOLECULES

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0-612-57082-7
In Memory of my Grand Aunt

V. Saraswathi
ABSTRACT

The use of plasmid DNA encoding antigens has given rise to a novel class of vaccines, that may overcome many of the disadvantages associated with classical antigen-based vaccines. This thesis examines specific methods of optimizing immune responses directed against the hepatitis B virus surface antigen (HBsAg) encoded by a plasmid expression system in BALB/c mice. Interaction of B7.1 and B7.2 with their receptor CD28/CTLA-4 molecules on T cells is known to facilitate progression of the T cell through the cycle of immune activation. We have determined whether coexpression of costimulatory molecules, B7.1 or B7.2, along with HBsAg from a DNA vaccine, administered as separate plasmids (codelivery) or encoded within the same expression vector (colinear expression), can augment HBsAg-specific humoral and cell-mediated responses. Intramuscular (IM) coexpression of B7.1 or B7.2 along with the DNA vaccine significantly enhanced cytotoxic T lymphocyte (CTL) responses whereas coexpression of B7.2 alone enhanced CTL responses with intradermal (ID) administration. However, there was no concomitant increase in humoral responses with coexpression by either route of administration. In contrast colinear expression of B7.1 or B7.2 significantly enhanced humoral as well as CTL responses with both IM and ID routes of administration. Furthermore, the Th2 bias that is seen with ID administration is skewed towards a Th1 response with B7 coexpression. The kinetics of plasmid DNA distribution in various anatomical compartments were also studied in order to address the differences in immune responses noted with IM and ID routes of administration. Collectively the results suggest that B7 coexpression can augment or alter responses to DNA vaccines and might prove effective for immunization in humans.
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INTRODUCTION

OVERVIEW

Humankind has witnessed tremendous progress in the field of vaccinology, and this has significantly reduced morbidity and mortality. Vaccine development may be classified into "generations" of progress with the first and second generations comprising live attenuated/whole killed and recombinant protein vaccines respectively. A recent, third generation approach to immunization is DNA-based vaccination, which is thought to hold enormous future potential in diverse fields.

This thesis focuses on the optimization of a DNA vaccine against hepatitis B with the use of costimulatory molecules as genetic adjuvants. The B7 family of costimulatory molecules are critical entities that contribute to the initiation and sustenance of productive immunity. In many studies the focus has been on manipulating the expression of costimulatory molecules in order to block the onset and progression of disease (e.g., allergic encephalomyelitis, non-insulin dependent diabetes mellitus). In contrast, here we are evaluating the use of costimulatory molecules for the possibility of transforming DNA vaccine transfected cells into professional antigen presenting cells (APCs). The possible mechanisms of immune enhancement with B7 coexpression will also be discussed herein. When I commenced my graduate work in this field, the use of costimulatory molecules as adjuvants for DNA vaccines had not been shown. However, three groups have since demonstrated the efficacy of using B7 molecules to augment immune responses in two infectious disease models, namely HIV and influenza.

This introductory chapter reviews three main subjects that are integral to this study: (i) DNA-based immunization, (ii) role of costimulatory molecules in immune
responses, and (iii) infection with the hepatitis B virus (HBV). At the end of the chapter, the experimental objectives and the hypotheses to this study are outlined.

1.1 Vaccines

The two public health interventions that have had the greatest impact on the world's health are clean water and vaccines. Indeed, the global impact of vaccination is profound, with an estimated 80% of the 130 million children born each year being immunized before their first birthday (1). Vaccination is a deliberate attempt to protect humans against disease by mobilizing the immune system to combat a specific disease.

Jules Bordet defines the process of immunization as follows:

Since a first attack, which strengthens the host, often provides valuable safeguards for the future, it must be considered desirable, on condition, of course that it does not produce too much serious damage. Artificially putting the host into a state comparable to that in which it would be if it had been cured of a spontaneous attack of the particular illness is the object of active immunization or vaccination

Jules Bordet,
*Traite de l'immunite dans les Maladies Infectieuses, 1920*

1.1.1 History

Attempts to vaccinate are almost as old as man's attempts to rid himself of disease (2). The first written record of vaccination comes from a Buddhist nun (1022 to 1063 AD) who recommended treating smallpox with dried scabs from mildly infected individuals (2). In addition, several other cultures including Indian Buddhists and Irano-Indians practiced toxoid-like immunization and variolation as preventative medicine (2). The first scientific demonstration of a vaccine was in 1796 when Edward Jenner, a British physician, infected a young boy with cowpox and found him protected from subsequent challenge of smallpox (3). Jenner's process came to be called vaccination,
after vacca, the Latin word for cow, and the substance used to vaccinate was called a vaccine. With the work by Louis Pasteur, who is credited with the development of the first man-made vaccine for rabies, the field of vaccinology gained momentum (2). The “Golden Age” of vaccine development began in 1949 with new discoveries and improved techniques, and involved an explosion of creative activity in vaccinology. Drs. Jonas Salk and Albert Sabin were credited with the development of inactivated and live-attenuated polio vaccines respectively – two of the most famous products developed during this period (1). Other discoveries include the widely used vaccines against measles, mumps, rubella, hepatitis B and *Haemophilus influenzae* type b (1). With the more recent advancement of molecular biology, the focus in vaccine development has been on genetic engineering of safer subunit vaccines to replace the conventional whole killed or attenuated vaccines. Now, DNA vaccines come as the most recent product of this molecular age of vaccine development.

Current vaccine development efforts are directed towards combating emerging diseases (e.g. AIDS) and reemerging diseases (e.g. antibiotic resistant bacteria) as well as improving vaccines to be safer, more efficacious and less expensive (3). Over a 10-year period prior to August 1995, United Nations Children's Fund (UNICEF) supplied 8 billion doses of vaccine to over 100 countries (1). The original six vaccines administered by the Expanded Programme on Immunization (EPI) of the World Health Organization (WHO) cost a total of US$1 per dose (4). Despite this tremendous success achieved by these joint programmes, approximately 2 million children still die each year of diseases that can be prevented by vaccines offered by the EPI (4). One important
challenge that researchers face today is to overcome the high cost-factor associated with the generation of new vaccines to make them accessible to developing countries.

1.1.2 Immune Responses to a Vaccine

Development of immunity is a complex, multifaceted phenomenon that involves the participation of several cell populations, soluble molecules and cell surface molecules. Immune responses can be broadly divided into humoral and cell-mediated immunity (CMI). Humoral immunity is associated with B cells, which function to recognize chemically diverse antigens (e.g., proteins, polysaccharides) in a variety of contexts (cell- or pathogen-associated or soluble) and secrete antigen-specific antibodies (5). B cells are stimulated upon meeting circulatory antigen, and this is further enhanced by cytokines that are secreted by T cells and APCs. Cellular immune responses are dependent upon the antigen being presented to the immune system by professional APCs (6). These cells engulf foreign antigen or organisms, enzymatically process that antigen and present it in the context of major histocompatibility complexes (MHC) (7). Subsequently, such activated APCs migrate to draining lymph nodes where specific activation of T lymphocytes occurs (8). Professional APCs that are of bone marrow origin, including dendritic cells and macrophages, express surface molecules that interact with T lymphocytes (7). The ligand for the T cell receptor (TCR) is a MHC molecule which has antigen-derived peptide bound into a specialized groove. Typically, synthesis of proteins within the cytoplasm results in MHC class I presentation while exogenous antigen, engulfed and processed in the lysosome leads to MHC class II presentation. T lymphocytes can be either (i) “helper” T cells (Th - CD4+) which secrete cytokines that augment both humoral and cellular immune responses or
(ii) “cytolytic” T cells (CTL - CD4+ or CD8+) that can act directly, by cytolysis or indirectly by production of cytokines which eradicate and control infected cells. T- helper cells are heterogeneous and are broadly classified on the basis of cytokines they produce: i) T-helper type 1 (Th1: interleukin (IL)-2, Interferon (IFN)-γ, lymphotoxin) and ii) T-helper type 2 (Th2: IL-4, IL-5, IL-6, IL-10, IL-13) (9). Th1 cells are typically associated with CMI (i.e., CTL, delayed-type hypersensitivity, certain isotypes of IgG such as IgG2a) and Th2 cells are associated with other types of humoral responses (i.e., IgG1 and IgE which are associated with asthma and allergy) (10). Several factors influence the polarization of the CD4+ cell response toward a Th1, Th2 or mixed immune response including i) genetic factors; ii) the type of APC; iii) the amount of stimulating antigen; and iv) the type of costimulatory molecule (11).

In order to develop an effective vaccine against a particular pathogen, it is desirable to understand the exact nature of immune responses required to combat that pathogen. Insofar as neutralizing antibodies are capable of providing protection against some pathogens, it is still preferable to induce cell-mediated immune responses (Th1), and in cases where pathogens reside intracellularly, these may be an absolute requirement. When antigen is presented by both class I and class II molecules, a combination of antibody-producing B cells and cytotoxic T cells are raised. However, if the antigen originated outside of a cell (i.e., exogenous antigen), and is presented only by class II, the immune responses are largely limited to T-helper cells and antibody production. Strategies for induction of strong cytolytic CD8+ mediated responses are still necessary (e.g., viral or bacterial vectors, DNA vaccines) as these CTLs can effectively eliminate pathogen-infected cells. Most recombinant proteins and synthetic
peptides are processed solely by the MHC class II pathway and as a result evoke a CD4+ cell response but not a CD8+ response (6).

1.1.3 Types of Vaccines

Rational design of vaccines is desirable so that they elicit protective immunity both in quantitative and qualitative terms, with minimal adverse effects. Current vaccines may be broadly classified as: i) live attenuated vaccines; ii) inactivated vaccines; iii) purified component vaccines; iv) recombinant proteins; and v) DNA vaccines, as outlined in Table 1 (3).

1.1.4 Adjuvants

Adjuvants (from the Latin, *adjuvare* = to help) have been vaguely defined as any substance that increases the immunogenicity of admixed antigens (12, 13). Adjuvants can be broadly classified into four categories based on the functions they fulfill, although most adjuvants serve more than one function (13). These are i) immune stimulation, ii) enhancement of antigen presentation, iii) targeting, and iv) depot generation. Such mechanisms may aid in delivery or proper exposure of antigens to APC or may serve to modulate the nature of the immune response being induced (13).
<table>
<thead>
<tr>
<th>Type of Vaccine</th>
<th>Type of Response</th>
<th>Strengths</th>
<th>Comment</th>
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<tr>
<td>Live attenuated vaccines</td>
<td>Th1  Th2  CTL  Ab</td>
<td>• cellular &amp; humoral immunity</td>
<td>• reversion to virulent forms</td>
</tr>
<tr>
<td>e.g., polio (oral) measles rubella</td>
<td>+    +    +    +</td>
<td></td>
<td>• safety in immunocompromised</td>
</tr>
<tr>
<td>Inactivated vaccines</td>
<td>Th1  Th2  CTL  Ab</td>
<td>• case of preparation</td>
<td>• poor immunogenicity</td>
</tr>
<tr>
<td>e.g., hepatitis A rabies</td>
<td>+    -    +    +</td>
<td>• low cost</td>
<td>• incomplete inactivation</td>
</tr>
<tr>
<td>Purified component vaccines</td>
<td>Th1  Th2  CTL  Ab</td>
<td>• humoral immunity</td>
<td>• limited or no CTL</td>
</tr>
<tr>
<td>e.g., typhoid (Vi) meningococcus</td>
<td>+    -/+   +    +</td>
<td>• safety</td>
<td>• risk of incomplete purification</td>
</tr>
<tr>
<td>Recombinant proteins</td>
<td>Th1  Th2  CTL  Ab</td>
<td>• humoral immunity</td>
<td>• poor cellular immunity</td>
</tr>
<tr>
<td>e.g., hepatitis B</td>
<td>+    -/+   +    +</td>
<td>• safety</td>
<td>• difficulty in preparation</td>
</tr>
<tr>
<td>DNA vaccines</td>
<td>Th1  Th2  CTL  Ab</td>
<td>• cellular &amp; humoral immunity</td>
<td>• requires adjuvants</td>
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<td></td>
<td>+    +    +    +</td>
<td>• case of preparation</td>
<td>• requires refrigeration</td>
</tr>
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<td></td>
<td>• low cost</td>
<td>• cost</td>
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Table adapted from Chatergoon et al. (14)
1.2 DNA-Based Immunization

1.2.1 Background

The immunological potential of DNA inoculation was first demonstrated by Atanasiu et al. (271) who showed that DNA transforming sequences from polyoma virus resulted in the generation of antibodies and regression of tumours. However, the demonstration by Wolff and colleagues, in 1990, of prolonged protein expression following intramuscular (IM) injection of plasmid DNA initiated a new era in vaccinology called genetic- or DNA-based immunization (15). It was subsequently shown that if the plasmid encoded an antigenic protein, antigen-specific immune responses were induced (16, 17). To date, preclinical studies have shown humoral and T cell responses protective against a wide range of viruses, bacteria and parasites as well as in various tumour models (reviewed in (18)). In infectious disease models, where it was possible to evaluate, these immune responses have provided protection from challenge (17, 19-23). DNA vaccines have been applied to a wide range of species including fish (24), rodents (25-28), domesticated farm animals (e.g., pigs, and cows) (21, 29, 30), companion animals (31-34), non-human primates (35-37) and humans (38-40). From a practical standpoint, they offer ease, speed and low cost of manufacturing, the possibility to make multivalent vaccines and stability at ambient temperature (41).

1.2.2 DNA Vaccine Design

DNA vaccines contain several essential features that enable expression of the antigen in vivo including: i) a gene encoding the antigen; ii) a ubiquitous promoter (usually derived from the cytomegalovirus [CMV] or Rous sarcoma virus [RSV]); iii) mRNA transcript polyadenylation/termination sequence (18, 42); iv) an origin of
replication for the amplification of the plasmid in bacteria and v) a gene for selection such as antibiotic resistance (Figure 1). A DNA vaccine is most effective when it is supercoiled (43).

**Figure 1: Components of a DNA Vaccine**
Plasmid DNA for immunization consists of plasmid backbone that delivers adjuvant and mitogenic activity via immunostimulatory sequences and a transcription unit comprising a promoter, antigen cDNA and poly-adenylation (A), which together direct protein synthesis.

1.2.3 DNA Uptake and Expression

There is evidence to suggest that DNA can enter cells by either receptor-mediated or non-receptor mediated endocytosis (44, 45). As such, the DNA captured in the phagosome may be expected to undergo degradation in the secondary lysosomes.
Yet, because the protein encoded by the plasmid or immune responses against it can subsequently be detected, this cannot be the only pathway of uptake. Two ways by which DNA may gain entry into the nucleus are (42):

(i) The DNA may escape from the lysosomotropic pathway to enter the nucleus, possibly via internal receptors, in which case, escape might proceed spontaneously or might be induced due to the presence of DNA.

(ii) There may be pathways to shunt the DNA from the phagosome to the cytosol, such as that by which endocytosed protein antigens are shunted to the cytosol from the phagosome.

Regardless of the mechanism, uptake of plasmid DNA into the host cells results in some being used for endogenous synthesis of antigen and this in turn allows effective induction of MHC class I restricted CTL response - a mechanism strongly mimicking antigen presentation following viral infection (46).

1.2.4 Antigen Presentation following DNA Immunization

One of the areas of focus with DNA-based immunization is the study of cells responsible for immune induction (i.e., APCs or cells at the site of administration). In the past, muscle was the preferred site for DNA administration because several studies had demonstrated its superiority over other sites of injection for uptake and expression of plasmid DNA (47, 48). Expression of a non-immunogenic reporter gene (e.g., luciferase) in post mitotic cells such as the muscle fibre may continue for 19 months in a mouse (49). However, recent studies demonstrate that when an antigen is expressed, the transfected muscle fibres are destroyed by an immune mediated mechanism within 10-20 days after DNA immunization (50). These observations lead to the question of the
importance of non-migratory cells at the site of injection in the induction of an immune response. Recent experiments show MHC class I priming can only proceed with bone marrow-derived APCs such as dendritic cells, although direct transfection of APC is not an absolute requirement (51-53). Some postulate that the plasmid or plasmid-containing cells that rapidly leave the site of vaccination induce primary responses (54, 55). In support of this, surgical ablation of the muscle bundle even 5 min after an IM injection does not impair the magnitude and longevity of the response (55). In contrast, following gene gun (GG) administration or injection by the ID route, the integrity of the skin was required to be maintained for at least 72 h (55). The differences in these observations have been attributed to the rate of plasmid migration from the site of injection to the proximal lymph nodes. Following IM injections, plasmid per se can leave muscle rapidly via the lymphatic and circulatory systems, however following ID injections, the APCs at the site of injection must migrate to the local lymph nodes which is dependent on the maturation of these cells. The role of myocytes or keratinocytes in the induction of an immune response following DNA injections appears to be minimal (52, 53).

