Regulating the regulators: Using CD25 depletion to enhance immune responses to a model plasmid-based vaccine

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

REGULATING THE REGULATORS: USING CD25 DEPLETION TO ENHANCE IMMUNE RESPONSES TO A MODEL PLASMID-BASED VACCINE

presented by Michelle C. Thoma, a candidate for the degree of Master of Science and hereby certify that, in their opinion, it is worthy of acceptance.

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Professor George P. Smith
DEDICATION

I dedicate this work to my loving parents, Ronald and Ellen Thoma, who have always been there to support me and to nurture me in my academic endeavors. Their wisdom has guided me through the good times and the not-so-good times, and my successes both scholarly and otherwise are a testament of their wonderful parenting. I love you both. Words cannot begin to express my appreciation. You are the most wonderful examples of what it means to be truly good people.

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Additionally, I dedicate this work to all of my wonderful friends. There are too many to name, but I love you all so much, and each of you has a special place in my heart. You are always there for me when I need you, and I hope that I am able to give you as much love and support in return. I would like to extend a special thank-you to Nathan McLeod and Curtis Pritzl, who have been my pillars of strength over the course of my graduate work, celebrating my successes and lifting me up during the rough times.

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<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate N-succinimidyl ester</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<tr>
<td>CTLA4</td>
<td>cytotoxic T lymphocyte antigen 4</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>ds</td>
<td>double stranded</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ELISpot</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Foxp3</td>
<td>forkhead/winged helix transcription factor</td>
</tr>
<tr>
<td>FR4</td>
<td>folate receptor 4</td>
</tr>
<tr>
<td>gB</td>
<td>glycoprotein B</td>
</tr>
<tr>
<td>GITR</td>
<td>glucocorticoid-induced tumor necrosis factor-receptor-related protein</td>
</tr>
<tr>
<td>GP</td>
<td>glycoprotein</td>
</tr>
<tr>
<td>HBsAg</td>
<td>hepatitis B surface antigen</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s buffered salt solution</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>HSV</td>
<td><em>herpes simplex virus</em></td>
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<tr>
<td>i.m.</td>
<td>intramuscular</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>ICCS</td>
<td>intracellular cytokine staining</td>
</tr>
<tr>
<td>IDO</td>
<td>indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iTReg</td>
<td>inducible T regulatory cell</td>
</tr>
<tr>
<td>LAG3</td>
<td>lymphocyte activating gene 3</td>
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</table>
LB    Luria-Bertani  
LCMV  lymphocytic choriomeningitis virus  
MHC   Major histocompatibility complex  
MOI   multiplicity of infection  
mRNA  messenger ribonucleic acid  
NK    natural killer  
NP    nucleoprotein  
nTreg natural T regulatory cell  
PBS   phosphate buffered saline  
PCR   polymerase chain reaction  
PE    Phycoerythrin  
pen   penicillin G sodium  
Pf/CSP Plasmodium falciparum circumsporozoite protein  
pfu   plaque forming unit  
PLG   polyactide co-glycolide  
RNA   ribonucleic acid  
RPMI  Roswell Park Memorial Institute media  
SARS  severe acute respiratory syndrome  
SCID  severe combined immunodeficient  
strep streptomycin sulfate  
TCR   T cell receptor  
Th1   T helper 1  
Th2   T helper 2  
TH3   T helper 3  
TLR   toll-like receptor  
TNF   tumor necrosis factor  
TR1   T regulatory 1  
Treg  T regulatory cell  
Ts cell T suppressor cell  
WNV   West Nile virus
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ABSTRACT

Due to their ease of production and safety, plasmid-based vaccines have become prime candidates for both prophylactic and therapeutic human vaccination. In experimental animal models, vaccination with plasmid DNA expression vectors has been shown to induce robust cellular and humoral immune responses against a variety of infectious diseases and some cancers. However, DNA vaccines have proven to be weakly immunogenic in human clinical trials. The poor immunogenicity of DNA vaccines in humans necessitates the development of novel methods to enhance both cellular and humoral immune responses against the plasmid-encoded antigen(s). Previous in vivo studies have shown that CD4+CD25+Foxp3+ T regulatory cells can repress antigen-specific immune responses. We demonstrate here that depletion of CD25+ cells prior to plasmid vaccination significantly enhances primary and memory T cell responses and antibody responses to a model DNA vaccine against *Lymphocytic choriomeningitis virus*. If this approach can be safely applied to humans it may not only improve the clinical utility of DNA vaccines but also improve conventional vaccines as well.
CHAPTER 1: T REGULATORY CELLS

