Induction of CTL responses
by plasmid DNA immunization

by
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A thesis submitted in conformity with the requirements of the degree of M.Sc.
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ABSTRACT

Vaccination is the most effective public health measure yet devised to decrease human morbidity and mortality. Despite success at controlling some pathogens, current vaccination techniques have proven ineffective for the treatment of many important diseases. A novel method of vaccination has recently been discovered, and is based on the ability of injected plasmid DNA to be taken up and expressed by cells in situ in such a way as to result in systemic immunity to the encoded gene product. The mechanisms by which DNA vaccines initiate cellular immunity are unclear. Bone marrow-derived antigen presenting cells are known to be responsible for the initiation of cellular immunity, but the question of how these cells acquire antigen remains to be solved. APCs are either directly transfected during the vaccination process, or they acquire antigen from an exogenous source and process it for direct presentation to T cells. Experiments described here address this question of induction of cellular immunity by DNA vaccines. The form of possible protein transfer was investigated: mice were immunized with influenza NP protein to elicit anti-NP antibodies, then immunized with plasmid DNA encoding the NP protein; the DNA vaccine-induced CTL response was found to be uninhibited by the presence of preexisting anti NP antibodies. These results suggest that protein is not transferred in a 'naked' form from transfected cells to APCs. A novel method for selecting in vitro transfected cells is described, based upon FACS sorting of fluorescently labeled gold particles used for transfection. To investigate the role of transfected APCs, cells with dendritic phenotype were cultured from murine bone marrow, transfected in vitro, and transferred to naive recipients. Attempts to induce immunity to the transfected gene product using this approach were unsuccessful. An experimental design is outlined for the determination of the balance between protein transfer and direct transfection of APCs in the case of gene gun immunization, utilizing bone marrow derived dendritic cells and in vitro gene gun transfection.
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LIST OF ABBREVIATIONS

Ab          antibody
Ag          antigen
APC         antigen presenting cell
bmAPC       bone marrow-derived antigen presenting cell
bmDC        bone marrow-derived dendritic cell
CD40L       CD40 ligand
CTL         cytotoxic T lymphocyte
DC          dendritic cell
ER          endoplasmic reticulum
GM-CSF      granulocyte macrophage colony stimulating factor
hsp         heat shock protein
IL-1β       interleukin 1 beta
IL-4        interleukin 4
IL-12       interleukin 12
LN          lymph node
LPS         lipopolysaccharide
MHC         major histocompatibility complex
NOG         neutravidin oregon green
PCR         polymerase chain reaction
TAP         transporter associated with antigen presentation
Th          T helper
TNFα        tumor necrosis factor alpha
CHAPTER ONE: INTRODUCTION

I. Vaccines: history and mechanisms

i) History of vaccines and their contribution to world health

The use of vaccines for the prevention of disease can be traced back to as early as 1000 BC, when medical practitioners attempted to protect patients from a serious disease by infecting them with a ‘mild’ form of the same disease (1). These initial attempts were based on infection of one patient with small amounts of the infectious byproducts (such as pus and scabrous material) from another patient suffering the disease in question; this technique was reasonably popular and came to be known as ‘variolation’ (reviewed in (2)). While occasionally successful, variolation suffered from relatively high rates of fatality and serious illness due to the fact that it was based on infection with small amounts of the exact same pathogen that causes the disease, and a truly safe method of inducing immunity to disease was not discovered until many centuries later.

In the late 1700s, Edward Jenner made a fortuitous discovery that infection with the mild cowpox virus protects human patients from infection by the often fatal smallpox virus. This discovery heralded the beginning of vaccinology as we know it today (reviewed in (2)). By using a different strain of virus, which caused only limited disease in humans, Jenner’s cowpox treatment represented what is now considered to be the first use of a ‘live attenuated’ vaccine. The range of immunization techniques was further expanded by Louis Pasteur in the late 1800s, who used heat to inactivate anthrax bacteria, rendering them non-pathogenic yet efficacious at inducing anthrax immunity when injected into naive sheep.

Today, the science of vaccinology has advanced beyond the use of whole killed or naturally occurring live attenuated pathogens to include such refined techniques as: recombinant genetic manipulation to specifically attenuate viruses; the use of non-pathogenic viral vectors to cause limited infections which can produce proteins from a
variety of pathogenic sources; and the use of purified whole protein or chemically synthesized protein subunit mixtures to elicit immunity to specific pathogenic proteins without any infectious agent (3).

Currently, more than 5 million doses of vaccine against some infectious organism are administered yearly in the US, making vaccines the most commonly administered immunotherapeutic (4). Presently, vaccinations against a number of diseases, including diphtheria, tetanus, whooping cough, measles, and polio, are estimated to prevent approximately 3 million deaths per year, making them the most effective public health measure to date in terms of decreasing human morbidity and mortality (5). Despite this success, a large number of devastating diseases have proven difficult or impossible to vaccinate against using these techniques (6), for reasons which can be traced to the mechanisms of action of current vaccines.

The vaccine discovered by Edward Jenner two hundred years ago was successful because when the vaccinia virus cowpox infects a cell, it delivers genetic information which allows the cell to produce viral proteins. The production of antigen within recipient cells has since proven to be an important component of successful vaccines. It has recently been discovered that pure DNA can be taken up and expressed by cells without the need for a viral vector. Instead of using a virus, researchers are now able to use a standard syringe to achieve the delivery of DNA to the cells of an individual. The objective of this thesis is to investigate the mechanisms by which this form of vaccination induces immunity. A review of the biology of antigen presentation is presented, and the mechanisms of action of current vaccine technology are described. Current knowledge about DNA vaccines and the cells which play important roles in immune induction are discussed. In light of this background information, the objective of the thesis is explained, and a set of experiments is outlined which is designed to determine the mechanisms of action of DNA vaccines.
ii) Basic presentation & immunity

In order to understand the mechanisms of action of vaccines, it is important to understand the way in which the immune system recognizes and responds to naturally invading pathogens. Proteins which are manufactured intracellularly in the normal life of a cell undergo some proteolysis into peptides. These peptides are then transported from the cytosol into the endoplasmic reticulum (ER) by the transporters associated with antigen presentation (TAP)-1 and TAP-2 proteins, and loaded on to the major histocompatibility (MHC) class I molecules, which are presented on the cell surface (reviewed in (7),(8)). Thus fragments of all proteins produced within cells are presented to the immune system through the MHC class I molecule, facilitating surveillance by MHC-I restricted, CD8+ cytotoxic T lymphocytes (CTLs) which are specialized in detecting and eliminating aberrant or infected cells (9) (10).

Extracellular antigens are detected in the body via antibodies (Ab). Ab bind foreign proteins and either neutralize them or draw them to the attention of specialized lymphocytes for ingestion and degradation. Ab-bound antigens are phagocytosed by antigen presenting cells (APCs), which present fragments of the protein in the context of MHC class II surface molecules. Antigen-MHC II complexes are recognized by MHC II-restricted, CD4+ T helper cells (Th) which stimulate antibody-producing B cells to expand (reviewed in (11), (10)). Thus extracellular viruses, bacteria and toxins are detected and eradicated by antibody mediated immune responses.

Historically, immune responses involving CTLs are referred to as ‘cellular’ while those involving Ab are known as ‘humoral’. Cellular interactions are required in the manifestation of the humoral immune response in the form of Th cells and APCs, however, and thus this terminology is not strictly accurate. Nonetheless, for the purposes of this discussion, immune responses which involve MHC I-restricted antigen presentation and CTLs will be referred to as cellular immune responses, while those involving MHC II-restricted antigen presentation and Ab will be designated humoral.
iii) Current vaccination methods and mechanisms

There are two broad categories of vaccines currently in use today; live attenuated organisms, and inactive material. The latter category includes inactivated virus, purified protein, and synthetic protein subunit preparations. Vaccines in each of these categories rely on different mechanisms of action, and subsequently have differing ability to elicit cellular and humoral immunity.

Live attenuated vaccines consist of viruses which have been engineered to replicate in a limited fashion within the host cells while exerting little pathogenic effect (reviewed in (5)). The limited intracellular replication of the attenuated virus leads to presentation of viral peptides on MHC I molecules of the infected cells. In addition, debris from infected cells and virus-Ab complexes are endocytosed and processed for expression on MHC II molecules by professional APCs. In this vaccination scenario, proteins which are produced by the attenuated virus-infected cells are exposed to the immune system such that both humoral and cellular immune responses to those proteins develop.

Inactive material, such as killed virus and purified protein preparations, collects in the extracellular space after administration because it can not actively enter cells. From the extracellular space, the antigenic material is phagocytosed by professional APC or antigen-specific B cells. Fragments of these antigens are then presented to T helper cells in an MHC class II restricted fashion, and humoral immune responses can be effectively generated (12) (13).

iv) Drawbacks of current vaccine technology

Despite their effectiveness at inducing both cellular and humoral immunity, live attenuated vaccines have associated with them a number of safety concerns, including a risk of reversion during replication or mutation of attenuated viruses to a more pathogenic strain while within the host (14). Some viruses, even in the attenuated state, may still induce disease in persons with weak or compromised immune systems (8). Non-pathogenic viral
vectors have limited effectiveness due to the fact that subsequent immunizations may be compromised by immune responses against the vectors themselves (15). Killed pathogen or protein subunit vaccines are safer than attenuated virus or viral vectors, but without the intracellular production of antigens these vaccines often cannot induce significant CTL responses (16) (8) (14).

For many infectious diseases, such as TB or malaria, humoral responses have been shown to be of little protective value against infection (4), and there is a striking absence or inadequacy of current vaccines for a number of infectious agents and diseases such as human immunodeficiency virus (HIV), hepatitis C, papillomavirus, rotavirus, dengue fever, tuberculosis, leishmaniasis, schistosomiasis and malaria (6).

II. DNA immunization

i) History of DNA immunization

Entering into a world frustrated by inadequate existing vaccination techniques, the announcement early in this decade of a novel strategy for vaccination and treatment of disease was met with much excitement: this novel strategy was referred to as 'genetic immunization', and is based on the use of 'DNA vaccines' (10). The initial discovery that naked DNA could be taken up by recipient cells and the encoded gene products expressed was made in the early 1960s. In this instance, it was shown that DNA from tumor cells, when transferred to recipient animals, could induce tumors (reviewed in (6)). The immunological significance of this finding was largely unrecognized until recently. In 1992, DNA vaccines sparked the avid attention of the scientific community when several groups reported that they were able to induce humoral and cellular immune responses to proteins encoded by plasmid DNA which had been injected into naive animals (reviewed in (5)).
ii) Advantages of DNA immunization

In the last six years, plasmid DNA vaccines have been shown to elicit promising humoral and cellular immunity to a broad range of pathogens, including those which cause influenza, hepatitis B, malaria, and tuberculosis (reviewed in (5)). Despite initial safety concerns (reviewed in (17)), plasmid DNA does not seem to replicate in the host, integrate into the host genome, or elicit general anti-DNA responses (18) (19). Plasmid DNA immunization offers a number of advantages over existing vaccination strategies (10). DNA can be easily, inexpensively, and rapidly produced and purified in comparison to the production of recombinant viruses or synthetic peptides. Dry DNA is heat stable, and only very small quantities are necessary for antigenic stimulation; this bodes well for delivery within developing countries where financial constraints and lack of refrigeration facilities challenge current immunization regimes. Indeed, cold chains and their administration costs may represent over 80% of the current expense involved in vaccine delivery (6). Immunization with DNA also offers conceptual advantages over whole killed, live attenuated virus, recombinant peptide, and protein-based vaccines because it allows specific genes to be expressed via nonreplicating vectors, and the sequence can be manipulated to present all or part of the genome of the pathogen (6) (5). This allows for the inclusion of immunologically relevant proteins or segments of proteins, while eliminating undesirable genes which could trigger unwanted immune responses, such as immunologic inhibition or autoimmunity (10). Genes can also be included which encode proteins from different pathogenic organisms to broaden the range of the vaccine. Cytokines can also be co-expressed to beneficially modulate immune responses (20).

iii) Mechanisms of DNA immunization

The two most common routes of DNA immunization are intramuscular (i.m.), via needle injection of plasmid DNA in a saline medium, or intradermal using the gene gun to
propel DNA-coated gold particles into skin. In the case of i.m. injection, muscle cells at the site of injection take up and express the DNA, and expression of reporter genes by transfected muscle cells can persist for up to 19 months (19). DNA that is not taken up by cells is thought to be rapidly degraded by nucleases present in serum and tissue fluids (12). Alternatively, the gene gun physically propels DNA-coated gold particles through the cell membrane and directly into the cytosol of epidermal cells. Epidermal cells transfected via gene gun bombardment are naturally sloughed within 10-14 days (12).

The humoral responses elicited by plasmid DNA injection are attributed to antigen being released by transfected cells into the circulation via secretion or cell death; these antigens are taken up by professional APCs (including B cells, macrophages, and dendritic cells) which are capable of initiating the T helper-dependent antibody response (21) (4). At the time of the first i.m. DNA immunization experiments, CTL responses were thought to be initiated by the transfected myocytes, which present MHC-I restricted antigen following endogenous proteasome- and TAP-dependent processing (4). More recent experimentation has shown, however, that transfected myocytes are not responsible for priming of cellular immunity in the case of DNA vaccines.

iv) Bone marrow-derived antigen presenting cells initiate immunity in DNA immunization

Further investigation into the initiation of cellular immunity raised questions as to the role of transfected myocytes following DNA immunization. Activation of naive T-lymphocytes requires both an MHC-restricted, antigen-specific signal and costimulatory signals which are usually provided by professional APCs (22). Myocytes lack these vital costimulatory molecules, thereby making them seemingly incapable of stimulating naive CD8+ T cells (23) (10) (8)). Further experiments using bone marrow chimeric mice showed that in the case of DNA immunization the CTL response is initiated by bone marrow derived-APCs (bmAPCs). When H-2d or H-2b bone marrow is used to reconstitute
irradiated H-2<sup>th</sup> mice, and the resulting chimeric animal is subsequently DNA immunized, it will mount CTL responses restricted to the haplotype of the donor bone marrow only (24) (25) (26), indicating that it is the bone marrow-derived APCs, and not the transfected muscle or skin cells at the site of inoculation, which initiate CTLs.

Given that bmAPCs are the cells which present antigen to the immune system to initiate a response, the question remains as to how the APCs can present antigenic material in an MHC class I-restricted manner. Two possibilities have been proposed: either the bmAPCs are themselves transfected during the vaccination, and induce immune responses by processing the encoded antigen through the classical endogenous MHC-I pathway; or they pick up exogenous protein which is produced by transfected cells and process it for presentation on their MHC-I via a non-classical pathway (4).