1.2.5 Immune Modifying Factors

A large number of factors appear to influence the Th-bias of the response following DNA-based immunization. These include the antigen and form of antigen (i.e., secreted or membrane bound) expressed, the dose, the immunostimulatory sequences of the plasmid backbone, other adjuvants used, the route and method of immunization, the number of administrations and the timing of boosts (discussed below).
1.2.5.1 Immunostimulatory motifs

The presence of unmethylated cytosine-guanine (CpG) dinucleotides in bacterial DNA has been shown to be a factor influencing the efficacy of DNA vaccines (56, 57). CpG dinucleotides in the context of specific nucleotide sequences (CpG motifs) have stimulatory or inhibitory properties (58, 59). These effects are not found with vertebrate DNA because of the greatly reduced frequency of CpG dinucleotides and methylation of the cytosines that renders the sequence non-stimulatory (60). Immune activation by CpG motifs appears to be an evolutionary adaptation that allows rapid responses in the presence of bacterial infection (61, 62). Immune activation can also be induced by oligodeoxynucleotides (ODN) that contain CpG motifs (CpG ODN). Stimulatory CpG (sCpG) motifs induce a spectrum of immune responses including: i) B cell proliferation and Ig secretion; ii) direct activation of monocytes, macrophages, and dendritic cells resulting in upregulation of costimulatory molecule expression and cytokine secretion (58, 63-65). These effects, in turn, stimulate natural killer cells to secrete IFN-γ and have increased lytic activity (66, 67). Overall, a Th1 pattern of cytokine production, predominated by IL-12 and IFN-γ, are induced with sCpG motifs (63). The presence of sCpG motifs within the plasmid backbone may explain, in part, the tendency of DNA vaccines to induce Th1 responses, although other factors may also contribute to this effect (detailed in following sections).

In recent years, several groups have shown that the addition of CpG motifs into the plasmid can augment the immune response to encoded antigen (56, 57, 68, 69). To take advantage of the adjuvant properties of CpG motifs, it seems necessary that they have to be cloned into the DNA vaccine as studies done by Weeratna et al. (70)
demonstrated that synthetic CpG ODN coadministered with DNA vaccines attenuates the efficacy of the vaccine. This appears to be due to competitive inhibition of DNA entry into the cell since synthetic phosphorothioate ODN bind with greater affinity to cell surface binding sites than natural phosphodiester DNA such as plasmids (71).

Neutralizing CpG motifs have been shown to reduce effects of sCpG motifs, in vivo and in vitro (59). In order to improve the efficacy of plasmid DNA, expression vectors have been reconstructed with the removal of the neutralizing motifs in the noncoding regions of the plasmid (59). Removal of the neutralizing motifs and further addition of stimulatory motifs into the expression plasmid enhances the antigen-specific Th1-like responses (59).

Immunostimulatory properties of CpG motifs suggest that these may be used as immune adjuvants for vaccines. Indeed, coadministration of ODN containing stimulatory CpGs along with antigenic components have been shown to be highly effective in inducing systemic (72, 73) as well and mucosal immunity (74, 75). In addition, the presence of CpG ODNs also contributed to the development of a predominantly Th1-like response characterized by a high IgG2a:IgG1 ratio and good CTL activity.

1.2.5.2 DNA vaccine delivery – route and method

The route and method of delivery of DNA vaccines influence the nature and strength of the induced immune response (76). Plasmid DNA can be delivered in an aqueous solution, or coated onto gold particles or with various delivery systems such as cationic liposomes or microspheres. Delivery of plasmid DNA is most commonly through direct IM or intradermal (ID) injection or by intraepidermal delivery of DNA-
coated gold particles using a GG (a biolistic device) (46) although other invasive and noninvasive routes have also been reported. These include (adapted from (77)):

   i) into the muscle by GG or IM injection (23, 78-83);
   ii) into the skin by epidermal GG delivery, ID (17, 84, 85) or subcutaneous injection (85, 86);
   iii) into the circulatory system by intravenous injection (85, 87, 88);
   iv) into the respiratory system by intranasal (85, 89-91) or intratracheal delivery (85);
   v) into the digestive system by oral feeding of microencapsulated (92, 93) or lipid-formulated DNA (26), injection into the oral mucosa (26), tongue (94), jejunum (95) or Peyer’s patches of the bowel (95) and GG delivery into the tongue (96) or oral mucosa (31);
   vi) into the genitourinary tract by intravaginal injection (95) or instillation (97, 98);
   vii) topical ocular administration (99).

The orientation of the CD4+ T cell responses is one aspect that is influenced by the method and site of DNA immunization (76). Generally, IM needle injections favour a Th1 response while the GG method promotes a Th2 response (78, 100-102). The amount of DNA used in these respective methods of immunization may, at least in part, account for the Th-bias that is observed. As needle injections comprise a larger quantity of DNA in comparison to gene gun delivery, there are relatively more CpG motifs, to promote Th1-responses (100). Evidence of this was provided by Barry et al. who demonstrated that very small amounts of DNA used by GG and IM needle injection
induce predominantly IgG1 – a Th2 associated isotype (100). However, a contradictory report by Feltquate et al. demonstrated that equivalent amount of DNA by injection and GG administration produce Th1 and Th2 type responses respectively (78). Thus, even when there is parity in the doses of DNA used, the two methods of immunization appear to invoke their preferential Th responses. Furthermore, needle injection by the ID route, even with large doses, induces a predominantly Th2 response (103, 104). Therefore, at present, most studies point to gene gun administration to the muscle or skin invoking a predominantly Th2 response. In contrast, with needle injections, administration to the muscle and skin induces Th1 and Th2 biased responses respectively.

1.2.5.3 Form of antigen

Earlier studies reported that the nature of the antigen (i.e., membrane bound, secreted) could influence the magnitude and Th-bias of an immune response. However, immune responses in mice against the rabies G-protein or HBsAg were similar with secreted and membrane-bound forms (105, 106). Other studies have shown that antigens that remain cytoplasmic induce lower antibody and CTL response in mice than secreted forms (107). Thus, evidence exists that the nature of the antigen may have an impact on the efficacy of the vaccine, but not necessarily in all situations.

1.2.5.4 Immunization regimen

The dose (100), the number of doses (103, 108, 109) and the resting period between immunizations (101, 110) are all factors that can affect the amplitude and nature of the immune response with DNA vaccines. However, there exists little consensus on the relative importance of these variables. Doses for injected DNA vaccines commonly range from 1 – 100 µg in murine models and 400 µg – 1 mg in
primate studies (76). It was initially assumed that DNA vaccines would behave like traditional vaccines and that an effective dose of vaccine would not be proportional to body size (38). This may, at least in part, explain why results in man and non-human primates have not been as good as in rodent models where dose per body weight is usually much lower than those used in mice. The optimal number of doses is a parameter that is thought to vary with the antigen and therefore requires individual, empirical evaluation (76). In mice, a single immunization is sufficient to induce strong responses with some antigens whereas other antigens require one or more boosts (25-28). In addition, the length of resting period between immunizations is thought to play a role. Immunization with two doses of an HIV-1 DNA vaccine spaced three-months apart induced stronger antibody titres than when spaced one month apart (101). Thus, the dose, the number of doses and the resting period may be factors that require further study especially in larger animal models, to optimize DNA vaccines for use in humans.

1.2.5.5 Combination studies

Recently, studies have employed a combination of DNA- and protein-based immunizations in prime-boost regimens to elicit stronger humoral and CTL responses to the antigen (76, 111). For example, this has been demonstrated in two recent studies that have used a DNA construct encoding the HIV envelope protein (HIV Env) for priming followed by a boost with HIV Env in mice (112) and monkeys (113).

1.2.6 Optimizing DNA Vaccines

Some preclinical studies with DNA vaccines are now aimed at finding ways to reduce the dose and the number of administrations while still achieving a long-lasting protective immune response appropriate to the pathogen in question. Current strategies
to enhance efficacy of DNA vaccines include: (i) targeting either the DNA vaccine itself or its expressed antigen to APCs (114-116), (ii) use of delivery systems such as cochleates (117), cationic liposomes (118) or microspheres (20, 86) to achieve higher efficiency of transfection, (iii) use of non-DNA adjuvants such as QS-21, cholera toxin and monophosphoryl lipid A (89, 119-121), (iv) use of cell-specific promoters (122, 123), (v) optimization of CpG content in plasmid DNA (56, 63) and (vi) coexpression of cytokines (91, 106, 124-130) or costimulatory molecules (127, 131, 132). This latter approach is discussed in further detail below.

1.2.7 Modulation of Immune Responses with DNA Vaccines

Early studies by several groups demonstrated that tumour cells transfected with cytokine expressing plasmids or cytokines inoculated as a protein can augment tumour-specific immune responses (reviewed in (133)). The use of naked plasmids encoding cytokine genes to modulate immune responses was first demonstrated by Raz et al. (134). In that study, cytokine genes (IL-2, transforming growth factor-β1 (TGF-β1) and IL-4) administered with exogenous transferrin, enhanced anti-transferrin immune responses. More recently, plasmid DNA expressing proinflammatory cytokines (e.g., tumour necrosis factor (TNF)-α, TNF-β, granulocyte macrophage colony stimulating factor (GM-CSF)) (127-129, 135, 136), Th1 associated cytokines (e.g., IL-2, IL-12, IL-18, IFN-γ) (91, 106, 128, 136) or Th2 associated cytokines (e.g., IL-4, IL-5, IL-10) (129, 136) coadministered with antigen encoding plasmids have been shown to augment a broad range of immune responses against a variety of antigens.
1.3 Costimulatory Molecules

The use of costimulatory molecules as an immune enhancer stems from the idea that these molecules that provide the ‘second signal’ for immune activation, may be used to manipulate the nature as well as the strength of the immune response. Discussed below are the fundamentals of second signals in immune responses and rationale for the use of these molecules as biological adjuvants.

1.3.1 Role of Costimulation in Immune Induction

In order for T cells to recognize foreign pathogens and to distinguish self from non-self, the immune system employs the TCR or the B cell receptor for defining the specificity of an immune response (137). Other mechanisms that have been adapted to restrict immune responses to foreign antigens include the unique architecture of the lymphoid tissue, the evolution of professional APCs, localized antigen deposition and costimulation (138). Initiation and amplification of immune responses involves two signals, a concept conceived of by Bretscher and Cohn (139) for B cells and Lafferty and Cunnigham (140) for T cells. The first signal is the recognition of the MHC by the TCR along with its co-receptors (e.g. CD4 and CD8). The second signal is mediated by soluble factors such as IL-2 or by the interaction of cell surface molecules expressed on APCs (141, 142). This second costimulus is complementary to the TCR engagement and is critical for effective lymphocyte activation and enhancement of immune responses (141). In the absence of costimulatory signals, naive T cells are not activated, but rather remain in a state of anergy (143). Recent studies have established that a major costimulatory signal for T cell activation is the interaction between B7.1 or B7.2 on professional APCs with CD28 and its homologue CTLA-4 receptors on T cells (144)
(Figure 2). CD28 interactions with the B7 family of costimulatory ligands are essential for initiating antigen-specific T cell responses, upregulating cytokine expression and promoting T cell expansion and differentiation (reviewed in (145)). It is also speculated that the role of B7 ligand interactions, during an ongoing immune response, may not be to initiate T cell effector function but rather to sustain a functional pool of T cells by upregulating anti-apoptotic factors such as bcl-xl (146, 147). Interaction of B7
molecules with CTLA-4 (CD152) is thought to mediate down-regulation of immune responses (137).

Induction of anergy is an active process that requires protein kinase activation, calcium mobilization and new protein synthesis – a process that is initiated following TCR ligation by the antigen without subsequent secretion of IL-2 (148). The lack of costimulation is thought to induce a state of anergy either by: i) absence of sufficient positive signal; or ii) activation of another distinct signal pathway (148). Anergy can be prevented by B7-mediated costimulation in vitro (149-152); the effect is also reversible with the addition of exogenous IL-2 (149, 152, 153).

1.3.2 The B7.1/B7.2 and CD28/CTLA-4 Glycoproteins

Yokochi et al. (154) first described the B cell activation molecule, B7.1 in 1981, as the ligand interacting with CD28 and later as a ligand for CTLA-4 (155, 156). B7.1 (B7/BB1, CD80) and B7.2 (CD86) are monomeric transmembrane glycoproteins coded for by genes located on human chromosome 3q13.3-3q21 (143). B7.1 expression is detected on activated B cells (157-160), dendritic cells (160-162), Langerhans cells (162, 163), activated monocytes (156, 158, 164, 165) and activated T cells (158, 159, 162). The B7.2 molecule first cloned by Freeman et al. (166) and Azuma et al. (158) was found to have structural similarities with B7.1, even though there is only a 25% homology in amino acid sequence (158, 166). Resting B cells and T cells express B7.2, and they are rapidly upregulated along with B7.1 after activation in other cell types (156, 158-160). Both B7.1 and B7.2 bear overall structural resemblance to the Ig-superfamily in that they have an extracellular domain (Ig-like domains) and a transmembrane domain followed by a cytoplasmic tail (158, 166). The cytoplasmic
domains of B7.1 and B7.2 are distinct with the B7.2 cytoplasmic domain being longer and containing potential sites for phosphorylation of protein kinase C and casein kinase II (148, 158, 166). CD28 is a disulphide-bonded dimer of 44 kDa polypeptide (chromosome 2q33-34) expressed on the surface of 95% of CD4+ and 75% of CD8+ T cells (144, 167). CTLA-4 is a homodimeric glycoprotein expressed on activated T cells (168). The B7s bind to CTLA-4 with 20- to 50- fold higher affinity (137).

1.3.3 Functional Outcome of B7/CD28 Signaling

Ligation of CD28 to B7s following TCR mediated signaling results in up-regulation of expression of IL-2 receptor (169, 170), IL-2 (171, 172), as well as other cytokines (172), CD40 ligand (173), CTLA-4 (174, 175) and T cell proliferation (176-178) (Figure 2).

For maximal induction of IL-2 gene expression, in human T lymphocytes, three distinct intracellular signals are required (179): (i) increased intracellular calcium; (ii) protein kinase C activation; and (iii) a signal derived from CD28. TCR triggering may induce the former two signals. In contrast, CD28 can couple to several signal transduction cascades (180) depending on the valency of stimulation and on the activation state of the T cell (143, 180). Tyrosine phosphorylation of substrates distinct from TCR induced phosphorylation are triggered by CD28 ligation with B7s (181-183). Protein tyrosine kinase (184), Tec family kinase (185) and PI-3 kinase (186) are thought to be involved in CD28 signaling although their role in triggering CD28 mediated signals at various stages of T cell activation is presently unresolved.

The differences in the temporal expression of B7.1 and B7.2 are often attributed to the distinct roles they play. B7.2 expression occurs within 6 h of stimulation with
maximal expression achieved between 18 to 24 h (159, 160), while B7.1 expression is not detected until 24 h post stimulation with peak expression noted between 48 and 72 h (159, 160). The spatial and temporal differences in the expression of B7.1 and B7.2 among different types of APCs are also dependent largely on the activation state of the cell (138). For instance, B7.2 is transiently up-regulated on activated B cells earlier than B7.1 (147) which is expressed later and is thought to associate with CD28 for a longer period of time, thereby sustaining IL-2 production and prolonging T cell responses (187, 188). In addition, *in vitro* studies indicate that B7.2 may be a requirement for providing decisive signals between immunity and anergy (144) while B7.1 might serve to amplify rather than initiate an immune response (158).

The region of CD28 and CTLA-4 interaction with B7 molecules are thought to be distinct (148) and these differences might contribute to distinct promotion of Th1 and Th2 type responses by B7.1 and B7.2 respectively. A fundamental role for CD28 has been demonstrated in the early development and differentiation of both Th1 and Th2 T cell subsets in both *in vitro* and *in vivo* studies (189). The B7-dependent T cell activation may vary in different disease models depending on the extent to which Th1 versus Th2 type responses are polarized (190, 191). For instance, in a *Heligmosomoides polygyrus* (a nematode parasite) disease model, blocking of both B7.1 and B7.2 caused significant down-regulation of the immune response (192). In contrast, in an autoimmune diabetes model, blocking B7.1 was protective while blocking B7.2 enhanced disease severity (245). Hence, the idea that B7.1 and B7.2 differentially regulate Th1 and Th2 cells remains controversial. It is also not clear if B7 expression is mandatory for induction of CD8+ mediated CTL responses since CD28 (194) or B7.1
(195) knockout mice show normal CTL function. However studies employing B7 molecules as enhancers of immune activation show clear augmentation of CTL activation (discussed below). These results indicate that, even though the exact role of B7 molecules in CTL induction is not known, they may be used as adjuvants for the purpose of vaccine development.