Introduction

T regulatory cells (Tregs) are a unique population of CD4+ T cells that benefit the host by maintaining peripheral tolerance and preventing immune-mediated tissue damage caused by an overly exuberant immune response. However, recent evidence has revealed that Tregs can also be detrimental to the host because they can prevent the immune system from adequately responding to an infection or cancer. This review will provide a brief history of Tregs, discuss their characterization, mode of action, and highlight their role during infection and vaccination.

History


R.K. Gershon is credited with the conceptual discovery of suppressor T cells (Ts cells). He described these cells as a unique population of T lymphocytes capable of inhibiting acquired immune responses, both antibody production by B lymphocytes and activation of T lymphocytes (1, 2). At the time, it was thought that Ts cells were able to suppress target cells by secreting antigen-specific suppressive factors encoded by the I-J region of the murine MHC and/or determinants encoded by immunoglobulin genes. T-cell-mediated suppression remained in the foreground of immunological research throughout
the 1970s as researchers in the field enthusiastically sought to identify and characterize the I-J region and the soluble suppressive factors produced by Ts cells.

**Phase II (Early 1980s): The Fall of T-cell-mediated Suppression**

By the early 1980s, however, the field of T-cell-mediated suppression began to be viewed as a sinking ship as many researchers abandoned their quest to characterize these cells. The demise of Ts cells was primarily due to the fact that these cells did not meet any of the requirements of molecular immunology. Researchers had failed to identify a gene or genes encoded within the I-J region (3, 4), messenger RNA for the β-chain of the T-cell receptor (TCR) in Ts hybridomas (5), or unique Ts cell markers. In addition, *in vitro* experiments attempting to determine Ts cell function led many investigators to propose unrealistic and highly complex regulatory circuits that relied upon largely uncharacterized suppressive factors. Fortunately, despite the decline in Ts cell popularity, the concept of T-cell-mediated immune regulation was never fully disregarded.

**Phase III (Mid 1980s-Mid 1990s): The Revival of T-cell-mediated Suppression**

The re-emergence of T-cell-mediated suppression came after researchers began reviewing two series of experiments performed more than 20 years earlier (6, 7). From these findings, came the concept that a deficiency or lack of regulatory T cells could result in autoimmunity.

In the first set of experiments, researchers from the lab of Yasutomi Nishizuka observed that thymectomized neonatal mice developed a wide range of organ-specific autoimmune
disease if the thymus was removed within the first three days of life (6). In addition, it was also found that CD4+ T cells were responsible for disease induction. Because mice thymectomized on day 7 did not develop autoimmune disease, researchers proposed that a population of “regulatory” T cells, capable of suppressing autoimmune T cells in the periphery, developed slightly later in ontogeny than self-reactive CD4+ T cells. Thus, thymectomy on day 3 prevented the migration of these T regulatory cells into the periphery and the subsequent inhibition of self-reactive CD4+ T cells. Based on this postulate, researchers were able to prevent the development of autoimmune disease in the day-3 thymectomized mice by either thymus transplant or by adoptively transferring CD4+ T cells from adult mice. Interestingly, the suppression of autoimmunity in the neonate was highly time dependent as the CD4+ T cell transfer or thymic transplants were only effective if they were performed within the first two weeks after birth.

In a similar set of experiments, W.J. Penhale et al. observed that thymectomy of adult rats, which are normally resistant to autoimmune disease, followed by fractional doses of irradiation to deplete peripheral lymphoid cells resulted in the development of autoimmune thyroiditis (7). In addition, the researchers found that reconstitution of the thymectomized, irradiated rats with lymphoid cells from normal donor rats prevented the development of autoimmune thyroiditis. To establish a role for Ts cells, the authors drew from several observations: (i) in previous studies, researchers found that Ts cells were relatively radiation sensitive as compared to other subpopulations of T cells (8, 9); (ii) a substantial population of T cells remained in the spleens of the rats; and (iii) the rats continued to produce helper-T-cell-dependent antibodies. Based on these observations,
the authors concluded that the development of autoimmune thyroditis was likely due to the fact that the procedure depleted only the most radiation-sensitive subpopulations of T cells, the Ts cells.