III. Cross priming in DNA immunization

i) APCs can utilize an exogenous MHC I pathway

Although class-I restricted presentation is often considered accessible only to endogenous proteins, some exogenous antigens can gain entry to this pathway by a mechanism known as cross-priming (27). This is known to be effective for CTL initiation for a variety of exogenous tumor, viral, and minor transplantation antigens (reviewed in (28)), and explains how the immune system can detect and respond to tissue-tropic viruses that do not infect professional APC (8).

Once the exogenous antigen is taken up by the APC, either by phagocytosis or macropinocytosis (27), two distinct pathways have been described for the presentation of antigens on APC class I molecules (29) (30). One is independent of the proteasome and TAP and is resistant to brefeldin A, suggesting that the presented peptides are generated in the endocytic compartment and then bind to class I molecules on the plasma membrane,
either via peptide replacement in the endosome or via regurgitation to the extracellular space (29) (31) (8). The other pathway requires the proteasome and TAP transporters and is sensitive to brefeldin A, suggesting that antigens are transferred from endosomes into the cytosol where they join endogenous antigens for classical MHC I presentation via the ER (32) (30).

ii) Evidence for protein transfer to bmAPCs

Compelling evidence shows the importance of cross-priming in DNA immunization. Ulmer and colleagues (33) have shown that transfection of nonmuscle cells is not required for the induction of MHC class I restricted CTLs and protective immunity. They stably transfected myoblasts with the influenza NP gene, and transplanted the NP-expressing myoblasts into naive syngeneic mice. The recipient mice were shown to develop NP-specific CTL responses which were initiated by bmAPCs and provided protective immunity to influenza virus. Since the myoblasts were stably transfected in vitro before transplanting into a naive recipient, transfection of host APC with the NP gene would be highly unlikely. APC mediated CTL priming must have occurred, at least in part, by transfer of antigen from the transplanted muscle cells to APCs in the recipient mice.

Doe et al (26) injected H-2\textsuperscript{b} or H-2\textsuperscript{d} scid mice with plasmid DNA encoding viral proteins. Because scid mice have very few mature T cells (34), the recipient mice were unable to mount CTL responses against the proteins. When the DNA immunized scid mice were infused with immunocompetent H-2\textsuperscript{b} bm bone marrow (bm), they developed both H-2\textsuperscript{b} and H-2\textsuperscript{d} restricted CTL responses to the DNA encoded viral proteins. The donor bm supplied the host with immunocompetent T, B, and antigen presenting cells. Because the CTL response was directed against both H-2\textsuperscript{b} and H-2\textsuperscript{d} restricted epitopes, this means that the donor APCs were presenting the DNA-encoded immunogen on their own MHC class I molecules. Since this response was seen even when the transfer of bm occurred three weeks after DNA immunization of the scid recipient, these results strongly suggest
that APC transfection need not occur for CTL initiation to result. It is highly unlikely that any plasmid DNA from the injection would remain available for transfection of the APCs after three weeks, given that the half life of plasmid in serum is less than 30 minutes in vitro (33). This leaves, of course, the possibility that the APCs were taking up exogenous antigen for cross priming.

It is possible that resident or circulating professional APCs, such as macrophages or dendritic cells, capture antigen which is released by transfected myocytes. Sites of injected muscle clearly become infiltrated with inflammatory cells following intramuscular DNA injection, and the effectiveness of i.m. injections is greatly enhanced by cytokines which may exert their effect in part by attraction of APCs (4). The antigen may then be processed by the infiltrating APC and appropriate peptides can be presented in the context of MHC I (via the exogenous MHC I pathway) and MHC II molecules, resulting in priming of both CD8+ and CD4+ T cells upon APC migration to the draining lymph node.

If this type of cross priming is a prominent factor in CTL initiation, the question arises as to how the protein transfer from non-APC to APC occurs. Plasmids with or without a leader sequence (required for secretion of the encoded protein) have proved equally successful at inducing immunity (23), raising the question of how the antigen leaves the myocyte to elicit an Ab response. Cell-associated proteins and particulate antigens are much more effective at class I-restricted CTL priming than are simple soluble molecules (27), leading to the speculation that these antigenic forms mimic the tissue debris associated with virus infections, and are taken up preferentially by APCs. Possible sources of antigenic material for cross priming include the shedding of antigenic vesicles from stressed cells and the release of immunogenic blebs from apoptotic cells (28).

**iii) Heat shock proteins**

Some attractive candidates for immunogenic factors which may be involved in cross priming are found in the family of proteins known as heat shock proteins (hsp). Hsp
bind and chaperone antigenic peptides within cells, and are upregulated during viral infection, inflammation, cell stress and tissue damage ((35, 36). Purified hsp-antigen preparations have been shown to be effective at inducing cellular immunity in tumor models, and when released extracellularly, hsps are known to be involved in directing peptides to the MHC class I pathway of professional antigen presenting cells (37). The detailed mechanisms leading to CTL activation after immunization with hsps are not completely understood. Among the hsps identified to induce tumor specific immunity when complexed with antigen are hsp70, hsp90, and the ER-resident gp96, which associates with MHC class I molecules. Of these, gp96-peptide complexes have been shown to be internalized by macrophages and the gp96-peptide complexes or the peptides alone are routed through the endoplasmic reticulum for presentation on MHC class I (38). Whether hsps are involved in the cross priming mechanism seen in the case of DNA immunization is unknown.

IV. Direct priming in DNA immunization

Despite the variety of evidence which suggests that cross priming is an important mechanism in DNA immunization, evidence also exists indicating that direct transfection of APCs and their subsequent migration to draining lymph nodes (LN) may be the critical event in induction of immune responses. This is particularly likely following gene gun plasmid delivery, as the dermal layers of the skin are rich in populations of Langerhans cells (LC) (6). LC are members of the dendritic leukocyte family, and are functionally similar to DC in that they are able to take up exogenous antigen and migrate to LN, where they initiate T cell responses (39).
i) 

Evidence for direct transfection of APCs

Investigations into the role of the cells at the site of inoculation have yielded some clues towards the importance of APC transfection in DNA immunization. Gene gun based immunizations require much less DNA than i.m. injections to elicit the same level of immunity (4). Torres and colleagues (40) showed through muscle ablation experiments that the injected muscle bundle at the site of i.m. inoculation need only be present for 10 minutes after injection for an immune response to develop to DNA encoded immunogens. Since this is too short a time for meaningful uptake, transcription, and translation of DNA by the myocytes, this suggests that the DNA is traveling away from the site and transfecting other cells. Assuming that the DNA reaches the spleen or draining LNs without being degraded, it could be transfecting APCs there.

Gene gun-mediated cutaneous immunization results in transfection of skin-derived DC which localize in draining lymph nodes within 24 hours of inoculation. Electron and light microscopy and fluorescent marker analysis has allowed the visualization of gold particle-containing, plasmid-expressing, skin-derived DC present within the lymph nodes of gene-gunned mice (21). In the case of gene gun immunization, bombarded skin is required to be present for at least 24 hours in order to achieve effective CTL initiation (40). Since this corresponds to the time required by APCs to migrate from the skin to the lymph node, this evidence suggests that transfected APCs play an important role in CTL initiation.

Porgador and colleagues (41) have recently performed DNA immunization experiments using the gene gun which demonstrate that the predominant antigen presentation resulting from this inoculation method is by directly transfected skin-derived APCs. They found that most antigen-bearing cells in the LN draining the site of DNA delivery appear to be directly transfected APCs, and that they numbered no more than 50-100 cells per node. In these experiments, mice were immunized with gold particles that had been co-coated with plasmids encoding β-galactosidase (β-gal) and human CD4. Depletion of cells expressing human CD4 obtained from the LN of these mice (i.e. directly
transfected APCs) reproducibly diminished presentation of antigen to β-gal specific T cells by 60 to 70%.

ii) Dendritic cells as APCs

The main candidate for the professional APC involved in the primary initiation of CD8+ T cell responses is the dendritic cell. Both macrophages and DCs can process exogenous antigens for MHC I presentation via the cytosolic pathway (42). Macrophages, however, have been shown to be less effective than DC at initiating T cells responses in vitro and in vivo (43) while DC have been shown to have exceptional ability to stimulate naive T cells in both cases (44).

In the last few years, insight into the role of dendritic cells has increased greatly, and it is now known that DC play a crucial role in the immune system. They are the professional APCs which are most efficient in the activation of naive T cells in vivo and, thus, for initiation of primary immune responses (45). An important feature of DC is their ability to migrate from peripheral tissue to lymphoid organs, where they can interact with naive T cells, facilitating their role as surveyors of peripheral antigens.

iii) DC maturation

In order to gain insight into the role which dendritic cells may play in DNA immunization, it is important to understand the development of these specialized antigen presenting cells, and the different roles which DCs might play at each stage of their development. Since APCs are capable of presenting exogenous foreign antigens in an MHC I restricted manner, it is likely that exogenous self antigens can also be presented by APCs in this fashion; yet reactivity to self antigens is a rare event. This indicates that there is another signal aside from constitutive expression of costimulation molecules and MHC-restricted antigen presentation by an APC which is required to stimulate naive lymphocytes. It is only at the mature stage that DC express the full complement of co-stimulatory
molecules, adhesion molecules, and antigen-presenting MHC molecules at the levels required for productive T cell activation (46), leading to the question of what factors trigger this final maturation.

Immature DC rest in peripheral tissue, where they capture and process antigens, but are poor activators of T cells. When triggered by cytokines or bacterial products that signal tissue disturbances, DC undergo a strictly defined maturation process which renders them potent APCs. The initial maturation steps include upregulation of MHC II surface expression, upregulation of adhesion and costimulatory molecules (including B7-2 and CD40), rearrangement of the cytoskeleton, and an increase in cell motility. This maturation reduces antigen-capture capacity and allows DCs to migrate to lymphoid organs, where the differentiation process is completed upon interaction with T cells. At this point, interactions with T cell surface molecules such as CD40 ligand (CD40L) and T-cell derived cytokines like IFNγ stimulate the final maturation of DCs. Phagocytic activity is lost, and more costimulatory molecules and cytokines are expressed by the DC (reviewed in (47), (45)). Mature DC are able to influence the T helper response by skewing it towards Th1 or Th2, through the release of cytokines such as TNFα, IL-1β and IL-12 (48) (49) (50).

Most CD8+ responses require CD4+ help in order to be initiated (22). It has been suggested that this help is delivered by stimulation of the presenting APC by Th cells recognizing MHC II restricted antigen. The APC receives a signal from the Th cell which allows it to subsequently activate naive CTLs (reviewed in (51)). Binding of CD40 on DCs by CD40L has been shown to upregulate IL-12 production and enhance the ability of DC to stimulate CTL responses in the absence of CD4+ help (52) (53) (54). These findings suggest that this critical signal given by Th cells is at least one possible stimulus for full maturation of DCs.

In addition to CD40 stimulation, exposure to certain bacterial products, including LPS and bacterial DNA, is known to activate DCs to mature and enhance their ability to prime CTLs (50) (55). In contrast to mammals, bacterial DNA is hypomethylated and
contains motifs with unmethylated CpG cores (56). These CpG motifs have been shown to cause simultaneous maturation of immature DC and activation of mature, MHC II\textsuperscript{hi} DC to produce cytokines and migrate to T cell areas (57) (46) '98), suggesting a CD40L-independent path of DC maturation.

iv) \textit{In vivo CTL initiation by DCs}

In light of this evidence showing the important role which DCs play in antigen presentation, costimulation, and T cell activation, much research has focused on the ability of DCs to initiate specific CTL responses \textit{in vivo}. Various studies in animal models have clearly shown that DC pulsed with tumor antigens \textit{in vitro} and then reinjected \textit{in vivo} induce protective immune responses that block tumor growth (58) (41) (59) (44) (60). Effective protection from tumor challenge can also be achieved by immunizations with DC that have been transfected \textit{in vitro} with plasmids encoding tumor antigens and then injected \textit{in vivo} (61) (62) (63) (64). These results serve to provide insights into the possible mechanisms of CTL induction via transfected DCs in the case of DNA immunization.

Following both i.m. and intracutaneous injection of DNA vaccines, PCR was used to look for DNA in DC, Langerhans cells, and B cells from immunized mice. DC and Langerhans cells, but not B cells, were found that contained the DNA sequences, and it was estimated that 0.4\% of DC from i.m. immunized mice were able to stimulate naive T cells (65). DC in the draining lymph nodes are the primary cell type bearing stimulatory levels of the βgal determinant associated with K\textsuperscript{b} after gene gun delivery of βgal encoding plasmid (41).

McArthur and colleagues (64) performed studies with βgal-transfected DC injected into mice which were then subjected to a β-gal expressing tumor of a different haplotype than the immunizing DC. H-2\textsuperscript{db} mice immunized with βgal-transfected H-2\textsuperscript{b} DCs were not protected from challenge with H-2\textsuperscript{d} tumor cells, indicating that cross-priming did not
occur in this system, although it is possible that the retroviral transduction of the DCs modified them is such a way as to abrogate their ability to cross-prime.

When naked DNA immunization is compared with transfer of in vitro peptide pulsed or transfected DCs, it has been shown that transfected DCs are superior to both peptide pulsed DC and direct DNA injection at initiating cellular immunity (63). This observation could explain the difference in efficiency between gene gun and i.m. inoculation. Intramuscular inoculations are much less efficient at transfecting DC (if indeed, DC even do get transfected using this method), and cross priming (i.e. from myocytes to DC) may be less effective than direct transfection.

Collectively, these investigations indicate that it is most likely that bone marrow-derived dendritic cells are responsible for initiating cellular immunity in the case of DNA vaccines. Whether the DC are picking up exogenous antigens or are being transfected themselves in the DNA vaccination process remains unclear; even when immunization with transfected or peptide pulsed DC elicits immunity, in many cases the induction of antigen-specific CTL could result from cross-priming from the transferred DC to recipient DC.

v) Antigen transfer between DCs vs. direct priming

Recent experiments investigating the possibility of cross-priming between DCs have revealed another layer of complexity in the role of transfected APCs. For optimal stimulation, DCs not exposed directly to antigen have been shown to be required. Knight and colleagues (66) investigated the ability of DC pulsed with peptide to prime DC-depleted naive T cell populations in vitro. Antigen present on all DC within a culture failed to activate naive T cells but antigen expressed by only a proportion of DC, where there was an antigen gradient between DC and Ag-DC, caused stimulation of primary responses. The optimal priming situation occurred when unpulsed DC were exposed to direct contact with pulsed DC, although limited transfer could occur through exposure of the unpulsed DC to medium from pulsed DC. These results suggest that antigenic material in some form must
be transferred between the antigen-exposed and the naive DC populations in order to initiate cellular responses.

V. Research objective

Given the current evidence on both sides of the cross priming vs. direct transfection question, it seems likely that both phenomena play a role in DNA immunization, perhaps with differing contributions depending on the site and nature of the inoculation. Quantitation of the number of transfected cells, be they APCs, skin cells, or myocytes, should help to define the contributions that each play in the initiation of an immune response. Towards this end, we designed a number of experiments to help elucidate the balance between protein transfer and direct transfection in the case of DNA immunization.

