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Dedication

This dissertation is dedicated to my parents, Karen and David Fletcher, whose love and support have made this achievement possible.
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List of Abbreviations

Ag – antigen
AIM2 – absent in melanoma 2
APC – antigen presenting cell
B4F – biotin-4-fluorescein
BpA – bovine growth hormone polyadenylation signal
CFA – complete Freund’s adjuvant
CFSE – 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester
CMV – cytomegalovirus
CpG – cytosine-phosphate-guanine
CR – charge ratio
CTLA-4 – cytotoxic t-lymphocyte antigen 4
DAMP – damage-associated molecular pattern
DC – dendritic cell
DC-SIGN – dendritic cell-specific ICAM-3 grabbing non-integrin
DNA – deoxyribonucleic acid
dsDNA – double-stranded DNA
E. Coli – Escherichia coli
eff – enhanced firefly luciferase
EMSA – electrophoretic mobility shift assay
FasL – Fas ligand
FBS – fetal bovine serum
FL – full-length
FL-Ag – full-length plasmid encoding the antigen cassette
FL-eff – full-length plasmid encoding enhanced firefly luciferase
GFP – green fluorescent protein
GVHD – graft-versus-host disease
HA2 – hemagglutinin 2
hUbC – human ubiquitin C promoter
ICAM3 – intracellular adhesion molecule 3
IFN-γ – interferon-gamma
IL – interleukin
IPTG – Isopropyl β-D-1-thiogalactopyranoside
KLRG1 – killer cell lectin-like receptor subfamily G member 1
LCMV – lymphocytic choriomeningitis virus
LCMV-Arm – Armstrong strain of the lymphocytic choriomeningitis virus
LCMV-cl-13 – clone 13 strain of the lymphocytic choriomeningitis virus
LM-OVA – Listeria monocytogenes bearing the ovalbumin antigen
LN – lymph node
Ln+dDC – langerin+ dermal dendritic cell
MC – minicircle
MC-Ag – minicircle DNA encoding the antigen cassette
MC-eff – minicircle DNA encoding enhanced firefly luciferase
MFI – median fluorescence intensity
MHC – major histocompatibility complex
**MR** – molar ratio  
**NFAT** – nuclear factor of activated T cells  
**OVA** – ovalbumin  
**PAMP** – pathogen-associated molecular pattern  
**PBS** – phosphate buffered saline  
**PD-1** – programmed death 1  
**PD-L1** – programmed death ligand 1  
**PD-L2** – programmed death ligand 2  
**pDNA** – plasmid DNA  
**PE** – phycoerythrin  
**PerCP** – Peridinin chlorophyll  
**pMHC** – peptide:MHC complex  
**PPAA** – poly(propylacrylic acid)  
**p/n** – positive/negative charge ratio  
**P/S** – penicillin/streptomycin antibiotic mixture  
**RLU** – relative light units  
**SDS-PAGE** – Sodium dodecyl sulfate polyacrylamide gel electrophoresis  
**SA** – streptavidin  
**siRNA** – small interfering ribonucleic acid  
**SS** – secretion signal  
**TCR** – T cell receptor  
**TLR** – Toll-like receptor  
**TNF-α** – tumor necrosis factor-alpha
TRAIL – TNF-related apoptosis-inducing ligand

VC – vector control plasmid

WT – wild-type
CHAPTER 1: Introduction

I. CD8+ T cell responses to infection

During an infection, CD8+ T cells respond in a well-characterized manner. Before encountering antigen (Ag), naïve T cells circulate through secondary lymphatic tissue (lymph nodes [LN] and spleen). Professional antigen presenting cells (APC), such as dendritic cells (DCs), in these secondary lymphatic tissues present pathogen-derived peptides to CD8+ T cells via class I major histocompatibility complexes (MHC) on their cell surfaces. These peptides derive either from pathogenic proteins present within infected APCs or from endocytosed extracellular proteins transported to the cytosol and cross-presented on class I MHC.

If a CD8+ T cell encounters the specific peptide:MHC (pMHC) complex for which its T cell receptor (TCR) is specific, it will become activated, but only if it receives additional activating signals. Co-stimulatory ligands, such as CD80 and 86 are upregulated on APCs in response to danger- or pathogen-associated molecular patterns (DAMPs/PAMPs) or in the presence of CD4+ T cell help. Co-stimulatory ligands bind to receptors on CD8+ T cells (CD28 in the case of CD80 and 86), delivering a necessary second signal for T cell activation (1,2). In addition, a third signal, typically in the form of interleukin-12 (IL-12) or tumor necrosis factor-α (TNF-α), is necessary to produce functional effector T cells. In the absence of these cytokines, which are secreted by APCs in response to DAMPs or PAMPs, T cells are deleted or become unresponsive to Ag, a condition known as anergy (3).
Once a CD8+ T cell has become activated by a mature APC during an infection, it undergoes massive clonal expansion and differentiates into an effector T cell capable of killing infected cells. Effector CD8+ T cells migrate through tissues until encountering infected cells that express their cognate Ags (4). When an effector CD8+ T cell detects a cell expressing a pMHC I complex for which its TCR is specific, it can kill the target cell. Killing results from the release of granules containing perforin, which forms pores in the cell membrane and aids in the entry of granzymes, which are serine proteases also present in the granules. The proteases induce apoptosis by activating caspases in the target cell (5). Effector CD8+ T cells also express the pro-apoptotic TNF receptor family proteins Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL). Both FasL and TRAIL induce apoptosis in targets via recruitment of caspases (6). In addition to killing targets, CD8+ effectors secrete anti-microbial and pro-inflammatory cytokines such as interferon (IFN)-γ and TNF-α, as well as IL-2, which promotes T cell proliferation (4).

The net result of this process is a large expansion of effector CD8+ T cells which occurs shortly after infection. Following pathogen eradication, this expansion phase is followed by a rapid contraction phase, in which 90-95% of effector cells die by apoptosis (4,7). The population that remains, although severely contracted, is larger than the original naïve population and differentiates into a population of memory CD8+ T cells. These memory cells persist long-term, sometimes for the lifetime of the animal, and are marked by their ability to rapidly proliferate and re-acquire effector function upon re-encounter with Ag (7,8). The kinetics of expansion and contraction following infection appear to be largely pre-programmed. That is, after being driven by an initial period of
Ag exposure lasting at least 24 hours, the presence or absence of Ag does not affect the timing of expansion and contraction (9-11).

It is important to note that in cases where pathogen burden is very high or the infection persists, formation of conventional memory does not occur. Instead, the contraction phase is followed by the emergence of a population of CD8+ T cells that differ from traditional memory cells in several significant ways. They do not respond robustly to encountered Ag. Their abilities to proliferate, secrete cytokines, and kill targets are impaired. Rather than persisting due to homeostatic signaling from IL-7 and IL-15 as memory cells do (12), they become Ag-dependent, requiring TCR stimulation for survival. Such cells are said to be exhausted (13).

Thus, successful CD8+ T cell responses not only clear an infection, but also generate a long-lived memory response capable of rapid eradication of the same pathogen upon re-infection. However, the absence of adequate inflammatory stimuli precludes the formation of such a response, resulting instead in anergy. Likewise, an effective T cell response is also subverted by persistent infection leading to exhaustion.

II. Vaccination

The goal of vaccination is to generate a long-lived memory population in the absence of illness to protect the host from infection in the future. Generating an effector response from naïve T cells requires 1-2 weeks due to their low initial frequencies, on the order of 15 – 1000 cells total in the body of a mouse (14-16). In contrast, generating effector cells from memory cells requires a few days. It is this difference between weeks and days that can determine whether an animal succumbs to a pathogen or eradicates it
Thus, it is this difference that vaccines seek to exploit. To do so, they must mimic a successful immune response to infection while minimizing danger and, ideally, significant discomfort, to the host. A vaccine that too accurately mimics an infection may offer little advantage over actually contracting the illness. However, a vaccine that is not inflammatory enough may fail to generate a long-term, protective immune response. The components and design of the vaccine determine how well it navigates this balance.

At its most basic level, a vaccine intended to activate CD8+ T cells must provide the three essential signals: TCR stimulation, co-stimulation, and cytokine activation. Different vaccine modalities provide these signals in different ways. Attenuated or killed pathogens contain many of the native PAMPs of the infectious organism, thereby activating APCs to upregulate co-stimulatory molecules and produce inflammatory cytokines. Furthermore, since attenuated pathogens are capable of replication and infection, they can prime CD8+ T cells in addition to stimulating production of neutralizing antibody. However, attenuated strains can be difficult to produce and to store long-term, as they must be kept viable. In addition, there is the risk of reversion to a virulent form of the pathogen. Even without reversion, attenuated vaccines are not safe for use in immunocompromised individuals (19,20).

Some vaccines use chemicals, heat, or radiation to inactivate or kill the pathogen. These vaccines do not share the risks associated with live, attenuated vaccines. However, they also stimulate weaker immune responses. While a killed virus cannot cause disease, it also cannot replicate or infect cells. Thus, it generally requires use of an added adjuvant and does not stimulate strong CD8+ T cell responses (19).
Subunit vaccines are protein vaccines composed of particular pathogen-associated Ags given alongside an adjuvant. Similar to inactivated vaccines, subunit vaccines often stimulate suboptimal immune responses and require multiple booster immunizations. Furthermore, because the protein is present extracellularly, it does not stimulate strong cellular (CD8+ T cell) responses. Instead, humoral, or antibody-mediated, immune responses are generated, since they are stimulated by extracellular antigens. In addition, protein production can be time consuming and difficult (21,22).

Recombinant vector vaccines deliver DNA-encoded Ags in heterologous carriers, often a viral vector although bacteria have been used as well. This approach mirrors a natural infection but the infecting agent is designed to be safer than the pathogen against which the host is being vaccinated. Vector vaccines, like attenuated vaccines, contain intrinsic PAMPs and stimulate strong immune responses, both humoral and cellular. However, as with attenuated vaccines, vector-based vaccines carry safety risks and are particularly dangerous to the immunocompromised. In addition, vectors are difficult to design and generally cannot be used again, either as a booster or a carrier for a different vaccine. Following vaccination with a vector vaccine, neutralizing antibodies to the vector are often generated that will prevent use of the same vector again (19,23).

DNA vaccines forgo the vector of recombinant vector vaccines and consist only of DNA, generally in the form of circular, double-stranded plasmid DNA (pDNA) that encodes chosen Ags. DNA vaccines are the focus of this thesis and are reviewed in detail in the next section.

III. DNA vaccines
The discovery that naked DNA is taken up and expressed by mammalian cells (24) led to interest in using DNA as a vaccine. In 1992, Tang and Johnston first reported immune responses to a protein produced in situ by a DNA vaccine when they demonstrated that pDNA encoding human growth hormone stimulated antibody production in mice (25). Other reports from Margaret Lui, Harriet Robinson, and David Wiener soon followed, showing that DNA vaccines could elicit protective immune responses against viral and tumor Ags in small animal models (26-28). These findings sparked tremendous interest in DNA vaccines.

The allure of DNA vaccines is related to the unique combination of advantages they offer compared to other vaccine platforms. Firstly, they are safe. Like subunit protein vaccines, they do not pose any risk of reversion and subsequent infection of the host. Furthermore, they do not detectably integrate into genomic DNA and are therefore not associated with an increased risk of malignancy (29,30). In addition, there was an initial fear that DNA vaccines would stimulate autoimmune responses against DNA, creating a Lupus-like pathology, but numerous human clinic trials have found no evidence that this occurs (31).

Secondly, DNA vaccines afford a great deal of flexibility in vaccine design. Generating and manipulating pDNA is straightforward and inexpensive. Simple cloning techniques permit the easy addition, removal, or alteration of encoded genes. A single vector can encode multiple Ag epitopes as well as reporter genes, cytokines, and/or protein adjuvants (31,32). Producing such a protein would be a technically challenging task and modifying it later could require an equally challenging effort.
The flexibility and ease of producing pDNA also make DNA vaccines increasingly attractive as genome sequencing data are used more and more to produce vaccines, a process known as reverse vaccinology (33). In particular, as algorithms for identifying antigenic epitopes improve (34,35), obtaining sequencing data, determining epitopes and encoding them in a DNA vaccine may offer a rapid way of generating vaccines against emerging pathogens (36).

Thirdly, pDNA is stable at room temperature. This makes it easy to store for long periods of time and easy to ship (32). Ease of shipment and storage, as well as the relative affordability of producing pDNA vaccines, make them potentially valuable tools to combat infectious disease throughout the world.

Lastly, because pDNA vaccines are taken up and expressed within mammalian cells, they drive cellular, in addition to humoral, immune response (31,37). Generating a safe, non-infectious vaccine that can elicit CD8+ T cell responses makes pDNA vaccines both unique and particularly valuable among vaccine modalities.

Due to the promise of DNA vaccines, there has been a great deal of work elucidating how they function and attempting to optimize them. Vaccine administration has generally been in either muscle or skin. Work by Bins et al. (38) showed that delivery to muscle yields significantly higher levels of gene expression but poorer immune responses than immunization in the skin. This discrepancy likely results from the role of skin as a barrier organ rich in APCs. Consequently, many groups deliver DNA vaccines to the skin. Methods for delivery to the skin can be as simple as intradermal injection, but also include scarification, bombarding the skin with DNA-coated particles using a gene
gun, using a mild current to the skin to create cellular pores via electroporation, and tattooing (39).

Regardless of the technique, once DNA has been delivered it must be taken up by cells in the skin. While certain delivery methods, such as scarification, deliver DNA only to the epidermis, most methods deliver to the dermis as well. The mechanism by which cells in the skin take up DNA is still incompletely understood. Endocytosis, proteoglycan-dependent macropinocytosis, and receptor-mediated uptake have all been proposed. Once in the cytosol, DNA must enter the nucleus rapidly before being degraded by endogenous nucleases. The mechanism of nuclear entry is also not understood, but DNA may gain entry via nuclear pore complexes, which enable bidirectional movement of macromolecules across the nuclear envelope. Following entry into the nucleus, pDNA genes are expressed using cell machinery (39,40).

Synthesized proteins elicit CD8+ T cell responses directly if the transfected cell is an APC. In this case, the proteins are processed and presented on class I MHC. Due to their relative abundance in the skin, however, it is much more likely for the transfected cell to be a keratinocyte. Proteins produced by keratinocytes that are secreted or released following cell death are taken up by APCs, processed and their derivative peptides cross-presented on MHC class I molecules. Whether presenting or cross-presenting Ag, APCs migrate to draining LN to prime naïve T cells (39).

The skin is home to a number of different APCs, particularly dendritic cells (DCs). There are three main types of DCs present in the skin: Langerhans cells, which are present in the epidermis, dermal DCs that express langerin (Ln+dDCs) and dermal DCs that are langerin negative. Ln+dDCs rapidly migrate to draining LNs following
inflammatory stimuli and are capable of cross-presentation. Furthermore, ablation of this DC subset markedly impairs CD8+ T cell responses in mice, indicating that these DCs are critical for mediating the CD8+ T cell response following DNA vaccination (41).

The ability of DCs to migrate to draining LNs and cross-present, as well as the ability to effectively prime naïve CD8+ T cells relies on the presence of inflammatory or danger signals. These are necessary to fully license the DCs and permit the production of signals 2 and 3 needed to produce effector T cells (42). However, an additional advantage found with DNA vaccines is that they function in the absence of a supplemental adjuvant. They have inherent adjuvanticity that mediates DC licensing. The mechanism underlying this adjuvanticity has not been definitively determined. Many of the delivery mechanisms induce damage in the skin, which may play an important inflammatory role (39). In addition, pDNA contains unmethylated cytosine-phosphate-guanosine nucleotide (CpG) sequences that bind to Toll-like receptor (TLR)-9. However, TLR-9 knockout mice respond to DNA vaccines as well as wild-type (WT) mice (43). It may be that DNA vaccines are stimulating inflammatory responses through alternative pathways, perhaps via cytosolic sensors of double stranded DNA (dsDNA). Two such sensors that have been recently identified are IFI16 and absent in melanoma (AIM2). Both of these proteins are interferon-inducible dsDNA sensors that have been shown to respond to microbial DNA (44,45).

Combining DNA vaccines with tradition adjuvants, such as aluminum salts, lipopolysaccharide, liposomes, or polysaccharides, often generates improved immune responses compared to immunization with DNA alone. However, improvements have been generally modest, motivating researchers to investigate the use of protein immune
stimulants that can be encoded in the DNA vaccine. These genetic adjuvants include cytokines, chemokines, co-stimulatory molecules, and signaling molecules. Of these, cytokines and chemokines have been most thoroughly studied and have been found to typically yield better immune responses than traditional adjuvants (46).

Based on the many advantages associated with pDNA vaccines and their early successes in small animal models, they have been investigated in numerous clinical trials. In fact, three DNA vaccines have been licensed for use in animals: one melanoma vaccine for dogs (47), a West Nile virus vaccine for horses (48), and a vaccine against infectious hematopoietic necrosis virus in salmonid fishes (49). In humans, there have been clinical trials exploring pDNA vaccine use for a variety of tumors as well as infectious diseases, including influenza (50,51), HIV (52,53), Hepatitis B and C (54,55), Hantaan virus (56), Dengue (57), H5N1 avian influenza (58,59), SARS (60), malaria (61), and Ebola virus (62). These trials have confirmed the safety and tolerability of DNA vaccines in people. However, they also illustrate that DNA vaccines as currently formulated generally fail to provide sufficiently protective immune responses in humans.

The failure of pDNA vaccines to translate effectively to use in humans stems from one major obstacle: poor immunogenicity. Although pDNA vaccines elicit cellular and humoral immune responses, those responses are generally too weak to provide substantial protection in humans (63). A major contributor to the poor immunogenicity of pDNA vaccines is insufficient Ag production. The reasons for insufficient Ag production are likely two-fold. Firstly, while mammalian cells take up naked pDNA, they do so inefficiently, leading to significant loss of DNA and low transfection rates (31). Secondly, encoded Ags are expressed only transiently following transfection (64). This initial robust
expression rapidly declines, potentially leading to inadequate priming of T cells. Chapter 2 of this thesis explores a novel solution to this latter problem of transient transgene expression by using minicircle (MC) DNA to circumvent the decay in gene expression that occurs following vaccination with standard pDNA.

**IV. Minicircle DNA**

MC DNA is circularized DNA derived from conventional plasmid DNA (pDNA) via removal of sequences required for propagation in bacteria. It contains only the mammalian expression cassette and a 36 bp recombination scar; all bacterially-derived elements, including the origin of replication and antibiotic resistance genes, are no longer present. Compared to pDNA, MC DNA permits higher levels and more prolonged duration of transgene expression *in vivo* (65). The smaller size of MC DNA compared to its parental full-length (FL) plasmid likely increases transfection efficiency (66), thereby improving expression. Prolonged transgene expression results from a decrease in silencing (67).