1.3.4 Coexpression of Costimulatory Molecules as Adjuvants for DNA Vaccines

It is unclear how transfection of B7 plasmids into professional APC, which natively express costimulatory molecules, would lead to immune augmentation. The kinetics in the expression of B7s within an immature APC, facilitated by vectors expressing B7s, might accelerate the maturing process of APCs or render the transfected cells more potent in delivering activation signals to T lymphocytes (127, 131, 132, 196). Another possible mechanism by which T cell activity might be enhanced is suggested from tumour studies that have used similar strategies to augment immune responses. Coexpression of B7s increases CTL activity even though tumour cells are incapable of directly activating naive CTLs (197-199). The authors suggest that the tumour cells may be more susceptible to lysis by natural killer cells and CD8+ T cells when expressing costimulatory molecules (200). Alternatively, CTL augmentation might involve a two-step process with initial T cell activation mediated by bone marrow derived APCs and subsequent enhancement by B7 expressing cells, at the site of inflammation, upon T cell recirculation (196). Indirect evidence for this is provided by two studies. First, in a herpes stromal keratitis model it was shown that T cells require costimulation at the site of inflammation because cytokine transcripts are labile in the absence of T cell restimulation (201). In a second study, Swain et al. (202) showed that
T helper cells have a short half-life during an immune response and that B7 molecules may serve to sustain a functional pool of Th cells by providing signals that inhibit the rapid exhaustion of these cells. Thus, a possible mechanism for B7 molecules expressed in myocytes or keratinocytes, at the site of injection, is to play a role not in the inductive phase of T cell responses but, in the effector phase of the response, at the site of inflammation.

As muscle cells are capable of expressing MHC class II molecules (203), past efforts have concentrated on transforming myocytes into professional APCs by providing them with the requisite costimulatory molecules. However, it is now becoming increasingly evident that bone marrow derived cells alone can function as APCs to induce immune responses after DNA immunization (52, 53). Regardless of the mechanism, coexpression of B7s has been shown to be effective in enhancing immune responses against antigens (Table 2). It was, therefore of interest to study the effects of B7 coexpression with a DNA vaccine in the hepatitis B disease model.

<table>
<thead>
<tr>
<th>Antigen Model</th>
<th>B7</th>
<th>Results</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-galactosidase</td>
<td>B7.1 \ B7.2</td>
<td>B7.1: ↑ CTL, ↑ IgG2a</td>
<td>codelivery</td>
<td>(196)</td>
</tr>
<tr>
<td>HIV-1 Env and Gag/Pol</td>
<td>B7.1 \ B7.2</td>
<td>B7.2: ↑ CTL and Th proliferation</td>
<td>codelivery</td>
<td>(132)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B7.1 &amp; B7.2: No effect on Ab response</td>
<td>IM</td>
<td></td>
</tr>
<tr>
<td>Influenza NP (mutated NP)</td>
<td>B7.1 \ B7.2</td>
<td>B7.2: ↑ CTL</td>
<td>codelivery</td>
<td>(127)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B7.1: No effect</td>
<td>colinear vectors</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>weak immunogen</td>
<td></td>
</tr>
<tr>
<td>HIV-1 Env and Rev</td>
<td>B7.1 \ B7.2</td>
<td>B7.2: ↑ CTL and DTH response</td>
<td>codelivery</td>
<td>(131)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B7.1: No effect</td>
<td>IM</td>
<td></td>
</tr>
</tbody>
</table>

25
1.4  Hepatitis B

1.4.1  Description

Advances in the understanding of HBV have progressed tremendously. Within a span of 15 years the antigen’s structure, mechanism of viral replication and transmission have been elucidated and consequently, this knowledge has enabled the development of an effective prophylactic vaccine. HBV is a noncytopathic, enveloped DNA virus classified under the family of hepadnaviruses (204). Immune responses against the virus can result in necroinflammatory liver disease with the duration and severity of the disease being variable, and dependent on the nature of the response (205). Vaccination is the most effective method of preventing HBV infection. If properly administered, the present vaccine induces protection in 90-95% of the recipients (206).

1.4.2  Epidemiology and Transmission

More than 2 billion people world-wide have evidence of past or current hepatitis B infection, 350 million of those being chronic carriers of the virus (1). Approximately 1 million individuals die each year as a direct result of HBV-induced cirrhosis or liver cancer (213). Three-quarters of the world’s population live in areas where the virus is endemic (1), which include sub-Saharan Africa, southern and eastern Europe, the Middle East, western Asia, the Indian subcontinent, and parts of central and South America (207). In developed countries most HBV infections result from parenteral exposure including contaminated needles and syringes, transfusion of unscreened blood products, sexual transmission and occupation exposure (4). In Canada, reported rates of HBV infection have doubled from 1980 to 1990 despite the introduction of the vaccine in high-risk populations (208). Less than 3% of the reported cases occur in children, but
the incidence increases dramatically in the 15- to 19-year age group and peaks in the 20-to 40-year age group, indicating that sexual transmission and injected drug use are major factors for HBV infection in Canada (208). In areas of the world where the virus is endemic, vertical (mother to child) and horizontal (between siblings) transmission is prevalent (4). Less frequently, the virus can spread through household contact, hemodialysis, transmission from a surgeon, and receipt of organs or blood products (207). Fecal-oral transmission does not occur with HBV, because its lipid envelope precludes the passage of viable virus from the liver through the biliary system to the intestinal tract as is common with hepatitis A and E (209). Respiratory, water-borne, or insect-related infections, with HBV, have not been documented.

1.4.3 Virus Structure and Replication

Three particles can be identified in partially purified human serum (Figure 3). A 43 nm particle, termed the Dane particle, is the intact virion. There are also 20 nm spheres and filaments of variable length that are non-infectious subviral particles (210). The HBV genome (reviewed in (211)) is a partially double stranded circular DNA of 3.2 kb length that encodes four overlapping open reading frames: S for the surface or envelope gene; C for the core gene; X for the X gene and P for the polymerase gene. The S and C genes have upstream regions termed preS and preC. The envelope protein encoded in the S gene has three distinct configurations namely the large, middle and major proteins (207). The large (L) and middle (M) proteins in addition to the major protein include the preS1+preS2 and preS2 peptides respectively and these are thought to be the more immunogenic portions of HBsAg. The core antigen (HBcAg) is the nucleocapsid that encloses the viral DNA (210).
The virus exhibits tropism to the liver although other sites of viral replication have also been documented (205). During chronic HBV infection, integration of the HBV DNA invariably occurs which leads to chromosomal rearrangement and deletions, and this, in turn, can result in the expression of oncogenes or deletion of genes regulating hepatocyte growth (205, 212).
1.4.4 Clinical Features and Immune Response

Infection with HBV can cause a wide spectrum of clinical manifestations and hepatic inflammation including (205):

- **Acute hepatitis** leading to complete recovery and subsequent protection, characterized with transient appearance of HBsAg and anti-HBc, and subsequent appearance of antibodies to HBsAg (anti-HBs);

- **Acute hepatitis** leading to **chronic hepatitis** where HBsAg and anti-HBc are usually persistent;

- **Chronic hepatitis** with symptoms of chronic liver disease which was not preceded by an episode of acute hepatitis; and

- "**Healthy**" **carrier state**, which is asymptomatic, but some biochemical abnormalities with persistent HBsAg and anti-HBc exist.

The incidence of acute or chronic disease development has strong relation to age in unimmunized individuals (205). Infection in childhood carries the highest risk of persistent viral carriage (~ 90%) but a small risk of acute hepatitis. In contrast, adult infection causes acute hepatitis in 30-50% of people and leads to carriage in less than 10% (213). HBV infection is a risk factor for hepatocellular carcinoma (214), however, no specific HBV-oncogene sequence appears to be responsible for this tendency. Rather, random insertional mutagenesis and chronic inflammatory processes seem to play a role. Epidemiological studies indicate that the risk of hepatocellular carcinoma is reduced with the resolution of chronic hepatitis B (215, 216).

The development of humoral immune responses to HBsAg is protective. The major B-cell epitope, which is encoded by the S domain, is called the group-specific
determinant ‘α’.

Two other determinants also exist on the S domain, (i) either ‘d’ or ‘y’ specificity; and (ii) ‘w’ or ‘r’ specificity. The combination of determinants can result in four subtypes namely adw, adr, ayw and ayr (217, 218) and antibodies against the S domain alone are protective against infection. In addition B cell epitopes are also found on pre-S1 and pre-S2 domains and these alone can protect against infection (206). The antibody response to HBV surface antigen is a T cell dependent process and it appears to have neutralizing capabilities (219). The cell-mediated response to HBV is a crucial element that functions to eradicate virus infected cells and control viral replication by secretion of antiviral cytokines (205, 212). Some fundamental differences in the manifestation of CTL responses are thought to contribute to the continuance of an infection to the chronic state (Table 3).

<table>
<thead>
<tr>
<th>Table 3: Development of CTL responses in acute and chronic HBV infections</th>
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<tbody>
<tr>
<td><strong>Acute Hepatitis</strong></td>
</tr>
<tr>
<td>• readily detectable during the infection</td>
</tr>
<tr>
<td>• strong, polyclonal, multi-specific for envelope, capsid and polymerase antigens</td>
</tr>
<tr>
<td>• major determinant of viral clearance and liver disease</td>
</tr>
<tr>
<td>• persists indefinitely after recovery</td>
</tr>
</tbody>
</table>

*Table adapted from Chisari et al. (205)*

The noncytopathic control of the HBV virus has been discussed widely by Chisari et al., (205, 212, 214) who suggest that intracellular pathways are activated to inhibit HBV gene expression and replication. This is thought to be mediated by IFN-γ and TNF-α, which are secreted by the antigen specific CD4+ and CD8+ T cells.
Figure 4: Immune Response to HBV Infection
The HLA class I pathway involves internal processing of HBcAg peptides within hepatocytes leading to their display on the hepatocyte surface. Presentation of externally derived HBV antigens may be presented to CD4+ cells, which results in cytokine secretion and T cell proliferation. The diagram is reproduced from (207) with the permission of the publisher.

This process mediated by a non-cytopathic mechanism, is thought to be superior as this avoids direct killing of infected cells by CTLs (212). Both in acute and chronic viral
hepatitis, the CD4+ mediated T cell response is critical (205, 214) (Figure 4). However, the pattern of CD4+ responses vary between acute, self-limiting hepatitis and responses mounted to the recombinant vaccine with respect to epitopes within the envelope protein (205). In acute infections CD4+ responses to the envelope protein are generally absent while in immunized individuals they exert a strong response, suggesting a correlation between antigen load and CD4+ responses to the envelope protein (205). To the nucleocapsid antigens (HBcAg and HBeAg – a soluble form of HBcAg), strong class II-restricted responses are mounted in acute, self-limiting infections but these are absent in chronic infections.

1.4.5 Vaccination against Hepatitis B

In 1960, Saul Krugman demonstrated that inoculation of heat-treated serum (drawn from HBV infected patients) could protect against subsequent viral challenge (220). Two types of HBV vaccines were first available in 1981: (i) heat-inactivated and (ii) chemically inactivated particles derived from plasma collected from chronic carriers of HBV (221). Presently HBsAg particles expressed from recombinant DNA in the yeast Saccharomyces cerevisiae expressing the S protein in its unglycosylated form (Engerix B) (222) or S and M protein produced by Chinese hamster ovary cells (Recombivax-HB) are used (223). Although the plasma-derived vaccine is still used in some countries, recombinant vaccines (Table 4, Appendix I) are now used in most areas of the world (224). Two commonly used vaccines are Recombivax-HB (Merck & Co, Inc., Whitehouse Station, NJ, USA) and Engerix-B (Smith Kline and Beecham Pharmaceuticals, Philadelphia, PA), which appear to be equally immunogenic (209). The HBV recombinant vaccines are among the safest vaccines available to date; mild
injection site reactions may occur while fever and other systemic symptoms are uncommon (209). The only contraindication to vaccine administration is hypersensitivity to yeast or to a component of the vaccine (209).

Despite the availability of a vaccine for hepatitis B since 1981, HBV still presents a serious health problem, particularly in developing areas of the world. The WHO estimates that the number of HBV carriers will reach 400 million by the year 2000 (4). It is likely that this number will continue to rise until early immunization is universally accepted and implemented. The current recombinant vaccine, although highly effective for prophylactic purposes, is not used in many areas where it is most needed owing to the high cost of production (224). Additional drawbacks to HBV vaccines are the requirement for three doses typical for a course of vaccination - poor compliance or difficult logistic situations in developing regions hinder optimal use (225); hypo-responses or non-response seen in approximately 5% of the healthy population; and the absence of a therapeutic vaccine for the 350 million HBV carriers (206). In 1991, the Global Advisory Group of the EPI recommended that hepatitis B vaccine be incorporated into the national immunization programmes of all countries (226). Even at a cost of US $0.50 per dose (plasma-derived vaccine), the poorest countries, most of which have high HBV endemicity, cannot afford the hepatitis B vaccine for their children (227). Thus it is crucial to find alternatives to the current vaccination strategies for HBV which are cost effective, safe and efficacious.

Current practice for treatment of babies born to mothers positive for HBeAg (a marker of active viral replication) is a combination of hepatitis B immunoglobulin (HBIG) and an HBV vaccine (226). However, use of HBIG is expensive ($US 25-50
per child), and requires serological testing of mothers to determine their HBsAg status (4). Unfortunately, screening and vaccination rates may not be feasible in many parts of the world. A preferred strategy might incorporate active immunization with a vaccine that will induce strong CTL responses to combat infected cells. DNA vaccines are an attractive candidate for this purpose as they are inexpensive and trigger strong humoral and CTL responses (228).

1.4.6 DNA Vaccines against Viral Hepatitis

Several studies have used DNA vaccines for prophylactic and therapeutic purposes against HBV (36, 80-82, 206, 229, 230). DNA vaccine studies in hepatitis B disease models have used genes encoding: (i) HBsAg (23); (ii) a combination of both envelope and capsid protein genes (231); and (iii) the pre-S region of the major surface protein for increased immunogenic responses (229). There are also reports of DNA immunization against viral hepatitis in many animal models that are susceptible to species-specific hepatitis. Vaccination with duck hepatitis virus surface antigens and woodchuck hepatitis surface and core antigens has been shown to elicit good immune responses and suppress hepadnavirus infection in challenge studies (232, 233).

The use of cytokines to manipulate the Th orientation of the immune response has been explored in the hepatitis B disease model. Coexpression of IL-2 to augment responses and break haplotype non-responsiveness was demonstrated by Chow et al. (234). Geissler et al. (235) used coexpression of IL-2 and GM-CSF to enhance responses to the large envelope protein of HBV. Collectively these studies suggested that manipulating the cytokine milieu enhances and facilitates responses against HBsAg that are otherwise suboptimal.
1.5 Scope of this Thesis

Our laboratory has developed a model for DNA vaccination against HBV whereby, strong systemic immunity is induced, in mice, by IM or ID injection (36, 236, 237). DNA encoding the major surface protein of the HBV has been shown to induce strong and long-lasting humoral and cell mediated responses (23, 237). To optimize these vaccines further, we explore the possibility of immune augmentation by coexpression of costimulatory molecules as adjuvants.

1.6 Hypothesis

The B7 family of costimulatory molecules are critical elements that dictate the functional outcome of immune responses. It is our hypothesis that coexpression of these genes with DNA vaccines will augment immune responses mediated by a DNA vaccine for HBV in mice. As the B7 interactions with their counter-receptors are concurrent with MHC-complex interaction with the TCRs, we also hypothesized that colinear expression of B7 should be superior to codelivery of the B7 and antigen encoding genes. In addition to enhancement, Th skewing towards Th1 and Th2 may be apparent with B7.1 and B7.2 coexpression respectively.
1.7  **Specific Objectives**

I.  **Coexpression of B7.1 or B7.2 with a hepatitis B DNA vaccine**
   - To augment immune responses to the HBV DNA vaccine by coexpressing B7.1 and/or B7.2.
   - To evaluate and compare the nature of the immune response with B7 coexpression
     - When the DNA is delivered by IM or ID routes of administration
     - When the B7 DNA is codelivered (delivery of 2 plasmids) or expressed colinearly

II. **Polymerase chain reaction (PCR) amplification to determine the distribution of plasmid DNA in anatomical compartments following injection**
   - To elucidate the differences in the magnitude of immune responses following IM and ID routes of DNA administration
   - To detect differences in access of plasmid DNA to secondary lymph organs with the different routes
MATERIALS AND METHODS

2.1 Animals

All in vivo studies were carried out using female BALB/c mice, 6-8 wk of age (Charles River, Montreal, QC). Experimental groups consisted of 5-10 animals. All procedures were performed in accordance with protocols approved by the Loeb Health Research Institute’s (Ottawa Hospital – Civic site, ON) Animal Care and Ethics Committee. BALB/c mice were chosen as the animal model based on studies done, both in our laboratory and by others, which show them to be good responders to the HBsAg antigen (238). Non-response to HBsAg is associated with the H-2 haplotype: H-2d and H-2b are considered good responders (the former responding more strongly) while H-2a and H-2f mice are classified as poor responders (239).