To address the issue of protein transfer between myocytes and APCs in the case of i.m. DNA immunization, we first investigated the possibility of ‘blocking’ antigen transfer. If protein is simply ‘leaking’ out or is secreted from transfected myocytes, to be picked up by APCs, then the presence of a high concentration of antibodies specific for the encoded protein should inhibit this transfer. To investigate this possibility, we immunized mice with whole influenza nucleoprotein (NP) under circumstances known to induce polyclonal anti-NP antibodies, but not CTL responses. We then injected plasmid DNA encoding the NP protein intramuscularly, a protocol which does prime CTL, and investigated whether the resulting CTL initiation was inhibited by the presence of pre-existing anti-NP antibodies. If CTL responses were inhibited in any way compared to those induced by DNA inoculation of naive animals, it would indicate that protein transfer does occur in i.m. DNA immunization, and that the form in which the protein is transferred is recognizable by anti-NP antibodies.
In order to address the role of transfected cells, it would be highly desirable to be able to detect and select transfected cells in a quantitative manner. Thus, a further goal was to transflect cells \textit{in vitro} under conditions similar to those found in \textit{in vivo} DNA inoculation, and then sort the transfected cells in a minimally intrusive fashion before reintroducing them into recipient animals. To facilitate this goal, we designed a novel method for sorting transfected cells without the use of detecting antibodies.

Finally, in order to determine the role of antigen transfer between transfected and untransfected APCs, we decided to focus our investigation on DC. DC can be preferentially expanded \textit{in vitro} from bone marrow and transfected for use in transfer experiments to investigate CTL initiation in naive recipients. We grew enriched DC populations from mouse bone marrow and investigated their ability to induce immune responses when transfected \textit{in vitro} and transferred to naive recipient mice.

Experimental procedures and results addressing each of these three objectives are described in the following three chapters of this thesis. At the end of each chapter, the results and future directions for research are discussed. Concluding remarks of the thesis are found in the final chapter.
CHAPTER TWO: INITIATION OF CTL RESPONSE BY I.M. DNA IMMUNIZATION IS NOT INHIBITED BY THE PRE-EXISTENCE OF POLYCLonal ANTIBodies AGAINSt THE ENCODED PROTEIN

I. Rationale

Protein transfer from plasmid DNA-transfected myocytes to bmAPCs may represent a key step in the induction of a CTL response by i.m. DNA immunization (outlined in Chapter 1). To investigate this mechanism, we designed an experiment to determine the possible form of protein transfer. It is possible that antigen is simply secreted from transfected cells into the extracellular milieu, and is then taken up by bmAPCs. If this is the case, antibodies should be able to bind the protein. This antibody binding may affect the protein transfer to APC, by inhibiting transfer or by modifying the response of the APC to the protein in such a way as to affect the CTL response initiated by the APC. In this experiment, influenza NP protein immunization was used to elicit high titres of anti-NP antibodies (but no NP-specific CTL) in individual mice and then these mice were further immunized with NP-encoding plasmid DNA. We investigated the kinetics of the subsequent anti-NP CTL response resulting from the DNA immunization. Comparing the CTL response to NP in the presence or absence of anti-NP antibodies allows one to investigate the nature of protein transfer, and thereby helps to assess this mechanism as a means of CTL induction by plasmid DNA immunization.
II. Materials and Methods

i) Animals

Female BALB/c mice, obtained at 6-8 weeks of age (Charles River Laboratories, Quebec), were housed in the Division of Comparative Medicine at the University of Toronto. All procedures with animals were carried out in accordance with institutionally approved protocols.

ii) Plasmid DNA and peptides

NPv.CMV consists of the NP gene from A/PR/8/34 (Genbank #V01084), cloned into the pRc.CMV vector (Invitrogen, San Diego, CA) (24). Plasmid DNA was amplified in the JM109 bacterial strain and purified through large-scale endotoxin free plasmid preparations, using Qiagen MaxiPrep columns (Qiagen, Santa Clarita, CA). The H-2K\textsuperscript{d} restricted peptide NP147-155 (TYQRTRALV) from the influenza nucleoprotein of A/PR/8/34 was synthesized and purified commercially (Alberta Peptide Institute).

iii) Cell lines and culture

P815 (H-2\textsuperscript{d}), a murine DBA/2 mastocytoma (TIB-64, ATCC, Rockville, MD), was cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Cansera, Rexdale, ON), 100 U/ml penicillin (GibcoBRL, Grand Island, NY), 100 \( \mu \)g/ml streptomycin (GibcoBRL) and 2 mM L-glutamine (GibcoBRL).

iv) Immunizations

BALB/c mice were immunized against NP by intraperitoneal injection of 200 HAU of influenza strain X-31 (67) in PBS. Splenocytes from mice immunized with influenza (flu) served as a source of anti-NP CTL for a positive control in CTL assays.
Groups of 2-4 female BALB/c mice (6-12 weeks of age) were injected subcutaneously with NP protein (partially purified NP from Dr. David Burt, Pasteur Merieux Connaught Canada) in Freund's complete adjuvant (Difco, Detroit, MI)(100 ug of NP in PBS emulsified at 1:1 volume with CFA) and reinjected four weeks later with the same amount of NP protein in Incomplete Freund's Adjuvant (Difco). Sera were prepared from mice bled intraorbitally 4 weeks post IFA boost and 1 day pre DNA inoculation.

Lyophilized closed circular plasmid DNA was resuspended in sterile PBS and 100 μg in a total volume of 50 μl was injected into a single hind leg quadriceps muscle.

v) ELISAs

96-well flat bottom PVC plate wells were coated at 37°C for 1 h with 10 μg/ml (100 μl/well) of NP antigen. 100 μl of blocking solution (5% milk) was then added to the wells, and the plates were incubated for 1 h at 37°C. After three washes with wash buffer (PBS plus 0.5% NP-40), 100 μl of diluted sera (diluted in PBS plus 1% BSA) were placed in triplicate wells for 1 h at 37°C, followed by 3 washes with PBS/Tween (PBS plus 0.05% Tween-20). Wells were then incubated for 1 h at 37°C with alkaline phosphatase-conjugated goat anti-mouse IgG (Caltag Laboratories, San Francisco), and once again washed 3 times with PBS/Tween. Finally, 100 μl of 1 mg/ml of p-nitrophenylphosphate in 10% diethanolamine was added to the wells, and the level of hydrolysis was measured by reading the O.D. at 405 nm using a Titertek Plus reader (Flow Laboratories, Canada).

vi) Cytotoxic T lymphocyte assay

Spleen cells were recovered from individual mice from immunization groups 1-5 weeks post inoculation, or 1-6 months post infection with X-31, and were restimulated in vitro to assess CTL effector function. Cells were cultured at 37°C, 5% CO₂ for 5-7 days in
RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 
μg/ml streptomycin, 2 mM L-glutamine, and 50 μM 2-ME (Sigma) at 5x10⁶ cells/ml in the 
presence of 2.5x10⁵/ml syngeneic normal spleen cell stimulators which had been irradiated 
and pulsed for 1 h with the H-2Kᵈ-restricted epitope, NP(147-155), at 0.1 μg/μl. Cell-
mediated cytotoxicity was assayed against P815 (H-2^d) cells pulsed with NP147-155 
peptide and labeled with 100 μCi of [⁵¹Cr]Na₂CrO₄ (Amersham, Oakville, Ont.). Target 
cells at 10⁵/well were incubated for 4 h in triplicate at 37°C with two-fold serial dilutions of 
effector cells. Plates were then spun briefly at 300xg and 25 μl of supernatant removed for 
counting in a TopCount scintillation counter (Canberra-Packard, Canada). Maximum and 
spontaneous release were determined from wells that contained either 2% Triton X-100 or 
medium alone respectively. Percent specific lysis was calculated as (experimental ⁵¹Cr 
release - spontaneous ⁵¹Cr release)(maximum ⁵¹Cr release - spontaneous ⁵¹Cr release)x 
100. Lysis of ⁵¹Cr-labeled targets in the absence of NP peptide was <10% at all effector to 
target ratios.

III. Results

i) Immunization with NPv.CMV elicits anti-NP specific CTL response

In order to be able to determine whether or not the preexistence of antibodies 
against an encoded protein would inhibit the priming of CTL responses by DNA 
immunization, it was necessary to first establish the typical response resulting from DNA 
immunization alone. Mice were injected i.m. with 100 μg NPv.CMV (Fig. 2.1), and 
boosted 3 weeks later with the same amount of NPv.CMV. Two weeks after the boost, 
splenocytes from immunized mice were harvested, restimulated in vitro in the presence of 
NP epitope-pulsed syngeneic naive splenocytes, and tested for NP-specific CTL in a CTL 
assay. Mice immunized and boosted using this protocol typically exhibit a CTL response
Figure 2.1. Structure of NPv.CMV

Immunization plasmid vector containing the influenza nucleoprotein (NP) gene, used for DNA immunization against NP.
which can be seen to approach that elicited by immunization with influenza virus itself (Fig. 2.2). The CTL assay reveals non-specific lysis, measured against a target cell which has not been pulsed with NP peptide, to be consistently less than 10% of specific lysis of peptide-pulsed targets.

ii) Immunization with NP protein elicits anti-NP antibodies

In order to raise high titres of anti-NP antibodies, BALB/c mice were immunized subcutaneously with 100 µg NP protein emulsified in CFA, and boosted 4 weeks later with 100 µg NP in IFA. 4 weeks post boost, serum from immunized mice was tested for anti-NP antibodies using ELISA. High titres of anti-NP antibodies were measured consistently in all individual mice treated with this protein immunization regime (Fig. 2.3). Titres of anti-NP antibody persisted at this level for at least 12 weeks post boost (data not shown).

iii) The presence of high-titre anti-NP antibodies does not inhibit the development of anti-NP CTL responses by subsequent DNA immunization

Having determined the maximum CTL response which is initiated by DNA immunization in naive mice, we next examined the kinetics of this CTL development in the presence of anti-NP antibodies. Mice which had been immunized with NP protein and were known to have high titres of anti-NP antibodies (as tested by ELISA) were injected with 100 µg NPv.CMV. Naive mice were injected with 100 µg NPv.CMV concomitantly so that the kinetics of CTL development could be compared in the two situations. A total of 11 NP protein immunized and 13 naive mice were injected with NP-encoding DNA and splenocytes were harvested 1, 2, or 3 weeks later to assay for anti-NP CTL (Fig. 2.4).

As can be seen at day 0, NP protein immunization alone does not elicit NP-specific CTL, but a CTL response does develop over a 3 week period following DNA immunization (Fig. 2.5). Although a broad range of responses was seen in the individual immunized mice, there was no detectable difference between the overall kinetics of the CTL
Figure 2.2. NP-specific lysis of target cells by NPv.CMV DNA immunized mice

Mice were injected i.m. with 100 μg NPv.CMV and boosted with the same amount 3 weeks later. Two weeks post boost, spleens from immunized mice, mice immunized with influenza virus (flu), or mice immunized with CMV vector alone, were harvested and tested for NP-specific CTL by CTL assay. (A) splenocytes tested against P815 target cells pulsed with NP peptide (NP147-155) (B) splenocytes tested against unpulsed P815 target cells. These representative results are from a CTL assay of two individual mice.
Figure 2.3. Anti-NP antibodies in serum of mice immunized with NP protein
Representative ELISA showing average of four individual NP immunized mice. Mice were injected with 100 µg NP in CFA and boosted with 100 µg NP in IFA four weeks later. Four weeks post boost, sera from individual animals were tested for anti-NP antibodies using ELISA. Absorbance shown is the average of sera from four individual mice.
Figure 2.4. Assay for NP-specific CTL after NPv.CMV plasmid DNA immunization in the presence of the anti-NP antibodies

High titres of anti-NP antibodies were raised in naive mice by immunizing with 100 μg NP protein in CFA, and boosting four weeks later with 100 μg NP protein in IFA. Four weeks post boost, and one day prior to DNA immunization, sera of protein immunized mice were tested by ELISA for the presence of anti-NP antibodies. Both protein immunized and naive mice were then injected with 100 μg NPv.CMV. 1, 2, and 3 weeks after DNA injection, splenocytes were harvested from mice and tested for NP-specific CTL using a CTL assay.
Figure 2.5. NP-specific CTL activity in previously unimmunized or NP protein immunized mice following NPv.CMV DNA immunization

Naive or NP protein immunized mice were injected i.m. with 100 µg NPv.CMV. Individual spleens were harvested 3 weeks post DNA immunization. Specific lysis shown at a 100:1 effector:target ratio. Percent lysis for flu in infected mice in these assays was 76% (range: 68%-86%). Percent lysis for CMV immunized mice was 3% (range: 1.4%-9.2%). (A) average from 10 individual CTL assays. (B) individual data points (NP immunized n=11, unimmunized n=13)
response between the two groups. The CTL response developed, on average, at the same rate and to the same level in protein immunized and unimmunized mice, indicating that CTL priming by NP DNA vaccination was not delayed or altered by the presence of polyclonal anti-NP antibodies.

IV. Discussion and Future Directions

The myoblast transfer experiments performed by Ulmer et al. (33), and chimeric scid mouse experiments by Doe et al (26) strongly suggest that the transfection of APCs need not occur in order for DNA vaccines to elicit CTL immunity. APCs can process exogenous antigen from transfected cells and present it in an MHC-I restricted manner to prime naive CTLs. Given this evidence, the question arises as to what the mechanism of protein transfer is that facilitates this cross priming.

The theory that protein is secreted by or leaks out of transfected cells at the site of DNA immunization, and is taken up and processed by APCs which go on to initiate CTL, is confounded by a very basic problem. If protein is secreted in an unencumbered or 'naked' fashion (ie. uncomplexed to cellular molecules or unbounded by some kind of vesicle) from transfected cells, then why are straightforward protein immunizations rarely capable of initiating CTL?

A closer look at the myoblast transfer experiments of Ulmer et al. (33) reveals some insight into the possibility of naked protein being cross presented by APCs. The quantity of transfected myoblasts which were transferred in these experiments was seen to produce approximately 20 ng of secreted NP protein over a 3 day period in vitro, yet injection of 15 μg NP protein alone into naive recipients did not induce CTL (33). Even when intracellular stores of NP were exposed to naive mice in the form of lysed transfected myoblasts, no CTL activity was detected against NP. Also, the antibody isotype profile induced by
transfer of transfected myoblasts was similar to that induced by NP DNA vaccination and live virus infection, but different from that after inoculation with recombinant NP protein (33). This strongly suggests that the transfer of antigen to APCs observed after transplantation probably did not involve simple uptake of naked exogenous antigen following release from muscle cells.