When mammalian cells are transfected with pDNA they initially express the transgene robustly. However, following this initial burst, expression rapidly wanes. Histone modifications generating transcriptionally-repressive heterochromatin initially form on the plasmid backbone, but spread *in cis* to cover the entire plasmid, including the expression cassette. These histone modifications are responsible for the loss in transgene expression following transfection with conventional pDNA. In the absence of a bacterial backbone, no such heterochromatin forms, leading to the high and persistent gene expression observed following transfection with MC DNA (67,68).
The elements on the plasmid backbone that serve as a nidus for initiation of the formation of heterochromatin are unknown. Silencing is independent of CpG or methylation status (69) and in fact, seems to be independent of any particular DNA sequence. Instead, the critical factor appears to be the length of extragenic DNA. If the length of DNA between the 5’ and 3’ ends of the transgene cassette is greater than 1 Kb, silencing occurs; if it is smaller than 0.5 Kb, silencing does not occur. This is true regardless of whether the extragenic DNA is of bacterial origin, mammalian origin, or is comprised of random nucleotides. Furthermore, when 1 Kb of extragenic DNA was moved into the 3’ untranslated region of the expression cassette, silencing no longer occurred, showing that it is only the size of the extragenic DNA that determines whether genes will be silenced (70). In MC DNA, the length of extragenic DNA is well below 0.5 Kb (36 bp, as mentioned above), thus accounting for the prolonged duration of transgene expression.

MC DNA production has been optimized to make it nearly as straightforward as standard pDNA preparation. Kay et al. modified a strain of *Escherichia coli* (*E. coli*) to contain inducible copies of the ϕC31 integrase and *I*-SceI homing endonuclease. MC DNA is produced from a parental FL pDNA that contains recognition sites for the *I*-SceI endonuclease along the backbone and recognition sites for the integrase (termed attB and attP sites) flanking the expression cassette. Upon induction with L-arabinose, the integrase mediates recombination at the attB and attP sites, producing two circular DNAs: one containing the expression cassette and another containing the backbone. The backbone is linearized via digestion with the *I*-SceI endonuclease and subsequently degraded by endogenous exonucleases. The remaining circular MC DNA is then purified
via standard pDNA preparation techniques with similar yields (71). Production of MC DNA thus differs from production of pDNA only in the addition of an induction step and the necessity of using a particular DNA backbone and producer strain of *E. coli*, therefore making it a practically viable alternative to pDNA.

The prolonged transgene expression afforded by MC DNA has been employed in several applications including gene therapy for the treatment of a lysosomal storage disease (72), insulin production to treat type I diabetes (73), stable gene knockdown to reduce cardiac damage following myocardial infarction (74), and production of adult human induced pluripotent stem cells (75). Chapters 2 and 3 of this thesis explore a novel use for MC DNA: as a vaccine. In addition, chapter 3 investigates the effect of prolonged expression of a MC-encoded Ag on immune responses.

V. Targeted gene delivery

Most current methods of DNA administration involve significant uptake by non-target cells, generally keratinocytes (dermal administration) or myocytes (intramuscular administration), with secondary transfection of targets occurring suboptimally. The ability to target genes is thus of considerable interest. For instance, as mentioned above, DNA vaccine-encoded Ags need to be presented by APCs to prime CD8+ T cells, but the majority of transfected cells are keratinocytes or myocytes. Ralph Steinman’s group designed a DNA construct that encoded a fusion protein containing the Ag joined to a single-chain Fv antibody specific for the DC endocytic receptor, DEC-205. Thus, protein produced by myocytes after an intramuscular injection would be targeted for improved uptake by DCs. They found that targeting their Ag significantly improved both T and B
cell responses and allowed for the generation of protective responses at a drastically reduced dose of vaccine (76).

Other groups have looked at targeting DCs through other receptors, such as the mannose receptor and the C-type lectin receptor, DC-specific intercellular adhesion molecule 3 [ICAM3]-grabbing non-integrin (DC-SIGN). These studies have shown that targeted DCs alone is not sufficient; their functionality must be modulated. If immature DCs are targeted, tolerogenic immune responses develop. Therefore, receptor choice as well as maturation agents are used to mediate DC maturation along with Ag delivery (77).

In addition to DC targeting, there has been interest in modulating T cells to improve vaccines. Antibodies against the negative regulatory proteins cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) have shown great success in overcoming tolerance in both infection and tumor settings (78-82). Both CTLA-4 and PD-1 are proteins expressed by CD8+ T cells that inhibit T cell activation. PD-1 binds to PD ligands 1 and 2 (PD-L1 and PD-L2), which are both found on APCs, although PD-L1 is also found on many other tissues (83). CTLA-4 competes with CD28 for binding to the co-stimulatory molecules CD80 and 86 expressed by APCs. However, unlike CD28, when CTLA-4 binds to CD80 or 86, it delivers an inhibitory signal to T cells (84). By blocking PD-1 and CTLA-4, these antibodies allow CD8+ T cells to become activated in otherwise tolerizing environments.

Similarly, T cell responses have been manipulated by the toxin-mediated deletion of Ag-specific CD8+ T cells. This approach has used class I pMHC tetramers linked to the saporin toxin (85). pMHC tetramers contain four biotinylated class I MHC molecules
bound to a target peptide and tetramerized via binding to the protein streptavidin (SA) which contains four biotin binding sites. The tetrameric form of the protein increases the avidity of the interaction between the tetramer and T cells (86). Following internalization of the complex through the TCR, T cells were killed by the toxin. Death occurred in an Ag-specific manner because only T cells bearing TCRs specific for the given pMHC complex on the tetramer would bind and internalize it. T cell deletion was not used in a vaccine setting, but rather as a therapy for T cell mediated disease, such as autoimmunity and graft-versus-host-disease (87,88).

As summarized above, T cell surface markers have been blocked and T cells have been deleted. However, to date, T cell modulation approaches have not involved targeting of genes to T cells, as they have with DCs. Such an application would not involve delivery of Ag directly to T cells, as they require interaction with Ag:MHC complexes on APCs. However, more subtle alterations in function could conceivably be achieved by delivering genes than by blocking cell surface markers or eliminating populations of cells. For instance, while CTLA-4 and PD-1 antibodies have shown great success in augmenting signal 2 for T cell activation in vaccination, attempts at administering IL-12 to provide a strong third signal have been associated with serious dose-limiting toxicities, including pulmonary edema, liver damage, gastrointestinal toxicity and bone marrow suppression (89,90). However, if a plasmid encoding IL-12 were targeted to T cells, or to Ag-specific T cells, these side effects could potentially be avoided.

Furthermore, as demonstrated by the work on Ag-specific T cell depletion, there are many applications for T cell targeting beyond vaccine development that bear brief
Autoimmunity, in which pathogenic T cells target healthy "self" tissues is a key example. Allogeneic graft rejection and graft-versus-host-disease (GVHD) are caused by destruction of donor graft tissue by host T cells or attack on recipient tissues by donor T cells, respectively. On the other end of the spectrum, inappropriately tolerized T cells in malignancy or exhausted T cells in chronic infection could benefit from delivery of genes designed to re-activate these cells. The fourth chapter of this thesis explores a method for targeted delivery of genes to T cells that would permit delivery to T cells in general as well as Ag-specific T cells.

**VI. Thesis Statement**

This thesis examines strategies for improving DNA vaccines. Despite their many advantages and the considerable promise shown in small animal models, poor immunogenicity resulting from low transfection efficiency and transient transgene expression limits the effectiveness of DNA vaccines in humans. Methods for circumventing transient transgene expression as well as for directly manipulating T cell responses are explored.

The next two chapters of this thesis (chapters 2 and 3) study the use of MC DNA as a vaccine. In chapter 2, a MC DNA vaccine is shown to generate larger CD8+ T cell responses and confer superior protection against an infectious challenge than a conventional pDNA vaccine. Chapter 3 investigates the mechanism by which MC DNA elicits superior CD8+ T cell responses. In particular, the role of prolonged Ag expression is explored and found to be an important component of MC DNA vaccine efficacy.
Chapter 4 proposes a method for targeted gene delivery to T cells in general, or to Ag-specific T cells. The targeting mechanism uses the SA platform modified with positively charged peptides to enable binding to nucleic acid. Targeting is mediated by addition of biotinylated targeting moieties, either an anti-CD7 antibody for targeting of T cells or pMHC molecules for targeting of Ag-specific T cells. We find that such an approach is promising, as it mediates uptake of both fluorescently labeled siRNA and DNA. However, gene expression did not occur, indicating that further modifications to the approach are needed before it can be used.
CHAPTER 2: Minicircle DNA is Superior to Plasmid DNA in Eliciting Antigen-Specific CD8+ T Cell Responses


¹Both first authors made equal contributions to this work. Figures 2.8-2.11 were generated by NEB Skinner.

INTRODUCTION

Plasmid DNA (pDNA) is an attractive platform for gene delivery in vivo because it is a non-viral, non-integrating vector that is safe, inexpensive, stable and easily manipulated (40). As such, it has been evaluated in hundreds of clinical trials (32,63). Collectively these trials reveal that while pDNA has promise, the major challenge to its widespread use for the prevention and treatment of human disease is poor protein production in situ. This results from both low transfection efficiency and low transgene expression.

Expression of the transgene cassette in pDNA vectors rapidly wanes because transcriptionally repressive proteins are initially deposited on extragenic DNA, the
backbone pDNA located between the 5’ and 3’ ends of the transgene expression cassette (64,65,67). Chromatin deposition is independent of the sequence, CpG content and methylation status of the extragenic pDNA but is dependent on its length (68-70,91). Chromatinization spreads in cis from the extragenic DNA to the transgene cassette, silencing its expression. This results in a brief burst of protein production in situ followed by its rapid loss.

MC DNA is similar to pDNA as both contain expression cassettes that permit transgene products to be made at high levels shortly after delivery in vivo (92). They differ in that MC DNA is devoid of essentially all prokaryotic sequence elements (e.g. origin of replication and antibiotic-resistance genes). Removal of these backbone pDNA sequences is achieved via site-specific recombination in E. coli prior to episomal DNA isolation (71,93). The lack of these prokaryotic sequence elements also reduces MC size relative to its parental Fl pDNA, leading to enhanced transfection efficiencies (66). The net result is that compared to their FL pDNA counterparts, MCs transfect more cells and permit sustained high level transgene expression upon delivery in vivo. Because of this, we hypothesized that immune responses elicited by MC vaccines would be more potent than responses to FL pDNA vaccines encoding the same Ags.

The two main routes for administering pDNA vaccines are intramuscular and intradermal. The intradermal route is preferred because the skin is a barrier organ relatively rich in immune sentinel cells that present Ag to the immune system (94,95). Keratinocytes are critical for maintaining the immunological barrier function of the skin by initiating inflammation via recognition of damage- and pathogen-associated molecular patterns by pattern recognition receptors (96). Dermal dendritic cells similarly initiate
proinflammatory responses but they also can internalize and present Ag to B and T cells (94). Intradermal delivery methods for DNA include injection with a hypodermic needle, bombardment of the skin with DNA-coated gold particles ejected from a gene gun, topical application, electroporation and tattooing (97-101). Tattooing has the advantage of being relatively inexpensive and rapid, and the infliction of thousands of perforations likely serves as an adjuvant. Work by the Haanen group demonstrated that pDNA delivery to the skin via tattooing elicited more rapid and robust immune responses specific for the pDNA-encoded Ags than did intramuscular delivery (38).

We used tattooing to deliver FL pDNA and their derivative MC vaccines intradermally to mice. We found that the level, duration and immunogenicity of the MC transgene-encoded proteins were all significantly greater than those elicited by the FL pDNA vaccines, and that the primary and memory immune responses elicited by MC vaccines conferred significant protection against bacterial infection in a model of listeriosis. We discuss these results relative to future strategies involving pDNA-based vaccines.

RESULTS

Efficient production of MC-based expression vectors.

We constructed two series of expression vectors. One encoded enhanced firefly (eff) luciferase as a reporter to track expression. The other encoded a model peptide to monitor Ag-specific T cell responses. We chose eff luciferase as a reporter because of its sensitivity; mouse cells expressing eff luciferase emit > 100 times more light than cells expressing standard firefly luciferase (102). The model peptide was the chicken
ovalbumin-derived peptide SIINFEKL (amino acid residues 257-264). When SIINFEKL is bound by the mouse major histocompatibility class I molecule H-2K\(^b\), it forms an Ag recognized by mouse CD8+ T cells bearing the Va2/Vb5 OT-I transgenic TCR (103). Ag-specific OT-I responses are easily tracked after these cells are adoptively transferred into C57BL/6 (H-2b) recipients subsequently immunized with the SIINFEKL peptide plus adjuvant (104). Responses of Ag-specific endogenous CD8+ T cells are also readily tracked by flow cytometry using fluorophore-labeled SIINFEKL/K\(^b\) tetramers (86).

Expression of the eff and Ag genes is controlled by the human ubiquitin C (hUbC) promoter, chosen because it efficiently drives transgene expression in many mouse tissues including skin (105).

MC constructs encoding eff or Ag (MC-eff and MC-Ag, respectively) were generated from FL pDNA precursors (Fig. 2.1a). Briefly, we cloned the hUbC-driven expression cassettes between the \(\phi\)C31 integrase recognition sites attB and attP in pMC.BESPX, the MC producer plasmid (71). Upstream of the attB site was a block of 32 tandem repeats of the I-SceI homing endonuclease recognition sequence (106). FL pDNA was transformed into an E. coli strain (ZYCY10P3S2T) engineered to express \(\phi\)C31 integrase and I-SceI endonuclease (71). Upon induction, \(\phi\)C31 integrase activity generated the MC and circular plasmid backbone; the latter was subsequently degraded by host bacterial exonucleases after linearization by the I-SceI endonuclease.

Densitometry analyses of ethidium bromide-stained agarose gels revealed that less than 2% of the parental FL-eff pDNA remained in the purified MC-eff preparations (Fig. 2.1b). MC-Ag preparations were comparably pure (data not shown).
Enhanced expression of MC-encoded eff luciferase in vitro.

To determine if the expression levels or transfection efficiencies of MC-encoded genes were enhanced as compared to FL plasmid-encoded genes, we transfected COS cells transiently with equimolar amounts of MC-eff or FL-eff DNA. Two days later the cells were analyzed by flow cytometry for intracellular luciferase expression (Fig. 2.2a). On average approximately 14% of cells transfected with MC-eff expressed luciferase, more than twice the frequency (6%) of luciferase-expressing cells transfected with an equimolar amount of FL-eff (Fig. 2.2b). However, the median fluorescent intensities (MFI) of the two luciferase-expressing populations were comparable ($P = 0.65$). Together these data suggest that under the conditions used, the smaller MC-eff (3348 bp) is superior to the larger FL-eff (7262 bp) in transfection frequency but not in gene expression level on a per cell basis as assessed two days post-transfection.

Enhanced expression of MC-encoded eff luciferase in skin.

Because our goal is to develop MC-based vaccines, we next determined the intensity and duration of MC gene expression in vivo following intradermal delivery via tattooing. Each mouse in the experimental cohort was tattooed on the left and right thighs while lying supine with equimolar amounts of FL-eff and MC-eff DNA, respectively. Mice in a separate cohort were tattooed with equimolar amounts of FL pDNA lacking an expression cassette (empty vector control: VC). The mice were then injected with saturating amounts of D-luciferin substrate on the indicated days and imaged for bioluminescence (Fig. 2.3a). Expression peaked two to three days post-delivery in both groups (Fig. 2.3b).
The groups differed, though, in that luciferase activity encoded by MC-eff was consistently and significantly higher than that of FL-eff. Luciferase activity decayed to background levels by day 42 in the FL-eff cohort but it remained detectable in the MC-eff cohort throughout the 63-day observation period. It is noteworthy that the MC-eff luciferase activity on day 63 was comparable to the FL-eff peak response on day 3. It also took twice as long for luciferase activity to decay to levels significantly lower (\(P < 0.05\); paired \(t\) test) than the peak response in MC-eff (56 days) versus FL-eff (28 days) mice. Together these data indicate that luciferase expression in the skin persists significantly longer and at significantly higher levels in mice tattooed with MC-eff relative to FL-eff.

**Enhanced presentation of MC-encoded Ag in vitro.**

As a prelude to vaccination, we assessed Ag secretion and presentation in vitro by transfecting a dendritic cell line, DC2.4 (H-2b haplotype) with equimolar amounts of VC, FL-Ag or MC-Ag DNA. The Ag cassette encoded the chicken ovalbumin-derived peptide SIINFEKL linked to a secretion sequence and a histidine tag (Supplementary Fig. 2.4). Western blot analysis of transiently transfected COS cells revealed the Ag was secreted (data not shown). To measure presentation of the SIINFEKL peptide, we analyzed DC2.4 cells one, three and five days after transfection for reactivity with 25-D1.16 (Fig. 2.5a). This monoclonal antibody specifically binds SIINFEKL only when presented by H-2K\(^b\)(107). The frequency of cells bearing detectable SIINFEKL/H-2K\(^b\) complexes was significantly higher and persisted longer in the MC-Ag transfectants as compared to the FL-Ag transfectants (Fig. 2.5b). These MC-Ag transfectants also
expressed significantly higher levels of SIINFEKL/H-2K\(^b\) complexes than the FL-Ag transfectants, as measured by MFI (Fig. 2.5c). This level of enhanced Ag expression in the MC-Ag versus the FL-Ag transfectants on a per cell basis contrasts with the comparable levels of eff expression level in the MC-eff and FL-eff transfectants noted above. We speculate that the SIINFEKL/H-2K\(^b\) complexes on the cell surface have a greater half-life than intracellular eff. An alternative, but not mutually exclusive, possibility is that the Ag-transgene encoded protein containing the SIINFEKL peptide and the eff protein are degraded at similar rates but the resultant pool of SIINFEKL peptides provides a reservoir for prolonged presentation by H-2K\(^b\).

Enhanced immunogenicity of MC-encoded Ag in vivo in an adoptive transfer model.

To determine if the enhanced presentation of MC-encoded Ag observed \textit{in vitro} corresponded to increased immunogenicity \textit{in vivo}, we adoptively transferred equal numbers of CD8+ OT-I (Thy1.1+) cells into congenic C57BL/6 (Thy1.2+) mice and tracked the OT-I cell responses following DNA immunization. One day after OT-I cell adoptive transfers, recipients were tattooed with equimolar amounts of VC, FL-Ag or MC-Ag DNA. At the peak of the response seven days later, flow cytometry was used to identify OT-I cells by their expression of CD8 and the allelic marker Thy1.1. The proliferative responses of OT-I cells, as measured by an increase in absolute OT-I cell numbers, in MC-Ag immunized mice were significantly greater than the responses in mice immunized with FL-Ag or VC. This was true in the spleen (Fig. 2.6a) and draining LN (data not shown). To assess their functionality, OT-I cells from immunized mice were
stimulated briefly in vitro with cognate Ag (syngeneic H-2b splenocytes pulsed with SIINFEKL peptide) and examined for IFN-γ expression. IFN-γ was expressed by significantly more OT-I cells from mice immunized with MC-Ag than from mice immunized with FL-Ag (Fig. 2.6b). Moreover, the levels of intracellular IFN-γ tended to be higher in OT-I cells from MC-Ag immunized mice than in FL-Ag immunized mice (MFIs of 370 versus 235, respectively).