2.2 Cell Culture

Cells lines P815 (ATCC, # TIB-64, Manassas, VA), P815/S, P815-preS1 (generously provided by Jör-g Reimann, University of Ulm, Germany) and EL-4 (ATCC, # TIB-39) were maintained in RPMI 1640 (Gibco-BRL, Burlington, ON) tissue culture medium supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), penicillin-streptomycin solution (final concentration of 1000 U/ml and 1 mg/ml respectively; Sigma, Oakville, ON), 5 X 10⁻⁵ M β-mercaptoethanol (Sigma). Mouse C2C12 cell line (murine myoblast, ATCC #CRL-1772) was maintained in Dulbecco’s Modified Eagle Medium (DMEM, Gibco-BRL) containing 10% FBS (Gibco-BRL), penicillin-streptomycin solution (final concentration of 1000 U/ml and 1 mg/ml respectively) (Sigma), 5 X 10⁻⁵ M β-mercaptoethanol (Sigma).
2.3 Plasmid DNA Vectors

The pMAS-S (Genbank accession number AF053407) plasmid expresses the major surface protein of the HBV (HBsAg - ay subtype). The pMAS construct, is an optimized plasmid vector derived from pUK21 (generously provided by Qiagen GmbH, Hilden, Germany). This contains the immediate early promoter of the human cytomegalovirus (CMV-IE), the bovine growth hormone (BGH) polyadenylation signal and the kanamycin resistance gene (59). The pMAS expression vector was created by removing CpG neutralizing motifs in the kanamycin resistant gene and the non-essential regions after the gene (59).

Murine B7.1 (generous gift of Dr. D. T. Curiel, University of Alabama at Birmingham, AL) and B7.2 (generous gift from Dr. G. J. Freeman, Harvard Medical School, Boston, MA) genes were isolated from their respective vectors by restriction enzyme digest and cloned into the pMAS vector. B7.1 was isolated from pGT59 by digestion with EcoRI (Gibco-BRL), blunt ended, with Klenow fragment (Gibco-BRL), and cut with KpnI (Gibco-BRL) and cloned into the pMAS expression vector (digested with XhoI (Gibco-BRL) blunt ended and cut with KpnI). Murine B7.2 (from pCDM8 expression vector) was excised with XbaI (Gibco-BRL), blunt-ended and ligated into the pMAS expression vector (digested with EcoRV (Gibco-BRL)). Murine genes B7.1 and B7.2 were also cloned in the reverse orientation into the pMAS expression vector and used as controls along with the pMAS backbone vector. For the cloning of B7.1 in the reverse orientation (B7.1 (R)), the fragment isolated from pGT59 (with KpnI and EcoRI digestion) was inserted into pMAS (digested with EcoRI and KpnI). As B7.2 was cloned as a blunt end ligation, a clone with the insert in the opposite orientation was also
isolated during the initial cloning and this will be referred to as B7.2(R). B7 cloning was verified by gene sequencing.

In addition, four colinear expression plasmids, pMAS-S-B7.1, pMAS-S-B7.2, pMAS-S-B7.1(R), pMAS-S-B7.2(R) were constructed. The expression vector pMAS-S was digested with HpaI (New England Biolabs, Beverly, MA) and StuI (Gibco-BRL) and the CMV-S insert was isolated. The CMV-S segment was inserted into the B7 plasmids, pMAS-B7.1 and pMAS-B7.2 and their respective reverse cloned controls after digestion with HpaI (New England Biolabs). Orientation of gene insertion was determined by restriction enzyme digest. E.Coli strain DH5α (frozen competent cells prepared using calcium chloride and magnesium chloride) was used as the bacterial host. Shown in Figure 1 is the strategy that was employed to clone the various plasmids.

2.4 DNA Purification

The DNA was purified on Qiagen anion-exchange chromatography columns (Qiagen) according to manufacturer's guidelines and resuspended in sterile saline (0.15M NaCl, Sigma-Aldrich, Oakville). The concentration of DNA was calculated based on absorbance of ultraviolet light (OD 260 nm) with final concentrations usually being 2-5 mg/ml. Protein contaminants in the DNA preparations were determined by the ratio of absorbance (OD 260 nm/280 nm). OD 260/280 ratios of >1.8 were considered acceptable (2.0 is ideal). DNA solutions were stored at -20°C until required for in vitro transfection and in vivo delivery.
Figure 1: Cloning of murine B7 genes in expression vectors

Murine B7.1 and B7.2 were isolated from their respective vectors by restriction enzyme digest and cloned into the pMAS vector comprising a CMV promoter (▲) and polyadenylation (A) terminator (●). B7.1 present in pGT59 was isolated by restriction enzyme digest, cloned into the pMAS expression vector, and named pMAS-B7.1. Murine B7.2 was excised from pCDM8, ligated into the pMAS expression vector, and named pMAS-B7.2. Murine genes B7.1 and B7.2 were also cloned in the reverse orientation into the expression vector and used as non-expressing controls and these were referred to as pMAS-B7.1(R) and pMAS-B7.2(R). For cloning of the colinear expression plasmids, CMV-S segment was isolated from pMAS-S and ligated into pMAS-B7 expression vectors. These colinear expression vectors have been identified as pMAS-S-B7.1, pMAS-S-B7.1(R), pMAS-S-B7.2, pMAS-S-B7.2(R).
2.5 *In Vitro* Assessment of Gene Expression

*HBsAg expression:* HBsAg gene expression was detected by indirect immunofluorescence. Mouse C2C12 cells were plated in six well plates (Becton Dickinson, Franklin Lakes, NJ) at 1x10^5 cells/ml. Prior to plating cells, ethanol sterilized cover slips (1oz micro cover glasses, VWR Scientific Inc., Media, PA) were placed on the bottom of the wells. Cells were grown to 60-80% confluence and transfected with the colinear plasmids, pMAS-S-B7.1 or pMAS-S-B7.2 (10 μg) using SuperFect® transfection reagent (Qiagen) according to manufacturer's protocol (240). Forty-eight hours post-transfection, cover slips were removed and washed three times by immersion for 5 min in 2 ml of Dulbecco's phosphate buffered saline (PBS, Gibco-BRL). After air drying the cover slips, cells were fixed with ice cold acetone for 5 min and further washed in PBS 3 times. Cells were then blocked for 15 min with 100 μl/cover slip of FBS and then washed in PBS three times. The cells were then incubated for 30 min at room temperature (RT) with 60 μl/cover slip of the primary antibody, anti-HBs in pooled sera from BALB/c mice immunized with the HBsAg protein (each animal had a total antigen-specific IgG ELISA titre > 150000). Following this incubation, cells were washed three times in PBS and then treated for 30 min at RT with 60 μl/cover slip of secondary antibody, sheep anti-mouse Ig-Fluorescein F[ab’]2 fragment (Boehringer Manheim, GmbH, Germany). The cover slips were washed a final time and mounted on a glass microscopic slide (Fisher Scientific, Pittsburgh, PA) using Permoun® (Fisher Scientific). The cells were examined by confocal microscopy (Olympus IX70; emission filter 522DF32; scale 0.824 μm/pixel) for HBsAg expression, which was evident as green fluorescence.
**B7.1 / B7.2 expression:** Mouse C2C12 cells were grown and transfected with plasmids (Table 1) as described in the previous section. Forty eight hours post transfection, cells were washed and treated for 30 min with biotin conjugated antibodies against B7s (Pharmingen, San Diego, CA) as indicated in Table 1. After 3 washes with sterile PBS, cells were incubated with secondary antibody (Extravidin® - Alkaline phosphatase, Sigma) for 30 min and treated with BCIP/NBT-buffered substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate, nitro blue tetrazolium, tris buffer and MgCl₂ (Sigma) to visualize antibody binding. The cover slips were washed a final time and mounted and preserved as outlined in the earlier section. The cells were examined by phase contrast microscopy (Nikon-eclipse E400, 20x magnification) for B7 expression, which was evident as purple staining against a transparent background.

<table>
<thead>
<tr>
<th>Table 1: Treatment of B7 antibodies</th>
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<tbody>
<tr>
<td><strong>Plasmid</strong></td>
</tr>
<tr>
<td>pMAS-B7.1</td>
</tr>
<tr>
<td>pMAS-B7.2</td>
</tr>
</tbody>
</table>

2.6 *Immunization of Mice with DNA Vaccines*

**IM injection:** HBsAg expressing DNA constructs suspended in 50 µl of 0.15M NaCl, were injected into the left *tibialis anterior* (*TA*) muscle of mice as described previously (239). All IM injections into the *TA* were through the skin using a 27 gauge needle (Becton Dickinson) fitted with a collar of polyethylene tubing (Becton Dickinson) which limited penetration to 2 mm.
**ID injection:** Mice were immunized with 50μl of DNA, which was injected in the skin of the lower back in two different sites using 0.3 cc insulin syringes (Becton Dickinson). All immunizations were carried out under general anaesthesia (Somnotol®, MTC pharmaceuticals, Cambridge, ON., 0.75 mg/kg, intraperitoneally).

Codelivery of various expression cassettes involved mixing relevant plasmids prior to injection. For administration of a combination of protein and DNA plasmids: i) DNA solution (50 μl volume) was injected into the TA muscle while the protein solution (50 μl volume) was administered in the upper quadriceps muscle (different sites); or ii) DNA plus protein were injected simultaneously in the TA muscle (total volume of 50 μl, same site). The protein component of the vaccine was the recombinant hepatitis B surface antigen (rHBsAg) (Medix Biotech Inc., San Carlos, CA).

### 2.7 Collection and Preparation of Sera

Blood was recovered by retro-orbital puncture from anesthetized mice at 2, 4, 8 and 12 wk post immunization using heparinized pipettes. Samples were centrifuged, plasma recovered and stored at -20°C until used for ELISA (enzyme-linked immunosorbent assay).

### 2.8 Evaluation of In Vivo Humoral Response

Anti-HBs were detected and quantified by end-point dilution ELISA on samples (in triplicate) from individual animals as described previously (73). Briefly, solid phase-derived HBsAg particles (αy subtype, 100 μl of 1 μg/ml per well in carbonate buffer – pH 9.6, overnight at RT) were used to capture anti-HBs antibodies in the plasma (1 h at 37°C). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1 or IgG2a (1:4000 in PBS-Tween, 10% FBS: 100 μl/well; Southern Biotech
Inc., Birmingham, AL), followed by o-phenylenediamine dihydrochloride solution (100 μl/well, 30 min at RT in the dark; Sigma) were used for detection. End-point titres were defined as the highest plasma dilution that resulted in an absorbance value (OD 450) two times greater than that of non-immune plasma, with a cut-off value of 0.05. In our laboratory we have previously determined that the relationship between endpoint titers in mice and those in milli-international units (mIU), as defined by the WHO, is close to 1:1 (241). A value of 10 mIU/ml is protective in humans.

2.9 Evaluation of CTL Response

Spleens were recovered under sterile conditions from mice 4 wk after immunization with DNA vaccines (n=5/group) and analyzed for CTL activity as previously described (75). Briefly, single cell suspensions were prepared and suspended in media (section 2.2) and supplemented with 3% EL-4 supernatant as a source of IL-2. Splenocytes (4.5 x 10^7) were cocultured with 1.5 x 10^6 syngenec HBsAg-expressing stimulator cells (P815-preS), which had been inactivated by irradiation (20 000 rads). The cultures were maintained for 6 days in 15 ml of RPMI media in upright 25 cm^2 tissue culture flasks (Becton Dickinson) in a humidified atmosphere (5% CO_2) at 37°C and then were harvested and washed in media. These effector cells were serially diluted and cultured with 5 x 10^3 ^51Cr-labelled HBsAg-expressing targets (P815/S) or control target cells (P815) at 37°C in round-bottomed 96-well microtiter plates (Becton Dickinson), with each sample in triplicate. After 4h of incubation, 100 μl of supernatant was removed for radiation (gamma) counting (Beckman Gamma 5500B). The percent lysis was calculated as:

\[
\frac{(\text{Experimental Release} - \text{Spontaneous Release})}{(\text{Maximum Release} - \text{Spontaneous Release})} \times 100
\]
Spontaneous release was determined by incubating target cells without effector cells and total release was determined by adding 100 µl of 2 N HCl to target cells. The percent specific lysis was calculated as: % lysis with P815/S cells - % lysis with P815 cells. Animals were considered positive for CTL when specific lysis was greater than 10%.

2.10 Plasmid Detection in Tissues after IM and ID Injection

Animal Treatment: Mice (n=3) were injected with 10 µg of pMAS-S either IM or ID as described in an earlier section (section 2.5). At two time points - 5 min, 5 h post injection, mice were sacrificed and the injected TA muscle or area of skin where DNA was injected (~ 1 cm²) was removed. In addition, for IM injections, the inguinal and popliteal lymph nodes were removed unilaterally and pooled. For ID injection both inguinal lymph nodes were removed after injection and pooled. Tissue samples were snap frozen and stored at -80°C until used for plasmid isolation. As controls, mice were injected with 50 µl of 0.15 M NaCl instead of DNA.

Plasmid Isolation: Genomic and plasmid DNA were isolated from the various tissue samples using the QIAmp Tissue Kit (Qiagen) according to manufacturer's protocol (242). Eluted DNA was stored at -20°C until used for PCR analysis.

PCR Analysis: The PCR amplification reactions were performed using primers from the pMAS region of the pMAS-S plasmid. The upstream and downstream primers (Gibco-BRL) amplifies a 711 bp product.

Upstream primer: 5'-GCTCTCTCCCTAACTAGAGAA - 3'

Downstream primer: 5' - GACTCGAGATCTATCGATGCA - 3'

The components of the reaction mixture were as indicated in the Basic PCR protocol supplied with Pwo DNA polymerase (Boehringer Manheim). The amplification was
performed in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT). After an initial denaturation at 94°C for 2 min, the samples were subjected to 25 cycles of the following: denaturation at 94°C for 20s, annealing at 56°C for 45s, and elongation at 72°C for 1 min. This was followed by a 7 min extension at 72°C. The presence of the specific PCR product in each reaction tube was assayed by electrophoresis on 0.8% agarose (Sigma) gel followed by staining with ethidium bromide (Gibco-BRL).

2.11 Statistics

Statistical analyses were performed on all treated animals without exclusion of non-responders. The statistical significance of differences between groups was determined by Student's t test (for 2 groups) or one-way ANOVA (for three or more groups) followed by Tukey’s post-test. Differences were not considered to be significant with P>0.05. All statistical tests were performed using InStat (GraphPad Software, SanDiego, CA) and Prism (GraphPad Software).
CHAPTER 3
RESULTS

3.1 *In Vitro* Assessment of Gene Expression

Prior to using vectors for *in vivo* studies, we examined expression of gene products in muscle cells transfected with the expression vectors. Immunohistochemistry on transiently transfected mouse C2C12 cells revealed that HBsAg (Figure 1, A & B), B7.1 (Figure 2A) and B7.2 (Figure 2B) gene products were expressed in muscle cells. Immunohistochemical staining was negative when myocytes transfected with the backbone plasmid (pMAS) or the reverse cloned plasmids (pMAS-B7 (R)) were treated with anti-HBs (Figure 1C) and anti-B7 antibodies (Figure 2, C & D).

3.2 Comparison of Codelivery and Colinear Expression by the IM route

To investigate the influence of coexpressing B7 costimulatory molecules on immune responses induced by plasmid DNA immunization, we designed a series of expression vectors. In a carcinoembryonic antigen model, Conry et al. (135) demonstrated that the effectiveness of this approach is contingent upon coexpression of B7 and antigen within the same cell. However, other studies have demonstrated effective immune augmentation with codelivery of B7- and antigen-encoding plasmids as it is likely that some cells are transfected with both the plasmids (131, 132), indicating that colinear expression of the two genes is not an absolute requirement. Hence in our model, we compared two situations: i) where it is assumed there will be some coexpression in the same cells (codelivery) and ii) it is guaranteed that the two genes of interest are expressed in the same cell (colinear expression).
Figure 1: Immunofluorescence staining for expression of HBsAg in muscle cells

Expression of HBsAg in C2C12 cells (murine myoblast) transfected with pMAS-S-B7.1 (panel A), pMAS-S-B7.2 (panel B), or pMAS (panel C). Expression of HBsAg was detected by immunofluorescence using mouse anti-HBs polyclonal antibody and fluorescein labeled sheep anti-mouse IgG (F[ab'']_2 fragment). HBsAg-expressing cells were identified by their green fluorescence under confocal microscopy.
Figure 2: Immunohistochemical assay for expression of B7.1 and B7.2 in muscle cells

Expression of B7.1 and B7.2 molecules in C2C12 cells (murine myoblast) transfected with pMAS-B7.1 (panel A), pMAS-B7.2 (panel B) or pMAS-B7.1(R) (panel C) and pMAS-B7.2(R) (panel D). Expression of B7.1 and B7.2 was detected by immunohistochemistry using biotin-conjugated mouse anti-B7.1 or anti-B7.2 monoclonal antibody and alkaline phosphatase labeled secondary antibody. The cells were examined by phase contrast microscopy for B7 expression, which is evident as purple coloration.
pMAS-B7.1 \(\Rightarrow\) anti-B7.1

pMAS-B7.1 (R) \(\Rightarrow\) anti-B7.1

pMAS-B7.2 \(\Rightarrow\) anti-B7.2

pMAS-B7.2 (R) \(\Rightarrow\) anti-B7.2
3.2.1 Effect of Coexpression on Humoral Responses

To investigate the impact of different costimulatory molecules on humoral and CTL responses by plasmid DNA immunization, it was necessary to establish a model system that was sufficiently suboptimal to permit detection of a net increase in immune responses. In the course of our experiments we found that responses induced with our standard model, which utilized 10 μg of the DNA vaccine, were indistinguishable from responses noted with addition of B7 encoding plasmids (Figure 3a, P>0.05), nor were there differences in Th-bias of induced response (Figure 3b). Consequently, a suboptimal dose (1 μg) of the HBsAg-expressing DNA vaccine admixed with B7 expressing plasmids were used. With this approach, rate of seroconversion in mice was 50% by 4 wk post immunization as indicated by anti-HBs titre > 10. However, codelivery of the B7 encoding plasmids with the DNA vaccine had no significant effect on HBsAg-specific antibody responses in comparison to their respective reverse cloned controls (Figure 4, P>0.05). These results, however, do not conclusively indicate that B7 coexpression is incapable of augmenting humoral responses because a minimum protein concentration that is needed to surpass the activation threshold of B cells and develop a functional Th cell response may not have been achieved with the suboptimal dose of the vaccine.