Although these experiments strongly supported the existence of a subset of CD4+ T cells capable of suppressing autoimmune CD4+ T effector cells, further progress in the field was hampered by the inability to distinguish regulatory T cells from effector T cells. However, in 1993, James D. Watson’s group showed that suppressor cells could be distinguished from effector cells on the basis of differential expression of CD45RB (10). CD4+ T cells that expressed high levels of CD45RB induced severe wasting disease when transferred into congenic, severe combined immunodeficient (SCID) mice, whereas the cotransfer of purified CD4+CD45RB^{low} T cells prevented disease development. Later that year, Powrie and Mason reported that inflammatory bowel disease could be induced in immunodeficient mice by the adoptive transfer of CD4+CD45RB^{high} T cells, while mice that received CD4+CD45RB^{low} T cells or a mixture of the two cell types failed to develop intestinal inflammation or intestinal lesions (11).

Although progress in understanding the role of regulatory T cells in preventing autoimmunity was made by a number of laboratories, the most important finding of this era was the demonstration by the Sakaguchi group that mouse CD4+ suppressor cells also expressed CD25—the high affinity α chain of the interleukin-2-receptor (IL2-Rα) (12). Transfer of CD4+CD25^{-} T cells from normal adult mice to immunodeficient recipient mice led to the development of a wide array of autoimmune diseases, similar to those
seen in day-3 thymectomized mice. Conversely, transfer of CD4+CD25+ T cells within 10 days of the initial transfer prevented the development of autoimmune disease. The finding that CD4+CD25+ T cells were capable of suppressing autoimmune disease was later confirmed by the Takahashi group, who showed that organ-specific autoimmunity could be induced in normal mice by the injection of a monoclonal antibody against IL-2Rα (13).

Phase IV (Mid 1990s- Present): Continued Progress

While experiments performed in the early 1990s firmly established the existence of a population of CD4+ T regulatory cells (Tregs) capable of regulating the activity of other T cells, many questions regarding the development, phenotypic characterization, and suppressive capabilities of Tregs remained unanswered. Partly due to the technical advances over the last 15 years—which have led to a clearer understanding of dendritic cell-T cell interactions, the biochemical actions of a wide variety of cytokines, new transgenic and knockout animal models, and more sophisticated flow cytometric techniques—some of the missing pieces in the T-regulatory-cell puzzle have been put into place.

Perhaps the most important experiments of the present era have been those identifying the forkhead/winged helix transcription factor Foxp3 as the master regulator of Treg function and as the most specific marker, to date, in distinguishing these cells from conventional T cells. The path that ultimately identified the role of Foxp3 in the biology of the immune response began in 1949 when animals in an inbred mouse line at Oak
Ridge National Laboratory spontaneously developed severe multi-organ autoimmune disease. These animals, termed “scurfy” mice, were born runted; had skin abnormalities on the ears, eyelids, feet and tail; and usually died within three weeks of birth. The mutated gene, which was initially named scurfin, was found to be X-linked, so only male mice were affected (14). The multiple signs of autoimmunity observed in the homozygous male mice were thought to be due to the absence of Tregs. In 2001, the scurfin gene was cloned and shown to encode a protein product that is a member of the forkhead/winged-helix transcription factor family (15). The gene was renamed Foxp3 to designate it as the third member of this protein family. Importantly, expression of the Foxp3 transgene or CD4+CD25+ T cell transfer could prevent the development of disease in scurfy mice. In 2003, the Rudensky group published the most definitive experiments highlighting the importance of Foxp3 in the development and function of CD4+CD25+ Tregs. Using bone marrow reconstitution experiments in irradiated mice, Rudensky and colleagues showed that Foxp3 is required for the development of CD4+CD25+ T cells in the thymus. Additionally, adoptive transfer of Foxp3+ cells could correct the disease syndrome induced by the scurfy mutation. Moreover, ectopically expressed Foxp3 could activate a program of suppression in peripheral naïve CD4+CD25- cells (16). Presently, Foxp3 is still the most definitive signature for identifying thymic derived or “natural” T regulatory cells.
Phenotypic Characterization