To assess in vivo cytotoxicity, C57BL/6 mice were adoptively transferred with OT-I cells and the next day immunized with equimolar amounts of VC, MC-Ag or FL-Ag DNA via tattooing. One week later these mice were injected with equal numbers of SIINFEKL-pulsed syngeneic splenocytes labeled with 5 μM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSEhigh) and unpulsed syngeneic splenocytes labeled with 10-fold less CFSE (CFSElow). The loss of CFSEhigh (Ag-positive) relative to CFSElow (Ag-negative) cells provided a measure of OT-I cytolytic activity in vivo (Fig. 2.7a) (108). Ag-specific cytolytic activity in MC-Ag immunized mice was significantly greater than that in FL-Ag immunized mice (Fig. 2.7b). OT-I cytolytic activity was comparable between mice immunized with MC-Ag DNA and SIINFEKL peptide plus complete Freund’s adjuvant (positive control; Fig. 2.7b). The latter is remarkable given the 3,700 molar excess of peptide (52 nmol) relative to MC-Ag DNA (14 pmol), and that pDNA transfection efficiencies in vivo are poor (101). Together these data indicate MC-Ag immunization elicits a significantly greater OT-I response than does immunization with FL-Ag pDNA.

Superior protection from a MC vaccine in model of listeriosis.
Vaccine efficacy is measured by the ability of endogenous Ag-specific immune cells to protect a vaccinated animal from a subsequent pathogen challenge. To determine if MC immunization enhanced the responses of endogenous Ag-specific CD8+ T cells, we first tracked the frequencies of SIINFEKL/Kb tetramer-binding CD8+ T cells in immunized C57BL/6 mice that had not been adoptively transferred with OT-I cells. Bins et al. showed that three intradermal immunizations three days apart were required for an optimal immune response to a pDNA-encoded Ag which peaked five days after the third immunization (38). We replicated this immunization scheme by tattooing mice three times, three days apart (on d0, d3, and d6) with equimolar amounts of VC, MC-Ag or FL-Ag DNA. One day after each immunization, mice were injected subcutaneously at the tattoo site with polyIC, a TLR 3 agonist that serves as an adjuvant by eliciting type I interferons (109). The peak response in our immunized mice also occurred five days after the third immunization (on d11), as measured by the frequencies of SIINFEKL/Kb tetramer-binding CD8+ T cells in the peripheral blood taken over time (data not shown). Mice immunized with MC-Ag had significantly higher frequencies of tetramer-binding CD8+ T cells on d11 than mice immunized with either the FL-Ag or VC pDNA (Fig. 2.8).

We next asked if vaccination with MC-Ag DNA protected mice from a challenge of *Listeria monocytogenes* engineered to express the model Ag, chicken ovalbumin (LM-OVA) (110). *Listeria monocytogenes* is a gram-positive intracellular bacterium that can cause fatal listeriosis in neonates, pregnant women, and immunosuppressed individuals (111). LM-OVA is particularly useful because it provides a well-defined T cell Ag (e.g. SIINFEKL peptide presented by H-2Kb) and because CD8+ T cells are necessary for
induction of protective immunity to the pathogen (112). Mice were immunized as described above except that polyIC was replaced with polyICLC because of the latter's enhanced pharmacokinetics (113). At the peak of the immune response five days after the last immunization (d11), all mice were challenged intravenously with LM-OVA bacteria. Mice were euthanized five days after bacterial challenge (d16) and the frequencies of peripheral blood and splenic CD8+ T cells binding the SIINFKEL/K\textsuperscript{b} tetramer were determined by flow cytometry. Bacterial burden was measured as the number of colony forming units (CFUs) of LM-OVA in the spleen.

Consistent with the d11 results, on d16 there were significantly higher frequencies of Ag-specific CD8+ T cells in the peripheral blood and spleens of mice immunized with MC-Ag than in mice immunized with FL-Ag or VC DNA (Fig. 2.9a and 2.9b, respectively; gating strategies shown in Fig. 2.10). Vaccination with MC-Ag DNA correspondingly conferred significant protection to challenge with LM-OVA. Bacterial burden was significantly reduced in mice immunized with MC-Ag as compared to FL-Ag or VC (Fig. 2.9c). Although the numbers of CFUs were not significantly different between the FL-Ag and VC groups, challenge with a lower dose of LM-OVA showed a protective effect of FL-Ag immunization (Fig. 2.11).

To test whether the memory response elicited by MC-Ag DNA was also superior to that elicited by FL-Ag DNA, mice were challenged with LM-OVA on d36, 30 days after the last DNA immunization. All mice were euthanized five days after bacterial challenge (d41) to measure bacterial burden in their spleens and the frequencies of peripheral blood and splenic CD8+ T cells binding the SIINFKEL/K\textsuperscript{b} tetramer. Although the frequencies of Ag-specific CD8+ T cells in the peripheral blood (Fig. 2.9d) and spleens
(Fig. 2.9e) of mice immunized with MC-Ag, FL-Ag or VC DNA were not significantly different, vaccination with MC-Ag DNA 30 days prior to LM-OVA challenge conferred significant protection (Fig. 2.9f). We speculate that infection leads to priming of naïve SIINFEKL-specific cells in all groups, which masks the proliferation of memory cells in immunized mice. Together these data suggest vaccination with MC DNA encoding a pathogen-associated Ag can provide protection from a subsequent challenge with the corresponding pathogen.

**DISCUSSION**

Our data indicate MC DNAs delivered via tattooing are superior to their FL counterparts in terms of the expression levels and duration, as well as the immunogenicity, of the proteins they encode. The enhanced levels and duration of protein expression we observe in the skin corroborate studies of MC DNA delivered to various tissues via other routes for gene therapy (93,114,115). The novelty of our report lies in the enhanced Ag-specific CD8+ T cell response to an epitope encoded by a MC delivered intradermally. This enhanced response conferred significant protection to mice challenged with *Listeria monocytogenes* expressing the MC-encoded Ag.

Standard pDNA has two components: the bacterial backbone required for plasmid propagation in bacteria and the transcription cassette for expression in mammalian cells. Removal of the bacterial backbone reduced the size of MC DNA relative to pDNA by approximately 50%. pDNA size is inversely related to transfection efficiency (66) and correspondingly, the transfection efficiency and expression levels of MC are higher than pDNA levels (92,115,116). Accordingly we showed transfection of
cells in vitro with MC resulted in higher rates of gene transfer (Fig. 2.2a). This was also true in vivo, as evidenced by the ≥ 10-fold levels of luciferase activity in mice tattooed with MC-eff compared to FL-eff at all time points measured (Fig. 2.3b).

Similarly, the significantly higher frequencies of effector Ag-specific CD8+ T cells measured five days following MC-Ag DNA immunization most likely reflected higher transfection efficiencies relative to FL-Ag DNA. We speculate that the Ag provided by the MC DNA and the adjuvant effect resulting from the thousands of needle pricks inflicted by the tattoo device combine to produce a local microenvironment conducive for naïve OT-I CD8+ T cell activation. It is unlikely anything other than inflammation induced by the needle pricks serves as an adjuvant because the needles are sterile, the tattoo site is wiped with ethanol to minimize introduction of skin commensals, and the DNA preparations have exceedingly low endotoxin levels (≤ 0.1 pg/μg DNA). However, the adjuvanticity of the needle pricks was insufficient to elicit significant numbers of endogenous Ag-specific CD8+ T cells (data not shown), necessitating the use of polyICLC. This need for a greater immunostimulatory microenvironment may reflect the much lower precursor frequency of endogenous SIINFKEL/Kb specific CD8+ T cells (100-200 cells/mouse) (14) as compared to the frequency of OT-I cells in adoptively transferred mice.

Because the majority of cells transfected by tattooing pDNA are likely keratinocytes (101), we included a secretory signal in the Ag expression cassette. Our rationale was that secreted Ag by transfected cells could drain to the regional LN for presentation or be internalized by resident APCs in the skin. These cells could then migrate to the draining LN and cross-present SIINFKEL/Kb to naïve CD8+ T cells. This is
consistent with reports that dermal DCs are required to stimulate naïve CD8+ T cells upon intradermal immunization with pDNA (117). Studies of pDNA-encoded Ags delivered intradermally suggest, though, that transfection of Ag-presenting cells themselves is the key event required to elicit specific CD8+ T cell responses. Ag expression in keratinocytes failed to elicit specific CD8+ T cell responses upon intradermal delivery of pDNA containing a K14 promoter, whereas Ag expression in dermal DCs (via a CD11c promoter) did (41). Moreover, depletion of Ln+ dDCs ablated the response of CD8+ T cells to Ag encoded by pDNA delivered intradermally, suggesting that specific delivery of MC to dermal DCs should further enhance immunogenicity upon tattooing. It remains to be determined if differential cellular uptake of MC versus FL DNA impacts both the length of expression and the nature of the immune response.

In addition to enhancing transfection efficiency, a second critical benefit to the removal of the plasmid backbone elements relates to their role in gene silencing. We have shown that the deposition of heterochromatin on the bacterial sequences causes the loss of expression that is independent of DNA methylation, CpG content, immune cell clearance, and plasmid copy number (68,69,72,91). More recent studies confirm silencing is sequence independent but unexpectedly is dependent on sequence length (70). Silencing requires the plasmid DNA separating the 5’ and 3’ ends of the transgene expression cassette to be ≥ 1 kb; silencing does not occur when this extragenic DNA is < 0.5 kb. In our MC constructs, the extragenic DNA is < 40 bp. It remains unclear why transgene silencing does not occur with shorter extragenic DNA.
The near complete absence of plasmid backbone elements most likely allowed expression of the eff transgene encoded by MC DNA to persist throughout the duration of the nine-week observation period. It is noteworthy that MC-eff expression at seven weeks was comparable to that at one day. This was unexpected because 33-44% of C57BL/6 keratinocytes are replaced weekly and keratinocytes are the predominant cell type in the skin (118). Moreover, van den Berg et al. reported that 99% of human skin cells transfected by pDNA delivered via tattooing were keratinocytes (101). The persistence of eff luciferase expression we observe is consistent with the transfection of longer-lived progenitor cells in the skin, such as hair follicle-associated dermal precursors (119).

The duration of Ag expression following intradermal immunization with pDNA affects CD8+ T cell responses to the encoded Ag (120). Hovav et al. reported that APC activity slowly increased over a two-week period following injection of pDNA into the ear pinnae of mice (121). Removal of the ear pinnae at various times post-immunization diminished this activity and the resultant primary specific CD8+ T cell responses. These data suggest a depot of Ag controls CD8+ T cell expansion at the level of Ag presentation. A persistent Ag depot afforded by MC immunization arguably could permit sustained CD8+ T cell responses through the initial activation of naïve CD8+ T cells and the persistent reactivation of resultant memory cells or through modification of the kinetics of memory CD8+ T cell differentiation, as Hovav et al. suggest (121). Our data showing enhanced protection to LM-OVA challenge 30 days after immunization with MC-Ag DNA is consistent with this possibility. Alternatively, higher Ag transgene expression following MC-Ag DNA immunization could have generated greater numbers of Ag-
specific CD8+ T cells which in turn, yielded higher frequencies of Ag-specific memory CD8+ T cells. Regardless, the combination of Ag and coadministered proinflammatory cytokines/chemokine all encoded by MC might establish a long-lived immunostimulatory microenvironment in the skin, leading to efficacious anti-pathogen and anti-tumor immunity.

In conclusion we show for the first time the applicability of MC DNA to mediate long-term expression of transgenes when delivered intradermally. Although the immunogenicity of the MC-encoded Ags undoubtedly needs to be increased for the MC platform to serve as a potent vaccine, this clinically relevant delivery method and the inherent safety benefit of the non-integrating MC DNA represents a novel approach toward DNA-based therapies.

MATERIALS AND METHODS

DNA constructs. The plasmid backbone for the FL constructs (pMC.BESPX.MCS1) contains the requisite sequences for bacterial propagation (e.g. the pUC origin of replication), the kanamycin-resistance gene, the φC31 integrase recognition sites attB and attP, and a block of 32 tandem repeats of the recognition sequence for the I-SceI homing endonuclease (71).

To generate the FL-eff construct, the eff gene was amplified from the pUltra Bright eff luciferase+ plasmid(102) and inserted into a vector containing the bovine growth hormone polyadenylation signal (BpA). The luciferase-BpA fragment was then PCR amplified. In a parallel PCR reaction the hUbC promoter (a generous gift from Michael Kyba, University of Minnesota, Minneapolis, MN) was amplified. The hUbC
The luciferase/BpA amplicon contains an overlap region with the pMC.BESPX plasmid downstream of the EcoRV site. The two PCR products and the EcoRI/EcoRV digested pMC.BESPX were subjected to a one-step isothermal DNA assembly protocol allowing for seamless joining of the overlapping fragments (122).

A similar strategy was utilized to generate the FL-Ag construct containing the hUbC promoter-driven Ag cassette. The cassette encodes a signal sequence, a 10x histidine tag, a linker and a region that includes the chicken ovalbumin-derived peptide SIINFEKL (Fig. 2.4). It was amplified from a codon-optimized synthetic gene (GenScript, Piscataway, NJ) and inserted upstream of BpA. Overlapping PCR products for cloning hUbC-Ag-BpA were generated and isothermally assembled into pMC.BESPX. Sequences of all amplifying primers are available upon request from the authors.

All PCR reactions were performed using Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA) under the following conditions: 98°C x 30 seconds, followed by 35 cycles of 98°C x 10 seconds, 60°C x 30 seconds, and 72°C x 120 seconds. Plasmids containing the eff or Ag cassettes were then transformed into the ZYCY10P3S2T bacterial strain. ZYCY10P3S2T bears ten copies of the φC31 integrase gene, three copies of the I-SceI homing endonuclease gene, and the araE and LacY arabinose transporter genes that constitutively express proteins importing arabinose to induce φC31 integrase and I-SceI endonuclease expression (71). For FL pDNA preparation, transformed bacteria were grown in LB-broth (Invitrogen, Carlsbad, CA) supplemented with kanamycin. pDNA was isolated using the GenElute Endtoxin-
free Plasmid Maxiprep Kit (Sigma-Aldrich, St Louis, MO) according to the manufacturer’s instructions. For MC generation, bacteria transformed with FL pDNA were grown overnight in Terrific Broth supplemented with kanamycin (Invitrogen). The following day MC induction media (fresh LB broth containing 0.04 volumes of 1N NaOH and 0.02% L-arabinose (Sigma-Aldrich, St Louis, MO)) were added and the culture temperature was decreased from 37°C to 32°C for 5-8 hours. The culture was centrifuged and MC DNA was purified with the GenElute Endtoxin-free Plasmid Maxiprep Kit by increasing the recommended volumes of buffers six-fold and using four columns. Following elution in water, the DNA was concentrated to 4-6 μg/μl by ethanol precipitation, resuspended in water and stored at -20°C.

Sequence analyses verified the genes encoded by the FL pDNAs and their derivative MCs were identical. The amounts of residual FL pDNA in the MC DNA preparations were evaluated by \(KpnI\) (FL pDNA) or \(StuI\) (MC DNA) restriction endonuclease-mediated linearization, followed by agarose gel electrophoresis and DNA visualization using ethidium bromide (Fig. 2.1b). For the GenElute Endotoxin-free Plasmid Maxiprep Kits, the manufacturer defines “endotoxin-free” as ≤ 0.1 endotoxin unit/μg DNA. The levels of endotoxin in MC and FL pDNA preparations were measured by the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ) and were all found to be < 0.001 endotoxin unit/μg DNA (< 0.1 pg endotoxin/μg DNA).

**Cell lines and transfections.** COS cells were maintained in complete Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and DC2.4 cells were maintained in complete RPMI 1640 (Invitrogen)
supplemented with 10% fetal bovine serum at 37°C with 5% CO2. Adherent cells were dissociated from tissue culture flasks with TrypLE Express (Invitrogen) and plated in 6 well tissue culture-treated plates (BD Biosciences, San Jose, CA) at equal numbers (2-5 x 10^5 cells) per well without antibiotics. Cells were transfected the next day using Lipofectamine LTX with PLUS Reagent (Invitrogen) according to the manufacturer's protocol. COS cells were transfected with 2.5 μg (1.1 pmol) per well of MC-eff or equimolar amounts of VC or FL-eFF and were harvested two days later for flow cytometry analysis. DC2.4 cells were transfected with 2.6 μg (1.8 pmol) of MC-Ag per well or equimolar amounts of VC or FL-Ag and were harvested one, three and five days later for flow cytometry analysis.

**Measurements of eff and Ag expression by flow cytometry.** Staining for intracellular eff luciferase in COS cells was carried out using the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's protocol. Fixed and permeabilized cells were incubated with an anti-luciferase-FITC conjugate (Lifespan Biosciences, Seattle, WA) for 20 minutes. The DC2.4 cells were analyzed for cell surface Ag presentation using a 25-D1.16-APC conjugate (eBioscience, San Diego). 25-D1.16 is an antibody specific for the SIINFEKL/H-2K^b complex (107). Cells were stained at 4°C for 30 minutes. Data were acquired on a BD FACSCalibur flow cytometer (BD Biosciences) using BD CellQuest Pro software (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). Data are reported as the percentage and median fluorescent intensity (MFI) of cells in a defined gated population.
Mice. Four to eight week old female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) or the National Cancer Institute (Frederick, MD). C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) Thy1.1+/Thy1.2+ mice were a generous gift from Dr. Stephen Jameson at the University of Minnesota (Minneapolis, MN). All mice were housed under specific pathogen free-conditions at the University of Minnesota (Minneapolis). All animal procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee.

OT-I adoptive cell transfer. All work was carried out under asceptic conditions using sterile reagents. OT-I mice were euthanized and LNs were harvested. Single cell suspensions were prepared and cells were washed in phosphate buffered saline (PBS) supplemented with 2% (v/v) fetal bovine serum (Invitrogen). Cells were counted and analyzed by flow cytometry to determine the percentage and number of OT-I cells with the following antibody-fluorochrome conjugates: CD44-fluorescein isothiocyanate (FITC), B220-phycoerythrin (PE), and CD8-allophycocyanin (APC) (eBioscience) and Thy1.1-Peridinin Chlorophyll Protein Complex (PerCP) (BD Pharmingen, San Diego, CA). The OT-I phenotype was B220-CD8+Thy1.1+. Additionally, the OT-I cells were CD44low, indicating they were naïve at the time of transfer. To determine the cell count, PKH26 Reference Beads (Sigma-Aldrich) were mixed with cells; 5,000 bead events were collected on the flow cytometer. The following equation was used to determine the number of lymphocytes: $\#$ cells/ml = ($\#$ cells acquired x dilution factor of cells x $\#$ singlet beads/ml) / ($\#$ beads acquired x dilution factor beads). Cell counts were verified manually using a hemocytometer. Data were acquired on a BD FACSCalibur flow
cytometer (BD Biosciences) using BD CellQuest Pro software (BD Biosciences) and
analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). Cells were washed in
PBS with 1% (v/v) fetal bovine serum and then in PBS before being resuspended at $10^6$
cells/ml in PBS. $10^5$ cells were transferred into each anesthetized mouse via retro-orbital
injection using 1ml tuberculin syringes and 27 gauge needles.