Since codelivery of separate plasmids cannot guarantee transfection into the same cells, vectors expressing both HBsAg and B7.1 or HBsAg and B7.2 were constructed. These plasmids were used to evaluate whether better augmentation could be seen when expression of antigen and costimulatory molecule was ensured in the same cell.
Figure 3: Effects of codelivery are masked at higher doses of antigen-encoding DNA

Strength of humoral response in BALB/c female mice immunized IM with 10 μg of DNA vaccine (pMAS-S) and 10 μg of plasmids encoding B7.1 or B7.2. Panel A shows HBsAg-specific total IgG titres and no significant differences were detected between groups. Panel B represents HBsAg-specific Ig-isotype titres. Numbers at the bottom of each bar in panel B represent the IgG2a:IgG1 ratio. A strong Th1 bias was produced with each administration. The height of each bar represents the geometric mean titre (GMT) for anti-HBs ELISA titre at 8 wk post administration (n=10). Vertical lines on bars represent SEM. End-point titres were defined as the highest plasma dilution that resulted in an absorbance value of (OD 450 nm) two times greater than that of control non-immune plasma, with a cut-off value of 0.05.
Figure 4: Humoral responses to the HBV DNA vaccine are unaffected by codelivery of B7 expression plasmids

No significant increase in the strength of humoral responses were detected in animals immunized IM with 1 μg of DNA vaccine (pMAS-S) and 10 μg of plasmids encoding B7.1, B7.2 or both (black bars). B7 genes cloned in the reverse orientation were used as non-expressing controls (hatched bars) along with the backbone vector pMAS. The height of each bar indicates the group GMT (n=10) for anti-HBs ELISA titre (total IgG) at 4 wk post administration; vertical lines represent the SEM. Numbers in brackets represent number of responders (end-point titre > 10). End-point titres were defined as the highest plasma dilution that resulted in an absorbance value of (OD 450 nm) two times greater than that of control non-immune plasma, with a cut-off value of 0.05.
In contrast to codelivery, colinear expression of B7.1 or B7.2 (10 μg) contributed to significant enhancement in HBs-specific antibody titres, compared to the reverse cloned controls (Figure 5, P<0.05) suggesting the extension of B7 effects on CD4+ T cells. Seroconversion in animals injected with pMAS-S-B7.1 and pMAS-S-B7.2 at a 10 μg dose was 90% and 100% respectively. This observation was dose-dependent, as a 10-fold decrease in dose of the plasmids did not result in any detectable antibody response. The adjuvant effect mediated by colinear expression of B7.1 or B7.2 was not remarkably different from each other, as the level of increase in antibody titres in the presence of B7.1 or B7.2 plasmids was approximately the same (Figure 5).

In order to determine if boosting with B7 encoding colinear plasmids could further enhance antibody titres, animals primed with pMAS-S or pMAS-S-B7.1 or pMAS-S-B7.1(R) were boosted with the same dose and regime at 8 wk post immunization. At 2 wk post-boosting, animals primed and boosted with B7.1 colinear plasmid yielded titres 15 times greater than the pre-boost titres (Figure 6). The reverse cloned plasmid also contributed to an increase in antibody titres after boosting, an effect that may be attributed to the presence of HBsAg encoding gene in these controls. Nevertheless the increase noted with pMAS-S-B7.1, in comparison to its reverse cloned control, was approximately 2 fold (P<0.05) indicating that B7.1 coexpression can contribute to augmentation during primary and secondary responses.

3.2.2 Ratio of IgG2a/IgG1 – an index of Th1-Th2 bias

The IgG2a/IgG1 ratio was used as an indicator of either a predominantly Th1 (IgG2a >> IgG1), predominantly Th2 (IgG1 >> IgG2a), or a mixed Th1/Th2 (IgG1~IgG2a) response (ratio: 0.5 to 2.0) (241). When B7 encoding plasmids (10 μg)
Figure 5: Colinear plasmids encoding B7 and HBsAg genes augmented humoral responses

Strength of humoral response in BALB/c female mice immunized IM with 10 μg of DNA vaccine (pMAS-S) or pMAS-S-B7.1 or pMAS-S-B7.2. B7 genes cloned in the reverse orientation (pMAS-S-B7.1(R), pMAS-S-B7.2(R)) within the colinear plasmids were used as non-expressing controls. Panel A shows HBsAg-specific IgG titre, which was significantly enhanced with colinear expression of B7.1 or B7.2 in comparison to the reverse cloned controls. Height of each bar represents the GMT (n=10) at 4 wk post administration. Vertical lines on bars represent SEM. Shown in panel B are the number of responders for the respective treatments, HBsAg-specific IgG-isotype titres ± SEM. IgG1 isotype titres were not detectable (ND) with the injection of the DNA vaccine or the colinear plasmids. End-point titres were defined as the highest plasma dilution that resulted in an absorbance value of (OD 450 nm) two times greater than that of control non-immune plasma, with a cut-off value of 0.05. *P< 0.05.
**A**

Anti-HBs ELISA GMT 4 wk post immunization

**B**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Responders</th>
<th>IgG2a</th>
<th>IgG1</th>
<th>Th-bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMAS-S</td>
<td>6/10</td>
<td>398 ± 695</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>pMAS-S-B7.1</td>
<td>10/10</td>
<td>944 ± 462</td>
<td>ND</td>
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<tr>
<td>pMAS-S-B7.1 [R]</td>
<td>4/10</td>
<td>33 ± 293</td>
<td>ND</td>
<td>Th1</td>
</tr>
<tr>
<td>pMAS-S-B7.2</td>
<td>9/10</td>
<td>218 ± 452</td>
<td>ND</td>
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</tr>
<tr>
<td>pMAS-S-B7.2 [R]</td>
<td>3/10</td>
<td>6 ± 64</td>
<td>ND</td>
<td></td>
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</tbody>
</table>

ND: Not determined

*: Significant difference compared to pMAS-S.
Figure 6: Effect of boosting with colinear plasmids encoding B7.1 and HBsAg genes

Strength of humoral response was significantly enhanced with a boost of the B7.1 colinear plasmid. Mice were immunized IM with 10 µg of DNA vaccine (pMAS-S), pMAS-S-B7.1 or pMAS-S-B7.1(R). At 8 wk post prime, mice were boosted with the identical regime. Height of each bar represents the GMT (n=5) for anti-HBs ELISA titre (total IgG) at 8 wk post immunization (hatched bars) and 2 wk post boost (solid bars). Vertical lines on bars represent SEM. End-point titres were defined as the highest plasma dilution that resulted in an absorbance value of (OD 450 nm) two times greater than that of control non-immune plasma, with a cut-off value of 0.05. *P< 0.05.
were codelivered with the DNA vaccine (1 μg), IgG-isotype antibodies were undetectable, therefore the Th-bias by this regime could not be determined. However, as shown in Figure 3b and 5b, IM administration of DNA, at higher doses, resulted in a Th1-biased response. The Th1-bias noted with codelivery and with colinear expression, however, could not be attributed to the over-expression of B7 molecules since the DNA vaccine administered alone also induced a strong Th1-bias indicating that this effect may be more strongly correlated with IM injections.

3.2.3 Cytotoxic T cell Responses

Codelivery of B7 plasmids augmented CTL responses. Mice immunized with 1 μg of the DNA vaccine (pMAS-S) in combination with 10 μg of B7 plasmids showed a significant enhancement in antigen-specific CTLs. It was previously observed that immunization of BALB/c mice with a control plasmid (not encoding HBsAg) did not induce detectable CTL responses (Davis et al. unpublished observations). Therefore, this control was not incorporated into our studies.

Shown in Figure 7 are CTL responses at an effector:target (E:T) ratio of 10:1 at 4 wk post immunization. Codelivery of B7.1 (Figure 7, Panel A) or B7.2 (Figure 7, Panel B) plasmids resulted in 4 and 6 fold increase in antigen-specific lysis respectively (P<0.01). However, there did not appear to be any additive effects with codelivery of B7.1 and B7.2 plasmids (Figure 7, Panel C), suggesting a redundancy in the role of B7s. Addition of both pMAS-B7.1 and pMAS-B7.2 with the DNA vaccine resulted in responses (84% specific lysis) comparable to treatment with pMAS-B7.2 alone (76% specific lysis). Furthermore, increasing the dose of B7 plasmids by 10-fold (100 μg) did not contribute to any additional enhancement; thus, the 10 μg dose of pMAS-B7 was
considered to be optimal (Figure 8). A suboptimal dose of the DNA vaccine (1 µg) was a requisite factor for observing any adjuvant activity of B7 coexpression. As seen in Figure 9, increasing the dose of the DNA vaccine by ten-fold (10 µg of pMAS-S) diminished the adjuvant effect of B7 coexpression. One of the reasons for the absence in the adjuvant effect of B7 DNA codelivery (1:1 of antigen:B7) may be the increase in CpG effect with increase in the total amount of DNA. In comparison to the colinear experiments, codelivery experiments utilized twice the amount of plasmid DNA (total of 20 µg) which proportionately increases the sCpG content. As sCpG motifs i) contribute to overall augmentation of immune responses (73); ii) induce an optimal cytokine milieu (58); and iii) upregulate costimulatory molecules (62), they may have contributed to an increase in immune responses. This effect, however, is not solely attributed to the increase in CpG content, as codelivery studies using 11 µg of total DNA (1 µg of DNA vaccine) do not have the same level of response noted with injection of the DNA vaccine (10 µg). Therefore, the amount of antigen encoding DNA still remains a strong determinant of the immune response that subsequently develops. The lack of any immune augmentation with codelivery, with a higher dose of the DNA vaccine, may be attributed to a synergistic effect between the antigen and sCpG motifs, which in turn may mask the effect of B7 coexpression.

Using colinear expression plasmids, pMAS-S-B7.1 or pMAS-S-B7.2, antigen-specific CTL responses were not as potent as with codelivery. Here, colinear expression of B7.2, but not B7.1 significantly enhanced CTL responses at E:T ratios of 250:1 and 50:1 (Figure 10, P<0.05). Since codelivery of B7.1 plasmids significantly enhanced CTL responses, but this effect was undetectable with colinear expression of B7.1, it is
Figure 7: Cytotoxic T cell responses are enhanced with codelivery of B7 encoding plasmids

BALB/c mice were immunized by IM injection with 1 μg of DNA vaccine (pMAS-S) encoding HBsAg and 10 μg of plasmids encoding B7.1 (Panel A), B7.2 (Panel B) or both B7.1 and B7.2 (Panel C). B7 genes cloned in the reverse orientation were used as non-expressing controls along with the backbone vector pMAS. Numbers at the bottom of the panel represent mice that responded as a fraction of the total number of mice. Height of each bar represents the mean HBsAg-specific lysis as a percentage of the total possible lysis (% specific lysis) at E:T of 10:1 in splenocytes from mice that responded (specific lysis > 10%) 4 wk after immunization. Vertical lines on bars represent SEM. *P< 0.05.
Figure 8: Higher doses of B7 encoding DNA do not contribute to additional enhancement in CTL responses

BALB/c mice were immunized by IM injection with 1 µg of DNA vaccine (pMAS-S, ◆) encoding HBsAg and 10 µg (Panel A) or 100 µg (Panel B) of plasmids encoding B7.1 (■) or B7.2 (▲). Each value represents mean HBsAg-specific lysis as a percentage of the total possible lysis (% specific lysis) at E:T of 250:1, 50:1, 10:1 and 2:1 in splenocytes from mice that responded (% specific lysis > 10) 4 wk after immunization. Vertical lines on bars represent SEM.
Figure 9: Higher dose of antigen-encoding DNA masks the effect of codelivering B7 plasmids

BALB/c mice were immunized IM with 10 μg of DNA vaccine (pMAS-S, ◆) encoding HBsAg and 10 μg of plasmids encoding B7.1 (□) or B7.2 (▲). Each value represents the mean HBsAg-specific lysis as a percentage of the total possible lysis (% specific lysis) at E:T of 250:1, 50:1, 10:1 and 2:1 in splenocytes from mice that responded (specific lysis > 10%) 4 wk after immunization. Vertical lines on bars represent SEM.
Figure 10: Colinear expression of B7.2 augments CTL responses

BALB/c mice were immunized IM with 10 μg of pMAS-S-B7.1 (panel A) or pMAS-S-B7.2 (panel B). B7 genes cloned in the reverse orientation (pMAS-S-B7.1(R), pMAS-S-B7.2(R)) were used as non-expressing controls (hatched bars). Numbers in brackets represent mice that responded as a fraction of the total number of mice. Height of each bar shows the mean HBsAg-specific lysis as a percentage of the total possible lysis (% specific lysis) at E:T of 250:1, 50:1, 10:1 and 2:1 in splenocytes from mice that responded (specific lysis > 10%) 4 wk after immunization. Vertical lines on bars represent SEM. *P<0.05.
possible that the level of B7.1 expression determines if any adjuvanticity of B7.1 is to be detected.

3.3 Comparison of Codelivery and Colinear Expression by the ID Route

In order to determine if effects of B7 coexpression could be extended to other routes, we also evaluated codelivery and colinear expression of B7 plasmids in the skin. Previous studies in our laboratory have shown a 10 μg dose of pMAS-S to be suboptimal for the ID route (Davis et al., unpublished). Based on these findings, we extended our codelivery studies to include this suboptimal dose of DNA vaccine with 10 μg of B7 encoding plasmids and colinear expression studies with a 10 μg dose. In addition we investigated if B7.1 and B7.2 coexpression could contribute to skewing from a Th2 biased response, usually seen with the ID route, to a Th1 biased response.

3.3.1 Effect of Coexpression on Humoral Responses

Upon codelivery of B7.1 or B7.2 encoding plasmids (10 μg) with the DNA vaccine (10 μg), we observed no enhancement in total IgG titres (Figure 11a, P>0.05). In contrast, colinear expression of B7.1 or B7.2 in the context of the DNA vaccine resulted in significant enhancement in total IgG titres (Figure 12a, P<0.05). It is evident from this comparison that colinear expression is superior to codelivery of B7's since the adjuvant effect in humoral titres, that was not detectable with codelivery, are seen with colinear expression. With the ID route of administration, B7.1 was found to be more potent in augmenting total IgG titres (7-fold greater) compared to B7.2. This was in sharp contrast to increases noted with the IM route where both B7.1 and B7.2 contributed to equivalent increases in antibody titres. Furthermore, in comparison to IM
Figure 11: Humoral responses are unaffected by ID codelivery of B7 plasmids with the DNA vaccine

Strength of humoral response in BALB/c mice immunized ID with 10 μg of DNA vaccine (pMAS-S) and 10 μg of B7.1 or B7.2 plasmids (grey bars). B7 genes cloned in the reverse orientation were used as non-expressing controls (hatched bars) along with the backbone vector pMAS. Shown in the panel A are HBsAg-specific total IgG titres which show no significant differences with codelivery of B7 plasmids. Panel B represents HBsAg-specific Ig-isotype titres and numbers at the top of each bar in panel B represent the IgG2a:IgG1 ratio. Height of each bar represents the GMT (n=5) for anti-HBs ELISA titre (total IgG) at 4 wk post administration. Vertical lines on bars represent SEM. End-point titres were defined as the highest plasma dilution that resulted in an absorbance value of (OD 450 nm) two times greater than that of control non-immune plasma, with a cut-off value of 0.05.
Figure 12: ID injection of colinear plasmids
encoding B7 and HBsAg genes increases IgG titres

Strength of humoral response in BALB/c mice immunized ID with 10 µg of DNA vaccine (pMAS-S), pMAS-S-B7.1 or pMAS-S-B7.2. B7 genes cloned in the reverse orientation (pMAS-S-B7.1(R), pMAS-S-B7.2(R)) were used as non-expressing controls. Panel A represents HBsAg-specific total IgG titres which are significantly enhanced with B7 colinear expression. Panel B represents HBsAg-specific Ig-isotype titres and numbers at the top of each bar in panel B represent the IgG2a:IgG1 ratio. Each bar represents the GMT (n=5) for anti-HBs ELISA titre (total IgG) at 4 wk post administration. Vertical lines on bars represent SEM. End-point titres were defined as the highest plasma dilution that resulted in an absorbance value of (OD 450 nm) two times greater than that of control non-immune plasma, with a cut-off value of 0.05.