Types of CD4+ Treg Cells

Several types of CD4+ T regulatory cells have been characterized on the basis of their origin, generation, and mechanism of suppression. There are two main subsets of CD4+ T regulatory cells: naturally occurring CD4+CD25+ regulatory T cells (natural Treg cells or nTregs), which develop in the thymus and suppress primarily self-reactive T lymphocytes in the periphery, and inducible regulatory T cells (iTregs), which develop in the periphery from conventional CD4+ T cells. Inducible Treg populations include T regulatory 1 (T_{R1}) cells, which secrete IL-10 and are Foxp3-; T helper 3 (T_{H3}) cells, which secrete TGF-β and are Foxp3-; and converted Foxp3+ regulatory T cells. Because Foxp3 is expressed both by natural Tregs and some inducible Tregs, distinguishing between regulatory cells generated in the thymus and those generated in the periphery is difficult.

Natural Treg Cells

Natural Tregs express a wide variety of membrane markers; however, none of these markers are specific for nTregs as they can all be expressed by activated conventional T cells. nTregs are classically defined by their constitutive expression of CD25. They also express cytotoxic T-lymphocyte antigen 4 (CTLA4) and the tumor necrosis factor (TNF)-receptor family members—glucocorticoid-induced TNF-receptor-related protein (GITR) and OX40 (17), as well as CD39, CD73 (18), and high levels of folate receptor 4 (FR4) (19). Other markers present on the surface include lymphocyte activating gene 3 (LAG3), low levels of CD45RB, and neuropilin (20).
Mechanism of Action

nTregs have been shown to suppress the actions of multiple immune cell types, including effector and memory CD4+ and CD8+ T cells (induction and proliferation), B cells (immunoglobulin (Ig) production and class switching), natural killer (NK) and NK T cells (cytotoxicity), dendritic cells (function and maturation), and neutrophils (function and survival) (21). The mechanisms by which nTregs mediate suppression in vivo are poorly understood. Initially, researchers believed that nTregs must make direct contact with either the antigen presenting cell (APC) or the responding T cell to induce suppression (22). However, these in vitro findings have not always held true for in vivo experiments, many of which have shown that secreted cytokines—such as IL-10 and TGF-β—can, in some instances, mediate nTreg-induced suppression (23). Another mechanism of suppression may involve CTLA4 on the surface of the nTreg interacting with a cognate ligand on the surface of the APC, causing the cell to secrete indoleamine 2,3-dioxygenase (IDO), a tryptophan degrading enzyme. A low concentration of tryptophan in the extracellular milieu blocks T cell activation and induces apoptosis among recently activated T cells (24). Additionally, adenosine and cyclic AMP have also been implicated in nTreg-cell-mediated suppression (18, 25). These are just some of the mechanisms by which nTregs may suppress responder cells. As mentioned previously, how nTregs exert suppression on responder cells in vivo remains poorly understood, but this is an area of intense research.
T Regulatory Cells and Infection

In order for the host to survive an infection, it must generate a well-orchestrated, highly controlled immune response that recognizes and eliminates the pathogen while at the same time limiting immune-mediated pathology. Conversely, most invading microorganisms must avoid being cleared by the immune system long enough to be transmitted to a new host. Therefore, immune regulation in the presence of an infection can be initiated by host factors in order to restore homeostasis or by microorganismal factors in order to promote pathogen survival.

Both nTregs and iTregs have been shown to play important roles in both primary and secondary immune responses. In some cases, the actions of these cells have been shown to be beneficial to the host, and in other cases, they have been shown to be detrimental to the host. Immune regulation by nTregs may be particularly important for protecting immune-privileged sites or select sites where a highly specified task is being carried out, such as the eye or the liver. For example, research has shown that in mice infected with ocular herpes simplex virus, nTregs protect against the development of virus-induced lesions (26). Also, researchers have demonstrated in mice that the removal of nTregs during chronic infection with Schistosoma mansoni increases immune-mediated damage to the liver (27).