**DNA immunizations.** Mice were anesthetized and their inner hind legs were shaved
and wiped with 70% ethanol. Based on pilot experiments, mice were immunized with 20
μg of MC DNA in 10-15 μl volumes. This mass converts to 9.2 and 13.8 pmol for MC-
eFF and MC-Ag, respectively. Equimolar amounts of VC and the corresponding FL
pDNA samples were diluted in sterile water to the same volumes. All DNA was delivered
intradermally over an area of approximately 1 cm² for 30 seconds using a Cheyenne
Hawk PU II tattoo device (Unimax Supply Co. Inc., New York, NY) set at 110 Hz using 9-
point needles adjusted to a depth of 0.5 mm. Mice were immunized once for adoptive
transfer experiments. For endogenous T cell tracking and LM-OVA challenge
experiments mice were immunized three times, three days apart and 10 μg of polyIC
(Sigma) or polyICLC (Oncovir) adjuvant were administered subcutaneously 24 hours
after each immunization in the same inner hind leg as the DNA.

**Bioluminescence measured in vivo.** Mice were imaged for bioluminescence using a
Xenogen IVIS Imaging System (Caliper Life Sciences, Hopkinton, MA). Animals were
imaged daily for 18 days and then weekly from days 21 to 63. Mice were injected
intraperitoneally with 100 μl of 30 mg/ml D-luciferin and anesthetized with isoflurane.
Bioluminescence was measured for 5 minutes. Data were analyzed using Living Image 2.5 Software (Caliper Life Sciences).

**CD8+ T cell proliferation and function assays.** Mice were adoptively transferred with \(10^5\) OT-I cells and the next day immunized with DNA using a tattoo device as described above. Splenocytes and the draining inguinal lymph nodes were harvested seven days later and analyzed by flow cytometry for percentages of OT-I cells. Functionality was assessed by IFN-γ production following a brief exposure of cells to Ag *in vitro*. Splenocytes were incubated with or without 1μM SIINFEKL for 4 hours at 37°C and then examined for extracellular expression of CD8 and Thy1.1 (to identify OT-I cells) and intracellular expression of IFN-γ using BD Cytofix/Cytoperm kit (BD Biosciences). Data were acquired on a BD FACSCalibur flow cytometer (BD Biosciences) using BD CellQuest Pro software (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

**In vivo cytotoxicity.** C57BL/6 mice were adoptively transferred with \(10^6\) OT-I cells and were immunized the next day with equimolar amounts of DNA using the tattoo device as described above. Eight days later these mice were adoptively transferred with equal numbers \((10^7\) each) of syngeneic splenocytes that had been pulsed or not with SIINFEKL peptide (1 μg peptide per 2.5 x 10⁷ cells for one hour at 37°C). To distinguish these cells from one another and from host cells, peptide-pulsed and unpulsed splenocytes were respectively labeled with 5 and 0.5 μM CFSE. Fourteen hours later, the mice were euthanized and their splenocytes harvested and analyzed by flow
cytometry for percentages of CFSE\textsuperscript{high} (peptide pulsed) and CFSE\textsuperscript{low} (unpulsed control) cells. The percent specific killing was determined using the following formula: [1-(ratio in VC-immunized mouse/ratio in FL-Ag or MC-Ag-immunized mouse)] x 100 (108).

**Endogenous T cell analysis.** Peripheral blood samples taken from the facial vein and single cell suspensions prepared from harvested spleens were incubated with CD44-FITC, CD8-APC (eBioscience) and SIINFEKL/H-2K\textsuperscript{b} tetramer formed with streptavidin coupled to PE. Splenocytes were counted manually using a hemocytometer. Data were acquired on a BD FACSCalibur flow cytometer (BD Biosciences) using BD CellQuest Pro software (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

**Infection with LM-OVA.** Mice were infected with 4-6 x 10\textsuperscript{4} CFU intravenously in the tail vein five days after the last immunization. Bacteria were grown in tryptic soy broth (TSB) with 50 μg/ml of streptomycin to an absorbance at 600 nm of about 0.1. Actual numbers of CFU injected were determined for each experiment by plate count. Five days following infection, mice were bled from the facial vein, sacrificed, and their spleens harvested. Half of the cells from each spleen were used for flow cytometric analysis and the other half were serially diluted and plated on tryptic soy agar plates containing streptomycin. Bacterial colonies were counted after plate incubation for 24 hours at 37 °C.
**Statistical analysis.** Data were graphed and analyzed using Prism v5.0c (GraphPad Software, Inc., La Jolla, CA). Statistical analyses were performed by the unpaired two-tailed Student’s *t*-test unless noted otherwise.
Fig. 2.1. Minicircle derivation. (a) Plasmid construct and minicircle generation. The expression cassette is cloned between the φC31 integrase recognition sites attB and attP in the minicircle producer plasmid pMC.BESPX, which also contains I-SceI homing endonuclease recognition sequences. Following transformation of the E. coli strain ZYCY10P3S2T with a full-length (FL) parental plasmid, φC31 integrase activity generates a minicircle (MC) containing the expression cassette and a circular plasmid backbone; the latter is subsequently degraded by host exonucleases following linearization by the I-SceI endonuclease. (b) Agarose gel electrophoresis analysis of linearized FL plasmid encoding eff (FL-eff) and its MC derivative (MC-eff). The arrow indicates residual FL plasmid in the purified MC-eff DNA preparation. Sizes of the DNA species are noted next to each band. eff, enhanced firefly luciferase; FL-eff, full-length plasmid encoding eff; I-SceI x32, 32 tandem repeats of the I-SceI homing endonuclease recognition sequence; Kan', kanamycin resistance gene; MC-eff, minicircle encoding eff; mw, molecular weight standards; pUC, plasmid origin of replication.
Fig. 2.2. Enhanced expression of minicircle-encoded eff in vitro. COS cells were transiently transfected with equimolar amounts (1.1 pmol; 2.5 μg MC-eff) of each DNA species. A luciferase-specific antibody was used to determine the frequencies of luciferase-expressing cells 48 hours later. (a) Flow cytometric analysis of transfected COS cells. These are representative flow cytometry contour plots used to determine the frequencies of eff-positive COS cells shown in b. (b) Frequencies of luciferase-positive COS cell transfectants. Data are presented as means ± SEM (n = 3)(P** < 0.01) and represent three experiments. eff, enhanced firefly luciferase; FL-eff, full-length plasmid encoding eff; MC-Ag, minicircle encoding eff; VC, empty full-length plasmid vector control.
Fig. 2.3. Enhanced intensity and duration of minicircle-encoded eff expression in the skin. The ventral skin on both thighs of C57BL/6 mice was tattooed with equimolar (9.2 pmol; 20 μg eff-encoding minicircle DNA) amounts of empty full-length plasmid vector control (VC), full-length (FL-eff) plasmid encoding enhanced firefly (eff) luciferase, or the eff-encoding minicircle (MC-eff) derivative. (a) Xenogen IVIS imaging of in vivo luciferase activity four days post-gene delivery. FL-eff and MC-eff luciferase activities are shown on the left and right thighs, respectively, of the first four mice as they lie in the supine position. (b) Quantitative long-term in vivo luciferase expression. The data for the FL-eff and MC-eff cohorts are presented as the mean ± SEM (n = 4), corrected for background bioluminescence. Values for the VC cohort (n = 4) never exceeded background and are not shown. Luciferase activity in the FL-eff and MC-eff cohorts was significantly different ($P < 0.05$) on each day analyzed except the first day ($P < 0.06$). This is representative of two similar experiments. eff, enhanced firefly luciferase; FL-eff, full-length plasmid encoding eff; MC-eff, minicircle encoding eff; VC, empty full-length plasmid vector control.
**Fig. 2.4. Single letter amino acid sequence of Ag insert.** The signal sequence is underlined. The 10x histidine tag is italicized. The ovalbumin-derived SIINFEKL peptide is bold faced. The SIINFEKL epitope is embedded in a larger peptide to facilitate Ag processing.
Fig. 2.5. Enhanced presentation of minicircle-encoded Ag by dendritic cells. Cells from the C57BL/6-derived dendritic cell line DC2.4 were transiently transfected with equimolar (1.8 pmol; 2.8 μg MC-Ag) amounts of empty full-length plasmid vector control (VC), full-length plasmid encoding the chicken ovalbumin-derived peptide SIINFEKL, or the SIINFEKL-encoding minicircle derivative of the full-length plasmid. The latter two samples are respectively abbreviated FL-Ag and MC-Ag, as SIINFEKL presented by the MHC class I molecule H-2Kb is an Ag recognized by specific CD8+ T cells. Cells were analyzed by flow cytometry for presentation of SIINFEKL by H-2Kb, as determined by reactivity with the monoclonal antibody 25.D1.16. (a) Flow cytometric analysis of transfected DC2.4 cells. These are representative flow cytometry contour plots (day three) used to determine the frequencies of Ag-positive DC2.4 cells shown in b. (b) Frequencies of Ag-positive DC2.4 cells. Data are presented as the means ± SEM (n = 3)(P*** < 0.001) and represent three experiments. (c) Median fluorescent intensities (MFI) of Ag-positive DC2.4 cells. Data are presented as the MFI ± SD (n = 3)(P*** <
and represent three experiments. Ag, antigen (SIINFEKL/H-2K\(^b\)); FL-Ag, full-length plasmid encoding SIINFEKL; MC-Ag, minicircle-encoding SIINFEKL; VC, empty full-length plasmid vector control.
Fig. 2.6. Enhanced activation of Ag-specific CD8+ T cells in vivo following immunization with MC-Ag. C57BL/6 mice were adoptively transferred with OT-I cells and 24 hours later tattooed with equimolar (13.8 pmol; 20 μg MC-Ag) amounts of vector control (VC), full-length plasmid encoding SIINFEKL (FL-Ag), or the SIINFEKL-encoding minicircle derivative (MC-Ag). Mice were sacrificed one week later and their splenocytes analyzed by flow cytometry. (a) OT-I cell quantification. OT-I cells were identified by their expression of CD8 and Thy1.1 (CD57BL/6 endogeneous CD8+ T cells express Thy1.2). (b) Frequencies of IFN-γ+ OT-I cells. Splenocytes from adoptively transferred, immunized mice were incubated with or without SIINFEKL peptide in vitro before analysis by flow cytometry for intracellular IFN-γ expression. Data are presented as the means ± SEM (n = 3) and represent three experiments. (P < 0.05). FL-Ag, full-length plasmid encoding SIINFEKL; MC-Ag, minicircle-encoding SIINFEKL; VC, empty full-length plasmid vector control.
Fig. 2.7. Enhanced cytolytic activity of Ag-specific CD8+ T cells following immunization with MC-Ag. C57BL/6 mice were adoptively transferred with OT-I cells and 24 hours later tattooed with equimolar (13.8 pmol; 20 μg MC-Ag) amounts of empty full-length plasmid vector control (VC), full-length plasmid encoding the SIINFEKL peptide (FL-Ag), or the SIINFEKL-encoding minicircle derivative (MC-Ag) of the full-length plasmid. Control mice were immunized subcutaneously with 50 μg SIINFEKL peptide emulsified in complete Freund’s adjuvant (CFA). Eight days later, mice were adoptively transferred with equal numbers of syngeneic splenocytes pulsed with SIINFEKL peptide (labeled with 5 μM CFSE) and unpulsed splenocytes (labeled with 0.5 μM CFSE). Mice were sacrificed 14 hours later and their splenocytes analyzed by flow cytometry for the frequencies of CFSE<sub>low</sub> (unpulsed) and CFSE<sub>high</sub> (peptide-pulsed) cells. (a) Representative histograms of CFSE<sub>low</sub> and CFSE<sub>high</sub> cells. The numbers show the frequencies of CFSE<sub>low</sub> and CFSE<sub>high</sub> cells in the CFSE<sup>+</sup> populations in immunized mice. (b) Ag-specific in vivo cytolytic activity. The frequency of unpulsed cells (CFSE<sub>low</sub>) was divided by the frequency of peptide-pulsed cells (CFSE<sub>high</sub>) to determine the ratio of Ag-negative to Ag-positive target cells in each mouse. These ratios were then used to calculate the percent Ag-specific cytolytic activity by the equation: [1-(VC ratio/FL-Ag or MC-Ag ratio)] x 100%. Data are presented as means ± SEM (n = 5)(P<sup>*</sup> < 0.05; P<sup>**</sup> < 0.01) and represent three replicate experiments. CFA, complete Freund’s adjuvant; CFSE, 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester; FL-Ag, full-length plasmid encoding SIINFEKL; MC-Ag, minicircle encoding SIINFEKL; VC, empty full-length plasmid vector control.
Fig. 2.8. Enhanced expansion of endogenous Ag-specific CD8+ T cells in vivo following immunization with MC-Ag. C57BL/6 mice were immunized via tattooing three times, three days apart (d0, d3, and d6) with equimolar (13.8 pmol per immunization; 20 μg MC-Ag) amounts of vector control (VC), full-length plasmid encoding SIINFEKL (FL-Ag), or the SIINFEKL-encoding minicircle derivative (MC-Ag). Twenty-four hours after each immunization, mice were injected subcutaneously on the ventral portion of the tattooed thigh with 10 μg of polyIC. Five days following the last immunization (d11), the mice were bled via the facial vein. (a) Representative flow cytometry plots showing the gates set on CD8+ SIINFEKL/Kb tetramer-binding subsets of lymphocytes. Cells were identified by reactivity with anti-CD8-APC monoclonal antibody and SIINFEKL/Kb-PE tetramer. (b) Frequency of Ag-specific CD8+ T cells. Data are presented as the mean ± SEM (n = 5) (P* < 0.05; Kruskal-Wallis test corrected with a
Dunn’s multiple comparison test) and represent three experiments. FL-Ag, full-length plasmid encoding SIINFEKL; MC-Ag, minicircle-encoding SIINFEKL; VC, empty full-length plasmid vector control.
Fig. 2.9. Enhanced protection from challenge with Listeria monocytogenes following immunization with MC-Ag. C57BL/6 mice were immunized via tattooing three times, three days apart (d0, d3, and d6) with equimolar (13.8 pmol per immunization; 20 μg MC-Ag) amounts of vector control (VC), full-length plasmid encoding SIINFEKL (FL-Ag), or the SIINFEKL-encoding minicircle derivative (MC-Ag). Twenty-four hours after each immunization, mice were injected subcutaneously on the ventral portion of the tattooed thigh with 10 μg of polyICLC. Five or 30 days following the last immunization (d11 or d36), mice were infected with LM-OVA intravenously in the tail vein. Five days following infection (d16 or d41), mice were bled by the facial vein, sacrificed, and their spleens harvested. Ag-specific T cells were identified by staining with anti-CD8-APC and SIINFEKL/Kb-PE tetramer. Bacterial burden was measured as the number of Listeria colony-forming units (CFUs) in the spleen. Frequencies of Ag-specific CD8+ T cells in the peripheral blood and spleen on d16 (a,b) and d41 (d,e). Data are presented as the mean ± SEM (n = 10) (P* < 0.05; P** < 0.01; Kruskal-Wallis test corrected with a Dunn’s multiple comparison test) and represent three experiments. (c) Bacterial burden in the spleen on d16. Mice were infected intravenously with 6x10^4 CFUs of LM-OVA on d11 and bacterial burden was measured five days later (d16). (P** < 0.01; Kruskal-Wallis test corrected with a Dunn’s multiple comparison test). (f) Bacterial burden in the spleen on d41. Mice were infected intravenously with 4x10^4 CFUs of LM-OVA on d36 and bacterial burden was measured five days later (d41). Data are presented as the mean ± SEM (n = 10) (P* < 0.05; Kruskal-Wallis test corrected with
a Dunn’s multiple comparison test). These are pooled data from two experiments. FL-Ag, full-length plasmid encoding SIINFEKL; MC-Ag, minicircle-encoding SIINFEKL; VC, empty full-length plasmid vector control.
Fig. 2.10. Gating strategy for detecting Ag-specific CD8⁺ T cells in mice challenged with LM-OVA. Mice were immunized with equimolar amounts of MC-Ag, FL-Ag or VC DNA three times, three days apart (on d0, d3, d6). One day after each immunization, mice were injected subcutaneously with 10 μg polyICLC. Peripheral blood and spleens were harvested on d11 from LM-OVA challenged mice that were immunized with the indicated DNA. The contour plots below were generated from lymphocytes, based on their forward and side scatter profiles. The gate indicates cells binding the anti-CD8-APC antibody and SIINFEKL-K⁺-PE tetramer.
**Fig. 2.11. Protection against lower dose LM-OVA challenge elicited by MC-Ag or FL-Ag immunization.** Mice were immunized with equimolar amounts of MC-Ag, FL-Ag or VC DNA three times, three days apart (on d0, d3, d6). One day after each immunization, mice were injected subcutaneously with 10 μg polyICLC. Five days after the last immunization (d11), each mouse was injected intravenously with 4.9x10⁴ LM-OVA bacteria. Five days later (d16), the mice were euthanized and bacterial burden was measured as the number of CFUs/spleen. Mice immunized with MC-Ag were significantly protected from LM-OVA challenge when compared to the VC control (P** < 0.01; Kruskal-Wallis test corrected with a Dunn’s multiple comparison test). There was a trend towards decreased bacterial burden among the FL-Ag immunized mice but the group’s mean bacterial burden was not significantly less than the burden in the VC cohort. LOD, limit of detection.
CHAPTER 3: Prolonged antigen expression following minicircle vaccination enhances protective immune responses.

INTRODUCTION

DNA vaccines are promising for disease prevention and treatment due to their safety, stability, affordability, and flexibility (32,40). However, suboptimal immune responses to the proteins they encode limit their clinical effectiveness (31,123). In chapter 2, we show that the immunogenicity of DNA vaccines is improved when using MC DNA, which lacks a bacterial backbone and thus produces higher and more prolonged levels of gene expression than conventional plasmids (64,65). Given that MC DNA induces both higher peak levels of gene expression and more prolonged expression than standard pDNA, it is unclear if the prolonged duration is a necessary or even beneficial component of MC DNA vaccines.

There has been considerable interest in the effect of prolonged T cell stimulation on immune responses. Work using lymphocytic choriomeningitis virus (LCMV) has compared how immune responses differ following acute and chronic infections. Rapidly cleared, acute infections mediated by the LCMV Armstrong (Arm) strain result in prototypic CD8+ T cell responses: expansion of newly activated Ag-specific effectors shortly after infection, rapid contraction, and the subsequent establishment of memory. Cells in the memory phase are marked by their ability to quickly proliferate and regain effector function upon Ag re-challenge (8,124).

Responses differ, however, in mice infected with LCMV clone 13 (cl-13). LCMV cl-13 is derived from LCMV Arm and only differs from it by two amino acids. These two
amino acids, however, alter known T cell epitopes. In cl-13, the glycoprotein GP260 has a Phe to Leu change and the L1079 polymerase protein has a Gln to Lys change. These two differences in LCMV cl-13 produce a chronic infection lasting up to 3 months (125,126). Chronic LCMV infection results in an expansion of effectors as well as a contraction phase, but induction of conventional memory cells does not occur. Instead, the Ag-specific CD8+ T cells that persist demonstrate an increasing impairment of their ability to respond to Ag.