*P<0.05.
injections, ID injections yielded higher IgG titres regardless of B7 coexpression (Figure 5a vs. 12a) indicating that the latter was a better route to induce strong antibody titres.

3.3.2 *Ratio of IgG2a/IgG1 – an index of Th1-Th2 bias*

Immune responses after ID administration of the DNA vaccine resulted in a mixed Th response as detected by approximately equal proportions of IgG1 and IgG2a. Codelivery of B7 plasmid pushed the Th response from a mixed to one that was favoring a Th1 response. As seen by the number (ratio IgG2a/IgG1) that is indicated on each bar (Figure 11b), codelivery of B7.1 or B7.2 gives ratios of 14.3 and 6.3 respectively, while their reverse cloned control give ratios less than 2. The trend in Th switch was also apparent with colinear expression of B7.1 or B7.2 (Figure 12b). Thus, both, codelivery and colinear expression of B7 plasmids were able to overcome the mixed Th-bias seen with ID injections of DNA vaccines.

3.3.3 *Cytotoxic T cell responses*

Both, codelivery and colinear expression of B7.2 resulted in significant enhancement in antigen-specific CTL responses. Codelivery of pMAS-B7.2 with the DNA vaccine pMAS-S, resulted in a 2-fold increase in CTL response at an E:T ratio of 50:1 (Figure 13, P<0.05). Similarly, colinear expression vector pMAS-S-B7.2, in comparison to the reverse cloned control, caused about a 3 fold increase in antigen-specific lysis at E:T ratios of 50:1 and 10:1 (Figure 14, P<0.05). Whereas, codelivery of pMAS-B7.1 with the DNA vaccine did not contribute to any significant enhancement in CTL responses (Figure 13), colinear expression of B7.1 resulted in augmented (3-fold increase) antigen-specific CTL responses (Figure 14, P<0.05). Thus, here again, CTL
**Figure 13:** Cytotoxic T cell responses are enhanced with

**ID codelivery of B7.2 encoding plasmids**

BALB/c mice were immunized ID with 10 μg of DNA vaccine (pMAS-S) encoding HBsAg and 10 μg of plasmids encoding B7.1 or B7.2 (grey bars). B7 genes cloned in the reverse orientation were used as non-expressing controls (hatched bars) along with the backbone vector pMAS (black bar). Numbers at the bottom of the panel represent mice that responded as a fraction of the total number of mice. Height of each bar represents the mean HBsAg-specific lysis as a percentage of the total possible lysis (% specific lysis) at E:T of 50:1 in splenocytes from mice that responded (% specific lysis > 10) 4 wk after immunization. Vertical lines on bars represent SEM. *P<0.05.
Figure 14: ID injection of colinear plasmids encoding

B7 and HBsAg genes augments CTL responses

BALB/c mice were immunized ID with 10 μg of pMAS-S-B7.1 (panel A) or pMAS-S-B7.2 (panel B). Also shown are results of ID immunization with 10 μg of DNA vaccine alone (black bars). B7 genes cloned in the reverse orientation (pMAS-S-B7.1(R), pMAS-S-B7.2(R)) in the colinear plasmids were used as non-expressing controls (grey bars). Numbers in brackets represent mice that responded as a fraction of the total number of mice. Height of each bar represents the mean HBsAg-specific lysis as a percentage of the total possible lysis (% specific lysis) at E:T of 50:1, 10:1 and 2:1 in splenocytes from mice that responded (% specific lysis > 10) 4 wk after immunization. Vertical lines on bars represent SEM. *P<0.05.
responses with colinear expression were superior to codelivery. Moreover, a notable similarity in these results was the potency of B7.2 over B7.1 in enhancing CTL responses both, by IM and ID routes of administration. Summarized in Table 5 are the results of all coexpression experiments.

3.4 Kinetics of DNA Passage after IM and ID Injections

To detect the trafficking of plasmid DNA from the injected site to regional lymph nodes, these tissues were removed from the mice after they were injected with pMAS-S (10 μg) and assessed for presence of plasmid at two time points. Plasmid DNA was detected in both the injected muscle and lymph nodes (popliteal and inguinal pooled) 5 min after an IM injection. In contrast, 5 min following ID administration, the injected skin had an abundance of the plasmid but none was yet detectable in the proximal lymph nodes (left and right inguinals) (Figure 15a). Preliminary results indicate the presence of the plasmid in both the injected site and the proximal lymph nodes 5 h post injection, either via IM or ID routes of administration (Figure 15b). These results indicated that the kinetics in plasmid arrival at the lymph nodes vary between the two routes of administration. Plasmid injected in the muscle reaches the lymph nodes more quickly, while DNA injected by the ID route reaches the lymph nodes gradually.

3.5 Combination Studies – Strategy to Augment Humoral Responses

Our initial codelivery experiments by the IM route showed strong increases in CTL responses when B7 plasmids were injected with a suboptimal dose of the DNA vaccine. However, this regime did not result in a concomitant increase in humoral titres. Hence, we explored the possibility of combining the above strategy with an additional
Table 5: Summary of Coexpression Experiments

<table>
<thead>
<tr>
<th></th>
<th>Codelivery</th>
<th></th>
<th>Colinear Expression</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dose of: Antigenic DNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B7 DNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µg/10 µg</td>
<td>IM</td>
<td>10 µg</td>
<td>ID</td>
<td>10 µg</td>
</tr>
<tr>
<td>10 µg</td>
<td>IM</td>
<td>10 µg</td>
<td>ID</td>
<td>10 µg</td>
</tr>
<tr>
<td><strong>CTL Responses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B7.1 - ↑</td>
<td>IM</td>
<td>B7.1 – No Effect</td>
<td>ID</td>
<td>B7.1 - ↑</td>
</tr>
<tr>
<td>B7.2 - ↑↑</td>
<td>IM</td>
<td>B7.2 - ↑</td>
<td>ID</td>
<td>B7.2 - ↑↑</td>
</tr>
<tr>
<td><strong>Ab Responses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B7.1 or B7.2</td>
<td>IM</td>
<td>B7.1 or B7.2</td>
<td>ID</td>
<td>B7.1 - ↑</td>
</tr>
<tr>
<td>B7.2 - No Effect</td>
<td>IM</td>
<td>B7.2 - No Effect</td>
<td>ID</td>
<td>B7.2 - ↑</td>
</tr>
<tr>
<td><strong>IgG Isotype Responses</strong></td>
<td></td>
<td>Th1</td>
<td>B7.1 - IgG2a &gt; IgG1</td>
<td>B7.1 &amp; B7.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IgG2a &gt; IgG1</td>
</tr>
</tbody>
</table>
BALB/c mice were injected IM or ID with 10 μg of pMAS-S. At 5 min post injection, target site of injection (TA muscle or the area of skin where DNA was injected) was removed. In addition, for IM injections, the inguinal and popliteal lymph nodes were removed and pooled. For ID injection both inguinal lymph nodes were removed after injection and pooled. Lanes 2-5 represent tissue samples following IM injection while lanes 6-9 represent tissue samples from ID injections. Animals injected with saline were used as negative controls (lanes 10-14). Positive and negative controls for the PCR reaction consisted of pMAS-S plasmid or H2O in the reaction mixture (lanes 15, 16 respectively). The presence of the specific PCR product in each reaction tube was assayed by electrophoresis on 0.8% agarose gel followed by staining with ethidium bromide.
Detection of plasmid DNA in different sites
5 minutes after injection

1 Kb DNA ladder

Muscle

Lymph Nodes

Skin

Lymph Nodes

Muscle

Lymph Nodes

Skin

+ control

- control

1 Kb DNA ladder

Intramuscular

Intradermal

Controls
Figure 15b: Detection of DNA plasmid 5 hours following IM and ID injections

BALB/c mice were injected with 10 µg of pMAS-S either IM or ID. At 5h post injection, target site of injection (TA muscle or the area of skin where DNA was injected) was removed. In addition, for IM injections, the inguinal and popliteal lymph nodes were removed and pooled. For ID injection both inguinal lymph nodes were removed after injection and pooled. Panel A: IM injection; panel B: ID injection. Positive and negative controls for the PCR reaction consisted of pMAS-S plasmid or H₂O in the reaction mixture (panel A: lanes 5,6 & panel B: lanes 4,5). The presence of the specific PCR product in each reaction tube was assayed by electrophoresis on 0.8% agarose gel followed by staining with ethidium bromide.
Detection of plasmid DNA in different sites 5 hours after injection

A: IM

1. Muscle
2. Lymph Nodes
3. + control
4. - control
5. 1 Kb DNA ladder

B: ID

1. Skin
2. Lymph Nodes
3. + control
4. - control
5. 1 Kb DNA ladder
element, rHBsAg, which could potentially boost the humoral response. Initially, a tricomponent vaccine including the HBsAg-expressing DNA vaccine (1 μg), the B7 plasmid (10 μg) and the rHBsAg protein (1 μg) were administered simultaneously, into the left TA muscle.

The animals immunized with the tricomponent vaccine showed 3-fold higher antibody titres than the animals immunized with only HBsAg-expressing DNA (1μg) + B7 plasmids (10 μg) (Figure 16). The isotype profile indicated a Th2 or mixed Th response with all combinations of rHBsAg and plasmids encoding DNA and B7 (Figure 16). However, the CTL augmentation that had been observed with the codelivery of HBsAg-expressing DNA vaccine (1 μg) + B7 plasmids (10 μg) was lost with the use of the tricomponent vaccine (Figure 17a).

The loss of CTL augmentation with codelivery of HBV DNA vaccine (1 μg) and B7 plasmids (10 μg), when used in the context of a tricomponent vaccine, may have been due to interference of the protein with plasmid DNA transfection. Therefore, in our next set of experiments, the plasmid DNA components (HBV DNA vaccine & B7 plasmids) were injected into the TA muscle while the protein component (rHBsAg at 1 μg) was injected into the upper quadriceps muscle thereby reducing possible interference. This approach to augment humoral responses with rHBsAg and CTL responses with DNA+B7 combination, however, was inferior; both humoral and CTL responses were equivalent to protein injected controls (Figure 18, 17b). Here again, the isotype profile indicated a Th2 or mixed Th response as evidenced by the Ig2a:IgG1 ratios.
Figure 16: Combination of DNA and protein: Effects of single site administration on humoral responses

Strength of humoral response in BALB/c mice immunized IM with a vaccine consisting of 1 μg of DNA vaccine (pMAS-S) and 10 μg of pMAS-B7.1 or pMAS-B7.2 with (black bars) or without (grey bars) rHBsAg (1μg). Both DNA and protein components were injected into the left TA muscle. Panel shows HBsAg-specific total IgG titres and numbers at the bottom of each bar represent the IgG2a:IgG1 ratio. Height of each bar represents the GMT (n=5) for anti-HBs ELISA titre at 4 wk post administration. Vertical lines on bars represent SEM. End-point titres were defined as the highest plasma dilution that resulted in an absorbance value of (OD 450 nm) two times greater than that of control non-immune plasma, with a cut-off value of 0.05.
Figure 17: Combination of DNA and protein: Effects of single site and multiple site administration on CTL responses

BALB/c mice were immunized IM with a tricomponent vaccine consisting of 1 μg of DNA vaccine (pMAS-S) and 10 μg of pMAS-B7.1 or pMAS-B7.2 with (black bars) or without (grey bars) rHBsAg (1μg). Panel A: All three components were mixed prior to injection and administered into the left TA muscle. Panel B: The DNA components were mixed prior to injection and administered into the left TA muscle and the protein component was administered into the upper quadriceps muscle. Height of each bar represents the mean HBsAg-specific lysis as a percentage of the total possible lysis (% specific lysis) at E:T of 50:1 in splenocytes from mice that responded (% specific lysis > 10) 4 wk after immunization. Vertical lines on bars represent SEM.
Figure 18: Combination of DNA and protein: Effects of multiple site administration on humoral responses

Strength of humoral response in BALB/c mice immunized IM with a vaccine consisting of 1 µg of DNA vaccine (pMAS-S) and 10 µg of pMAS-B7.1 or pMAS-B7.2 with (black bars) or without (grey bars) rHBsAg (1 µg). The DNA components were mixed prior to injection and administered into the left TA muscle and the protein component was administered into the upper quadriceps muscle. Panel represents total IgG titres and numbers at the bottom of each bar represent the IgG2a:IgG1 ratio. Height of each bar represents the GMT (n=5) for anti-HBs ELISA titre at 4 wk post administration. Vertical lines on bars represent SEM. End-point titres were defined as the highest plasma dilution that resulted in an absorbance value of (OD 450 nm) two times greater than that of control non-immune plasma, with a cut-off value of 0.05.
CHAPTER 4
OVERVIEW

In this chapter, I have included a brief explanation of the rationale for the approach taken and discussed the results presented in the previous chapter. Also discussed are (i) the current understanding of immunity induced by DNA immunization and possible modes by which B7 coexpression might augment immune responses, (ii) the implications and future directions of this investigation and (iii) the safety issues that concern B7 coexpression in particular and DNA vaccines in general.

4.1 Rationale for B7 Coexpression

The purpose of this investigation was to study the effects of modifying the environmental context of antigen presentation on antigen-specific humoral and cell mediated responses. More specifically, this thesis examines a strategy for optimization of an HBV DNA vaccine by coexpression of the ligands for CD28, namely B7.1 and B7.2 with either IM or ID delivery.

Initially, we believed that B7 coexpression in muscle cells could augment immune responses in a different manner (i.e., transformation of myocytes into APCs) than that mediated by the CpG motifs contained in plasmid DNA (i.e., to provide a "danger signal" that would increase B7 expression in APCs). But during the course of our investigation, other groups demonstrated that with DNA-based immunization, cells of non-bone marrow origin, cannot act as APCs, even if they are engineered to express B7s (53). Rather, it was shown that only APCs can induce immune responses following DNA immunization (51, 52). Nevertheless, in those studies, B7 coexpression did bring about pronounced increases in CTL responses (127, 132, 196). This suggested that enhancement with B7 coexpression: (i) occurred through APCs, and/or (ii) occurred
through non-APCs, but by a mechanism other than transformation of those cells into APCs. We therefore used a similar strategy in a mouse HBV DNA vaccine model to determine if humoral and CTL responses could be augmented by coexpression of B7 molecules.

Both codelivery (132, 196, 243) and colinear expression of B7s had previously been shown to augment antigen-specific CTL responses (127), but the relative efficacies of these approaches were not addressed. B7 and MHC interaction with counter-receptors on T cells must occur simultaneously, thus it was hypothesized that colinear expression of B7 and antigen genes with obligate expression of both gene-products in the same cells would induce better augmentation of responses than codelivery of separate plasmids encoding antigen and B7.

We also compared the two approaches of coexpression by two routes of administration namely IM and ID. The original plan was to evaluate B7 coexpression with only IM injection of the DNA vaccine. However, the finding that immune enhancement with B7 coexpression is not mediated by transformation of myocytes into APCs indicated an alternate mechanism that might involve APCs themselves (detailed in section 4.8). Given this alternate mode of enhancement, it is possible that in the skin, where there are more APCs than in muscle, B7 coexpression may exert a greater influence.

In this body of work, we demonstrate that coexpression of B7 molecules can augment antigen-specific immune responses by both IM and ID routes of injection. Our comparison between codelivery (at a 1:1 ratio to allow comparison with colinear expression where there is obligate 1:1 expression) and colinear expression revealed that
the colinear approach was superior for both IM and ID routes, as hypothesized. However, the separate plasmid approach allowed different ratios of B7 and antigen to be tested and we discovered that increasing the ratio of antigen : B7 encoding plasmids could be advantageous, particularly at very low doses of the DNA vaccine.

Hence it is evident that B7 coexpression may be used as an optimization strategy for DNA vaccines, even if it does not involve transformation of non-APCs into APCs. Our studies also indicate that B7 coexpression can not only augment T cell responses, but is also capable of positively influencing antigen-specific B cell responses.

4.2 Comparison of Codelivery and Colinear Expression

We have tested two different approaches to optimize immune responses to an HBV DNA vaccine in mice by coexpression of B7.1 or B7.2 costimulatory molecules: (i) codelivery of the B7 and antigen encoding plasmids, where the two plasmids are mixed prior to injection; and (ii) colinear expression of the B7 and antigen genes from the same plasmid. These two approaches were each tested by two routes of administration, namely IM and ID injection. Since B7 interactions with CD28 must occur simultaneous with MHC-TCR interactions, the assumption with the codelivery approach is that the B7- and antigen-encoding plasmids transflect, largely, the same cells. Our results support the hypothesis that with codelivery, plasmids encoding antigen and B7 are transfected into at least some of the same cells since CTL responses by both IM and ID routes are enhanced with B7 codelivery. We also predicted that obligate coexpression of B7 and the antigen within the same cell by using colinear expression (both genes on same plasmid) would further increase the effects of B7 coexpression than the responses seen with the codelivery approach. For this comparison we also employed
the strategy of colinear expression (both genes on same plasmid) where there would be obligate coexpression of antigen and B7 proteins in the same cell.