Although a tightly controlled immune response has been shown to prevent immunopathology, this dampening of the immune response can lead to enhanced pathogen survival and long-term pathogen persistence. In a mouse model of chronic
*Leshmania major* infection, nTregs accumulated at the infection site, suppressed the local effector cells, and prevented the parasite from being eliminated (28). Although somewhat counterintuitive, parasite persistence in this model equipped the host with a level of protective immunity against the parasite. In some cases, however, the tight control of the immune response by Tregs can lead to increased damage to the host and/or decreased survival. For example, mice infected with lethal rodent malaria (*Plasmodium yoelii*) that were depleted of nTregs were able to eliminate the parasite and survive infection (29). Likewise, mice infected with a filarial nematode displayed extensive accumulation of nTregs in the thoracic cavity, and depletion of nTregs using antibodies against CD25 and GITR led to clearance of the parasite and protected the animals from disease (30). In addition, researchers have observed that mice depleted of nTregs were able to mount enhanced acute and memory CD8+ T cell responses to infection with *herpes simplex virus* (HSV) (31).

**T Regulatory Cells and Vaccination**

nTregs not only dampen acute and memory responses against pathogens, but they have also been implicated in preventing vaccines from inducing efficient protective immune responses. In one study, researchers observed that when using a vaccine against *Listeria monocytogenes*, nTregs reduced the magnitude of antigen-specific CD8+ T cell responses upon secondary challenge with either the bacterium or the vaccine (32). In another study, researchers found that depletion of CD25+ Tregs prior to DNA vaccination against *herpes simplex virus* type 1 (HSV1) significantly enhanced the antigen-specific CD8+ T cell response during both the acute and memory phases (33).
References


CHAPTER 2: PLASMID-BASED DNA VACCINES

Introduction

Vaccines are considered one of the most effective medical breakthroughs in history. The global campaign to vaccinate against smallpox has successfully rid the world of variola infections, and another ancient scourge, polio, is on the verge of being eliminated. Plasmid-based deoxyribonucleic acid (DNA) vaccines are one of the most facile vaccine embodiments. Plasmid DNA vaccines are simply a bacterial plasmid engineered to express a disease-specific antigen(s). In addition, they contain both mammalian-specific promoter elements and a transcription terminator. The finding that plasmid-based DNA vaccines were able to induce both cellular and humoral immune responses, against the encoded gene product(s), has lead to an intensive effort to develop plasmid-based vaccines for use in prophylactic and therapeutic immunization scenarios involving infectious diseases and certain forms of cancer. This review will serve to highlight the advancements and challenges in the field of plasmid-based DNA vaccine research, with particular focus on strategies aimed at improving the immunogenicity of these vaccines.

The Need for New Vaccines

Every year, millions of people around the world die from infectious diseases for which there is no effective vaccine. These deadly diseases include emerging infections such as human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS), severe acute respiratory syndrome (SARS), and West Nile Virus (WNV) as well as re-
emerging ancient diseases including malaria and tuberculosis. In addition, immunotherapeutic vaccines for diseases such as cancer are critically needed to augment conventional chemotherapeutic approaches. Traditional vaccine development strategies may not be successful in combating these diseases for a variety of reasons: (i) the strategies fail to elicit the appropriate protective immune responses; (ii) stringent manufacturing requirements make global vaccination against these agents difficult if not impossible; and (iii) some types of vaccines (i.e. live attenuated viruses) may have the ability to revert to a pathogenic form within the vaccine recipient and are therefore too risky to be used against certain pathogens such as HIV. Plasmid-based DNA vaccines, however, have many positive attributes that make them excellent candidates for the development of both prophylactic and therapeutic vaccines: (i) they are able to activate both humoral and cytotoxic T cell responses; (ii) they can be designed to induce a tailored immune response (i.e., either a Th1 or Th2 T cell response); (iii) they can direct the immune response toward a specific chosen antigen(s); (iv) they are highly stable (unlike protein-based vaccines); (v) they are simple and inexpensive to manufacture; and, most importantly, (vi) there is a large body of data indicating that plasmid-based DNA vaccines are safe.