The degree of impairment is proportional to viremic load and duration of viremia (13,126,127) and develops in characteristic stages. Functions such as IL-2 production, ex vivo killing, and proliferative capacity are lost early. Next, cells lose the ability to produce TNF, followed by an inability to produce sufficient quantities of IFN-γ or degranulate. Eventually, T cells are physically deleted (13,124,126,128). This state of T cell dysfunction in response to persistent Ag stimulation is known as exhaustion and is associated with the characteristic expression of certain phenotypic markers. Among many such markers identified, Wherry et al. (129) found that starting early in exhaustion and continuing long-term, the marker PD-1 is upregulated on exhausted CD8+ T cells while the marker killer cell lectin-like receptor subfamily G member 1 (KLRG1) and the commonly used marker of Ag experience, CD44, are both downregulated.

Given these results as well as data showing that less than 48 hours of Ag stimulation is needed to effectively prime immune responses (9,10,130), it could be argued that prolonged Ag expression in a vaccine would be both unnecessary and possibly deleterious to immune responses. However, it is important to distinguish between a chronic infection in which the immune system is exposed to sustained Ag in
the context of a highly inflammatory milieu, and a vaccine occurring in the presence of minimal or short-term inflammation. There has been a relative paucity of data investigating the effect of Ag duration on CD8+ T cell responses in the absence of extensive inflammation.

To determine the effect of Ag duration on immune responses, Hovav et al. (121) immunized mice on the ear with a DNA vaccine and then removed the ears at various times points. They found that truncation of Ag expression before peak immune responses resulted in an earlier and reduced expansion of effector CD8+ T cells followed by an accelerated contraction phase. Despite the diminished priming, they observed that equivalent numbers of memory cells were formed in mice with truncated Ag expression and that these cells expanded more robustly upon challenge than mice without truncation. However, due to the kinetics of their system, where peak CD8+ T cell responses did not occur until three weeks after vaccination, these authors and others investigating Ag duration (131) did not investigate the effect of truncating Ag expression at time points past peak expansion, a system more analogous to chronic infections.

We take advantage of an immunization scheme designed by Bins et al. (38) involving three immunizations three days apart that produces robust immune responses that peak 11 days after the first of three DNA immunizations. In addition, by using MC DNA, we investigate how prolonged Ag stimulation continuing well past the peak of an immune response affects Ag-specific CD8+ T cells in the absence of infection. We found that following MC vaccination, sustained Ag expression (as opposed to antigen expression that was truncated 3, 8, 15, or 22 days post-immunization) improved the ability of T cells to clear infection. We did not see evidence of exhaustion. Thus, we
demonstrate that prolonged duration of gene expression contributes to the enhanced immunogenicity of MC vaccines, although enhanced peak levels of expression may contribute as well. These findings have important implications for vaccine development.

RESULTS

**DNA construct.**

We constructed a vector (CMV-Ag.6) encoding three class I and three class II MHC-restricted Ags driven by the cytomegalovirus (CMV) promoter (Fig. 3.1a). For these experiments, we tracked Ag-specific T-cell responses to one of the three class I-restricted Ags: the SIINFEKL peptide, derived from the chicken ovalbumin protein (amino acid residues 257-264). SIINFEKL presented by H-2Kb is recognized by the OT-I transgenic TCR(103). The construct also contains a Kozak consensus sequence for initiation of translation, a secretion signal (SS) to ensure that transfected cells will secrete Ag for uptake and cross-presentation by APCs, a 3X repeat of the sequence for the FLAG protein affinity tag, and an encoded reporter protein followed by the bovine growth hormone polyadenylation signal (BpA). The reporter, eff, is produced as a separate protein due to its placement downstream of the *tetraviridae* T2A self-cleaving peptide, which induces ribosomal skipping during translation (132). MC DNA was produced from parental FL pDNA of this construct as described(71).

**Gene expression following immunization and ear excision.**

Before assessing the effect of truncating Ag expression on immune responses, we first needed to establish the kinetics of gene expression. We immunized mice via
tattooing three times, three days apart (Fig 3.1b) in the ear with MC CMV-Ag.6 and
determined levels of gene expression in vivo over 32 days by injecting mice with a
saturating amount of D-luciferin, the substrate for eff, and measuring bioluminescence
(Fig 3.2a-b). Background luminescence was measured on the unimmunized ear.

Interestingly, expression levels peaked on day -1, two days after the second
immunization rather than peaking after the third immunization. This may have been due
to saturation of the cells’ ability to take up DNA or loss of DNA due to damage from
repeated tattooing on the thin tissue of the ear. Levels of eff decayed to background or
near background levels by around day 20.

Three days after the last immunization, mice intended for ear excision that day
(D3 mice) were imaged pre- and post-excision to confirm loss of gene expression
following excision (Fig. 3.2c-d). Before excision, gene expression is clearly detectable.
After excision, however, levels do not differ significantly from levels of background
luminescence.

**Truncation of Ag expression before, but not after, the peak CD8+ T cell response
causes early contraction of Ag-specific CD8+ T cells.**

Following immunization, Ag expression was terminated in some animals at
different time points by ear excision (Fig. 3.1b) and frequencies of Ag-specific CD8+ T
cells tracked for six weeks. Removing Ag before the peak immune response, on day 3,
resulted in an accelerated contraction (Fig. 3.3a), consistent with results reported by
Hovav et al. (121). In contrast, removing Ag 3 days after the peak, on day 8, did not
result in a similar accelerated contraction (Fig. 3.3b). Hovav et al. (121) also reported
that removal of Ag decreased the size of the contracted population. We did not see a statistically significant difference in the frequency of Ag-specific CD8+ T cells between D3 mice and any other group at day 42 (Fig. 3.3c). However, when we pooled data from mice with ears removed later than D3, we observed a statistically significant decrease in frequencies of Ag-specific CD8+ T cells in D3 mice compared to the mice with Ag removed after the peak immune response (Fig. 3.3d).

*Prolonged Ag expression is associated with increased proliferative capacity and protection following infection.*

We next assessed the ability of T cells to respond to infection with *Listeria monocytogenes* bearing the ovalbumin protein (LM-OVA) after prolonged or shortened Ag expression. We infected mice 42 days after the last immunization then, 5 days after the infection, we analyzed peripheral blood and spleen-derived CD8+ T cells and determined bacterial burden in the spleen (Fig. 3.1b). We detected fewer Ag-specific CD8+ T cells in the peripheral blood (Fig. 3.4a) and spleens (Fig. 3.4b) of animals with ears removed at day 3 following infection. In addition, frequencies of Ag-specific T cells in the peripheral blood in mice with Ag removed at day 8 were lower in the peripheral blood than in mice without Ag removed (Fig. 3.4a). Furthermore, we compared the fold increase of Ag-specific CD8+ T cells in the blood from pre-infection on day 42 to post-infection on day 47 and found that, in general, truncating Ag expression via ear excision resulted in a decreased expansion of T cells (mean fold increase of 66.8) compared to mice without ear excision (mean fold increase of 93.1) (Fig. 3.4c). In addition, when we quantified bacterial burden in the spleens of mice, we found that ear excision at any time
point resulted in impaired clearance, while eight of ten mice without Ag excision showed no detectable bacterial burden.

**Truncation of Ag expression is associated with increased upregulation of PD-1 following infection.**

Given the role of prolonged Ag expression during chronic infection in inducing exhaustion of CD8+ T cells, we were interested in comparing PD-1 expression in mice with and without early truncation of Ag expression. At day 42, before the infection, we saw no difference in PD-1 expression between mice expressing Ag for different lengths of time (Fig. 3.5a). However, five days after infection, ear excision was associated with a subtle but statistically significant increase in PD-1 expression in the peripheral blood (for all days except D22) (Fig. 3.5b) and the spleen (for all days) (Fig. 3.5c,d).

**Long-term gene expression following MC immunization is not associated with markers of exhaustion.**

Since MC DNA is expressed for prolonged periods of time (Fig. 3.2a), we asked if this long-term expression would increase markers of exhaustion compared to the short burst of Ag associated with protein immunizations. Therefore, we immunized mice with MC or VC DNA three times, three days apart on the leg and compared them to mice immunized with four consecutive, subcutaneous injections of whole ovalbumin protein (OVA) and the TLR-3 agonist, polyIC, as an adjuvant (133). The protein/adjuvant immunization was timed based on pilot experiments so that the peak response would coincide with the peak of the response in MC-immunized mice. At the peak response on
day 5 following the last immunization, OVA induced more Ag-specific CD8+ T cells than MC, but the two groups contracted with similar kinetics to a similar baseline that was significantly above values seen in the VC group (Fig. 3.6.a). At day 120 following the last immunization, we stained peripheral blood lymphocytes for expression of PD-1 (Fig. 3.6b), KLRG1 (Fig. 3.6c) and CD44 (Fig 3.6d), comparing expression in MC-immunized mice, OVA-immunized mice, and naïve CD8+ T cells from VC mice. There was no significant difference between T cells in MC-immunized or OVA-immunized mice for any of these markers.

**FL immunizations yielding similar peak expression levels as MC produce similar immunologic responses.**

Previous work has shown that immunization with MC DNA results in more protective immune responses than immunization with FL pDNA (134). We have primarily investigated the role of duration of gene expression in mediating this effect, but MC DNA also generally produces significantly higher peak levels of transgene expression (Fig. 2.3b), which could contribute to the efficacy of immune responses. When immunizing with the CMV-Ag.6 construct in the ear, we seemed to have encountered a ceiling effect, whereby the ability of the skin to take up our DNA was saturated and increasing the dose of MC DNA had no effect on gene expression (Fig 3.7). When we immunized with FL DNA in the ear, we observed a less dramatic difference between MC and FL peak levels of gene expression (Fig 3.8a) than we had previously observed (Fig. 2.3b). In fact, at day 3, there was no statistical difference between the two curves. After that point, as expected, FL expression rapidly declined to background levels while MC levels
persisted. When the differences between the two peaks was minimized by immunization in the ear, there were no differences in frequencies of Ag-specific CD8+ T cells in the peripheral blood at any time measured in the 6 weeks following immunization (Fig. 3.8b). Furthermore, there were no differences in the frequencies in the peripheral blood (Fig 3.7c) or numbers in the spleen (Fig. 3.8d) five days after infection with LM-OVA and no differences in bacterial burden (Fig 3.8e).

DISCUSSION

We investigated the effect of prolonged MC DNA-driven Ag expression on CD8+ T cell responses. We found that prolonged expression is an important contributor to the efficacy of MC DNA vaccines. Previous work on Ag duration in DNA vaccines evaluated truncating Ag expression at time points before the peak in expansion of CD8+ T cells (121,131). When we removed Ag just before the peak of expression, we found acceleration of contraction and evidence of a decrease in the size of the contracted population (Fig. 3.3a,d). This indicates that in contrast to infection, the presence or absence of Ag during the peak T cell expansion affects the kinetics and, subtly, the extent of contraction following DNA vaccination.

These initial findings are consistent with previously described results (121). However, those authors also found that despite this accelerated and increased contraction, T cells in animals with Ag removed shortly before the peak immune response proliferated more robustly in response to a boost with recombinant adenovirus. In contrast, we found that excision prior to the peak resulted in poorer proliferation and a reduced ability to protect against *Listeria* infection (Fig. 3.4). It is possible that the
differences in our results arise from differences in the kinetics inherent in our immunization schemes. In their system, prolonged Ag expression does not induce a peak immune response for approximately three weeks, whereas we see a peak 5 days after the last immunization (11 days after the first immunization). Perhaps when there is a prolonged delay in producing a peak T cell response, truncating Ag expression to induce a faster contraction provides a benefit not present when the peak response naturally occurs sooner.

We were particularly interested in investigating the effect of Ag removal after the peak response, a situation that has not been studied in a vaccine setting to our knowledge. We found that excision at any time tested impaired the ability of animals to clear an infection (Fig. 3.4d). Surprisingly, this was as true in the mice with Ag removed at day 22 as those with Ag removed at day 3 or 8. By day 22 and later, the levels of gene expression occurring with MC vaccination as detected by in vivo bioluminescence are fairly low (Fig. 3.2a). Nevertheless, it appears that the gene expression that continues after day 22 is significant, since without it, mice have a reduced ability to clear infection. It is notable that when immunizing with FL DNA, levels of eff fall to background levels at approximately day 20 (Fig 3.8a), yet the FL vaccine shows no diminished efficacy compared to the MC vaccine (Fig. 3.8b-e). This may indicate the importance of even minimal Ag expression. It is likely that when eff can no longer be detected, there is still low level, residual gene expression. However, when the ear is excised, any Ag expression occurring in the ear abruptly ends. The difference between very low levels of gene expression and no gene expression may account for the difference that we see in response to Listeria infection.
Given how short a time is necessary to prime an immune response, and given that we are investigating effects occurring after the prime, it is surprising that the continued presence of Ag should be important for generating an optimally protective secondary response. The reason for this may be closely tied to levels of Ag encountered by CD8+ T cells as well as levels of inflammation. In a plasmid or MC-based vaccine, there is poor transfection of cells with DNA and no replication within the host (32). Thus, levels and kinetics of Ag expression differ dramatically between these vaccines and typical infections.

Furthermore, an infection is associated with levels of inflammation not approached by standard vaccines. For instance, our vaccine did not encode an adjuvant and was not administered with one. Our method of administration (tattooing) likely has an adjuvant effect due to the production of many small perforations in the skin (101). However, this effect is limited to the time of vaccine administration and likely is not as inflammatory as a conventional infection. DNA vaccines themselves can function as adjuvants by stimulating TLR-9, however this is only a minor effect as immune responses to DNA vaccines in TLR-9 or MyD88 knockout mice were shown to be indistinguishable from those in wild-type mice (43). The reduced inflammation associated with DNA vaccines as well as the different kinetics of Ag expression may prime T cells less optimally than an infection would. In that case, prolonging Ag expression may compensate for the suboptimal prime, thereby improving immune responses when it is present.

Similarly, we speculate that the lower levels of Ag and inflammation associated with our vaccine explain why we saw no evidence of exhaustion with prolonged Ag
expression. As late as 120 days after vaccination, we detected no difference in markers of exhaustion (PD-1, KLRG1, CD44) between MC-immunized mice and mice given a standard protein/adjuvant immunization (Fig. 3.6b-d). Furthermore, proliferative capacity is one of the functions lost early during exhaustion, however at 42 days after immunization we observed robust proliferation in response to an infectious challenge (Fig. 3.4a-c).

Interestingly, we observed an increased upregulation of PD-1 expression following infection in mice with Ag expression truncated via ear excision (Fig. 3.5b-d). This difference was noticeable only as a subtle shift in median fluorescence intensity (MFI) relative to PD-1 MFI in mice with no ear excision. The significance of such an increase, given its subtlety (Fig. 3.5d), is unclear. However, it is possible that the modest increase in PD-1 is related to the reduced ability of Ag-specific CD8+ T cells in mice with shortened Ag expression to proliferate or provide protection in response to infection with *Listeria* (Fig. 3.4c-d). Further work is needed to elucidate by what mechanism truncating Ag expression would induce such an upregulation. It is important to note that truncation of Ag expression did not affect PD-1 levels prior to infectious challenge (Fig 3.5a) and is therefore not likely indicative of exhaustion, which would be particularly surprising to see in mice with decreased Ag expression. PD-1 levels normally increase immediately following infection as a homeostatic mechanism (135,136). The increase in PD-1 observed in mice with excised ears appears to reflect an increased negative regulatory response following challenge.

In this study, we have primarily focused on the role of prolonged Ag expression resulting from MC vaccines on immune responses. However, as mentioned above, MC
DNA also typically produces increased peak levels of gene expression. When tattooing in the ear, we obtained less striking differences in peak expression between MC and FL than generally seen (65,134), while still maintaining differences in duration (Fig. 3.8a). This was likely due to the inability of the thin tissue of the ear to take up an increasing amount of DNA, leading to saturation of expression (Fig. 3.7). We predict that immunizing at significantly lower doses or immunizing in the leg would have recapitulated the difference seen between MC and FL in chapter 2 of this thesis.

However, under the present circumstances, we detected no differences in frequencies or number of CD8+ T cells, or protection from infectious challenge (Fig. 3.8b-e). These data indicate that differences in peak expression, in addition to duration of expression, are likely important in determining vaccine efficacy. Thus, it is probable that both prolonged gene expression and increased gene expression contribute to the success of MC DNA as a vaccine compared to pDNA. These findings are important for the development of future vaccines and furthermore, provide valuable insight into how CD8+ T cells respond to persistent Ag in a DNA vaccine setting.

MATERIALS AND METHODS

DNA constructs. The plasmid backbone for the FL constructs (pMC.BESPX.MCS1) contains the requisite sequences for bacterial propagation (e.g., the pUC origin of replication), the kanamycin-resistance gene, the φC31 integrase recognition sites attB and attP, and a block of 32 tandem repeats of the recognition sequence for the I-SceI homing endonuclease (71). To generate the FL CMV-Ag.6 construct, we used the isothermal enzymatic DNA assembly strategy described by Gibson et al (122). Briefly,
60-nucleotide oligonucleotides were synthesized with 20-base overlapping/complementary regions that together encoded the secretion signal, Ags, 3X FLAG, and T2A sequences. The overlapping oligonucleotides were grouped into fragments containing 8 oligonucleotides each and assembled via splice overlap extension PCR using Phusion High-Fidelity DNA Polymerase and associated buffers (New England Biolabs, Ipswich, MA). The resulting products were then cloned into pCR4 TOPO TA vectors (Invitrogen, Carlsbad, CA) via TA cloning, using Taq polymerase (Invitrogen) to append a single deoxyadenosine to the 3' end of the DNA sequences, generating an overhang compatible with the pCR4 TOPO TA vector. These fragments were then sequence verified, excised from the pCR4 TOPO TA vector via digestion with the EcoRI restriction endonuclease (Invitrogen) and assembled together via PCR as above. This product was likewise cloned into pCR4 TOPO TA and sequenced verified before being excised and gel purified. The CMV promoter was amplified from the pJ603 vector (DNA2.0, Menlo Park, CA) and the eff gene and BpA signal were amplified as one product from an existing construct, FL-eff (134). The internal fragment, the CMV promoter, the eff-BpA sequence, and EcoRI-digested pMC.BESPX vector were subjected to the one-step isothermal DNA assembly procedure (122), which takes advantage of engineered overlaps between DNA fragments to generate a single, seamlessly joined construct. Empty pMC.BESPX was used as a vector control.

For FL pDNA preparation, transformed Top10 bacteria (Invitrogen, Carlsbad, CA) were grown in LB broth (Invitrogen) supplemented with kanamycin. pDNA was isolated using the GenElute Endtoxin-free Plasmid Maxiprep Kit (Sigma- Aldrich, St Louis, MO)
according to the manufacturer’s instructions, then concentrated via ethanol precipitation to 8-12 μg/μl, resuspended in water and stored −20°C.