Our results are in agreement with the suggestion that antigen is not being transferred in a ‘naked’ form from transfected cells to APCs. No difference was seen in the kinetics of anti-CTL response between NP protein immunized and unimmunized mice. We measured high titers of NP-specific polyclonal Abs in recipients’ serum, but we did not specifically measure the presence of anti-NP Abs at the site of DNA injection. In humans, immunoglobulin levels in afferent lymph are 15 - 30% of the levels found in serum (68). Furthermore, in conditions of inflammation such as that which occurs following DNA inoculation in muscle, increased permeability of the capillary wall results in a greater influx of antibodies to the tissue. Although this suggests that we could expect relatively high concentrations of NP-specific Ab in the muscular interstitium, we cannot be sure that Ab was present at such high concentrations at the site of inoculation as it was in the serum. If naked protein is transferred at a very close range between myocytes and APCs, then we cannot rule out the possibility that there may not have been enough Ab present at the muscle to inhibit this transfer.

Our experimental design does not rule out the possibility that direct transfection of APCs may still be occurring, and therefore any inhibition of protein transfer which may have resulted from antibody binding could have been obscured by the direct priming of CTL by transfected APC. In order to be sure that this is not the case, it would be desirable to repeat the protein immunization experiment using transferred transfected myoblasts. Even without this conclusive evidence, however, the fact that no inhibition whatsoever occurred in our experiment strongly suggests that protein transfer was not inhibited by antibody.
If cross priming is not the result of uptake of secreted naked protein by APCs, we are still left with the question of what form the protein transfer takes. Protein could be being transferred in a form that is not recognizable by antibody; possibly in the form of immunogenic blebs released by stressed cells, or in apoptotic bodies from dying cells. It has also been suggested that protein or peptide may be complexed to chaperone molecules such as heat shock proteins due to the fact that purified hsp-antigen preparations have been shown to be effective at inducing cellular immunity in tumor models.

The hsp gp96 was considered a likely candidate for the role of transferring processed peptides (reviewed in (54)), but Schoenberger et al. (54) have recently shown that gp96 does not represent an essential component of the in vivo cross-priming pathway for cell-associated antigens. Their experiment demonstrated that immunization with TAP+ tumor cells enabled efficient cross-priming of CTL responses in vivo, concluding that the mechanism of cross-priming does not depend upon the presence of peptides in the ER of immunizing cells as a source of antigen for transfer to host APC. Tumor cells in which antigenic peptides do not reach the ER, and therefore cannot endogenously associate with gp96, are nevertheless able to efficiently lead to cross-primed CTL responses (54). Their results do not argue against the possibility that other hsps (such as hsp 70) may play a direct role in Ag transfer to APCs, but they do support the theory that a different mechanism of protein transfer is involved in cross priming, which involves processing of the transferred protein within APCs.

The recent discovery that DC preferentially phagocytose apoptotic cellular material and process it for presentation on MHC I molecules to initiate CTLs (69) has provided new insight into the possible mode of cross priming in DNA immunization. While it is possible that myocytes transfected in vivo may undergo some apoptosis, expression of reporter genes by transfected muscle cells can persist for up to 19 months (19), indicating that apoptosis is not a predominant response to transfection. Therefore, there may be different
mechanisms involved in the cross priming seen upon DC phagocytosis of apoptotic cells versus that seen upon DNA immunization.

It is important to understand the phenomenon of protein transfer in the case of DNA immunization in order to be able to manipulate it as an effective aspect of immunization strategies. If cross priming is shown to be the most effective method of inducing CTL immunity \textit{in vivo}, then understanding the mechanisms by which it occurs will allow researchers to tailor future vaccines to take full advantage of this biological phenomenon. Protein-based vaccines could be rendered effective by the inclusion of hsps if this turns out to be the mechanism of cross priming; genes whose products trigger apoptosis could be included in DNA vaccines if apoptosis is the goal. Clearly, more research is needed in this area before such tailoring of vaccines can be expected to be productive, and any future insights will likely have many positive repercussions on the field of vaccinology and immunotherapy.
CHAPTER THREE: NON-DRUG BASED IMMEDIATE SELECTION OF TRANSFECTED CELLS BASED ON FLUORESCENT BALLISTIC TECHNIQUES

I. Rationale

In order to gain insight into the mechanisms of DNA immunization, we were interested in examining the specific contribution of gene gun-transfected cells to the initiation of cellular immunity. Our ultimate goal was to transfect different cells in vitro and introduce them to naive recipient animals to study their ability to initiate CTL responses. We were interested in devising a method of selecting transfected cells immediately following transfection. Most techniques used to select transfected cells are dependent upon in vitro expression of the transfected DNA and subsequent selection for specific expressed genes by antibody-mediated fluorescent staining or by drug selection. These sorting techniques require time for significant expression of the transfected DNA. We considered that it may be important to perform the transfer of cells immediately after transfection. By transferring cells quickly, any early cellular responses to the transfection procedure would take place within the context of the recipient animal and thus have effects which were relevant to the case of in vivo inoculation. To facilitate our objective, we devised a method of labeling gold particles such that particle-containing cells could be quantitatively sorted immediately following gene gun bombardment in vitro.
II. Materials and Methods

i) Plasmids and antibodies

A eukaryotic expression plasmid encoding the HLA-A2 heavy chain gene cloned into pcDNA3 (pcDNA3.HLA-A2) was kindly provided by Charles Dela Cruz (University of Toronto). Biotinylated mouse anti-HLA-A2 antibody (HB82, ATCC) was used to detect surface expression of HLA-A2. Biotinylated mouse anti-human IgG,Fd (Caltag, San Francisco, CA) served as control antibody for FACS. pRc.CMV vector (CMV) was from InVitrogen (San Diego, CA).

ii) Cell lines

P815, a murine DBA/2 mastocytoma (TIB-64, ATCC, Rockville, MD), and COS-7, a CV-1 simian fibroblast-like cell line (ATCC; CRL 1651), were routinely cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum (Cansera, Rexdale, ON), 100 U/ml penicillin (GibcoBRL, Grand Island, NY), 100 μg/ml streptomycin (GibcoBRL) and 2 mM L-glutamine (GibcoBRL). COS-7 cells constitutively express wild-type SV40 large T-antigen, which enables them to replicate plasmids which contain the SV40 origin of DNA replication. Transfection of COS-7 cells with SV40 origin of replication-containing plasmids, such as pcDNA3, results in extensive replication of the transfected plasmid and high levels of expression of encoded genes (70). Primary splenocytes were obtained from 6-12 week old female BALB/c mice (Charles River Laboratory, Quebec), filtered through a 70 μm nylon screen, washed and maintained in tissue culture medium (described above).

iii) Fluorescent dyes

Fluorescent dyes tested consisted of different fluorophores conjugated to streptavidin or neutravidin. Neutravidin is avidin which has been processed to remove the
carbohydrate and lower its isoelectric point (71). Streptavidin-oregon green, streptavidin-fluorescein, streptavidin-phycoerythrin, and neutravidin-oregon green (Molecular Probes, Eugene, OR) were tested. Oregon green (oregon green 488) is a dye with excitation and emission spectra similar to fluorescein but with greater photostability and a higher fluorescence.

iv) Gene gun particle preparation

Gold particles were prepared as per the manufacturer’s instructions (Helios Gene Gun, BioRad, Hercules CA). In brief, 25 mg gold particles (1.0 or 1.6 μm diameter) were suspended in 100 μl of 0.05 M spermidine (Sigma)(diluted either in H2O or in 2-4 mg/ml fluorescent dye), and briefly vortexed and sonicated in an ultrasonic cleaner. DNA (62.5 - 250 μg) was added to the gold mixture and vortexed to mix. While vortexing, 100 μl 1 M CaCl2 was added to the mixture to precipitate the DNA onto the gold. The mixture was allowed to sit at RT for 15 min, then the pellet was washed three times in 100% EtOH, and finally resuspended in 3 ml 0.1 mg/ml PVP in 100% EtOH. Particle cartridges (‘bullets’) were prepared from this slurry as per manufacturer’s instructions, resulting in a final concentration of 1.25-5 μg DNA and 3.6-7.2 μg dye on 0.5 mg of gold particles per cartridge.

v) In vitro transfection with gene gun

Cells to be transfected were resuspended at 10⁶ cells/20 μl PBS or culture medium, and spread with a pipette tip to evenly cover a 2.5 cm diameter area on a dish. The sterile nozzle of the gene gun was placed against the dish surrounding the cells and the gun was discharged at 100-300 psi. Cells were immediately resuspended in 0.5 -1 ml PBS and washed twice in PBS before return to fresh culture medium or immediate analysis by FACS.
vi) Surface immunofluorescence sorting and staining

Immediately following bombardment, cells were washed 3 times in PBS and analyzed on a FACscan flow cytometer (Becton Dickinson, San Jose, CA) for the presence of fluorescent gold particles (Fl-1hi). In the case of sorting, COS-7 cells were washed 3 times in PBS following bombardment and Fl-1hi cells were sorted out and incubated in fresh medium. Three days after gene gun transfection, COS-7 cells were washed twice with PBS and stained for 30 min on ice with anti-HLA-A2 antibody at 1-3 μg/10^6 cells in 100 μl PBS, 0.1% BSA, 0.01% NaN3 (PBS/BSA). Streptavidin-conjugated phycoerythrin (0.5-1.0 μg) (Molecular Probes, Eugene, OR) in 100 μl PBS/BSA was added, and cells were incubated for a further 30 min on ice, followed by 2 washes with PBS/BSA. The cells were then analyzed within 1 h on a FACscan (Becton Dickinson, San Jose, CA).

III. Results

i) Preparation of bullets for gene gun

The gene gun uses compressed helium to propel microscopic gold particles ("bullets") directly into the cytoplasm of cells. The gene gun apparatus (illustrated in Figure 3.1, and detailed in (72)) employs a high velocity stream of helium to accelerate gold particles to velocities sufficient to penetrate cells, both in vitro and in vivo. As the helium travels through the gene gun, it enters one of the bores of the cartridge holder, which contains a teflon tube (cartridge) lined with gold particles. The gold particles on the inside of the cartridge are pulled from the surface and are carried along in the helium stream, accelerating through the barrel. The barrel slopes outward to form a cone, which serves to expand the helium stream. This expansion, while maintaining particle velocity, results in a lower velocity pulse of gas and wider diameter of impact at the target site. The gold particles are coated with DNA, which solubilizes from the gold surface in an aqueous
Figure 3.1. Gene gun apparatus
The gene gun uses compressed helium to propel microscopic gold particles directly into the cytoplasm of cells. Helium gas is pulsed through a cartridge which is loaded with DNA-coated gold particles. This pulse sweeps the gold from the inside wall of the cartridge. The gold particles accelerate through the barrel, borne on the helium stream which spreads out as it leaves the barrel. The outward spread of the helium stream results in particle delivery over an approximately 12 mm diameter target area.
environment (such as the cytoplasmic milieu), and is subsequently expressed by the recipient cell.

The preparation of bullets for the gene gun is outlined schematically in Figure 3.2. To prepare the bullets, DNA is suspended in spermidine and precipitated onto gold particles (1.0 or 1.6 μm diameter) with 1 M CaCl₂. The particles are then washed in 100% EtOH and resuspended in 0.01% PVP/100% EtOH. The PVP/EtOH slurry is then injected into a teflon tube for coating. After 3-5 minutes, the gold particles have settled onto the bottom of the teflon tube, and the PVP/EtOH is gently withdrawn, leaving the gold behind. The tube is then rotated for 3-5 minutes under nitrogen gas to allow the gold to evenly coat and dry on the inside of the tube. When completely dry, the tube is cut into segments of approximately 12 mm; these cartridges can be stored with desiccant at 4°C for up to one month before use in the gene gun. Storage for longer than one month can lead to less effective clearing of gold particles from the cartridges upon discharge.

**ii) In vitro transfection of cells using the gene gun**

Cells for in vitro transfection by gene gun are suspended in 20 μl medium or PBS, and spread in a thin layer using a pipet tip to cover a 2 cm diameter circle. The gene gun nozzle is placed over the circle of cells, and a helium pressure discharge propels the gold bullets from the cartridge into the cells (illustrated in Fig. 3.3).

**iii) Optimization of gene gun transfection parameters**

For optimization of gene gun transfection parameters, including helium pressure discharge and the size of gold particles, cell survival and transfection efficiency were analyzed using P815 cells. Cell survival was calculated 24 hours after bombardment, with survival rates determined as (number of viable cells in bombarded population)/(number of cells in non bombarded population) x 100. Mock bombardments served as controls in some
Figure 3.2. Preparation of bullets for gene gun transfection
DNA in spermidine is precipitated onto gold particles by addition of CaCl₂. After 3 washes in 100% EtOH, the particles are resuspended in EtOH/PVP and coated on the inside of a teflon tube by rotating the tube. Ethanol solution is withdrawn and the tube is rotated under nitrogen gas to dry. Once the gold has evenly coated the inside of the teflon tube and is completely dry, the tube is sliced into individual cartridges which can then be loaded into a gene gun and used for transfection via high pressure helium propulsion.
cells spread in 2 cm diameter circle using a pipettor

gene gun abutted to dish, nozzle surrounding circle of cells, and discharged

Figure 3.3. In vitro transfection of cells using the gene gun
Cells are spread in a very thin layer on the surface of a tissue culture dish and bombarded with gold particles from the gene gun. The gold particles are coated in DNA which solubilizes in the cytoplasm of the bombarded cells and is expressed.
experiments, where cells were spread into a dish and subjected to helium discharge of empty cartridges without gold particles.

Examination by simple light microscopy allows for the visualization of cells containing gold particles immediately following bombardment (Fig. 3.4). For determination of optimum pressure of helium discharge, P815 cells were suspended at 1-3.5 x10^6 cells/20 μl and bombarded with bullets using pressures of 100 - 400 psi. The efficiency of transformation was determined by microscopic examination (Table. 3.1). A pressure of 200 psi was ultimately determined to be optimum, with transfection percentages averaging around 40% as assessed by microscopic visualization. Increasing the helium pressure above 200 psi resulted in poorer recovery and survival of cells and did not increase the efficiency of transformation, and changing the cell concentration of the 20 μl sample did not affect the efficiency of transfection (data not shown).

Survival of cells following bombardment was determined by counting viable P815 cells using trypan blue staining 24 hours after bombardment. Cells containing gold particles were seen during these visual examinations, indicating that the shot cells do survive the bombardment procedure. As can be seen from the data presented in Table 3.2, the degree of P815 cell survival was dependent on the size of the gold bullets, with a greater survival rate when using 1.0 μm than 1.6 μm gold when compared to mock bombardment.