A Brief History of Plasmid-Based DNA Vaccines

The application of plasmid-based expression vectors as a way of inducing an immune response against a foreign antigen is a relatively recent development in the field of vaccinology. In 1990, using a mammalian-specific promoter to drive expression of betagalactosidase, Phillip Felgner and colleagues were the first to show in vivo that
plasmid DNA could be taken up and the encoding protein expressed by muscle cells (1). The following year, members of the Sanford lab demonstrated that a needle-less injection apparatus was able to deliver plasmid DNA to cells of the liver and skin, and these cells subsequently expressed the encoded protein (2). The most surprising discovery during these early years was made in 1993 when researchers observed that intramuscular (i.m.) immunization of mice with plasmid DNA encoding an influenza protein could generate CD8+ cytotoxic T lymphocytes (CTL) as well as antibodies (Abs) (3). In addition, and perhaps most importantly, researchers found that the CTL response was able to protect mice from an otherwise lethal challenge with a heterosubtypic strain of influenza. These findings were incredibly surprising for several reasons: (i) i.m. injection would not likely result in the transfection of professional antigen presenting cells (APCs); (ii) only low amounts of protein were produced by the transfected cells; and (iii) no replication of the plasmid could have occurred in mammalian cells. Traditionally, neutralizing antibodies were thought to play a dominant role in protecting the host against influenza and, because neutralizing epitopes are subject to rapid mutation, a new influenza vaccine is required every year. The observation that CTL responses directed against the influenza nucleoprotein, a highly conserved viral antigen, could protect mice against a heterosubtypic strain of virus was astounding and very promising for human influenza vaccine development. Together, data from these and other studies led to a rapid boom in research on plasmid-based DNA vaccines and, in the coming years, many laboratories demonstrated the immunogenicity of vectors that encoded a wide variety of antigens while, in some cases, also highlighting their ability to protect the host from disease.
The next major findings came in the late 1990s when a series of researchers examined the expression and processing of the plasmid-encoded antigen. They found that while myocytes were the primary cell type initially transfected following i.m. injection, activation of naïve T cells resulted from T cell cross-priming by non-transfected, professional APCs (4-7). However, the researchers could not rule out that in rare occurrences the direct transfection of professional APCs resulted in T cell activation. The exact mechanism by which myocytes provide antigen for cross-priming of CD8+ T cells and the identification of the APC remains to be determined. The mode of action of plasmid-based DNA vaccines will be discussed in greater detail in the next section.

Soon after the initial discovery by Felgner and colleagues, which demonstrated that plasmid DNA could be taken up and expressed by muscle cells following i.m. injection, the first human clinical trial examining the safety and efficacy of plasmid-based DNA vaccination against HIV-1 infection began (8). In this study, asymptomatic HIV-infected patients who were not using anti-retroviral drugs and who had CD4+ T lymphocyte counts greater than or equal to 500 per microliter of blood were vaccinated with plasmid DNA encoding HIV-1 env and rev proteins. In an initial phase I dose escalation trial, patients received 30, 100, or 300 micrograms of plasmid DNA at 10-week intervals. Researchers observed that a subset of patients had increased gp120-specific Ab levels and/or elevated CTL activity against gp160 and/or heightened lymphocyte proliferative activity at the conclusion of the study period. Importantly, researchers also noted that DNA vaccination produced no local or systemic adverse reactions, did not result in the production of anti-double stranded (ds) DNA antibodies, and did not elevate systemic
levels of muscle enzymes, indicative of muscle damage. Although the immune responses induced in vaccinated humans were very low, the fact that this plasmid DNA vaccine was shown to be safe was seen as a very promising result and spurred a relatively large number of clinical trials against a wide variety of human diseases including HIV, malaria, influenza, hepatitis B, and cancer. However, despite their safety and their ability to induce both cellular and humoral immune responses, plasmid-based DNA vaccines have unfortunately been shown to be only weakly immunogenic in humans. The human clinical trials using plasmid-based DNA vaccination and the efforts to improve their immunogenicity will be discussed in greater detail in the following sections.