As expected, colinear expression was superior to codelivery of antigen and B7 encoding plasmids when these plasmids were delivered at a ratio of 1:1 to mimic the ratio with colinear expression. However, codelivery allows varying the ratio of antigen:B7 encoding plasmids and better CTL enhancement was noted by the IM route at a 1:10 ratio of antigen:B7 encoding plasmids than at the 1:1 ratio, suggesting that more costimulation relative to antigen expression may be advantageous. Another advantage associated with the codelivery approach is the simple mixing of plasmids versus the requirement for cloning of two genes into a single plasmid, as is necessary with colinear expression. In addition, with codelivery there is significant reduction in the amount of plasmid DNA required to produce strong immune responses. For example, CTL responses seen with codelivery of antigen- (suboptimal dose – 1 μg) and B7-encoding plasmids were equivalent to the responses seen with the optimal dose (10 μg) of the DNA vaccine administered alone. The possibility to use lower doses (10-fold and 2-fold reduction in antigen-encoding and total DNA respectively) is attractive as it reduces the risk of plasmid DNA integration into genomic DNA of the host. Thus, the two notable points from the comparison made between the two approaches are that: (i) Colinear expression of B7s can augment immune responses and this is superior to codelivery at a 1:1 ratio of antigen : B7 encoding DNA; (ii) Codelivery of the two plasmids allows manipulation of the ratio of antigen:B7 encoding DNA and a surplus of B7 encoding DNA appears to be advantageous.
Similarly with the ID route, there is evidence that B7s are useful adjuvants for the HBV DNA vaccine. By this route, a comparison between the efficacies of codelivery and colinear expression was also possible. B7.2 but not B7.1 codelivery caused moderate increases in antigen-specific immune responses whereas both B7.1 and B7.2 colinear expression augmented both humoral and CTL responses. Thus, like with IM results, B7 colinear expression is better for enhancing immune responses to an HBV DNA vaccine in mice than is coinjection of plasmids.

Colinear expression of B7s (pMAS-S-B7.1 and pMAS-S-B7.2) significantly augmented antigen-specific antibody titres in comparison to their respective reverse cloned controls (pMAS-S-B7.1(R) and pMAS-S-B7.2(R)). However the antiHBs response was not significantly greater with B7-expressing vectors than with the standard DNA vector, pMAS-S, and those with the reverse-cloned B7 controls were lower than with pMAS-S. Although it is not clear why the reverse cloned colinear plasmids (pMAS-S-B7(R)) were not as efficacious as the standard plasmid vector (pMAS-S) the increased size of the colinear plasmid may have been a factor. As indicated in section 2.3, the colinear plasmids consist of an insert containing the CMV promoter and the B7 gene, resulting in an additional 1.7 kb added to the standard plasmid vector (4 kb) and this increase in size may have impaired transfection. It would therefore be desirable to compare levels of expression of the colinear plasmids and the standard vector using a reporter gene such as luciferase, which could ascertain the levels of expression induced with these expression plasmids. Nevertheless, although colinear expression of B7 significantly augmented antibody levels relative to an appropriate molecular control, it did not relative to a standard vector. As such, the ultimate goal to produce a more
effective DNA vaccine through co-expression of B7 molecules was not attained, at with respect to the humoral response.

4.3 Effects of B7s – Similarities and Differences

Other investigators (141), who have used B7 coexpression IM for optimizing DNA vaccines, found that augmentation of CTL responses were obtained with B7.2 but not B7.1, whereas we found that codelivery of either B7.1 or B7.2 contributed to increases in CTL responses. A comparison of the study design might explain, at least in part, the differences in results. In the former studies, a 1:1 ratio of antigen:B7 encoding plasmid delivery was employed (122, 123); whereas, we used a 1:10 ratio for the codelivery experiments. Thus it is possible that, CTL augmentation is seen only if the levels of B7.1 expression with respect to antigen expression are sufficiently high. With respect to the observation that colinear expression of B7.1 augments humoral and CTL responses by the ID route suggest that this effect may also be due to the disease model that was used.

The dependency of B7.1 effects on the antigen model is exemplified by several tumour studies. Gajewski et al. (189, 190) have shown that B7.1 expressing tumour cell lines were effective in inducing tumour rejection and offered subsequent protection from tumour challenge. Similarly, B7.1 was shown to be superior to B7.2 in its capacity to protect against wild-type tumour challenge in a murine model of acute myeloid leukemia (244). Collectively these studies indicate that coexpression of B7s can have variable effects, the reasons for which remain unknown.

Overall, coexpression of B7.2 augmented immune responses more than coexpression of B7.1. Both B7.1 and B7.2 associate with the same counter-receptors,
CD28 and CTLA-4, but they bind to different peptide motifs of CD28 and with differing kinetics (242). It is therefore possible that the differences in the level of augmentation may be due to the differences in the period of ligation with the counter-receptors. Furthermore, antibody-blocking studies indicate that B7s differentially regulate disease development in allergic encephalomyelitis and non-obese diabetic disease models (131, 239) suggesting that B7.1 and B7.2 may induce different intracellular signal transduction pathways. This, in turn, could affect the magnitude of increase in immune responses.

Since codelivery of either B7.1 or B7.2 encoding plasmids significantly enhanced CTL responses, we predicted that these responses could be further augmented with the addition of both B7.1 and B7.2 expressing plasmids. However, there was no evidence of an additive effect with B7.1 and B7.2 codelivery since, the CTL levels were comparable to those obtained with codelivery of B7.2 plasmid alone. This again may be due to competition between B7.1 and B7.2 for the interaction with CD28 on T lymphocytes or the redundancy of B7 costimulation, with either B7.1 or B7.2 being able to support generation of CTL responses (236). There is also evidence that B7.2 might function as a dominant costimulatory molecule. For example, blocking of B7.2 but not B7.1 in MHC class-II deficient mice abrogated CTL responses (236). In addition, B7.1 knockout mice have relatively normal Th1- and Th2- dependent responses while B7.2 knockout mice are severely immune compromised (195). Thus if B7.2 plays the dominant role of costimulation for development of immune responses it may overpower the role of B7.1 when they are expressed in equivalent amounts (168).
4.4 Effects on Immune Responses

4.4.1 Humoral Responses

The lack of enhancement in humoral responses with B7 codelivery could indicate that costimulation requirements for antibody and CTL responses differ. However, this does not appear to be the case since colinear expression of B7s augmented antibody and CTL responses. Rather, the differences are likely to be related to the higher efficiency in antigen and B7 expression within the same cell or the use of a higher dose of antigen encoding DNA with colinear expression.

With colinear expression, both B7.1 and B7.2 enhanced total IgG titres against HBsAg and this was true when the plasmids were given by either IM or ID injections. Although no previous studies using B7 coexpression with DNA vaccines had noted an increase in antigen-specific antibody responses (122, 123, 187), blocking with anti-B7.1 or anti-B7.2 antibodies did abrogate humoral responses (237, 238) and abolish germinal centre formation (183) in regional lymph nodes, indicating a role for B7 in antibody production. Thus, B7 interactions are essential for initiation of immunoglobulin responses and progression of B cell differentiation that occurs in the germinal centres (182). Moreover, in a recent report, using anti-B7 antibodies, B7 costimulation following DNA immunization, was shown to be an absolute requirement for development of antigen-specific antibody responses (196). These studies demonstrate that B7 costimulation is essential for the induction of humoral responses against natural infections as well as for protein or DNA vaccines.

It is also possible that the nature of the antigen plays a role in realizing the adjuvant effect of B7 coexpression. HBsAg is strongly antigenic for humoral responses,
owing, perhaps, to the spontaneous formation of virus-like particles in the host. Coexpression of B7s, in turn, might nurture a milieu that further supports strong humoral responses dictated by HBsAg. In comparison, in a recent report, Iwasaki et al. used coexpression of B7s with a mutated form of the influenza nucleoprotein (N-terminus mutation of the wild-type nucleoprotein), and observed no enhancement of antigen-specific antibody responses (118). Hence, weak antigens may not allow the effects of B7 coexpression to be realized. As such, the antigen would be the master-switch that dictates the strength of the humoral response and if of sufficient strength, B7 coexpression could aid in augmenting this response.

4.4.2 CTL Responses

The observation that codelivery and colinear expression of B7s augmented CTL responses while colinear expression alone enhanced humoral responses is noteworthy. One possible explanation for this is that to detect augmentation in humoral responses there may be a requirement for greater number of cells expressing both antigen and B7, which is achieved with greater efficiency by the colinear approach. In addition, the threshold of antigen concentration that is required to seroconvert may be more feasible with colinear expression and this, in turn, can be augmented with B7 coexpression. In contrast, the portion of cells needed to facilitate enhancement of CTL responses may be sufficiently met with codelivery. Alternatively, B7 expression in cells might render them more susceptible to killing by CTLs thereby allowing for expansion of this population of T cells. Indeed, this is the current model used to explain the augmentation in CTL activity upon B7 coexpression in tumour models (198, 199). A similar mechanism may be prevalent in our model where myocytes expressing antigen and B7s
may be susceptible to attack by CTLs to a greater degree than those expressing only antigen, thereby facilitating the expansion of the restimulated CTLs (detailed in section 4.8).

4.5 Differences in Routes

B7 coexpression, by the two approaches had similar effects by the IM and ID routes of administration; although, colinear expression of B7s was generally superior to codelivery. The varying of the ratio of antigen to B7 encoding plasmids for the codelivery approach was not done for the ID route. The reason for this is that our initial experiments with 10 μg dose of the DNA vaccine was sufficiently suboptimal to note the effects of B7 (10 μg) coexpression.

The most noteworthy effects of B7s that were distinct for the two routes of administration is their contribution to the Th bias assessed in our experiments by the IgG2a:IgG1 ratio. By the IM route, a strong Th1 biased response was seen and B7 coexpression did not affect this bias. However, by the ID route, colinear expression of B7.1 and B7.2 contributed to skewing from a mixed (Th1-Th2) response, typically seen with the ID route, to a more Th1 biased response. These results suggest that B7 coexpression generally contribute to Th1 biased responses and further that a predisposed Th1 bias is unaffected by B7 coexpression.

In the literature, there is evidence that B7.1 and B7.2 contribute to development of Th1 and Th2 type responses respectively (245). However, a number of studies in vivo have shown that B7.1 and B7.2 can regulate both Th1- and Th2-biased responses (246, 247)). It is suggested that (i) the polarization of Th responses for a given
pathogen, (ii) the antigen load and (iii) the timing of B7 expression are all factors that can influence the Th responses that develop (168). We observed that colinear expression of B7.1 or B7.2 preferentially augmented Th1 type responses. This may be related to the antigen concentration and consequently the intensity of T cell signaling which can affect the balance of Th1/Th2 subsets. Under conditions of low antigen concentration CD28 ligation appears to be more important because of weak interaction between TCR and the antigen/MHC complex (138, 168). For example, B7 mediated costimulation exerts a stronger influence on CTL and Th responses with the weakly replicating vesicular stomatitis virus while, strongly replicating viruses such as lymphocytic choriomeningitis virus or vaccinia virus were able to trigger antigen-specific immune responses even in the absence of B7-mediated costimulation. Similarly, in the case of DNA immunization, antigen load is low (pg – ng) relative to protein vaccines (μg) (41). Thus, the lower antigen load and the higher level of B7 expression might explain the influence B7 coexpression exerts in Th1 differentiation by the ID route.

4.6 Overall Superiority of ID Delivery

When IM and ID routes were compared for a given immunization strategy, humoral responses were generally greater with the ID route of administration. This effect was noted whether DNA was administered alone or in combination with B7 coexpression, hence, the better antibody responses could not be attributed to B7 coexpression. Rather, the differences in antibody responses with the two routes may be due to the differences in: (i) efficiencies of priming, (ii) the level of circulating antigen, and/or (iii) cell types or cell numbers at the different sites of DNA delivery. On the other
hand, improved CTL responses correlated more closely with B7 coexpression than to the route of administration.

Approximately 5% of epidermal cells are bone marrow-derived Langerhans cells (55), which are dendritic cell-like professional APCs. In contrast, there are very few resident dendritic cells in muscle tissue (203). The predominant cell types (myocytes and keratinocytes) transfected following DNA injections by the IM and ID routes are capable of secreting cytokines and expressing cell adhesion molecules and thereby facilitating some form of immune amplification (203, 248). However, only the immunostimulatory properties of keratinocytes can have direct effects on APCs (i.e., Langerhans cells) (55). This may account in part for the better results with ID than IM DNA injection.

The primary site of immune induction following IM and ID routes of injection are the lymph nodes and skin respectively (54, 55). This has been demonstrated by removal of the injected tissue. When the muscle bundle was removed 5 min following IM injection there was no effect on the rate or magnitude of immune response. In contrast, removal of the skin 24 h after ID injections resulted in loss of humoral responses. Since the muscle tissue contains low numbers of APCs, it is likely that the APCs that are responsible for immune induction following IM injections are transfected remote from the site of injection, possibly in the lymph nodes. Moreover, plasmid DNA injected into muscle can readily access regional lymph nodes via lymphatics. By the ID route, DNA remains in the skin for a longer period of time and immune induction is likely facilitated by cells that are transfected near the site of injection. To investigate if the overall superiority of antibody responses by the ID route, was due to this difference
in the site of immune induction, we designed experiments to detect DNA in various tissues following IM and ID injections.

Plasmid DNA was detected in the lymph nodes 5 min after IM injection but was detectable at 5 h following ID injections. This reflects the fact that lymphatic drainage is better in muscle than skin (272) and thus may allow faster movement of the DNA from the interstitial spaces to the lymph nodes after IM injections. In contrast, since most of the DNA is localized in the skin immediately following ID injection, and is not detected in the lymph nodes until 5 h post injection, it is possible that APCs are directly transfected in the skin and the later detection of DNA in the lymph nodes may reflect the subsequent movement of APCs to the lymph nodes. It may be that this difference in the site of APC transfection may account, in part, for the differences in the level of antibody responses seen with IM and ID administration.

However, another study done in our laboratory by McCluskie et. al. (104) showed that IM delivery of a DNA vaccine was superior to ID injections for inducing antibody responses in mice. In that study, DNA solution was delivered ID as 3 separate injections (50 µl / injection site - total volume of 150 µl). Our studies, on the other hand, used only two sites in the skin with a total volume of 50 µl. It is therefore possible that the volume used for ID injection may also influence the strength of the immune response. There are likely many such factors that can influence the strength of immune responses after IM or ID delivery and as such no one route would be overall superior to other; it would depend more on the exact method that is employed.
4.7 Combination of DNA and Protein Vaccines

One possible explanation for the observation that codelivery of B7-encoding plasmids (10 μg) with the HBV DNA vaccine (1 μg) augmented antigen-specific CTL responses but not antibody titres is that the level of circulating antigen may not have been sufficient to induce a strong humoral response. In order to investigate if an increase in the level of circulating antigen can boost the humoral response, we included an additional component, rHBsAg to the plasmid DNA for immunization. Although, plasmid DNA immunization regime with B7 coexpression induced strong T cell mediated responses the antibody response induced by this method was virtually undetectable. We predicted that including the rHBsAg element with the plasmid DNA immunization strategy would increase the level of antigen-specific antibody responses while the CTL augmentation that was seen with injection of antigen and B7 encoding plasmids would be simultaneously maintained.

Mice immunized with the tricomponent vaccine (pMAS-S + pMAS-B7 + rHBsAg) did not exhibit the CTL augmentation that had been seen with codelivery of pMAS-B7 and pMAS-S without protein antigen. Humoral responses with the tricomponent vaccine were comparable to the titres seen with injection of rHBsAg alone. The lack of any enhancement in CTL responses was not due to interference in DNA transfection by the protein because even when the plasmid DNA and protein were injected into different muscles, results were no better than when the tricomponent vaccine was injected in a single site. Rather the loss of CTL augmentation that was originally noted with the plasmid DNA injections may have been due to differences in the kinetics of immune priming. Priming with protein may predispose the immune
system more towards a Th2 response, due to the presence of increased circulating antigen thereby overriding the Th1 response that would have been evident with B7 coexpression. To establish this Th2 predisposition the recombinant protein antigen must have been detected by the host's immune system, prior to protein secretion resulting from plasmid DNA expression. Indeed, it is generally thought that protein production with DNA immunization occurs in the first 3 days, with expression detectable in the lymph nodes as early as 24 h post immunization (54). However, protein introduced as an antigen is likely to be cleared from circulation in 24 h. Thus, even a simultaneous injection of protein and DNA probably results in the protein priming an immune response thereby dictating the immune system to develop a response that is more characteristic of protein-based immunization (i.e., Th2-biased). This pre-established response may not be skewed when the proteins of plasmid DNA components (antigen and B7) are subsequently expressed. Thus an alternative that may be a better approach to maintaining strong CTL responses while concurrently augmenting humoral responses would be to employ a regime where DNA vaccine and B7 plasmid are used to prime and rHBsAg is used to boost the immune response. Indeed, studies done by Cormanita et al. using a similar strategy have shown the development of good T cell and B cell responses (unpublished data).

4.8 Possible Modes of Immune Augmentation with B7 Coexpression

The mechanism of immune enhancement with coexpression of costimulatory molecules still remains unknown. Shown in Figure 1, is a summary of three possible modes by which APCs and myocytes/keratinocytes might contribute to the development
and sustenance of immune responses following DNA immunization. Also shown are three non-exclusive mechanisms by which B7 coexpression might augment responses.