**iv) Labeling bullets with fluorescent dye**

In order to be able to detect gold particle-containing cells using FACS, fluorescent dyes were co-coated onto the particles during the bullet preparation process. Streptavidin-phycoerythrin (SAPE), streptavidin-fluorescein (SAF), streptavidin-oregon green (SOG), or neutravidin-oregon green (NOG) (180 μg each), was added to the spermidine mixture used to suspend the DNA before precipitation onto particles (resulting in a dye

41
Figure 3.4. Visibility of gold particles within cells following gene gun bombardment
Micrograph of splenocytes which have been bombarded with 1.0 μm gold particles using the gene gun. Photograph taken at 1 h post bombardment. Gold particles are readily visible within the cytoplasm of bombarded cells (a single gold particle can be seen here within the cell indicated by the arrow). (1000X)
Table 3.1. Transfection efficiency of gene gun bombardment at different helium discharge pressures

Shown are percent of P815 cells which are seen to contain gold particles upon microscopic examination following bombardment with 1.0 μm gold particles at varying pressure. Data collected 30 min post bombardment. Transfection efficiency did not vary with amount of cells used between 1 and 3.5 x10^6.

<table>
<thead>
<tr>
<th>cell concentration</th>
<th>discharge pressure (psi)</th>
<th>% with gold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3.5x10^6</td>
<td>100</td>
<td>26 +/- 1.1 (n=5)</td>
</tr>
<tr>
<td>1-3.5x10^6</td>
<td>150</td>
<td>25 +/- 2.0 (n=3)</td>
</tr>
<tr>
<td>1-3.5x10^6</td>
<td>200</td>
<td>41 +/- 3.0 (n=6)</td>
</tr>
</tbody>
</table>

Table 3.2. Survival rates of P815 cells following gene gun bombardment at 200 psi with 1.0 μm vs. 1.6 μm gold particles

Cells were counted using trypan blue staining 24 hours after bombardment, and percent survival was calculated as (number of viable cells in shot population)/(number of viable cells in unshot population)x100. Data are presented as an average survival rate for 3 different populations at each pressure.

<table>
<thead>
<tr>
<th>bullet size</th>
<th>% survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>none (mock bombardment)</td>
<td>100 +/- 13%</td>
</tr>
<tr>
<td>1.0 μm</td>
<td>70 +/- 6%</td>
</tr>
<tr>
<td>1.6 μm</td>
<td>45 +/- 5%</td>
</tr>
</tbody>
</table>
concentration of 7.2 μg/mg gold). Initial visual examination of bullets revealed that SAPE was not detectable on the bullets, but SAF, SOG and NOG-coated bullets were visualizable using fluorescent microscopy (Fig. 3.5 and data not shown).

Further examination into the FACS suitability of the different fluorescent dyes took into account the possible toxicity and the detectability of the different dyes. Primary splenocytes were used to measure survival in anticipation of their being more susceptible to dye toxicity due to their less hardy nature in vitro. Microscopic examination using trypan blue to count viable cells 24 h post-bombardment revealed that cell survival was roughly equivalent between dyes, but FACS analysis showed that a greater number of transfected cells were detectable when using NOG bullets (Table 3.3). It was decided that NOG bullets, with their greater ease of detection, would be used in subsequent experiments.

v) Optimizing DNA/dye ratios on NOG bullets

The amount of DNA and dye was varied in different bullet preparations, and the efficiency of transfection was determined by FACS (Table 3.4). It was seen that increasing the amount of dye used (above 7.2 μg/mg gold) did not increase the detectability of cells, suggesting that this amount of dye saturated the bullets. Increasing the DNA in the bullet preparation was seen to decrease the detectability, however, suggesting that the DNA at high concentrations could compete with the dye for space on the gold particles. It was decided to use 7.2 μg of dye and 5.0 μg of DNA per mg gold for maximum detectability by FACS. This ratio results in approximately 3.6 μg dye and 2.5 μg DNA per cartridge (0.5 mg gold/cartridge).

vi) DNA is coated on and released by gold particles in the presence of fluorescent dye

In order to ensure that the presence of the fluorescent dye did not inhibit the precipitation of DNA onto the gold particles or its release in an aqueous environment, NOG
Figure 3.5. Visibility of fluorescently labeled gold particles within bombarded cells

Micrographs of splenocytes which have been bombarded with 1.0 μm gold particles using the gene gun. Photographs taken 1 h post bombardment. A1 and B1 are phase contrast light micrographs; A2 and B2 are fluorescent micrographs of A1 and B1 respectively. Gold particles have been coated in neutravidin oregon green and are readily distinguishable under fluorescent stimulation, when free (dashed arrows) or within cells (solid arrows). Cells which do not contain gold particles are not visible under fluorescent light (X) (1000X)
Survival was determined by trypan blue staining and microscopic examination. Percent survival was calculated as (number of viable cells in shot population)/(number of viable cells in unshot population)×100. Selectability by FACS was determined as the proportion of cells which were Fl-1 expressed as compared to mock bombarded populations. Gold particles were 1.6 μm in diameter. Data collected 24 hours post bombardment, represented as average of 3 separate samples each; FACS populations were pooled from 3 separate samples.

<table>
<thead>
<tr>
<th>fluorescent dye</th>
<th>% survival</th>
<th>% selectable by FACS</th>
</tr>
</thead>
<tbody>
<tr>
<td>none (mock bombardment)</td>
<td>100 +/- 13%</td>
<td>0</td>
</tr>
<tr>
<td>streptavidin fluorescein</td>
<td>50 +/- 2%</td>
<td>7</td>
</tr>
<tr>
<td>streptavidin oregon green</td>
<td>62 +/- 16%</td>
<td>14</td>
</tr>
<tr>
<td>neutravidin oregon green</td>
<td>51 +/- 4%</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 3.3. Survival and selectability of primary splenocytes bombarded with gold particles coated in different fluorescent dyes
Table 3.4. Selectability of P815 cells transfected with gold particles co-coated with different ratios of fluorescent dyes and DNA

<table>
<thead>
<tr>
<th>NOG (µg/mg gold)</th>
<th>DNA (µg/mg gold)</th>
<th>% selectable by FACS (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1.6 +/- 0.0%</td>
</tr>
<tr>
<td>7.2</td>
<td>2.5</td>
<td>17.0 +/- 1.9%</td>
</tr>
<tr>
<td>7.2</td>
<td>5.0</td>
<td>18.4 +/- 0.3%</td>
</tr>
<tr>
<td>7.2</td>
<td>10.0</td>
<td>12.9 +/- 0.7%</td>
</tr>
<tr>
<td>15.0</td>
<td>2.5</td>
<td>17.0 +/- 2.2%</td>
</tr>
</tbody>
</table>

P815 cells were bombarded at 200 psi with 1.0 µm gold particles coated with differing amounts of DNA and neutravidin oregon green dye (NOG). Selectability was determined by FACS, performed immediately following bombardment. Selectability by FACS was determined as the proportion of cells which were Fl-1<sup>hi</sup> as compared to mock bombarded populations; average values of 3 individual samples are shown here.
bullets which had been prepared with the optimum ratios of dye and DNA as determined were prepared. The cartridges were discharged into 20 µl PBS; 1 ml PBS was added to the dish immediately following bombardment and the dish was agitated gently for 10 minutes, whereupon the PBS was collected and centrifuged to remove the gold particles. Supernatant was analyzed by spectrophotometry for the presence of DNA (Table 3.5). DNA was present at the expected concentrations, with approximately 2.2 µg DNA being retrieved from dishes which were shot with 2.5 µg DNA (1 cartridge). A small number of gold particles become embedded in the dish after bombardment, which could account for some of the unretrieved DNA. These results indicate that most if not all of the DNA is being precipitated onto and released from the gold particles as anticipated, despite the presence on the bullets of fluorescent dye.

vii) Cells transfected in vitro using fluorescent bullets are sortable by FACS and express transfected DNA

In order to ensure that shot cells do survive bombardment with fluorescent bullets, are sortable, and are able to express the transfected DNA, bullets made with NOG and pcDNA3.HLA-A2 (HLA/NOG) were used to transfect cells. COS-7 cells were used for transfection due to their ability to express high quantities of the transfected plasmid DNA. Immediately after bombardment, cells were washed and sorted by FACS (Fig. 3.6). Fl-1 hi cells were separated and cultured for 3 days to allow for maximum expression of HLA-A2. Due to imperfect sorting there was some contamination of the Fl-1 hi population with Fl-1 lo cells (approximately 8% of the sorted population was Fl-1 lo). After 3 days, FACS analysis was performed on sorted cell populations to test for HLA-A2 expression (Fig. 3.7). Due to differing degrees of expression seen in the transfected cells, it is difficult to ascertain the exact number of HLA-A2 expressing cells in each population, however the sorted Fl-1 hi population is definitely enriched for HLA-A2 expression. The positively
Table 3.5. Recoverability of DNA coated onto gold particles in the presence of fluorescent dye

<table>
<thead>
<tr>
<th>bullet type</th>
<th>DNA/cartridge (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA</td>
<td>2.2 +/- 0.3</td>
</tr>
<tr>
<td>HLA/NOG</td>
<td>2.2 +/- 0.2</td>
</tr>
</tbody>
</table>

Cartridges containing gold particles which were coated with pcDNA3.HLA-A2 DNA alone (HLA) or DNA along with neutravidin oregon green (HLA/NOG). For quantification of soluble DNA per cartridge, three cartridges were discharged into 20 µl PBS. 1 ml PBS was added to each well immediately after bombardment. After 10 minutes of gentle rocking, the amount of DNA which had become disassociated from the gold particles and suspended in the PBS was determined by measuring the OD$_{260}$ of the PBS. OD values were converted to DNA concentration (1 OD = 50 µg/ml DNA), and then divided by three to determine the amount of DNA per cartridge. Data represents average of three separate trials. Cartridges were prepared with 2.5 µg DNA/cartridge.
Figure 3.6. FACS mediated sorting of cells which have been transfected with fluorescently labeled gold particles

Representative data from FACS sort of COS-7 cells not shot (I) or shot (II-IV) with gene gun bullets that had been co-coated with HLA-A2 DNA and neuravidin oregon green. Column 1 shows the forward and side scatter of the individual populations and gating used to exclude non viable cells (region A). Column 2 shows the same populations' fluorescence in Fl-1 (NOG); populations which had not been shot (I) were Fl-1<sup>+</sup> (region F), while shot populations (II) had a distinct subpopulation of Fl-1<sup>+</sup> cells (region E). Column 3 shows histograms of Fl-1 populations I-IV. The population shown in II was subsequently sorted on the basis of Fl-1; cells from regions E and F in population II are shown in III and IV, respectively. Contamination of Fl-1<sup>+</sup> cells in the Fl-1<sup>+</sup> sorted population (III) can be seen in region F.
Figure 3.7. Expression of DNA by cells which had been transfected using fluorescently labeled gold

Representative FACS analysis of HLA-A2 expression by COS-7 cells 3 days after transfection by gene gun. Cells were bombarded with bullets coated in pcDNA.HLA-A2 plus neutravidin oregon green (HLA/NOG) and either not sorted (HLA-NOG no sort) or sorted (HLA/NOG pos sort) immediately after bombardment for the presence of NOG bullets. (A) shows a histogram of HLA-A2 expression on day 3, and (B) summarizes the fluorescence parameters of the populations shown in (A).
sorted population had approximately twice as many HLA-A2<sup>hi</sup> cells, and a higher mean and median fluorescence than the unsorted HLA-A2 transfected cells.

Since almost all of the DNA is known to be present in the cartridges, it is unlikely that there were bullets which contained NOG but not DNA. The Fl-1<sup>hi</sup> population was contaminated during sorting with 8% Fl-1<sup>lo</sup> cells, which presumably would expand during culture to represent a greater proportion of the population at day 3. Untransfected COS-7 control cells expanded in number 250-400% (Fig. 3.8) during the 3 day period. If the 8% negative cells expanded by the same amount, they would be expected to number 2-3x10<sup>5</sup>. There were approximately 5-7x10<sup>5</sup> HLA-A2<sup>+</sup> cells in the day 3 positively sorted population, indicating that this contaminant negative population was not responsible for all the HLA-A2<sup>+</sup> cells. To account for the other HLA-A2<sup>+</sup> cells (roughly half of the Fl-1<sup>hi</sup> population), it was hypothesized that there were either 'false positives' selected during the sorting procedure, or not all of the cells which contained bullets were expressing the DNA.

When survival and expansion of negatively and positively sorted populations are compared (Fig. 3.8), it is clear that cells which contain gold particles do not expand significantly in culture compared to those which do not contain gold. COS-7 cells which are transfected by most transient transfection procedures do not expand significantly following transfection; this may be due to large-scale amplification and transcription of plasmid within transfected cells interfering with normal cellular function (70). Long term survival and expansion of cells transfected by gene gun bombardment should be ascertained using different cells than COS-7 in order to determine to what extent this method of transformation affects cellular growth potential.

False positives could theoretically result from fluorescent bullets adhering to the outside of the COS-7 cells, which would result in their Fl-1<sup>hi</sup> phenotype but not result in DNA uptake and expression. In order to test the 'stickiness' of the COS-7 cells, CMV/NOG cartridges were fired into 20 µl medium, and 1x10<sup>6</sup> COS cells in 20 µl medium were added to the well after bombardment. After 1 minute, cells were removed, washed
Figure 3.8. Survival and expansion of cells bombarded and sorted for the presence of fluorescent gold particles

Survival and expansion of COS-7 cells gene gun transfected (shot) at day 0 with different bullets: CMV plasmid with or without neutravidin oregon green (CMV, CMV/NOG); HLA-A2.CMV plasmid with or without NOG (HLA, HLA/NOG), or untransfected (not shot). Some populations were sorted immediately after bombardment for the presence (pos sort) or absence (neg sort) of Fl-1st NOG bullets (see Fig. 3.5). 3 days after transfection, cells were counted using trypan blue; survival and expansion is given here as percent cell recovery (total # cells at day 3/total # cells at day 0)x100. Data with error bars represent an average of three separate transfections.
twice in PBS, and analyzed by FACS (Fig. 3.9). FACS analysis revealed that less than 2% of cells which had been mixed with NOG bullets were above background for FL-1, indicating that very few bullets stick to the outside of cells in a manner that renders them selectable by FACS. Although this experiment does not rule out the possibility that the bombardment procedure itself somehow renders the cells 'stickier' than when the cells are simply mixed with bullets, it does suggest that bullet adherence to the cell surface is not a prominent phenomenon. As a result of this observation, it was concluded that most of the HLA-A2+ cells in the Fl-1hi sorted population of COS cells represented cells which had received a bullet but were not expressing the DNA.

IV. Discussion and Future Directions

The gene gun uses helium pressure to propel microscopic gold particles directly into the cytoplasm of cells, and has been shown to be an effective means of administering DNA vaccines (73). When compared to saline injection, gene gun immunizations require much less DNA (as little as 0.5-1.0 μg, approximately 1/100th that conventionally used in i.m. injection) to generate equivalent protection (74). Due to the requirement for much less DNA, and the practicality of targeting skin, gene gun bombardment has a great attraction for future use in vaccine application (75).