To produce MC DNA from the parental FL construct, we transformed the ZYCY10P3S2T bacterial strain with FL CMV-Ag.6. ZYCY10P3S2T contains ten copies of the φC31 integrase gene, three copies of the I-SceI homing endonuclease gene(106), and the araE and LacY arabinose transporter genes that constitutively express proteins importing arabinose to induce φC31 integrase and I-SceI endonuclease expression. For MC generation, bacteria transformed with FL pDNA were grown overnight in Terrific Broth (Invitrogen) supplemented with kanamycin. The following day MC induction media (fresh LB broth containing 0.04 volumes of 1 N NaOH and 0.02% L-arabinose (Sigma-Aldrich, St Louis, MO)) was added and the culture temperature was decreased from 37°C to 32°C for 5–8 hours. The culture was then centrifuged and MC DNA was purified with the PureLink HiPure Maxiprep Kit using buffers from the Purelink HiPure BAC Buffer Kit (Invitrogen). Following elution in water, the DNA was concentrated to 4–6 μg/μl by ethanol precipitation, resuspended in water and stored at −20°C.

Sequence analyses verified the genes encoded by the FL pDNA and its derivative MC were identical. The amounts of residual FL pDNA in the MC DNA preparations was evaluated by StuI (New England Biolabs) restriction endonuclease-mediated linearization, followed by agarose gel electrophoresis and DNA visualization using ethidium bromide.

Mice. Four- to eight-week-old, female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) or the National Cancer Institute (Frederick, MD). All mice
were housed under specific pathogen free conditions at the University of Minnesota. All animal procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee.

**Immunizations.** For DNA immunizations, mice were anesthetized with ketamine/xylazine and their right ears wiped with 70% ethanol before being immunized with 30 μg DNA (13.5 pmol) in 15 μl sterile water. Equimolar amounts of VC and the corresponding FL pDNA samples were diluted in sterile water to the same volumes. In other experiments mice were immunized on the leg. They were first anesthetized with ketamine/xylazine and their right inner hind leg shaved and wiped with 70% ethanol. They were then immunized with 30 μg MC DNA or equimolar amounts of VC as above. All DNA was delivered intradermally via tattooing over the ear pinna for 30 seconds using a Cheyenne Hawk PU II tattoo device (Unimax Supply, New York, NY) set at 110 Hz using 9-point needles adjusted to a depth of 0.5 mm. Mice were immunized three times, three days apart.

For protein immunizations, the vaccination scheme developed by Wick et al (133), was followed. Briefly, mice were immunized with 100 μg whole ovalbumin protein and 10 μg polyIC adjuvant in 150 total μl PBS subcutaneously in the right leg. Immunizations were administered for four consecutive days and timed so that the peak of the primary response occurred simultaneously with the peak of the response resulting from the DNA immunizations being compared. Both peaks occur five days after the last immunization.
**Ear pinna excision.** For removal of the immunized ear, mice were anesthetized with ketamine/xylazine and the immunized ear wiped with 70% ethanol. The pinna was then removed using sterile scissors, with sparing of the cartilage. The area was then cleaned again with 70% ethanol.

**In vivo Bioluminescence.** Mice were imaged for bioluminescence using a Xenogen Spectrum Imaging System (Caliper Life Sciences, Hopkinton, MA). Animals were imaged two days after the first and second immunization, three days after the last immunization, then weekly thereafter until day 35. To image, mice were injected intraperitoneally with 100 μl of 30 mg/ml D-luciferin and anesthetized with isoflurane. Bioluminescence was measured for 5 minutes. Data were analyzed using Living Image 2.5 Software (Caliper Life Sciences).

**Flow cytometric analysis.** Peripheral blood samples taken from the facial vein and single cell suspensions prepared from harvested spleens were incubated with CD8α-AlexaFluor 700, CD62L-FITC, CD127-APC, PD-1-PE-Cy7, KLRG-1-eFluor 450 (eBiosciences, San Diego, CA), CD44 Horizon V500 (BD Biosciences, San Jose, CA), and SIINFEKL/H-2Kb tetramer formed with SA coupled to PE. To determine the cell count, PKH26 Reference Beads (Sigma-Aldrich) were mixed with cells and 5,000 bead events were collected on the flow cytometer. The following equation was used to determine the number of lymphocytes: \( \text{Number of cells/ml} = (\text{number of cells acquired} \times \text{dilution factor of cells} \times \text{number of singlet beads/ml}) / (\text{number of beads acquired} \times \text{dilution factor beads}) \). Data were acquired on a BD LSR II flow cytometer (BD.
Biosciences) using BD FACSDiva software (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

**Infection with LM-OVA.** Mice were infected with \( \sim 6 \times 10^4 \) colony-forming units intravenously in the tail vein six weeks after the last immunization. Bacteria were grown in tryptic soy broth with 50 \( \mu \)g/ml of streptomycin to an absorbance at 600 nm of about 0.1. Actual numbers of colony-forming units injected were determined for each experiment by plate count. Five days following infection, mice were bled from the facial vein, killed, and their spleens harvested. Half of the cells from each spleen were used for flow cytometric analysis and the other half were serially diluted and plated on tryptic soy agar plates containing streptomycin. Bacterial colonies were counted after plate incubation for 24 hours at 37 °C.

**Statistical analysis.** Data were graphed and analyzed using Prism v5.0c (GraphPad Software, La Jolla, CA). Statistical analyses were performed using the Mann-Whitney nonparametric T-test except where indicated.
**Fig. 3.1. DNA construct and experimental design.** (a) Schematic of elements in the CMV-Ag.6 vector. The construct contains the CMV promoter, the Kozak consensus sequence, a secretion signal (SS) to permit secretion of produced proteins, three class I MHC-restricted epitopes (the p14 epitope from LCMV, the melanoma-associated pmel epitope, and the ovalbumin-derived SIINFEKL epitope recognized by the OT-I transgenic TCR), three class II MHC restricted epitopes (the class II-restricted ovalbumin epitope, AAHAEINEA, recognized by the OT-II transgenic TCR, the TEα epitope from the alpha chain of the murine I-E class II MHC molecule, and the 2W1S epitope derived from alpha chain of the murine I-E\(^d\) MHC II molecule), three FLAG tag repeats, and the eff reporter separated by the T2A ribosomal skipping peptide. (b) Experimental design. Mice were immunized 3 times, 3 days apart, infected with LM-OVA 42 days after the last immunization, and sacrificed 5 days after infection. Some groups had the immunized ear removed on day 3, 8, 15, or 22 after the last immunization. Day 5 after the last immunization denotes the peak primary response.
**Fig. 3.2. Duration of gene expression following immunization with MC CMV-Ag.6.**

Mice were immunized in the ear three times, three days apart with 30 μg MC DNA (13.5 pmol) and imaged *in vivo* for 35 days using the Xenogen Spectrum. (a) Xenogen Spectrum imaging of *in vivo* luciferase expression at the peak of expression, on day -1 (2 days after the second immunization) in mice immunized with MC CMV-Ag.6 on the right ear. The left ear represents background bioluminescence. (b) Kinetics of luciferase expression following immunization with MC CMV-Ag.6 in the absence of ear excision. Background levels were obtained by measuring luminescence in the unimmunized ear. Data are pooled from two similar experiments and are presented as mean ± SEM (n = 10) (**P < 0.01; ***P < 0.001). (c) Xenogen Spectrum imaging of luciferase expression in mice immunized with MC CMV-Ag.6 pre- (top) and post- (bottom) ear excision 3 days after the last immunization. (d) Quantification of bioluminescence before and after ear excision on day 3. Mice were imaged prior to ear excision and again the same day, after ear excision. Background levels were obtained by measuring luminescence in the unimmunized ear. Data are pooled from two similar experiments and are presented as mean ± SEM (n = 10) (**P < 0.01; Kruskal-Wallis test corrected with a Dunn’s multiple comparison test)
Fig. 3.3. **Truncating Ag expression before the peak immune response induces early contraction.** Mice were immunized in the ear three times, three days apart with 30 μg MC DNA (13.5 pmol). Certain groups had the immunized ear removed at 3, 8, 15, or 22 days following the last immunization. (a) Frequencies of Ag-specific CD8+ T cells in the peripheral blood from day 5 to day 42 following the last immunization in mice that had the immunized ear removed at day 3 (D3) and mice with no ear excision. Data are pooled from two similar experiments and are presented as mean ± SEM (n = 10) (*P < 0.05). (b) Frequencies of Ag-specific CD8+ T cells in the peripheral blood from day 5 to day 42 following the last immunization in mice that had the immunized ear removed at day 8 (D8) and mice with no ear excision. Data are pooled from two similar experiments and are presented as mean ± SEM (n = 10). (c) Frequencies of Ag-specific CD8+ T cells in the peripheral blood on day 42 after the last immunization in mice with ears removed on day 3 (D3), day 8 (D8), day 15 (D15), day 22 (D22) or in unexcised mice. Data are pooled from two similar experiments and are presented as mean ± SEM (n = 10) (ns = not significant). (d) Frequencies of Ag-specific CD8+ T cells from (c) with D8, D15, and D22 pooled together (later excision; n = 30) for comparison to day 3 (D3; n = 10). Data are pooled from two similar experiments and are presented as mean ± SEM. (*P < 0.05).
Fig. 3.4. Duration of Ag expression affects proliferative capacity and protection from infection. Mice were immunized in the ear three times, three days apart with 30 μg MC DNA (13.5 pmol) and infected with LM-OVA 42 days after the last immunization. Mice were infected IV with 6 x 10⁴ CFU LM-OVA in the tail vein and sacrificed 5 days later. Certain groups had the immunized ear removed at days 3, 8, 15, or 22 days following the last immunization. (a) Frequencies of Ag-specific CD8+ T cells in the peripheral blood 5 days following infection in mice with ears removed on day 3 (D3), day 8 (D8), day 15 (D15), day 22 (D22) or in unexcised mice. Data are pooled from two similar experiments and are presented as mean ± SEM (n = 10) (*P < 0.05; **P < 0.01). (b) Absolute number of Ag-specific CD8+ T cells in the spleen 5 days following infection in mice with ears removed on day 3 (D3), day 8 (D8), day 15 (D15), day 22 (D22) or in unexcised mice. Data are pooled from two similar experiments and are presented as mean ± SEM (n = 10) (*P < 0.05). (c) Fold increase in frequency of Ag-specific CD8+ T cells in the peripheral blood before (day 42) and after infection (day 47) in all mice with excised (n = 40) or unexcised (n = 10) ears. Data are pooled from two similar experiments and are presented as mean ± SEM (**P < 0.01). (d) Bacterial burden in the
spleen on day 47. Data are pooled from two similar experiments and are presented as mean ± SEM (n = 10) (*P < 0.05; **P < 0.01; ***P < 0.001).
Fig. 3.5. **PD-1 expression following infection in mice with truncated Ag expression.**
Mice were immunized in the ear three times, three days apart with 30 μg MC DNA (13.5 pmol). Certain groups had the immunized ear removed at 3 (D3), 8 (D8), 15 (D15), or 22 (D22) days following the last immunization. All mice were infected IV with $6 \times 10^4$ CFU LM-OVA 42 days after the last immunization and sacrificed 5 days later. Expression of PD-1 was assessed pre- and post-infection. (a) Median fluorescence intensity of PD-1 on Ag-specific CD8+ T cells in the peripheral blood pre-infection, 42 days after the last immunization. Data are pooled from two similar experiments and are presented as mean ± SEM ($n = 10$) (ns = not significant). (b) Median fluorescence intensity of PD-1 on Ag-specific CD8+ T cells in the peripheral blood 5 days after infection with LM-OVA. Data are pooled from two similar experiments and are presented as mean ± SEM ($n = 10$) ($**P < 0.01; ***P < 0.001$). (c) Median fluorescence intensity of PD-1 on Ag-specific CD8+ T cells in the spleen 5 days after infection with LM-OVA. Data are pooled from two similar experiments and are presented as mean ± SEM ($n = 10$) ($***P < 0.001$). (d) Representative histogram depicting PD-1 expression on Ag-specific CD8+ splenocytes of a mouse with Ag expression truncated on day 3 (D3; open, blue) and an unexcised mouse (shaded, red).
Fig. 3.6. Markers of exhaustion following MC or protein immunization. Mice were immunized on the inner hind leg three times, three days apart with 30 μg MC DNA (13.5 pmol) or an equimolar amount of VC DNA. As a positive control, some mice were immunized with 100 μg whole ovalbumin protein and 10 μg polyIC for 4 consecutive days (OVA). (a) Frequencies of Ag-specific CD8+ T cells in the peripheral blood from day 5 to day 120 following the last immunization. Data are presented as mean ± SEM (n = 5) (*P < 0.05) P-values compare MC and OVA; both MC and OVA frequencies were
significantly above VC on all days tested ($P < 0.05$). (b) Median fluorescence intensity of PD-1 on Ag-specific CD8+ T cells or naïve CD8+ T cells (defined as tetramer-/CD62Lhi/CD44lo) in the peripheral blood 120 days after the last immunization. Data are presented as mean ± SEM ($n = 5$) (*$P < 0.05$; ns = not significant). (c) Median fluorescence intensity of KLRG-1 on Ag-specific CD8+ T cells or naïve CD8+ T cells (defined as tetramer-/CD62Lhi/CD44lo) in the peripheral blood 120 days after the last immunization. Data are presented as mean ± SEM ($n = 5$) (*$P < 0.05$; ns = not significant). (d) Median fluorescence intensity of CD44 on Ag-specific CD8+ T cells or naïve CD8+ T cells (defined as tetramer-/CD62Lhi/CD44lo) in the peripheral blood 120 days after the last immunization. Data are presented as mean ± SEM ($n = 5$) (*$P < 0.05$; ns = not significant).
Fig. 3.7. In vivo gene expression following immunization with different doses of MC DNA in the ear. Mice were immunized in the ear three times, three days apart with the 30 μg (13.5 pmol) standard dose of MC DNA (MC-1X), a 50% reduced dose (15 μg, 6.75 pmol) of MC DNA (MC-0.5X), or a 5X increased dose (150 μg, 67.5 pmol) of MC DNA (MC-5X). *In vivo* bioluminescence was measured during and following immunizations and normalized to background levels in each mouse by subtracting luminescence in the unimmunized ear. Data are pooled for two similar experiments.
Fig. 3.8. Immune responses following immunization with FL DNA yielding similar peak expression levels as MC DNA. Mice were immunized in the ear three times, three
days apart with 30 μg MC DNA (13.5 pmol) or an equimolar amount of FL or VC DNA. No ears were excised for these experiments. Mice were infected IV with 6 x 10^4 CFU LM-OVA 42 days after the last immunization and sacrificed 5 days later. (a) In vivo bioluminescence during and following MC and FL immunization. Background levels were obtained by measuring luminescence in the unimmunized ear. Background levels for MC and FL mice did not significantly differ and were therefore combined. Data are pooled from two similar experiments and are presented as mean ± SEM (n = 10 for MC and FL; n = 20 for background). P-values compare MC to FL (*P < 0.05; **P < 0.01; ***P < 0.001). (b) Frequencies of Ag-specific CD8+ T cells in the peripheral blood from day 5 to day 42 following the last immunization in mice immunized with MC, FL, or VC DNA. Data are pooled from two similar experiments and are presented as mean ± SEM (n = 10). No significant differences exist between MC and FL on any day. Both MC and FL are significantly different from VC on all days (P < 0.05, P-values not shown). (c) Frequencies of Ag-specific CD8+ T cells in the peripheral blood 5 days following infection. Data are pooled from two similar experiments and are presented as mean ± SEM (n = 10) (**P < 0.001; ns = not significant; Kruskal-Wallis test corrected with a Dunn’s multiple comparison test). (d) Absolute number of Ag-specific CD8+ T cells in the spleen 5 days following infection. Data are pooled from two similar experiments and are presented as mean ± SEM (n = 10) (**P < 0.01; ns = not significant; Kruskal-Wallis test corrected with a Dunn’s multiple comparison test). (e) Bacterial burden in the spleen on day 47. Mice were infected IV with 6 x 10^4 CFU LM-OVA in the tail vein and sacrificed 5 days later. Data are pooled from two similar experiments and are presented as mean ± SEM (n = 10) (**P < 0.01; ***P < 0.001; ns = not significant; Kruskal-Wallis test corrected with a Dunn’s multiple comparison test).
CHAPTER 4: Targeted delivery of nucleic acid to T cells using a TAT-streptavidin fusion protein

INTRODUCTION

A method for specifically manipulating T cells would be immensely useful both in the investigation and treatment of T cell-mediated disease. Although B cells can be depleted using a monoclonal antibody against CD20, no analogous FDA-approved reagent exists for T cell-specific depletion. Instead, current FDA-approved methods for modulation of T cells generally involve systemic manipulation of IL-2, a necessary cytokine for T cell survival and proliferation. For instance, both allogeneic graft rejection and GVHD are T-cell mediated pathologies, treated via suppression of T cells using calcineurin inhibitors such as tacrolimus or cyclosporine. Calcineurin inhibitors block IL-2 production by interfering with the transcription factor, nuclear factor of activated T cells (NFAT). Conversely, metastatic melanoma and renal cell carcinoma can be treated with administration of recombinant human IL-2 to stimulate anti-tumor T cell responses (137,138).

These approaches are problematic because the methods of altering IL-2 are often indirect and are thus accompanied by unwanted drug activities. For instance, calcineurin inhibitors are associated with nephrotoxicity, thrombocytopenia, hypertension, and hyperlipidemia, among other side effects (139-142). Furthermore, IL-2 has a complex and multifaceted role in the modification of immune responses. It does not constitute a binary survival switch for T cells. Levels of IL-2 affect not just numbers of T cells, but their phenotypes, functionality, and differentiation fates. In addition, IL-2
affects other lymphocytes; NK cell proliferation as well as B cell proliferation and antibody production are influenced by IL-2 signaling (138). Thus, it is not surprising that IL-2-related therapies are associated with a wide array of serious immune-related side effects as well (143-146).

A reagent that targeted T cells in a more specific manner and allowed for both depletion and modulation of T cell responses would have tremendous clinical utility. Perhaps even more alluring than targeting all T cells would be targeting populations of Ag-specific T cells. This would allow for deletion of pathogenic clones with sparing of normal populations. Conversely, particular populations could be stimulated with growth factors or cytokines to augment anti-microbial or anti-tumor responses. We propose using the protein SA as a scaffold for targeting both T cells in general and Ag-specific subpopulations of T cells.

SA is a tetrameric protein that has four high affinity binding sites for biotin (147). Since biotin is a small molecule that can be easily appended to many other proteins, SA is a versatile adaptor and promising potential delivery platform. For example, Albarran et al. generated a SA-based delivery system that delivered a biotinylated cargo, enzymatically-active alkaline phosphatase, non-specifically to cells in vitro. Entry into the cells was achieved by modifying the SA so that each monomer had the basic 11-amino acid sequence from the HIV-1 TAT peptide (YGRKKRRQRRR) appended to the amino terminus (148). TAT has long been known to mediate intracellular uptake of a variety of cargoes (149-152).