1. *Engineering myocytes / keratinocytes to express B7 molecules might enable them to amplify immune responses*

Little is known about the role of occasional APCs, that is, cells that are not professional APCs (e.g. myocytes or keratinocytes), that are induced to express MHC class II following an inflammatory response. In contrast to what was predicted in the early reports of DNA immunization, transfected muscle cells do not play a role in the induction of immune responses (53, 55). However, immunobiology of myocytes/keratinocytes indicate that they can contribute to a microenvironment that can influence the outcome of an immune response (203). As such, it is conceivable that these cells function to amplify signals that were initiated in the lymph nodes. Studies that examined the possibility of priming immune responses by engineering non-bone marrow cells to express B7s have demonstrated that such a mode of immune induction is not possible (52, 53). This is not a surprising finding as naïve lymphocytes are restricted from entry into peripheral tissue such as the skin or muscle (8). However, as the classic experiments by Frey and Wenk demonstrate, once the T cells are sensitized in the local lymph nodes, they are capable of recirculating and readily migrating from the vasculature into peripheral solid tissue (8, 249). Moreover, Chen *et al.* (201) demonstrated that costimulation, at the site of inflammation, is a requirement both in the inductive and effector phases of T cell responses. The effector phase of the T cell response to infectious agents is initiated when infiltrating T cells are restimulated by microbial antigens in infected tissue (201) and is also likely the case
**Figure 1: Possible mechanisms of immune augmentation**

with B7 coexpression

Immune enhancement to the hepatitis B DNA vaccine might proceed by three mechanisms: 1) Transfected myocytes/keratinocytes that express B7 molecules might enable them to amplify immune responses; 2) Accelerated maturation of APCs due to B7 coexpression might enable presentation of engulfed antigen secreted by myocytes/keratinocytes leading to a greater population of mature APCs inducing an immune response; 3) Direct transfection of B7 encoding DNA to resident APCs or APCs present in the proximal lymph nodes thereby lowering activation threshold.
1) Amplification of responses at site of inflammation

2) Accelerated maturation of APCs

3) Direct transfection of plasmid to APCs

Muscle/Skin

Lymph Nodes

↑ CTL activity

↑ Th1 cytokines

Transfected B7
Endogenous B7
Naïve T cell
Primed T cell
with cells presenting antigenic sequences derived from vaccines. This restimulation is necessary because T cells that mediate inflammation secrete cytokines, which are labile in the absence of T cell stimulation (201). In a similar manner, myocytes/keratinocytes engineered to present antigen and express B7 molecules may serve to amplify the immune response at the site of inflammation. Evidence that muscle cells can, at least under certain conditions, fulfill costimulatory function is provided by a recent finding that muscle cells express a costimulatory molecule distinct from B7.1 or B7.2 in certain inflammatory myopathy diseases (250).

Studies show that myoblasts expressing surface molecules (MHC class II and ICAM-I) act as ‘facultative APCs’ and facilitate immune responses within the local setting (251). In addition, it has been demonstrated that keratinocytes can secrete cytokines such as GM-CSF, IL-1 and TNF-α, which, in turn, can recruit leukocytes to the site leading to a faster inflammatory response (252). Similarly, myocytes / keratinocytes expressing B7s may attract and facilitate interaction of the MHC-antigen and TCR complexes between themselves and the primed T cells. This interaction may also serve to stimulate secretion of cytokines and other surface molecules, which serve to amplify the inflammatory response. Several other cell types such as astrocytes (253-255), microglial cells in the central nervous system (256, 257) and pigment epithelial cells in the retina (258-260) have also been shown to be capable of enhancing immune responses after exposure to inflammatory cytokines such as IFN-γ. It is therefore possible that non-APCs engineered to express B7s might interact with primed CD4+ and CD8+ cells, thereby amplifying T cell responses upon recirculation to the site of inflammation.
2) Accelerated maturation of APCs due to B7 coexpression might enable better presentation of engulfed antigen secreted by myocytes / keratinocytes leading to a greater population of mature APCs inducing an immune response.

Another possibility to explain the augmentation of immune responses with B7 coexpression is that APCs manipulated to express B7s might enable them to mature faster and in greater numbers.

Maturation of APCs, particularly dendritic cells are crucial for the initiation of immune responses. Immature dendritic cells possess the capacity to capture and process antigen in vitro, and there is a gap of approximately 24 h before T cell stimulatory functions are acquired (i.e., expression of CD40, CD54 and B7s). Since protein expression as a result of plasmid DNA transfection is detectable as early as 1 hr post injection (271), coexpression of B7s from plasmid DNA might serve to reduce the time between antigen capture and APC maturation. Moreover, the interaction between dendritic cells and T cells is a two-way dialogue where the dendritic cells also respond to interaction with the T cells (261). Several surface molecules including CD40 and TRANCE / RANK receptors expressed on dendritic cells are ligated by the TNF family of proteins expressed on T cells, which in turn leads to increased dendritic cell survival (262-264). In a similar manner, transfection of B7-expressing plasmids into APCs might not only enhance the rate of maturation but also prolong the survival of this pool of cells.

Furthermore, HBsAg, an exogenous antigen can be processed by an alternate pathway allowing for MHC class I loading (265-267). This mechanism is favourable for the induction of class I induced CTL responses. As discussed above, since transfection
of B7 encoding plasmids might facilitate APCs to mature faster, these cells might be capable of capturing antigen secreted by non-APCs sooner, and this antigen may be presented in the context of MHC class I molecules to prime CTL responses. Thus, the unique property of exogenously acquired HBsAg to be presented in the context of MHC class I does not restrict immune augmentation to cells that are transfected with antigen and B7 encoding plasmids but is also permissive to cells transfected with B7 plasmids alone. Thus, APCs manipulated temporally and quantitatively to express B7s might mature faster and in greater numbers, which, in turn, could enable better presentation of engulfed or endogenously synthesized antigen and strengthen the immune response.

3. Direct transfection of B7 and antigen encoding DNA to resident APCs or APCs in the lymph nodes thereby lowering activation threshold

It is also possible that there may be direct transfection of antigen and B7 encoding DNA into APCs, which might augment CD4+ and CD8+ T cell responses. As discussed under mechanism 2, both the kinetics and the magnitude of B7 expression in directly transfected APC might help in strengthening the immune response. However, sCpG motifs in the DNA plasmid provide a strong danger signal and upregulate endogenous expression of B7s (62). Therefore, immune augmentation facilitated by direct transfection of B7 plasmids into APCs is probably not the predominant mechanism.

4.9 Implications of this Study

This thesis evaluates the possibility to use B7 molecules as genetic adjuvants for DNA vaccines. Our results demonstrate that both the Th-bias and magnitude of the immune response induced by DNA vaccines may be modulated by B7 coexpression.
Furthermore, our comparison between codelivery and colinear expression indicate that while the latter approach might be considered desirable owing to the obligate expression of antigen and B7 in the same cells, we show that former approach may also be used and in fact may be preferable as it more easily allows manipulation of the ratio between antigen and B7 encoding genes. For instance, codelivery of B7 plasmids was found to be more effective for augmenting CTL responses while colinear expression, particularly by the ID route, may be useful for inducing a wider spectrum of immune responses (i.e., humoral and CTL). Thus, our comparison of the approaches did demonstrate that each one had its own advantages. Depending on the correlates of protection for a disease, one of the two approaches may be more suited to combat that particular disease.

HBV is one of many viral infections that can persist after primary infection (212). Chronic HBV infection can cause significant morbidity through development of cirrhosis or hepatocellular carcinoma (212). The T cell response to HBV is vigorous, polyclonal and multi-specific in acutely infected patients who successfully clear the virus, while is weak and narrowly focused in chronically infected patients (212, 214). It is thought that preferential activation and commitment towards Th1 subset would influence the clinical consequences of HBV infection as spontaneous exacerbation of the virus in chronic carriers is thought to be associated with this type of response (268). In light of the results obtained with B7 coexpression, it seems plausible that they may be employed as a therapeutic vaccine wherein a Th1 response with strong CTL can effectively combat viral persistence. This effect may be true for many viral infections that have a tendency to persist as chronic or latent infections such as HIV, CMV and hepatitis C virus.
As discussed under mechanism II of section 4.8, the acceleration of APC maturation may in turn alter the kinetics of CTL responses. It is conceivable that with B7 coexpression, CTL responses may have been accelerated. This is particularly attractive for immunization of children in endemic areas where development of rapid, strong responses is invaluable because these children are exposed to a reservoir of viruses during or soon after birth. In addition, the promising results obtained from coexpression by the ID route suggest that this route of immunization is suitable for prophylactic vaccinations.

4.10 Safety Concerns

High levels of costimulation coupled with TCR occupancy may contribute to attenuated responses (141), based on an extension of the high zone tolerance and clonal exhaustion theory (138). This is thought to result from extensive signaling by the TCR and CD28 ligation with their respective ligands. Coexpression of increased B7s may lead to hyperstimulation of the immune system such that immune responses may be terminated prematurely. In addition, as the kinetics in the expression of endogenous versus engineered B7s differs significantly, CTLA-4 expression may also be altered. Since CTLA-4 mediated signals downregulate immune responses, earlier expression of B7s might result in interaction with this suppressive counter-receptor sooner. Therefore, it would be necessary to monitor the effects of B7 coexpression, through challenge models, so that detrimental effects such as exhaustion or premature termination are prevented. An often-addressed concern about manipulating expression of immune regulators is the long-term effects of plasmid expression in the organism. One of the most attractive advantageous of targeting over-expression of B7 molecules arises from
the existence of two counter-receptors each fulfilling antagonistic roles. Consequently, the effects of B7 interaction are realized in a tightly regulated manner whereby any deleterious effects of prolonged B7 expression are regulated by CTLA-4 delivering negative signals. Moreover, the use of bi-cistronic vectors, which concomitantly express antigen and B7, will likely shorten expression of B7s in the target site, as cells expressing antigen are susceptible to immunological destruction once CTL are induced (50).

Potential dangers of plasmid DNA immunization itself include (i) integration, (ii) immunologic tolerance, and (iii) autoimmunity, such as the induction of anti-DNA antibodies. An integrative event of the plasmid in host cells may be harmful when it is phenotypically mutagenic. Integration is more likely when the replication of the host genome and the plasmid DNA is concurrent and there are large closely spaced regions of homology (18). As the plasmid employed for DNA vaccines have origin of replication functional only in prokaryotes and have very limited sequence homology with mammalian DNA, an integrative event is highly unlikely and an integrative event that is mutagenic is even less likely.

The potential for immunologic tolerance and autoimmunity was considered possible as it was thought that small amounts of the antigen would be secreted for weeks after injection. However, there has been no evidence of this and in fact tolerance to antigens can be broken with DNA vaccines (269).

The development of autoimmunity, again is a theoretical concern, which may arise with destruction of muscle fibres by immune cells. However, muscle fibres are routinely destroyed during viral and bacterial infections, and this does not pose a great
risk for autoimmunity (18). Lastly, the possibility of inducing anti-DNA antibodies resulting in autoimmune diseases is a concern. However, antibodies to double stranded DNA can be detected in normal mice and humans. These antibodies were most likely the result of prior exposure to bacterial infection (18). In any event, these antibodies have been shown to be specific for bacterial DNA and are not cross-reactive to mammalian DNA (18, 270). Thus the evidence suggests that none of these factors are likely to contribute to any detrimental effects of DNA immunization in the host.

4.11 Future Directions

In order to attempt to further enhance immune responses by B7 coexpression, it would be helpful to better understand the mechanism by which immune enhancement occurs. In addition, the induction of memory responses in animals coexpressed with B7s must be evaluated both in terms of the rate and magnitude at which anamnestic responses develop. While the Th-bias for the various B7 treatments were assessed on the basis of the IgG-isotype ratios, a more accurate picture of the Th-bias can be established when the cytokine profiles with B7 coexpression are determined.

A fairly straightforward experiment could shed some light on the contribution of non-hemopoetic cells to enhancement of immune responses following B7 coexpression. Codelivery of two plasmids, one expressing the B7 gene and the other expressing the antigenic component could be injected IM. Here, a leak-proof muscle-specific promoter must be used to drive B7 expression and a ubiquitous promoter to drive antigen expression. If CTL augmentation is noted, then it is evident that B7 expression in the muscle serves to amplify T cell responses while the predominance of APCs in serving as
both inductors and amplifiers will be evident if no enhancement is noted with this strategy.

In addition, the combination studies have implications on the design of future studies. It is evident that the tricomponent vaccine, consisting of DNA encoding antigen and B7 and recombinant HBsAg, abrogates the augmentation noted with coexpression of B7 and antigen encoding plasmids. Whether this is due to deficiency in DNA transfection or a fundamental problem in the mechanism of immune induction is a vital question that must be addressed. Not only will a detailed study provide alternatives to the use of DNA-protein combinations but will also offer explanations to the kinetics and the mechanism of immune induction with DNA vaccines.

4.12 Summary

Coexpression of B7 molecules can augment immune responses mediated by the HBV DNA vaccine. Both codelivery and collinear expression have their selective advantages in enhancing the type and the magnitude of the immune response. Codelivery of B7s had a notable effect in enhancing CTL responses by the IM and ID routes of injection. While collinear expression by the IM route contributed to a significant increase in antigen-specific antibody titres and moderate increases in CTL responses, by the ID route both humoral and CTL responses were significantly increased in addition to Th1 skewing of the response. The significant increase in CTL responses and skewing of Th responses that can be attained with B7 coexpression, can be used for therapeutic vaccination for diseases requiring strong cell mediated immunity. Furthermore, while all experiments in this work have been performed using the hepatitis B disease model, the results may be applicable to emerging and existing viral infections.
APPENDIX I

Immunization schedule for current hepatitis B vaccines

Immunization with the hepatitis B vaccine is considered to have occurred if concentrations of surface antibody greater than 10 mIU/ml develop (213). However, successful immunization will not result in persistent protective antibody levels (221). Despite the loss of antibody it seems that individuals are still protected against associated chronicity or onset of acute liver disease owing to the strong memory response that develops in immunized individuals (213, 221).

<table>
<thead>
<tr>
<th>Group</th>
<th>Schedule</th>
<th>Recombivax HB$^1$</th>
<th>Engerix-B$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg (-) mother</td>
<td>0-2, 1-4, &amp; 6-18 mo</td>
<td>2.5 μg / 0.5 ml</td>
<td>10 μg / 0.5 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$22.50</td>
<td>$27.18</td>
</tr>
<tr>
<td>HBsAg (+) mother</td>
<td>At birth (&lt;12 h)</td>
<td>5.0 μg / 0.5 ml</td>
<td>10 μg / 0.5 ml</td>
</tr>
<tr>
<td></td>
<td>With HBIG, 1-2, &amp; 6 mo</td>
<td>$28.84</td>
<td>$27.18</td>
</tr>
<tr>
<td>Children (1-10 yr)</td>
<td>0, 1-2, 4-6 mo</td>
<td>2.5 μg / 0.5 ml</td>
<td>10 μg / 0.5 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$22.50</td>
<td>$27.18</td>
</tr>
<tr>
<td>Adolescents</td>
<td>0, 1-2, 4-6 mo</td>
<td>5.0 μg / 0.5 ml</td>
<td>10 μg / 0.5 ml</td>
</tr>
<tr>
<td>(11-19 yr)</td>
<td></td>
<td>$28.84</td>
<td>$27.18</td>
</tr>
<tr>
<td>Adults (&gt;20 yr)</td>
<td>0, 1-2, &amp; 4-6 mo</td>
<td>10 μg / 1.0 ml</td>
<td>20 μg / 1.0 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$59.50</td>
<td>$54.35</td>
</tr>
<tr>
<td>Immunocompromised adults</td>
<td>0, 1, &amp; 6 mo</td>
<td>40 μg / 1.0 ml</td>
<td>40 μg / 2.0 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$167.91</td>
<td>$108.70</td>
</tr>
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</table>

Table adapted from (221)

$^1$ Recombivax – Merck & Co., Inc., Whitehouse Station, NJ, USA.
$^2$ Engerix B – Smith Kline and Beecham Pharmaceuticals, Philadelphia, PA.
### APPENDIX II

**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HBs</td>
<td>Antibodies to HBsAg</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BGH</td>
<td>Bovine growth hormone</td>
</tr>
<tr>
<td>CMI</td>
<td>Cell-mediated immunity</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-guanine</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>E:T</td>
<td>Effector:Target</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPI</td>
<td>Expanded Programme on Immunization</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GG</td>
<td>Gene-gun</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage stimulating factor</td>
</tr>
<tr>
<td>HBCAg</td>
<td>Hepatitis B core antigen</td>
</tr>
<tr>
<td>HBeAg</td>
<td>Hepatitis B e antigen</td>
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<td>HBG</td>
<td>Hepatitis B immunoglobulin</td>
</tr>
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<td>HBsAg</td>
<td>Hepatitis B surface antigen</td>
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<td>HBV</td>
<td>Hepatitis B virus</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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APPENDIX III

Permission to Publish
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