In saline-DNA immunizations, cells acquire DNA from extracellular spaces by poorly understood uptake mechanisms (33). In gene gun DNA immunizations, cells are directly transfected by DNA-coated gold beads. These different methods of DNA transfection may conceivably alter the resulting immunological response; different types of cells may be transfected, and different signals may derive from cells which have been transfected actively or passively (76). The pneumatic pressure from gene gun bombardment versus the hydrostatic pressure resulting from saline injection may also
Figure 3.9. Adherence of non transfecting gold particles to cells
FACS analysis of cell populations which had been mixed or shot with NOG bullets. COS-7 cells were mixed with 1.0 μm gold particles coated in neutravidin oregon green (NOG), or transfected by gene gun (shot) with the same gold particles. Data here are representative of 3 separate trials. (A) shows a histogram of the different populations. (B) summarizes the fluorescence parameters of the populations shown in (A).
influence the cytokine release profile of cells at the site of inoculation. The presence of much greater quantities of DNA in the extracellular spaces of tissues which have undergone saline injection may have a biologic effect as well (76). Indeed, when the immune responses induced by gene gun and saline injection methods are compared, qualitative differences are seen (49) (76) which suggest that the cellular responses to these transfection methods are different.

When antibody isotype profile and cytokine production by Th cells are investigated, it can be seen that saline injections of DNA appear to preferentially induce Th1-like immune responses in mice, while the gene gun appears to induce immune responses with a Th2-like phenotype (49) (76). These differences are manifest regardless of the site of inoculation, indicating that it is the transfection method, not the inherent characteristics of the cells at the site of transfection, which are responsible for the difference in immune response.

Keeping in mind the popularity of gene gun bombardment as a means of administering DNA vaccines, and in light of the unique immune response seen to gene gun mediated DNA vaccination, we wanted to investigate the role of transfected cells in the case of gene gun inoculation in particular. In order to mimic the in situ scenario as much as possible, we planned to transf ect cells in vitro using the gene gun, and then transfer transfected cells to recipient mice in order to investigate their actions.

Immature DC reside in epithelial and connective tissue compartments and after activation they migrate to draining lymph nodes and undergo final maturation upon interaction with T cells (77). Experiments which involved the transplantation of gene-gunned skin to naive recipients and investigation of the resulting immune response revealed that the transfer of immunized skin to naive recipients could induce immune responses if the skin was transferred within 5-12 h of immunization, but was ineffective at inducing immunity if transferred later (78). These results suggest that there is a critical time period for the events which result in induction of immunity following gene gun transfection of skin, and that it occurs soon after bombardment.
Given the possibility that critical events occur very soon after gene gun transfection, we were interested in being able to transfer transfected cells into naive recipients as soon as possible after bombardment. This would not be possible if the selection of transfected cells was based on drug selection or fluorescent antibody staining of expressed surface markers using FACS techniques, as both of these methods require waiting at least days for significant expression of the transfected DNA.

With a view to investigating the role of transfected cells in the case of gene gun transfection, all of the above parameters had to be taken into consideration. We were able to satisfy these requirements by developing a method of selecting gene gun transfected cells immediately following bombardment by labeling the gold used in bombardment with a fluorescent label which could be detected by FACS.

We were able to create gold particles for use in gene gun cartridges that were coated in plasmid DNA and neutravidin oregon green, a fluorescent dye. Our experiments showed that cells which were transfected in vitro by gene gun using the fluorescently labeled gold were selectable by FACS and expressed the DNA which had been co-coated on the gold.

It was difficult to precisely assess the proportion of bullet-containing cells which expressed the plasmid DNA due to the growth of contaminating untransfected cells, and a range of degree of expression of the DNA in our experimental system. Ultimately, it would be useful to more accurately quantify the number of gold-containing, DNA expressing cells obtainable by this transfection and selection method. A method which could be used to address this need would be the transfection of cells with a plasmid encoding a marker such as β-galactosidase, which may be more sensitive to detection due to its enzymatic activity, and is visible by light microscopy upon suitable staining. Transfected cells that had been sorted for the presence of gold particles could be analyzed soon after bombardment, as the catalytic activity of even a small number of β-gal molecules is sufficient for detection (41). β-gal expressing cells could be easily visualized and
simultaneously analyzed for the presence of gold particles by light microscopy, allowing for a more precise analysis of what proportion of cells which receive bullets also express the DNA.

We were able to achieve up to 50% transfection efficiencies using fluorescent gold bombardment, and we found that transfected cells could be immediately sorted away from untransfected cells in a quantitative manner. Cells which had been sorted for the presence of gold particles went on to express the DNA coated on those particles. This method of transfection and selection should prove very useful in investigation of the functional capabilities of transfected cells in the case of gene gun mediated DNA immunization, due to the fact that this manoeuver mimics the transfection event in vivo, and allows for immediate selection of transfected cells with minimal manipulation. It may also have applications in other areas where long culture periods, viral vector-mediated transfection, or drug selection are unfeasible or undesirable.
I. Rationale

To study the role of transfected APCs in the case of DNA immunization, we chose to investigate gene gun transfected bone marrow-derived dendritic cells. As outlined in Chapter 1, bmDCs can be grown and transfected in vitro, then transferred to naive recipient mice to elicit CTL against the transfected gene product. Our intent was to investigate the relative contributions of direct priming by transfected APCs versus possible cross priming resulting from antigen transfer in vivo to recipient APCs. Towards this end, we designed an experiment to differentiate between the two scenarios: by using transfected cells which were unable to directly prime naive CTL (specifically, MHC I-deficient cells), we hoped to be able to distinguish direct priming from cross priming. In experimental terms, our intention was to transfec wild type and MHC I-deficient bmDCs (β₂m⁺ and/or TAP⁺) with the NP-encoding plasmid NPv.CMV, then transfer the transfected cells to naive recipient mice and investigate the resulting anti-NP CTL response.
II. Materials and Methods

i) Animals

The following (female) mice were obtained at 6-8 weeks of age and were housed in the Division of Comparative Medicine at the University of Toronto: BALB/c and C57Bl/6 (Charles River Laboratories, Quebec); B6,129F2/J (wild type/B6,129), B6,129-Tap1<sup>m1Am</sup> (TAP<sup>−/−</sup>) and C57Bl/6J-B2m<sup>m1Unc</sup> (B<sub>2</sub>m<sup>−/−</sup>) (Jackson Laboratory, Bar Harbor, Maine); and BALB/c B<sub>2</sub>m<sup>−/−</sup> (kindly provided by Dr. Pamela Ohashi, University of Toronto). All procedures with animals were carried out in accordance with institutionally approved protocols.

ii) Plasmids and peptides

The HindIII/StyI fragment of pUHC13.3 (kindly provided by Dr. Tania Watts, University of Toronto), containing the luciferase gene, was cloned into the pRc.CMV vector (InVitrogen, San Diego, CA) between the HindIII and XbaI sites (luc.CMV). The GM-CSF and IL-4 plasmids consisted of murine GM-CSF and murine IL-4 respectively, cloned into pcDNA3 (24). The IL-12 plasmid consisted of murine IL-12 in pcDNA3 (24). Plasmid DNA was amplified in the JM109 bacterial strain and purified through large-scale plasmid preparations, using Qiagen MaxiPrep columns (Qiagen, Santa Clarita, CA). Peptides used in CTL assays and cell pulsing were: the H-2K<sup>d</sup> restricted peptide NP147-155 (TYQRTRALV) in the case of BALB/c mice, and the H-2D<sup>b</sup> restricted peptide NP366-374 (ASNENMETM) in the case of C57Bl/6 and B6,129 mice; both peptides are from the influenza nucleoprotein of A/PR/8/34 (Alberta Peptide Institute).

iii) Cell lines and culture

Target cells for CTL assays were P815 (H-2<sup>d</sup>), a murine DBA/2 mastocytoma (TIB-64, ATCC, Rockville, MD), and EL4 (H-2<sup>b</sup>), a murine C57Bl/6 lymphoma (TIB-39,
ATCC). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Cansera, Rexdale, ON), 100 U/ml penicillin (GibcoBRL, Grand Island, NY), 100 \( \mu \text{g/ml} \) streptomycin (GibcoBRL) and 2 mM L-glutamine (GibcoBRL).

\textit{iv) Cytokines}

COS-7 cells were transfected with the above described plasmid encoding GM-CSF or IL-4 (DEAE dextran mediated transfection (70)), and supernatants were harvested at day 3-5. After two 15 min 1600xg spins, supernatants were stored at -20°C. Aliquots were thawed for subsequent cytokine quantification as per manufacturer's directions using an Endogen GM-CSF or IL-4 ELISA kit (Endogen, Woburn, MA). Quantified supernatant was stored at -20°C. IL-12 was from unpurified supernatants of COS-7 transfected with IL-12 plasmid (murine IL-12 in pcDNA3 (24)). For some experiments, commercially manufactured GM-CSF and IL-4 (Pharmingen, Missassauga, ON) were used instead of the above preparations, as indicated in the Results.

\textit{v) Isolation and propagation of bone marrow-derived DC}

Based on a protocol by Inaba and colleagues (79), bone marrow was collected from the femurs and tibia of donor mice on day 0, by flushing bones using a 25-gauge needle with bm medium (RPMI 1640 supplemented with 5% FCS, 50\( \mu \text{M} \) 2-ME, 10 mM HEPES (Sigma), 2 mM glutamine, 100 U/ml penicillin and 100 \( \mu \text{g/ml} \) streptomycin). Bone marrow tissue was passed through a 70 \( \mu \text{m} \) nylon mesh and red blood cells were lysed for 5 min at RT with ammonium chloride buffer (0.15 M \( \text{NH}_4\text{Cl} \), 10 M KHCO\(_3\), 0.1 mM \( \text{Na}_2\text{EDTA} \), pH 7.2-7.4). After rinsing with bm medium, cells were resuspended at 10\(^7\)/ml in complement-fixing mAb cocktail (1-10 \( \mu \text{g/ml} \) each of anti-CD4, anti-CD8, and anti-B220 antibodies in bm medium), and incubated for 30 min on ice. After one rinse with fresh bm medium, cells were resuspended at 10\(^7\)/ml in Low-Tox-M rabbit serum complement (Cedarlane, Hornby, ON) and incubated for 60 min at 37°C, 5% CO\(_2\). Cells
were then rinsed 3 times in bm medium and plated at 10⁶/ml in DC medium (bm medium supplemented with 100 ng/ml GM-CSF +/- 10 ng/ml Il-4) at 3 ml/well in 6-well tissue culture dishes. On days 2 and 4, plates were gently swirled and 75-90% of medium was aspirated and replaced with fresh DC medium. On day 6, wells were vigorously pipetted to remove all loosely adherent and non-adherent cells, which were transferred to 60 mm tissue culture plates at 10⁶/ml, 5 ml/plate in fresh DC medium. Non adherent and loosely adherent cells were collected for transfection or analysis on day 7-12 by gentle pipetting.

**vi) Antibodies**

Unconjugated anti-murine CD4 and anti-murine CD8 monoclonal antibodies were supernatants from YTS191 and YTS169 rat hybridomas, respectively (80). Phycoerythrin conjugated purified rat anti-mouse antibodies against CD3, CD4, CD8, CD90.2, and CD19 were obtained from Pharmingen (San Diego, CA). Kindly provided by Dr. Tania Watts (University of Toronto), purified from supernatants and either unconjugated, FITC-conjugated (Molecular Probes, Eugene, OR) or biotinylated were: rat anti-mouse, CD11b (TIB 128), B220 (TIB 146), I-Aβ² (MKD6), CD8α (53.6.72), HSA (M169), B7-2 (HB253); and hamster anti-mouse CD11c (HB224), CD3 (CRL1975), B7-1 (16.10A1). Also provided by Tania Watts was gp39-CD8 supernatant and anti-human CD8 (53.6.72 (81). Gp39-CD8 supernatant is a COS cell supernatant which contains murine CD40 ligand (CD40L; gp39) fused to human CD8; coincubation with anti-human CD8 antibody will cross link CD40 and stimulate APC to upregulate expression of B7 costimulatory molecules (81). Antibody controls included FITC conjugated, biotinylated, or PE-conjugated hamster Ig and rat Ig (Sigma).

**vii) Surface immunofluorescence staining**

Cells were washed twice with PBS and stained for 30 min on ice with the appropriate antibodies at 1-3 µg/10⁶ cells in 100 µl PBS, 0.1% BSA, 0.01% NaN₃.
(PBS/BSA), followed by two washes with PBS/BSA. Streptavidin-fluorescein (Molecular Probes, Eugene OR) was added at this stage to biotinylated antibodies, and incubated for a further 30 min on ice, followed by 2 washes with PBS/BSA. The cells were analyzed within 1 h on a FACscan flow cytometer (Becton Dickinson, San Jose, CA).

viii) Luciferase assay

12 -24 h after transfection, cells were lysed and assayed for luciferase activity as per manufacturer’s instructions using a Promega Luciferase Assay kit (Promega, Madison WI). Briefly, cells were washed twice in PBS and resuspended at 0.5-3 x 10⁷ cells/ml in 1X lysis buffer and then incubated at RT for 15 min. Following 15 min centrifugation at 16,000xg, aliquots of supernatant were transferred to a fresh tube and mixed with substrate buffer at a ratio of 3.5:1 substrate:supernatant. Luciferase activity was measured using a TopCount scintillation counter (Canberra-Packard, Canada)

ix) Gene gun particles, in situ bombardment, and bmDC transfection

Bullets were prepared (without fluorescent dye) and bmDCs were transfected in vitro using the gene gun as described (see Chapter 2). To inoculate mice in situ, abdominal hair was removed using an electric shaver, the gene gun was abutted to the abdominal skin, and two cartridges were discharged per mouse (400 psi ) at non-overlapping sites.

x) bmDC transfer and treatments

After transfection, bmDC were washed 3 times and resuspended at 0.3-2 x 10⁶ cells/500 µl in HBSS or PBS, and injected i.p. or i.v. into naive syngeneic (or wild type in the case of knockout bmDC) recipient mice. In cases where stimulation was used, bmDC were transferred to fresh medium containing 1% IL-12, 1 µM calcium ionophore (Sigma A23187), or 10% (v/v) gp39-CD8 supernatant plus 10 µg/ml anti-human CD8, and incubated overnight at 37°C before transfection and transfer.
xi) Peptide pulsing

bmDCs were washed in bm medium and resuspended at 1-4x10⁶ cells/ml with 100 µg/ml peptide (82). After 3h incubation at 37°C, with gentle shaking every 30 min, cells were washed twice in HBSS or PBS and resuspended at 0.5-2x10⁶ cells/500 µl PBS/HBSS for injection into naive recipients.

xii) CTL assays

CTL assays were performed as described (Ch. 2); the target cells for C57/Bl6 and B6,129 were EL4 (H-2b), with the H-2Dᵇ restricted (NP366-374) peptide for in vitro stimulation and target pulsing.