SA has also been used as the core of pMHC tetramers, which enable identification of Ag-specific CD8+ or CD4+ T cell populations (86,153). pMHC tetramers
contain fluorophore-labeled SA bound to biotinylated class I or class II pMHC. The pMHC interact specifically with TCRs, allowing for tracking of Ag-specific clones. Following the advent of tetramers used for tracking T cells, groups have investigated using pMHC tetramers to deplete Ag-specific T cell populations. The first successful such attempt used class I pMHC tetramers conjugated to a radioactive particle (154). Other groups took advantage of the fact that tetramers are internalized upon binding to TCRs and used class I pMHC tetramers conjugated to the potent ribosome inactivating toxin, saporin, to kill Ag-specific clones. The saporin fusion protein showed successful specific killing of target CD8+ T cells in vitro and in vivo (85). Furthermore, the toxic tetramer showed utility in treating mouse models of autoimmune diabetes (88), T-cell induced choriomeningitis, and T-cell mediated hepatitis (87).

These advances are promising and exciting. However, one potential limitation to using tetramer fusion proteins is a lack of versatility. Each new manipulation requires the creation of an entirely novel protein that must be tested anew for the ability to bind biotinylated targeting moieties and be internalized by T cells. Also, the protein must be functional when fused to SA. This process is time-consuming and expensive. In contrast, delivering DNA instead of protein would only require one delivery platform and would enable the expression of any number of desired proteins. In addition, working with DNA allows for a great degree of ease and flexibility. Simple cloning allows for the addition, removal, or alteration or delivered genes. Furthermore, the use of tissue or cell-specific promoters permits an additional level of specificity not afforded by protein-based systems.
We investigate delivery of DNA specifically to T cells using a biotinylated anti-CD7 monoclonal antibody complexed to the TAT-SA fusion protein described by Albarran et al (148). CD7 is a rapidly internalized marker found on most T cells (155,156). The TAT peptide is highly cationic and thus binds DNA efficiently due to electrostatic interactions. When bound to DNA, it can mediate non-specific uptake into cells via endocytosis (150). However, when bound to DNA, efficient uptake is dependent on the charge ratio resulting from the TAT/DNA complexes. Maximal non-specific uptake occurs when there is a 5:1 positive/negative (p/n) charge ratio, likely because excess free TAT molecules are necessary to mediate uptake (150). Thus, we anticipate that by controlling charge ratio, this approach will allow for specific targeting using biotinylated targeting moieties, such as anti-CD7.

We found that TAT-SA/αCD7 complexes deliver both siRNA and DNA to CD7+ T cells but that delivered genes are not expressed. We also use TAT-SA to form class I pMHC tetramers, but likewise fail to achieve production of encoded proteins. We discuss possible obstacles to gene expression in these systems and propose potential solutions that could enable further development of this promising technology.

RESULTS

TAT-SA production and functionality

Our approach is summarized in Fig. 4.1. It is important to note that plasmid DNA (pDNA) is vastly larger and more charged than TAT-SA (Fig. 4.1b), such that uptake likely involves coating of DNA with many TAT-SA molecules. Thus, finding a charge ratio
that allows for, at a minimum, neutralization of the negatively charged DNA, without permitting non-specific uptake is an important consideration.

We produced the TAT-SA protein solubly in E. coli and, as expected, found its monomeric size to be approximately 15 kDa, slightly larger than the WT core SA upon which it was based (148,157) (Fig. 4.2a). For TAT-SA to effectively deliver DNA to targeted cells, it must be bifunctional, capable of binding DNA via the TAT peptide and capable of binding biotin. To assess DNA binding, we performed an electrophoretic mobility shift assay (EMSA). When DNA and TAT-SA are complexed together at a 25-fold molar excess of TAT-SA, there is a slight retardation in migration of DNA through the agarose gel as determined by ethidium bromide staining. When TAT-SA is present at a 50-fold molar excess, the ethidium bromide signal is lost entirely. Molar excesses of up to 200 were assayed and showed a similar loss of signal (data not shown). This is likely due to an inability of ethidium bromide to intercalate between base pairs of DNA condensed onto the positively charged TAT peptides (158). In contrast, when DNA is combined with as much as a 200-fold excess of WT SA there is no loss of ethidium bromide signal and no retardation of DNA migration through the gel (Fig. 4.2b). Thus, the addition of TAT to the SA molecule enables DNA binding.

To determine if TAT-SA was capable of binding biotin, we performed a quenching assay using the molecule biotin-4-fluorescein (B4F), as previously described (159). Unbound B4F fluoresces. However, upon binding to SA, B4F undergoes a conformational change that results in loss of fluorescence. As seen in Fig. 4.2c, as the concentration of SA (gray line) is increased (while maintaining a constant concentration of B4F), fluorescence drops steadily until it reaches a baseline, background level.
Fluorescence quenching following incubation with TAT-SA (black line) follows an identical trend. Thus, TAT-SA appears to bind biotin as well as WT SA.

**TAT-SA mediates internalization of siRNA in a charge ratio-dependent manner**

We next assessed the ability of TAT-SA to mediate targeted uptake of nucleic acid *in vitro* into T cells via CD7. We used the human T cell line, MOLT-13, which highly expresses CD7 (Fig. 4.3a). Given the much larger size of DNA to TAT-SA (Fig. 4.1b), we initially investigated TAT-SA-mediated uptake of siRNA. We hypothesized that the charge ratio formed by DNA/TAT-SA complexes would be important in mediating uptake and were interested in examining this effect with the 21 bp siRNA. To test this, we coated MOLT-13 cells in biotinylated anti-CD7 or isotype control antibodies, then incubated them with complexes of TAT-SA, followed by Cy3-labeled siRNA. These incubations were performed at 4°C to prevent internalization. The TAT-SA was present at either a 25X molar excess (leading to a positive charge ratio of 16.67) or at an equimolar concentration, (yielding a negative charge ratio of 0.67). After a 4 hour incubation at 37°C, we found that the labeled siRNA was only present in cells coated with the anti-CD7 antibody (not the isotype control antibody) and only when the TAT-SA was present in excess of the siRNA (Fig. 4.3b,c).

**TAT-SA mediates internalization of DNA via the anti-CD7 antibody, but not gene expression**

Since our primary interest is the targeted delivery of DNA, rather than siRNA, we next assessed the ability of TAT-SA to mediate uptake of labeled DNA to MOLT-13 T
cells. For these experiments, we first formed TAT-SA/αCD7 dimers by combining a 2X molar excess of biotinylated anti-CD7 antibody with TAT-SA. We chose to form dimers, rather than tetramers, because we wanted to ensure that all antibody would be bound to TAT-SA. We were concerned that free antibody would compete with TAT-SA/αCD7 for binding to cells, and reasoned that given the high affinity of antibody-Ag interactions, having two antibodies present on TAT-SA complexes would be sufficient for successful binding.

We incubated TAT-SA/αCD7 dimers or TAT-SA alone with Cy5-labeled pDNA at a charge ratio of 2 (p/n). We chose this charge ratio because the siRNA experiments confirmed that negative charge ratios inhibit uptake, possibly due to repulsion of negatively charged complexes by the negatively charged phospholipids on the cell surface. However, we wanted a charge ratio low enough to minimize non-specific uptake (150). As our plasmid, we used pmaxGFP, a 3,486 bp plasmid that robustly expresses green fluorescent protein (GFP). To assess the ability of TAT-SA/αCD7 to bind to cells, we incubated complexes with MOLT-13 cells at 4°C, a temperature that inhibits internalization. We washed samples with DNAse and heparin, which degrade DNA and cause dissociation of complexes from the cell surface, respectively, to ensure that the Cy5 signal we were detecting derived from binding at the cell surface, rather than internalization. We determined that both TAT-SA alone and TAT-SA/αCD7 bound to cell surfaces when carrying labeled DNA. The DNAse/heparin treatment showed that at 4°C there was minimal internalization of complexes, but slightly more internalization with TAT-SA/αCD7, than with TAT-SA alone (Fig. 4.4a).
When this same experiment was carried out at 37°C to permit internalization, however, differences between the TAT-SA and TAT-SA/αCD7 groups emerged (Fig. 4.4b). Without DNase/heparin treatment, there was Cy5 signal in both groups, but substantially more in the TAT-SA/αCD7 group. The TAT-SA/αCD7 samples treated with DNase/heparin showed no diminution of Cy5 signal, indicating that all of the complexes were internalized and thus not exposed to the DNase and heparin. However, the TAT-SA samples treated with DNAse/heparin showed significant loss of Cy5 signal, demonstrating that the majority of the signal seen was from DNA present on the cell surface and not internalized. Thus, while TAT-SA and TAT-SA/αCD7 both bind to the cell surface when carrying DNA, TAT-SA doesn’t mediate significant internalization, while TAT-SA/αCD7 does. We postulate that TAT-SA binds to the cell surface via free TAT arms, which are present given the excess of TAT-SA to DNA. However, the TAT is likely unable to mediate significant non-specific internalization due to insufficient free TAT. TAT-SA/αCD7, however, takes advantage of the specific anti-CD7/CD7 interaction to mediate internalization, which likely accounts for the ability of these complexes to achieve internalization of DNA.

We next wanted to determine if TAT-SA transfections could result in gene expression. For these experiments we used unlabeled pDNA that either encoded GFP or eff. For both experiments, DNA was complexed with TAT-SA or TAT-SA/αCD7 dimers at a charge ratio of 2. MOLT-13 cells were then transfected with the TAT-SA or TAT-SA/αCD7 complexes. In addition, a group of cells were transfected with lipofectamine as a positive control. While lipofectamine transfection resulted in GFP or eff expression,
neither TAT-SA nor TAT-SA/αCD7 transfections resulted in any detectable gene expression (Fig. 4.4c, 4.4d).

**TAT-SA/pMHC tetramers are functional but do not mediate gene expression in Ag-specific cells**

Based on the success of pMHC tetramer toxins (85,87,88), we produced a TAT-SA/pMHC tetramer for potential DNA delivery. We used the FliC pMHC II monomer, a biotinylated I-A\(^b\) molecule presenting the FliC peptide (amino acids 427-441 of the Salmonella flagellin protein (160)), to produce TAT-SA/pMHC tetramers. We tested the ability of TAT-SA/pMHC tetramers to bind target cells in a competition assay with conventional PE-labeled tetramers using splenocytes from the SM1 mouse, a TCR transgenic mouse in which all CD4+ T cells are specific for FliC presented by I-A\(^b\). We stained SM1 splenocytes with SA-PE tetramer combined with increasing amounts of TAT-SA tetramer, hypothesizing that the TAT-SA/pMHC tetramer would compete for binding to SM1 cells, resulting in a loss of PE signal that would increase with the relative concentration of TAT-SA tetramer. We found this to be the case, demonstrating that the TAT-SA/pMHC tetramer bound target cells (Fig. 4.5).

We next assessed whether TAT-SA/pMHC tetramers could transfect Ag-specific cells. We transfected SM1 cells, WT C57BL/6 cells (the majority of which would not be specific for FliC/I-A\(^b\)), or an equal mixture of SM1 and WT cells with TAT-SA alone or TAT-SA/pMHC tetramers complexed with pmaxGFP at a charge ratio of 2. We added DNA alone to cells as a negative control and nucleofected cells with DNA as a positive control. The SM1 cells expressed the congenic marker CD45.1 and could thereby be
distinguished from WT cells, which were negative for CD45.1. We detected no GFP expression in cells treated with either TAT-SA or with TAT-SA/pMHC tetramers (Fig.4.6).

**DISCUSSION**

Targeted gene delivery to T cells, or to Ag-specific T cells, is an exciting concept with widespread applicability to the study and treatment of human disease. We explore using a bifunctional TAT-SA fusion protein to mediate targeted gene delivery to a human leukemic T cell line and to Ag-specific mouse primary T cells. The TAT-SA approach is attractive due to its extraordinary flexibility. Any targeting moiety could be used, provided it is biotinylated (a straightforward chemical modification) and binds to an internalizable receptor. T cells are not by any means the only cells that could potentially be targeted. Furthermore, any plasmid or other nucleic acid could be delivered without requiring any modification to the TAT-SA scaffold. This allows for gene silencing as well as the potential delivery of encoded toxins, cytokines, growth factors, reporters, transcription factors, or nearly any other proteins of interest. DNA vaccines could be delivered directly to cells of interest, for instance.

We produced TAT-SA and show that it was indeed bifunctional, capable of binding to both DNA and biotin (Fig. 4.2). We also found that TAT-SA/αCD7 mediated uptake of both siRNA and pDNA into CD7+ T cells, while TAT-SA alone did not (Fig. 4.3, 4.4). Despite uptake of pDNA into CD7+ cells, we did not observe any gene expression. We formed functional TAT-SA/pMHC tetramers (Fig. 4.5), but likewise achieved no gene expression in Ag-specific T cells using these tetramers (Fig. 4.6).
It is interesting that TAT-SA alone appears capable of binding to cell surfaces when complexed with DNA, but that it does not efficiently mediate internalization of that DNA. We postulate that this may be related to the charge ratio (2, p/n) at which we conducted those experiments. Ignatovoich et al. (150) found that non-specific uptake of DNA into cells via TAT alone was dependent on charge ratio. In particular, free TAT peptide was involved in mediating the internalization, requiring an excess of TAT and thus a positive charge ratio. We reasoned that a similar phenomenon might occur with TAT-SA and wanted to avoid the non-specific uptake associated with higher charge ratios. However, we found that at negative charge ratios, we lost internalization of nucleic acid (Fig. 4.3), possibly due to repulsion between complexes and negative membrane phospholipids. Our data indicate that a charge ratio of 2 strikes an appropriate balance, allowing for receptor-mediated internalization, but minimizing non-specific uptake. Likely at this charge ratio, there is enough excess TAT to allow complexes to adhere to cell surfaces, but not enough to mediate significant non-specific internalization.

There are a number of possible reasons for poor gene expression despite robust plasmid uptake. Once the complexes have been internalized, the DNA must escape from the endosome into the cytosol and then enter the nucleus. Future studies are needed to determine which of these steps TAT-SA-based delivery fails, so that appropriate steps can be taken to overcome the obstacles present.

Studies using TAT-SA to deliver biotinylated cargo found that a large percentage of the protein was sequestered in endosomes, a problem circumvented by addition of the endosomal releasing polymer, poly(propylacrylic acid) (PPAA) to complexes (148,161).
This problem could also potentially be overcome by modifying the TAT to contain HA2, a 20 amino acid portion of the influenza hemagglutinin protein known to direct transport of DNA out of endosomes (162). A TAT-HA2 fusion protein has already been shown to enable enhanced escape from macropinosomes (163). If entry to the nucleus is impaired, a nuclear localization signal could be appended to the TAT peptide to aid in translocation (164). Additionally, more work could be done investigating the use of siRNA, rather than pDNA, which has fewer barriers to functionality. In conclusion, using TAT-SA for targeted gene delivery has exciting potential, but ultimately, more work is needed before this technology becomes a reality.

MATERIALS AND METHODS

**TAT-SA protein production and purification.** Electrocompetent BL21(DE3) *E. Coli* were transformed via electroporation with the pJExpress vector containing the TAT-SA sequence. We produced TAT-SA solubly based on a protocol for soluble SA production published by Gallizia *et al* (165). One liter of sterile TP media (20 g tryptone, 2 g Na₂HPO₄, 1 g KH₂PO₄, 8 g NaCl, 15 g yeast extract in 1 L water) was seeded with 10 ml overnight culture of TAT-SA-transformed BL21(DE3) and grown at 25°C with 250 rpm shaking until the OD600 reached 0.8. Next, protein production was induced for 4 hours using 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG). Cells were then pelleted, resuspended in ice-cold water, and sonicated for 3-4 1 minute cycles (1 second on, 1 second off) on ice. Samples were then centrifuged at 16,000xg for 20 min. at 4°C and the soluble fraction was saved and filtered through a 0.45 μm filter.
Filtered samples were affinity purified using a column containing iminobiotin agarose (Pierce, Rockford, IL). The column was equilibrated in 50 mM ammonium carbonate, 500 mM sodium chloride, pH 11 and TAT-SA was eluted in 50 mM ammonium acetate, 500 mM sodium chloride, pH 4 before being immediately dialyzed into PBS and stored at 4°C. Recombinant WT streptavidin was purchased from Sigma-Aldrich (St. Louis, MO).

**SDS-PAGE.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of TAT-SA and commercial recombinant SA was performed. SDS and 2-mercaptoethanol were added to samples and they were boiled for 5 minutes before being loaded on a NuPAGE 4-12% Bis-Tris gradient gel (Invitrogen, Carlsbad, CA) and run for 1 hour at 150 V. Gels were stained with SimplyBlue SafeStain (Invitrogen) for protein visualization.

**Electrophoretic mobility shift assay.** A 0.7% agarose gel was pre-run at 93V for 1 hour at 4°C to remove any contaminants that might alter migration of TAT-SA/DNA complexes. TAT-SA/DNA complexes were formed by incubation of 120 ng of the pmaxGFP plasmid with TAT-SA or WT SA at variable molar ratios for 30 min. at 25°C. Complexes were then electrophoresed on the pre-run gel at 93 V for 100 minutes at 25°C in the absence of loading dye. Following electrophoresis, gels were stained with 50 ml of 0.6 μg/ml ethidium bromide for 15 minutes and analyzed using a UV light box.
**Biotin binding assay.** Biotin binding was assessed using biotin-4-fluorescein, as described by Kada *et al* (159). Varying concentrations of TAT-SA or WT SA were combined with 5.15 ng B4F in 1 ml total volume of a buffer containing 100 mM NaCl, 50 mM NaH$_2$PO$_4$, 1 mM EDTA (pH 7.5 with NaOH) and 0.1 mg/ml BSA. From each sample, 300 μl aliquots were pipetted into a 96-well plate and incubated for 30 min. at 25°C protected from light. Fluorescence was measured on a 96-well plate reader (485 nm excitation wavelength, 525 emission wavelength).

**MOLT-13 cell transfections.** All work was completed using sterile reagents and equipment. MOLT-13 is a human T cell acute lymphocytic leukemic cell line. These cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 5% penicillin/streptomycin (P/S) at 37°C with 5% CO$_2$. During transfections, cells were maintained in unsupplemented RPMI 1640 (no FBS or P/S).

For siRNA transfections, 1.5×10$^6$ cells were suspended in 160 μl cold media in FACS tubes and incubated with 1.6 μl biotinylated anti-CD7 or isotype control antibody (biotinylated mouse IgG1, kappa) for 20 min. at 4°C, then washed with cold media. Cells were then incubated with varying amounts of TAT-SA for 20 min. at 4°C, and washed again. Finally, 100 pmol of Cy3-labeled GAPDH siRNA (Invitrogen) was added to the cells, they were incubated at 4°C for 20 min., washed again with cold media, then plated in a 6-well plate and placed at 37°C in a tissue culture incubator. Lipofectamine (Invitrogen) transfections were carried out according to the manufacturer’s instructions using 100 pmol siRNA. After a 4-hour incubation, cells were harvested and analyzed via flow cytometry for uptake of Cy3-labeled siRNA on a BD FACSCalibur flow cytometer.
using BD CellQuest Pro software (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, Inc., Ashland, OR).