III. Results

i) Generation of cells with DC characteristics from bone marrow cultures

Figure 4.1 outlines the procedure which was used for the culture of bone marrow cells. Briefly, bone marrow tissue was harvested from the tibia and femurs of 6-10 week old female mice, and depleted of red blood cells. B220⁺, CD4⁺, and CD8⁺ cells were removed by antibody-mediated complement depletion, and the remaining cells were cultured in vitro in the presence of 100 ng/ml GM-CSF, with or without 10 ng/ml IL-4. The presence of IL-4 in the growth medium did not affect the rate of cell growth or physical morphology as visually assessed upon harvest. As a result, and considering that IL-4 is not strictly necessary for successful bmDC culture (82), IL-4 was not always used in the culture of bmDCs. Experiments in which IL-4 was included in the culture medium are indicated. Non-adherent cells, including granulocytes, were washed away and fresh medium was added every 2-3 days until the culture was 6-7 days old, whereupon non-adherent cells were collected and replated in fresh medium. On day 7-12, non-adherent
Figure 4.1. In vitro culture of mouse bone marrow cells

Culture of bone marrow depleted of red blood cells, B cells and T cells, in the presence of 100 ng/ml GM-CSF (with or without 10 ng/ml IL-4) promotes the development of cells with the characteristics of dendritic cells. Cytokines are replenished and non-adherent granulocytes are depleted by replacement of 75% of culture medium at day 2 and 4, and by day 6-7, DC-like cells are only loosely adherent and can be separated on this basis from strongly adherent macrophages.
cells from the replated population were collected and analyzed for cell surface markers and morphology or use in transfer experiments. Microscopic analysis revealed that the majority of cultured cells were dendritic in morphology, with prominent dendritic processes and ruffled edges (Fig. 4.2).

The surface phenotype of the cultured bone marrow cells was characterized by immunofluorescence and flow cytometry. Individual fluorescence histograms for a typical population are shown in Figure 4.3. All cultures lacked significant numbers of T cells (CD3+ and B cells (B220+); most were low in expression of CD11c and B7-1, and expressed medium to high levels of MHC class II and B7-2. Expression of these levels of markers by our bm-derived culture indicates that these cells are dendritic in phenotype, as described by other investigators (79) (63) (83) (84) (64).

ii) bmDCs can be transfected in vitro using the gene gun and express transfected DNA

Cultured bmDCs were transfected with luc.CMV in vitro using the gene gun, and analyzed 24 hours post bombardment for expression of luciferase (Fig. 4.4). Luciferase expression of luc.CMV shot cells was far greater than in controls, indicating that the bmDCs were capable of surviving bombardment and able to express the transfected DNA.

iii) Transfected bmDCs, when transferred to naive recipients, do not elicit NP-specific CTL or antibody responses

Cultured bmDCs were transfected in vitro with NPv.CMV using the gene gun, and transferred to naive recipient mice (Fig. 4.5). Different mouse strains, quantities of cells, routes of transfer, boosting and harvesting regimes, and in vitro cytokine growth parameters were investigated (Table. 4.1). Although at least 12 different cell transfer experiments were performed, no CTL activity against NP was detected in the recipient mice.
Figure 4.2. bmDC morphology
Micrographs of typical bmDC cells cultured in the presence of 100 ng/ml GM-CSF from mouse bone marrow tissue (1000X, phase contrast microscopy). Dendritic morphology is characterized by prominent dendritic processes (arrows).
FlTC biotin-RatIg, FITC biotin-HSA, PE-CD1, biotin-87.2.

FlTC fluorescence intensity

PE fluorescence intensity

A

B

FITC biotin-RatIg

FITC biotin-HSA, PE-CD11b

FITC biotin-B7.2, PE-CD11b

FITC fluorescence intensity

FITC-RatIg

FITC-B220

FITC-CD11b

PE-CD11b

FITC-CD8a, PE-CD11b

FITC-MHCII, PE-CD11b

0.41, 0.27

0.32, 0.01

0.00, 0.09

0.57, 0.59

0.99, 0.99

28.19, 73.72

99.58, 0.42

0.00, 0.00

98.48, 0.27

28.92, 70.19

84.41, 15.31

0.10, 0.04

60.42, 39.05

0.53, 0.00

0.27, 0.01
Figure 4.3. Cell surface phenotype of cultured bmDCs

FACS analysis of BALB/c bone marrow cells cultured in 100 ng/ml GM-CSF, arranged by antibody type, with appropriate isotype controls (FITC-rat Ig, biotin-FITC-rat Ig, FITC-hamster Ig and biotin-FITC-hamster Ig) (A) shows FITC-conjugated rat anti-mouse B220, CD11b, and CD8a along with PE-conjugated rat anti mouse CD11b. (B) shows biotin-conjugated rat anti mouse HSA, and B7-2 (detected with FITC streptavidin) along with PE-conjugated rat anti mouse CD11b. (C) shows FITC-conjugated hamster anti mouse CD11c and CD3 along with PE-conjugated rat anti mouse CD11b. (D) shows biotin-conjugated hamster anti mouse B7-1 (detected with FITC streptavidin) along with PE-conjugated rat anti mouse CD11b. Populations are gated to exclude dead cells on the basis of side and forward scatter. Quadrant ratios are shown.
Figure 4.4. Expression of plasmid DNA by transfected bmDCs
Cultured C57Bl/6 or B6,129 bmDC cells were transfected by gene gun with luc.CMV or CMV (vector) only. 24 hours after transfection, equal numbers of cells from each transfection group were lysed and luciferase expression was measured using a standard luciferase assay.
Figure 4.5 bmDC transfer protocol

bmDCs were transfected in vitro using the gene gun. After 2 washes in HBSS, cells were resuspended at 0.5-2x10^6 cells in 500 μl HBSS, and injected i.v. into naive syngeneic recipient mice. Some mice were boosted 8-25 days later and their splenocytes were harvested for CTL assay 9-21 days post boost, while other mice were harvested for CTL assay 10-34 days after initial injection with no boosting.
Table 4.1. Immunization regimes for transfer of transfected bmDCs to naive recipients

<table>
<thead>
<tr>
<th>strain</th>
<th>n</th>
<th>treatment</th>
<th>bm DC harvest</th>
<th>transfer regime (x10^6 cells)</th>
<th>route</th>
<th>CTL</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>2</td>
<td>100 ng/ml GM-CSF</td>
<td>d 9</td>
<td>1.3, d 0</td>
<td>i.p.</td>
<td>d 9</td>
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<tr>
<td>BALB/c</td>
<td>2</td>
<td>100 ng/ml GM-CSF</td>
<td>d 9</td>
<td>1.0, d 0</td>
<td>i.p.</td>
<td>d 15</td>
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<tr>
<td>BALB/c</td>
<td>1</td>
<td>100 ng/ml GM-CSF</td>
<td>d 9</td>
<td>1.3, d 0</td>
<td>i.p.</td>
<td>d 34</td>
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<td></td>
<td></td>
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<td></td>
<td>2.0, d 13</td>
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<tr>
<td>BALB/c</td>
<td>3</td>
<td>100 ng/ml GM-CSF</td>
<td>d 9</td>
<td>2.0, d 0</td>
<td>i.p.</td>
<td>d 21</td>
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<td></td>
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<td>1.0, d 13</td>
<td></td>
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<tr>
<td>BALB/c</td>
<td>1</td>
<td>100 ng/ml GM-CSF</td>
<td>d 9</td>
<td>1.3, d 0</td>
<td>i.p.</td>
<td>d 34</td>
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<td>1.5, d 25</td>
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<td>BALB/c</td>
<td>2</td>
<td>100 ng/ml GM-CSF</td>
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<td>1.0, d 0</td>
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<td>d 21</td>
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<td>1.0, d 15</td>
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<tr>
<td>C57Bl/6</td>
<td>2</td>
<td>100 ng/ml GM-CSF</td>
<td>d 9</td>
<td>2.0, d 0</td>
<td>i.v.</td>
<td>d 10</td>
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<tr>
<td>C57Bl/6</td>
<td>2</td>
<td>100 ng/ml GM-CSF</td>
<td>d 9</td>
<td>1.0, d 0</td>
<td>i.p.</td>
<td>d 21</td>
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<td>1.0, d 15</td>
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<tr>
<td>C57Bl/6</td>
<td>2</td>
<td>100 ng/ml GM-CSF 10 ng/ml IL-4</td>
<td>d 9</td>
<td>2.0, d 0</td>
<td>i.p.</td>
<td>d 14</td>
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<tr>
<td>C57Bl/6</td>
<td>2</td>
<td>100 ng/ml GM-CSF 10 ng/ml IL-4</td>
<td>d 9</td>
<td>2.0, d 0</td>
<td>i.v.</td>
<td>d 14</td>
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<td>1.0, d 8</td>
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<td>C57Bl/6</td>
<td>2</td>
<td>100 ng/ml GM-CSF 10 ng/ml IL-4</td>
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<td>C57Bl/6</td>
<td>2</td>
<td>100 ng/ml GM-CSF 10 ng/ml IL-4</td>
<td>d 9</td>
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<td></td>
<td>2.0, d 8</td>
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</table>

bmDCs were grown in culture in the presence of GM-CSF, with or without IL-4, and harvested on day 9 for transfection in vitro with NPv.CMV using the gene gun. 1.0-2.0 x 10^6 transfected bmDCs were transferred i.p. or i.v. to naive mice, and mice were or were not boosted with 1.0-2.0 x 10^6 of identically grown and transfected bmDCs 8 to 25 days later. 9-34 days post priming (or boost), individual recipient mouse spleens were harvested and cultured in vitro for testing by CTL assay for the presence of NP-specific CTL. No CTL activity was detected for any of the immunization regimes.
(Fig. 4.6), nor was an NP specific antibody response induced by the transferred transfected bmDCs, as measured by anti-NP ELISA (for a typical assay, see Fig. 4.7).

A number of treatments have been shown to stimulate bmDCs to mature or prime CTL. Calcium ionophore has been shown to induce DC activation/maturation in human peripheral monocytes (85), and murine splenic DC (86) (87). CD40 stimulation of DC can bypass the need for CD4+ T cell help in CTL initiation (52) (53) (54). IL-12 administered in vitro for activation of DC prior to peptide pulsing can substitute for the T helper peptide in initiating DTH following transfer of tumor antigen-pulsed DC (88). BmDCs were stimulated in vitro in overnight cultures with these reagents in an attempt to enable them to prime naive CTL (Table 4.2). Despite these attempts to stimulate bmDCs in vitro before transfer, no CTL initiation was seen in recipient mice for any of these experiments (see Fig. 4.8 for a representative CTL assay).

Under the hypothesis that the supernatant sources for cytokines used in our previous experiments may have negatively effected our bmDC cultures, β2m and TAP knockout bmDCs were grown in culture in the presence of 100 ng/ml GM-CSF and 10 ng/ml IL-4 from commercial sources. FACS analysis confirmed a similar phenotype to our previously cultured wild type bmDCs (refer to Fig. 4.3 for typical FACS analysis). Luciferase assays performed on the knockout bmDCs showed expression of transfected DNA comparable to wild type bmDCs previously tested (see Fig. 4.4 for typical luciferase assay of luc.CMV gene-gun transfected bmDCs). When transferred to naive recipients, no CTL initiation was seen (see Fig. 4.9 for a representative CTL assay). Unfortunately, due to the lack of ability of wild type bmDCs to initiate CTL in our experimental system, we were unable to draw any conclusions from the knockout bmDC data regarding the balance between cross priming and direct priming in the case of transfected APCs.
Figure 4.6. Lack of NP-specific CTL in mice receiving transfected bmDC
CTL Assay of C57Bl/6 mice injected i.v. or i.p. on days 0 and day 8 with $1 \times 10^6$
NPv.CMV-transfected bmDCs, or infected with influenza. Spleens were harvested from
individual mice on day 15 and assayed for NP-specific CTL. Naive mice received no
treatment; mice which had been infected with influenza (flu infected) provided NP-specific
CTL. Non-specific lysis in this assay was less than 5% in all cases.
Figure 4.7. Lack of NP-specific antibodies in mice receiving transfected bmDC

C57Bl/6 mice were injected with $1 \times 10^6$ NPv.CMV-gene gun-transfected bmDCs. 21 days after injection, serum was collected and analyzed for anti-NP antibodies by ELISA. Serum from a mouse which had been immunized by direct cutaneous bombardment with NPv.CMV served as a positive control for anti-NP antibodies.
<table>
<thead>
<tr>
<th>strain</th>
<th>n</th>
<th>treatment</th>
<th>bmDC harvest</th>
<th>transfer regime (x10^6 cells)</th>
<th>route</th>
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<td>d 6</td>
<td>1.5, d 0</td>
<td>i.v.</td>
<td>d 12</td>
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<td>100 ng/ml GM-CSF IL-12</td>
<td>d 11</td>
<td>4.0, d 0</td>
<td>i.v.</td>
<td>d 10</td>
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<tr>
<td>C57Bl/6</td>
<td>2</td>
<td>100 ng/ml GM-CSF IL-12</td>
<td>d 11</td>
<td>3.0, d 0</td>
<td>i.v.</td>
<td>d 10</td>
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<td>3 weeks restim in vitro</td>
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<td>100 ng/ml GM-CSF IL-12</td>
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<tr>
<td>C57Bl/6</td>
<td>2</td>
<td>100 ng/ml GM-CSF IL-12</td>
<td>d 8</td>
<td>2.0, d 0</td>
<td>i.v.</td>
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<tr>
<td>C57Bl/6</td>
<td>2</td>
<td>100 ng/ml GM-CSF IL-12</td>
<td>d 8</td>
<td>2.0, d 0</td>
<td>i.v.</td>
<td>d 10</td>
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<tr>
<td>C57Bl/6</td>
<td>2</td>
<td>100 ng/ml GM-CSF gp39-CD8 + αCD8</td>
<td>d 8</td>
<td>2.0, d 0</td>
<td>i.v.</td>
<td>d 10</td>
</tr>
<tr>
<td>BALB/c</td>
<td>2</td>
<td>100 ng/ml GM-CSF (c)* 10 ng/ml IL-4 (c)</td>
<td>d 8</td>
<td>1.0, d 0</td>
<td>i.v.</td>
<td>d 10</td>
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<tr>
<td>BALB/c beta-2-m K.O.</td>
<td>2</td>
<td>100 ng/ml GM-CSF (c) 10 ng/ml IL-4 (c)</td>
<td>d 8</td>
<td>1.0, d 0</td>
<td>i.v.</td>
<td>d 10</td>
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<tr>
<td>C57Bl/6</td>
<td>2</td>
<td>100 ng/ml GM-CSF (c) 10 ng/ml IL-4 (c)</td>
<td>d 8</td>
<td>1.0, d 0</td>
<td>i.v.</td>
<td>d 10</td>
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<td>C57Bl/6 beta-2-m K.O.</td>
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<td>100 ng/ml GM-CSF (c) 10 ng/ml IL-4 (c)</td>
<td>d 8</td>
<td>1.0, d 0</td>
<td>i.v.</td>
<td>d 10</td>
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<td>B6,129</td>
<td>2</td>
<td>100 ng/ml GM-CSF (c) 10 ng/ml IL-4 (c)</td>
<td>d 8</td>
<td>1.0, d 0</td>
<td>i.v.</td>
<td>d 10</td>
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<tr>
<td>B6,129 TAP K.O.</td>
<td>2</td>
<td>100 ng/ml GM-CSF (c) 10 ng/ml IL-4 (c)</td>
<td>d 8</td>
<td>1.0, d 0</td>
<td>i.v.</td>
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* (c) cytokines were from commercial source
Table 4.2. Immunization regimes for transfer of *in vitro* stimulated, transfected bmDCs to naive recipients

bmDCs were cultured in the presence of 100 ng/ml GM-CSF, with or without 10 ng/ml IL-4, and harvested on day 6-11 when various reagents were added for overnight incubation. Stimulation consisted of overnight incubation with either 1% IL-12 (COS supernatant), 1 μM A23187 (calcium ionophore), or 10% gp39-CD8 supernatant plus 10 μg/ml anti CD8 antibody to cross link CD40 (see materials and methods). The next day, 1.0-4.0 x 10^6 stimulated or unstimulated transfected bmDCs were then transferred i.v. to naive mice. 10-22 days post transfer, individual recipient mouse spleens were harvested and cultured *in vitro*, with or without repeated restimulation with peptide-pulsed naive splenocytes, for testing by CTL assay for the presence of NP-specific CTL. No CTL activity was detected for any of the immunization regimes.
Figure 4.8. Lack of NP-specific CTL in mice receiving stimulated transfected bmDC