For transfections with Cy5-labeled DNA, the pmaxGFP plasmid (Lonza, Allendale, NJ) was labeled using the Label IT Cy5 Labeling Kit (Mirus, Madison, WI), following the manufacturer’s instructions. TAT-SA/αCD7 complexes were formed by combining a 2X molar excess of biotinylated anti-CD7 antibody (eBiosciences, San Diego, CA) with TAT-SA and incubating for 1 hour at 25°C followed by a 12-hour incubation at 4°C. TAT-SA/αCD7 complexes or TAT-SA alone was then added to 1 μg labeled DNA (at a charge ratio of 2) and incubated at 25°C for 45 min. Complexes were added to 1.5x10⁶ cells in 200 μl RPMI 1640 and incubated at 4°C or 37°C for 4 hours. Lipofectamine (Invitrogen) transfections were carried out according to the manufacturer’s instructions using 1 μg DNA. After incubation, cells to be treated with DNase/heparin were then washed in 250 μl FACS buffer with 1.25 μl heparin three times, washed in FACS buffer, and incubated in 250 μl FACS buffer with 2 μl DNase for 10 min at 25°C. All cells were then washed in FACS buffer and analyzed via flow cytometry for uptake of Cy3-labeled siRNA on a BD FACSCalibur flow cytometer using BD CellQuest Pro software (BD Biosciences) and FlowJo software (Tree Star, Inc.). Transfections to determine GFP gene expression occurred as above except pmaxGFP was unlabeled, cells were not treated with DNase/heparin, and cells were incubated for 48 hours prior to analysis.

Transfections assaying luciferase expression used a MC DNA encoding the eff reporter. MC DNA is circular DNA that lacks a bacterial backbone and thus achieves higher levels of transgene expression (65). It is explored in more detail in chapters 2 and
3 of this thesis. Briefly, to produce the MC-eff we amplified eff from the pUltra Bright eff luciferase+ plasmid (generously provided by Patrick Hwu, MD Anderson Cancer Center (Houston, TX) (102) and inserted it into a vector containing the hUbC promoter (kindly provided by Michael Kyba, University of Minnesota, Minneapolis, MN). For MC generation, bacteria transformed with parental pDNA were grown overnight in Terrific Broth supplemented with kanamycin (Invitrogen). The following day MC induction media (fresh LB broth containing 0.04 volumes of 1 N NaOH and 0.02% L-arabinose (Sigma-Aldrich) were added and the culture temperature was decreased from 37°C to 32°C for 5–8 hours. The culture was centrifuged and MC DNA was purified with the PureLink HiPure Maxiprep Kit using buffers from the Purelink HiPure BAC Buffer Kit (Invitrogen). Transfections using MC-eff occurred just as for transfections with pmaxGFP. For analysis of luciferase expression, the Luciferase Assay System (Promega, Madison, WI) was used according to the manufacturer’s instructions.

**Tetramer competition assay and mouse primary cell transfections.** All work was completed using sterile reagents and equipment. TAT-SA/pMHC tetramers were preformed by combining 95 μl (1.9 mg, 61 μM biotin) FliC/I-Aβ monomer (kindly provided by Marc Jenkins, PhD, University of Minnesota, Minneapolis, MN) with 10 nM TAT-SA in 100 μl and incubating for 1 hour at 25°C and 12 hours at 4°C. For the competition assay, splenocytes from a SM1 TCR transgenic mice (a generous gift from Stephen McSorley, PhD, UC Davis, Davis, CA) were incubated with SA-PE tetramer (also a gift from Dr. McSorley) and TAT-SA tetramer at varying concentrations, then analyzed by flow cytometry for binding of the SA-PE tetramer.
For transfections, TAT-SA tetramers were formed as above, then incubated with 1 μg pmaxGFP (at a charge ratio of 2) for 30 min. at 25°C. Splenocytes were isolated from a CD45.1+ SM1 mouse or a CD45.1- C57BL/6 mouse, single cell suspensions prepared in RPMI 1640, and red blood cells lysed. DNA, DNA and TAT-SA, or DNA and TAT-SA/pMHC tetramer were added to 3x10⁶ cells in 300 μl and incubated for 20 min. at 4°C. Cells were transferred to 2 ml RPMI 1640 with 10% FBS in a 6-well plate and incubated at 37°C for 24 hours. Nucleofections were performed using the Amaza Mouse T Cell Nucleofector Kit (Lonza) according to the manufacturer’s instructions. Flow cytometry was then performed to detect GFP+ cells using a BD FACSCalibur flow cytometer with BD CellQuest Pro software (BD Biosciences). FlowJo software was used for data analysis (Tree Star, Inc.).

**Mice.** Four to eight week old female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) or the National Cancer Institute (Frederick, MD). All mice were housed under specific pathogen free-conditions at the University of Minnesota (Minneapolis). All animal procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee.
**Figure 4.1. Schematic of TAT-SA delivery platform.** (a) TAT-SA (not drawn to scale) contains the tetrameric core SA with TAT peptides appended to each monomer but retains the ability to bind biotinylated targeting moieties. (b) Approximate representation of relative size of TAT-SA to plasmid DNA. A 3000 bp plasmid is approximately 40 times more massive than TAT-SA and 200 times more negatively charged than TAT-SA is positively charged.
**Figure 4.2. TAT-SA can be produced and is functional.** (a) Reducing SDS-PAGE analysis of TAT-SA (15 kDa) and WT recombinant core SA (13.2 kDa). (b) Agarose gel electrophoresis analysis of DNA (pmaxGFP) and TAT-SA complexes formed at the indicated molar (MR) and charge (CR) ratios. DNA incubated with WT SA was used as a negative control. (c) Fluorescence quenching of B4F in the presence of WT SA (gray line) or TAT-SA (black line). WT SA or TAT-SA were incubated at the indicated concentrations with 8 nM B4F and 0.1 mg/ml BSA for 30 min and fluorescence was measured (485 nm excitation wavelength, 525 nm emission wavelength). Samples were prepared in triplicate.
**Figure 4.3. TAT-SA mediates uptake of siRNA into MOLT-13 cells via the anti-CD7 antibody in a charge ratio-dependent manner.**

(a) Histogram demonstrating CD7 expression on MOLT-13 cells. Cells were untreated (shaded), or treated with either a biotinylated anti-CD7 antibody (red, bold) or a biotinylated isotype control antibody (blue) followed by SA-PE. (b) Flow cytometric analysis of siRNA uptake. Cells were incubated with an anti-CD7 (left panel) or an isotype control (middle panel) antibody, followed by TAT-SA, then Cy3-labeled siRNA (25X excess of TAT-SA to siRNA) at 4°C to prevent internalization and enable formation of complexes at the cell surface. Cells were then incubated at 37°C for 4 hours and analyzed Cy3 expression by flow cytometry. Lipofectamine was used to transfect cells as a positive control (right panel). (c) Histograms showing Cy3 expression in MOLT-13 cells following transfections as in (b).
using TAT-SA and an anti-CD7 antibody (bold, red) or an isotype control antibody (blue) at a positive charge ratio (left panel) or a negative charge ratio (right panel).
Figure 4.4. TAT-SA mediates binding and internalization of DNA into MOLT-13 cells via the anti-CD7 antibody but not gene expression. (a) Binding of TAT-SA/αCD7 complexes to the cell surface. MOLT-13 cells were incubated with pre-formed TAT-SA/αCD7 dimers complexed with Cy5-labeled pmaxGFP plasmid (top row) or TAT-SA/DNA complexes without antibody (bottom row) at 4°C to prevent internalization. Samples were treated (right column) or untreated (left column) with DNAse and heparin to degrade and dissociate DNA on the cell surface. (b) Internalization of TAT-SA/αCD7 complexes. MOLT-13 cells were incubated with pre-formed TAT-SA/αCD7 dimers complexed with Cy5-labeled pmaxGFP plasmid (top row) or TAT-SA/DNA complexes without antibody (bottom row) at 37°C to enable internalization. Samples were treated (right column) or untreated (left column) with DNAse and heparin to degrade and dissociate DNA on the cell surface. (c) Flow cytometric analysis of GFP expression in MOLT-13 cells transfected with 1 μg pmaxGFP using lipofectamine as a positive control (left panel), TAT-SA/αCD7 preformed complexes (middle panel), or TAT-SA with no targeting antibody (right panel). (d) Luciferase expression in relative light units (RLU).
MOLT-13 cells transfected with 1 μg of luciferase-encoding DNA using lipofectamine, TAT-SA/αCD7 preformed complexes, or TAT-SA with no targeting antibody.
Figure 4.5. TAT-SA pMHC tetramers bind to Ag-specific target cells. (a) Gating strategy for detecting FliC-specific CD4+ T cells bound to SA-PE tetramer. Splenocytes from SM1 mice were stained with FliC SA-PE tetramer. Forward and side scatter parameters were used to gate on lymphocytes (left most plot). CD4+ T cells were determined to be gated lymphocytes that expressed CD4 but did not express CD11b, CD11c, B220, or class II MHC (middle plot). Tetramer+ cells were gated CD4+ T cells that were positive for PE (right most plot). (b) Flow cytometric analysis of TAT-SA competition for SA-PE tetramer binding to SM1 cells. The FliC-specific TAT-SA tetramer was combined with the SA-PE tetramer at the indicated molar ratios (TAT-SA tetramer : SA-PE tetramer) and incubated with freshly isolated SM1 splenocytes. (c) PE median fluorescence intensity from (b), normalized to the sample with no TAT-SA tetramer (MR = 0).
Figure 4.6. Absence of gene expression following FliC-specific TAT-SA tetramer transfections of SM1 cells. Flow cytometric analysis of GFP expression in splenocytes from SM1 or C57/BL6 mice transfected with 1 μg pmaxGFP using no transfection agent (DNA only, first column), nucleofection (second column), TAT-SA/pMHC tetramer (third column), or TAT-SA without a targeting moiety (fourth column). Transfected cells were splenocytes from CD45.1+ SM1 mice (first row), CD45.1- WT C57BL/6 mice (second row), or an equal mixture of SM1 and WT cells (third row).
CHAPTER 5: Conclusions

Since Edward Jenner first used scrapings from cowpox blisters to provide protection against smallpox in the 1770s, vaccines have saved countless lives. The knowledge that weakened or inactivated pathogens, and even pathogen-derived particles, could be used to confer protective immunity spurred the development of dozens of vaccines leading to the eventual eradication of smallpox from the globe and the near eradication of polio. After clean drinking water and antibiotics, vaccination is often cited as the most cost-effective and life-saving intervention in the history of medicine (19,20).

Unfortunately, however, there are a number of pathogens for which developing efficacious vaccines has been difficult: HIV, influenza, hepatitis C, tuberculosis, and malaria, among others. For these diseases, more sophisticated approaches and a more extensive understand of the biology of immune responses will be necessary to produce an effective vaccine (20). To that end, this thesis has examined one promising immunization approach, the use of DNA vaccines. In particular, we investigated a method for improving the immunogenicity of DNA vaccines by prolonging and enhancing Ag expression and studied the effect of prolonged Ag expression on immune responses. In addition, we discussed a method for augmenting or manipulating T cell responses via targeted DNA delivery.

The second chapter of this thesis demonstrated the use of MC DNA as a DNA vaccine. MC DNA lacks nearly all extragenic DNA, thereby avoiding silencing and resulting in enhanced and prolonged gene expression. Although MC DNA had been
shown to be effective in many experimental models, it had never before been tested as a vaccine. An important distinction between the previous models using MC DNA and our vaccine experiments is the ambiguity about whether prolonged Ag expression in a vaccine would be beneficial for immune responses. Previous models involving gene therapy or gene knockdown have rested on the certainty that prolonged gene expression is optimal for successful therapy. However, when using MC DNA as a vaccine, the situation was less clear. Prolonged Ag expression during chronic infections in both mice and humans leads to exhausted CD8+ T cells, rather than protective memory cell formation (13). Furthermore, studies using *Listeria monocytogenes* showed that for successful priming of CD8+ T cell responses to occur, only 24 hours of exposure to Ag was required (9).

We hypothesized that the situation with a MC DNA vaccine would differ from an infection due to the lower levels of Ag and inflammation present in a vaccine compared to an infection. Therefore, we predicted that immunizing with MC DNA would improve the immunogenicity of DNA vaccines since poor Ag expression is known to occur following DNA vaccination and MC DNA would generate increased levels of Ag. As other groups had observed, we obtained significantly higher levels of gene expression and more prolonged gene expression using MC DNA compared to FL DNA (Fig. 2.3). Furthermore, consistent with our hypothesis, we found that MC DNA vaccination yielded significantly increased frequencies of Ag-specific CD8+ T cells in the peripheral blood compared to FL DNA vaccination (Fig. 2.8). In addition, immunization with MC DNA provided superior protection following an infectious challenge with *Listeria* compared to FL DNA (Fig. 2.9).
The knowledge that MC DNA can be used to improve the immunogenicity of DNA vaccines is valuable. Low immunogenicity has plagued DNA vaccines in clinical trials, limiting their success despite the many advantages they have and the great promise they have shown in preclinical models (31). MC DNA is nearly as straightforward and easy to produce as standard pDNA (71), and therefore offers a simple way to circumvent the low immunogenicity of DNA vaccines. However, it is perhaps more important to understand why MC DNA generates superior CD8+ T cell responses so that these mechanisms could potentially be more fully exploited in vaccine design.

The reduced size of MC DNA likely aids in vector uptake, increasing transfection efficiency (66). However, the absence of a large extragenic portion also abolishes transcriptional silencing mechanisms normally responsible for the rapid decline in gene expression following transfection of pDNA into mammalian cells (64,67). These two attributes together accounted for the increase in gene expression seen with MC DNA transfection. However, the reason why the increase in gene expression seen with MC DNA vaccines yielded a superior CD8+ T cell response compared to pDNA vaccines was less clear, and was the subject of chapter 3 of this thesis.

As seen in Fig. 2.3, MC DNA vaccination yields both higher absolute levels of gene expression and an increased duration of gene expression. We were interested in whether the prolonged gene expression was necessary, or even beneficial, for the CD8+ T cell response generated by MC vaccines. By immunizing mice on the ear and excising the ears at various time points, we regulated duration of Ag expression. We found that the prolonged Ag expression associated with MC DNA vaccines is an important component contributing to their efficacy. Removing the ear at any time point tested,
including 22 days after the last immunization, resulted in a diminished ability to clear infection with *Listeria* (Fig. 3.4d). In animals with ears excised at later time points, such as day 22, this effect did not appear to derive from a diminished pool of responders prior to infection (Fig. 3.3c). Rather, the cells failed to proliferate as robustly following challenge (Fig. 3.4 a-c), indicating a functional deficit. On-going experiments will determine whether other functional deficits exist as well, such as a diminished ability to secrete IFN-γ, TNF-α, or granzyme B.

It is unclear what advantage persistent Ag confers on CD8+ T cells in our model. In chronic infection, persistent Ag induces exhaustion, rather than improving immune responses. However, following DNA vaccination, there is significantly less Ag and inflammation, which may account for the difference we see. Nevertheless, further experiments would be needed to determine the role that Ag is playing in enhancing the ability of T cells to respond to challenge. It would be important to know whether Ag presentation was still occurring at the time of infection in mice without ear excision. It may be that the presence of vaccine-encoded Ag in LN at the time of challenge allows for more robust expansion upon infection.

Whether or not the effect we see is dependent on Ag being present at the time of infection is a separate question from why prolonged Ag expression, either continuing until the point of infection or merely lasting well past the prime but terminating before challenge, is beneficial for the secondary response. As mentioned above, this is a novel finding, not seen in infectious settings, where prolonged infection is detrimental. Therefore, it may be that the prolonged Ag may compensate for weak or suboptimal Ag
stimulation early in priming, explaining why it would be beneficial in a weakly immunogenic DNA vaccine setting as compared to an infection.

The finding that excision before the peak of the CD8+ T cell response leads to early and more severe contraction (Fig. 3.3) is also interesting as it contradicts the finding that only 24 hours of Ag stimulation pre-programs the kinetics of CD8+ T cell expansion and contraction. We hypothesize that the difference in this case is also the level of Ag expression and/or inflammation. The presence of high levels of Ag and inflammation that occur during natural infection likely initiate a program of expansion and contraction that weaker immunizations may be insufficient to initiate as fully. In those cases then, there would be room for additional manipulation and augmentation of the response after the early initiating stimulus. This indicates that one must use caution when applying principles learned from infection experiments to vaccine experiments, particularly vaccines not using pathogen. Our data provide support for the idea that low immunogenicity can be overcome after the peak by continued Ag expression.

Of course, another solution would be to increase levels of Ag at the peak of expression, which MC DNA also generally does due to its reduced size. When we immunized with a FL vector that had similar levels of gene expression at the peak as MC, we found no differences in frequencies of CD8+ T cells or protection against *Listeria* (Fig. 3.8). This indicates that expression levels at the peak are likely also an important contributor to the development of protective immune responses. Further work is needed to delineate which approach, prolonged Ag expression or enhanced peak expression, is better at augmenting CD8+ T cell responses.
Furthermore, it remains to be determined whether the difference seen in our vaccination model and in infectious settings has to do specifically with levels of inflammation, levels of Ag, kinetics of T cell exposure to Ag and/or inflammation, or some combination of the above. It is possible that a vaccine generating high levels of Ag in the presence of low levels of inflammation would yield results very different than a vaccine that produced low levels of Ag and high levels of inflammation. Moreover, those results might change if the Ag or inflammatory stimuli were introduced to the host rapidly or slowly, over the course of weeks as can happen with certain DNA vaccine delivery methods.

In addition to investigating methods of improving DNA vaccines by overcoming transient transgene expression, this thesis explores a method for manipulating T cell responses with the long-term goal of augmenting T cell responses to improve vaccination. We found that while our TAT-SA construct mediated uptake of DNA, we were unable to achieve gene expression (Fig.4.4, 4.6). Despite this setback, this approach is a promising one worthy of further investigation. Gene targeting to DCs has already been shown to improve the efficacy of DNA vaccines (77), but no one has yet been able to test the effect of manipulating how T cells respond to Ag to generate optimal immune responses. Such an approach would have applications not just in vaccine development, but potentially in autoimmunity, graft rejection, GVHD, and cancer therapy. To reach these goals, however, much work must be done.

For our system, initial experiments would be needed to determine at what stage expression fails to occur: endosomal escape, entry to the nucleus, transcription, or translation. Modifications to overcome the barrier or barriers preventing expression
would then need to be integrated into the platform. Transitioning a product that functioned *in vitro* to one that functioned *in vivo* would potentially involve additional modifications. However, it is promising that SA-based tetramer depletion approaches have functioned *in vivo* (85,87,88).

The work in this thesis provides useful knowledge for the field of DNA vaccine design. In it, we show that one of the major obstacles hindering the translation of DNA vaccines to human use, poor immunogenicity, can be improved by using MC DNA instead of pDNA. MC DNA retains the advantages of pDNA, including ease and affordability, but drives stronger immune responses. Secondly, we demonstrate that the prolonged Ag expression associated with MC DNA is a necessary component contributing to its enhanced immunogenicity. This finding has important implications for vaccine design in general, which has often focused on early Ag levels, but also provides interesting insights into the nature of CD8+ T cell responses. Future avenues of research could further explore the limits that determine when responses are pre-programmed and when they are sensitive to on-going Ag expression, as well as differentiating the roles of Ag and inflammation. Lastly, this work provides a potential method for targeted gene delivery to T cells, a goal with a myriad of promising applications.
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