CTL Assay of C57Bl/6 mice injected i.v. with 1x10⁶ NPv.CMV transfected bmDCs, or infected with influenza. bmDCs were stimulated overnight with IL-12, A23187 (calcium ionophore), or anti-CD40 antibody, as outlined in materials and methods. Following overnight treatment (or no treatment), cells were transfected by gene gun in vitro with NPv.CMV and transferred to naive mice (NPv.CMV bmDC). Recipient mouse spleens were harvested 10 days after injection of bmDCs and assayed for NP-specific CTL. Each treatment was performed on two individual mice; data is represented as an average of the two. Naive mouse received no treatment. Non-specific lysis in this assay was less than 5% in all cases.
Figure 4.9. Lack of NP-specific CTL in mice receiving transfected wild type or knockout bmDC

CTL Assay of B6.129 mice injected i.v. with 5x10^5 bmDCs which had been gene-gun transfected \textit{in vitro} with influenza NP encoding NPv.CMV plasmid (NPv) or CMV vector only (CMV only). bmDCs were from wild type B6.129 mice (NPv wt bmDC) or from TAP knockout B6.129 mice (NPv (TAP-) bmDC). Mice which had been bombarded intradermally by gene gun \textit{(in situ)} with NPv.CMV or CMV only served as controls for the gene gun transfection; mice which had been infected with influenza (flu infected) provided NP-specific CTL. Spleens were harvested from individual mice on day 10 and assayed for NP-specific CTL. Non-specific lysis in this assay was less than 5% in all cases.
iv) Peptide pulsed bmDCs, when transferred to naive recipients, do not elicit NP-specific CTL responses

To further analyze the functional abilities of our cultured bmDCs to initiate CTL immunity, we decided to examine peptide pulsed bmDCs, transferred to naive recipients. We pulsed the bmDCs with NP peptide (H-2Kd restricted NP147-155 for BALB/c, and H-2Dd restricted NP366-374 for C57Bl/6 mice) at 100 μg/ml peptide for 3 hours before washing and transferring i.p. or i.v. to naive syngeneic mice. The same populations, parameters and number of trials as those used in the transfected bmDC transfer experiment (see Table 4.2) were used for the peptide pulsed bmDC transfer. Despite numerous attempts using different mouse strains, quantities of cells, routes of transfer, boosting and harvesting regimes, and in vitro cytokine growth parameters, no NP-specific CTL response was seen in recipient mice (see Fig 4.10 for representative assay).

IV. Discussion and Future Directions

The inability of our cells, though matching in phenotype to cells used by other investigators in similar experiments, to elicit either Ab or CTL responses in naive recipient mice following peptide pulsing or transfection in vitro, indicates that something was probably wrong with our cultured cells, and is not a reflection of bmDC's general inability to induce these responses (see introduction for review of similar experimental protocols). Several possibilities were considered in our efforts to determine the reason for lack of success in our experiment.

Given the fact that at least some of our bmDCs expressed luciferase, and that gene gun transfection of bmDCs has been successfully used by other investigators in similar transfer experiments, it is unlikely that insufficient expression of the transfected NPv.CMV plasmid is the problem in our experiment. Nonetheless, it would be useful to conclude this
Figure 4.10. Lack of NP-specific CTL in mice receiving peptide pulsed bmDC

CTL Assay of BALB/c mice injected i.v. on days 0 and day 14 with 1x10⁶ NP peptide pulsed bmDCs (NPpep bmDC). Spleens were harvested from individual mice on day 21 and assayed for NP-specific CTL. Naive mouse did not receive any injections; mice which had been infected with influenza (flu infected) provided NP-specific CTL. Non-specific lysis in this assay was less than 5% in all cases.
by using a reporter gene such as β-galactosidase to quantify the proportion of expressing cells in the transfer population, by visual or FACS analysis of the transfected cells.

It is possible that the stage of maturity at which the bmDCs were transfected and transferred was inappropriate for immune induction. Immature DC reside in epithelial and connective tissue compartments and after activation they migrate to draining lymph nodes and undergo final maturation upon interaction with T cells (77). Immature DCs express low levels of MHC class I, class II, and costimulatory molecules including B7-1 and B7-2 and poorly initiate immune responses (89). Upon stimulation by one or more of a variety of agents such as bacterial DNA, LPS, and inflammatory cytokines, DC undergo activation (90) (77) (84) (89). During the maturation process, DCs lose the ability to take up Ags while up-regulating MHC molecules and adhesion and costimulatory signals. Final maturation is thought to occur upon interaction with T cells, probably by the ligation of CD40 with CD40L on Th cells (90) (89), and enables DC to prime naive CTLs (77).

A wide range of expression of MHC II and B7-2 in our bmDC suggests that there was probably a range of maturity levels in our population, but the low expression of B7-1 indicates that fully mature cells may not have been represented. Given that CpG motifs are able to stimulate maturity in DCs (84), the act of transfection itself should have provided this stimulus. Even if the CpG motifs in the plasmid were unable to induce sufficient maturity, if fully mature cells were needed to initiate an immune response, then using the older (day 12) and/or in vitro stimulated (IL-12, CD40, calcium ionophore) populations should have relieved this problem. It would be useful to analyze the phenotype of the stimulated cells to determine the success of these maturation treatments in regards to upregulation of costimulatory molecules before concluding that the maturity level of the transferred population is not the problem. However, in light of the fact that APCs which are transfected during in situ gene gun inoculation of skin are probably in an immature stage, the use of an immature population of bmDCs should more accurately mimic the in vivo situation than the use of mature cells.
It seems more likely that our culture conditions somehow abrogated the ability of the bmDCs to initiate immunity. In order to determine the functional abilities of our bmDCs, they could be tested in an MLR (79). BmDCs which were grown by other investigators in associated labs, in the same culture conditions as our own, however, have been shown to be functional in MLR assays (Edward Bertram, personal communication). Another way to assess the functional abilities of our bmDCs would be to perform an antigen-specific cytokine release assay (62). Specifically, this assay could measure the ability of the NPv.CMV transfected bmDCs to cause IFNγ release by splenocytes isolated from influenza-immunized mice. Such secondary stimulation of T cells would indicate that the bmDCs are presenting influenza NP T cell epitopes in an immunogenic format.

It is conceivable that the transferred cells are killed upon transfer to the host due to uncharacterized features resulting from culture conditions, which render them immediately recognizable as foreign; but one imagines that this would be the case in experiments by other investigators as well. This possibility would seem more likely if non-specific lysis was seen in the CTL assays, where the immunized splenocytes are exposed to cultured cells; non-specific lysis would indicate a response by the recipient APCs to cultured cells in general. Also, the recent discovery that DC preferentially phagocytose apoptotic cells for purposes of cross priming would suggest that this is not occurring. Alternatively, the transferred bmDCs could passively die for other reasons upon transfer to recipients. Phagocytosis of necrotic cells has been shown not to lead to cross presentation of antigen by DC lines in vitro (89). One would assume that bmDC necrotic death would be a factor encountered by other researchers, although for some reason it may be a phenomenon peculiar to our own bmDC cells. In order to determine the in vivo survival of our bmDCs, it would be desirable to search for the cells some time after transfer. Harvesting the LN and spleens of mice which had undergone bmDC transfer, and searching for the presence of the immunizing plasmid (by PCR), or even the cells themselves (with the use of β-gal as a marker) would serve to answer the question of whether or not (and for how long) the
transferred bmDCs are surviving in the host. While this is a technically difficult and time-consuming proposition, it has been proven possible by the dedicated work of Porgador and colleagues (41).

It is difficult to determine the problem with our bmDC culture. Further manipulation of the details of culture conditions, such as the source of serum in medium, and the nature and source of reagents used to stimulate the cells before transfer may resolve their inability to induce immunity when transferred to naive recipients.

β₂m knockout cells do not express MHC class I on their cell surface, due to lack of the essential MHC I component β₂m (91). TAP knockout cells express very low levels of MHC class I on their cell surface due to their inability to effectively load and stabilize MHC I molecules with epitopes in the endoplasmic reticulum; in the absence of high affinity peptide ligands, cell surface class I molecules are unstable at 37 °C and rapidly denature (92). By using these knockout mice as sources of transfected bmDCs in transfer experiments, we planned to assess the ability of transfected DCs to initiate cellular immunity when they are incapable of directly priming naive CTL.

This question was designed to determine the balance between cross priming and direct priming in the case of transfected APCs. If no CTL priming was seen upon transfer of transfected knockout bmDCs, this would strongly suggest that cross priming does not occur in the case of transfected APCs. If CTL priming is uninhibited even when the transferred transfected bmDCs are unable to prime CTL directly, then it would point to cross priming being the dominant mechanism of CTL priming even in the case of APC transfection. A diminished CTL initiation would indicate that a balance between direct priming and cross priming is occurring in the case of transfected APCs.

Assuming that there is a balance between cross priming and direct priming, there are a number of avenues which could be pursued to elucidate the precise nature of this balance. Use of our previously developed fluorescent gold transfection/selection technique, in conjunction with the knockout bmDCs, would help to quantify the
contribution of protein transfer versus direct priming. By analyzing the number of directly presenting cells required to restore full priming in the system, both with and without the presence of cross priming cells (i.e. knockout bmDCs), we should be able to determine the contribution that each of these mechanisms makes to the priming of CTL. Analysis of the resulting immune response in regards to antibody isotype profile would also help determine the possible role that each mechanism has in inducing Th1 vs. Th2 type responses.

If one is able to establish that effective cross priming does occur between transfected and naive APCs, one could dissect the mechanisms involved in cross priming using this bmDC transfer experimental design. Knockout bmDCs could be manipulated in vitro before transfer by a number of possible treatments in an attempt to abrogate or enhance the resulting cross priming: promoting apoptotic or necrotic death immediately before transfer would shed light on the importance and manner of cell death in cross priming; adding additional 'danger' signals such as LPS or exogenous CpG sequences could show the effect of these on enhancement of immune initiation; including antisense sequences which could target different cellular components such as hsp70 could reveal the role they play in cross priming. All of these experiments have the potential to reveal important information about the mechanisms involved in DNA immunization in particular, and also with respect to cross priming and initiation of immunity in general.
CHAPTER FIVE: CONCLUDING REMARKS

Plasmid DNA has a number of advantages over more traditional vaccination strategies such as synthetic peptide or attenuated pathogen immunization. While protein or peptide immunization and the use of whole killed pathogens can be an effective method of stimulating humoral immunity for some diseases, these approaches are usually not useful for eliciting effective CTL responses, which are a necessary part of immunity for a number of diseases. Recombinant viral vaccines and attenuated pathogens are better able to stimulate cellular immunity, but carry the inherent risk of infection, particularly in immunocompromised individuals. In contrast, plasmid DNA vaccines have been shown to elicit protective humoral and cellular immunity to a very broad range of pathogens, with no apparent dangerous side effects. Plasmid DNA does not seem to replicate in host cells, nor does it appear to integrate into the host genome, or elicit general anti-DNA responses. Plasmid DNA can also be easily, inexpensively, and rapidly produced and purified in comparison to the production of recombinant viruses or synthetic peptides. The heat-stable nature of dry DNA makes it a feasible approach to vaccination in Third World countries where lack of refrigeration facilities has made immunization difficult in the past.

Results of clinical trials of DNA vaccines in humans are now reaching publication. Administration of a plasmid encoding the *P. falciparum* circumsporozoite protein has been reported to induce antigen-specific, genetically restricted CTLs (93). Despite such ongoing progress in the development of DNA vaccines, after more than six years of intensive research, the mechanism by which DNA immunization elicits cellular immunity still remains unclear. Considerable insight has been gained into the basic immunology of CTL priming, with the emerging importance of dendritic cells and their role in immune initiation being one of the most promising avenues of future research. Some experiments have been
outlined in this thesis which would help elucidate the role and action of dendritic cells in gene gun immunization and in the initiation of immunity in general.

It is only through understanding of the mechanisms action of DNA immunization that we can reasonably expect to improve upon current vaccine designs. There are numerous possible means of improving DNA vaccines. Plasmids which encode the immunogen of interest along with cytokines such as IL-12 or IFNα (49) can direct the quality and the quantity of the subsequent response, presumably by attracting to and influencing APCs at the site of antigen production (45). The use of intercellular targeting to direct transcribed gene products to specific cells has proven to enhance immunity, as in the case of fusion of immunogens with the APC ligand CTLA4 (94). Another promising approach to tailoring DNA vaccines is the addition of intracellular targeting motifs, such as ER and ubiquitination sequences (95) to direct processing within transfected cells (96). Understanding the different possible outcomes of DNA vaccination will also help in applications other than prophylaxis. The identification of methods that can inhibit or skew established cytokine profiles may enable effective intervention with ongoing responses to autoantigens, alloantigens, or infectious agents (97).

DNA vaccines have great potential as a widely applicable immunization strategy. We look to other researchers to continue the investigations of its mechanism of action, in anticipation of more discoveries in the fields of cross priming and dendritic immunology leading to improved understanding and administration of this promising novel vaccine technology.
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