Mode of Action of Plasmid-Based DNA Vaccines

There have been extensive studies to determine how plasmid-based DNA vaccines are taken up, how the encoded antigen is processed and presented, and how various immune responses are induced. From the earliest studies, researchers noted that the method of delivery was very important in determining the types of cells that were transfected and how the immune response was induced. Researchers found that the “gene gun” method of delivery, which bombarded cells of the epidermis with plasmid-coated microparticles, led to the direct transfection of professional APCs—epidermal keratinocytes and Langerhans cells, which then traveled to the regional lymph nodes and presented antigen to T cells (9). However, i.m. injection was only found to directly transfect professional APCs in rare cases and most often transfected myocytes that lacked co-stimulatory molecules. Nevertheless, this route of delivery still produced a robust CD8+ CTL response. In several studies during the late 1990s, using parental > F1 bone marrow chimeric mice,
researchers showed that bone-marrow–derived APCs were required for the induction of a major histocompatibility complex (MHC) class I-restricted CD8+ CTL response after i.m. plasmid-based DNA vaccination (4, 6, 10). Despite the fact that this data pointed directly to myocytes as providing cross-priming of CD8+ T cells, the precise mechanism by which this occurs has eluded scientists. Several possible mechanisms have been proposed, including the uptake of apoptotic cells by professional APCs, the transfer of processed peptide, and the transfer of protein (11). This remains an area of interest as elucidating how cross-priming occurs may lead to alterations or inclusions in the plasmid to increase cross-priming efficiency. Scientists have already applied methods to increase antigen uptake by professional APCs, which will be discussed in greater detail later in the review.

**Preclinical and Clinical Trials Using Plasmid-Based DNA Vaccines**

Before plasmid-based DNA vaccines could go to human clinical trial, they needed to first undergo various preclinical safety and efficacy trials using small animal models and non-human primates. Numerous animal studies carried out by various groups all showed that direct administration of plasmid DNA was safe and well tolerated. In one study, mice and cynomolgus monkeys received repeated intravenous injections of an anti-cancer vaccine expressing human class I MHC HLA-B7 heavy chain and the beta 2-microglobulin light chain. The animals showed no abnormalities in clinical chemistry, hematology, or organ pathology (12). In another study, mice and rabbits received repeated i.m. injections of a plasmid encoding the circumsporozoite protein from *Plasmodium falciparum* (PfCSP), the causative agent of malaria. The study indicated concurrent results regarding safety;
animals displayed no abnormalities in clinical chemistry, hematology, or organ pathology (13). Importantly, polymerase chain reaction (PCR) analysis of various tissues at various time points showed that although the plasmid was initially found in all of the highly vascularized tissues, within a few weeks time, it was only detected in the injected muscle.

One of the major safety concerns regarding this vaccine modality has been that the plasmid DNA may integrate into the host genomic DNA, which could induce mutagenesis and/or insertional carcinogenesis. This concern has been exhaustively evaluated in several animal studies. In one such study by the Troiolo group, mice were vaccinated with a plasmid encoding influenza nucleoprotein. Using PCR analysis, shown to have a sensitivity of 1 to 7.5 plasmid copies per 150,000 nuclei, the researchers examined integration at four and eight weeks post-vaccination. The researchers could not detect integration into the host genome at either time point (14). Another study by the Lew lab found that even the highest level of integration detected, 30 copies per 150,000 mouse genomes, was 3,000 times less than the spontaneous mutation rate for mammalian genomes (15).

Another concern regarding plasmid-based DNA vaccines has been that the host may produce anti-DNA Abs or Abs against other nuclear antigens, thus initiating or accelerating the development of autoimmune diseases such as lupus erythematosus. One study, conducted by Dennis Klinman’s group, using a strain of lupus-prone mice revealed that plasmid DNA injections did not accelerate renal dysfunction or histological deterioration relative to control animals receiving saline injections (16). Another study,
conducted by the Norman lab, demonstrated that mice and rabbits injected with plasmid encoding P/CSP did not show production of either anti-nuclear Abs or anti-dsDNA Abs (13).

The results from these preclinical animal studies—which demonstrated the overall safety of plasmid-based DNA vaccines, their lack of or minimal integration into the host genome, their failure to accelerate autoimmune disease, and their inability to induce the production of anti-nuclear or anti-dsDNA Abs—were very promising and served as the impetus for the initiation of multiple human clinical efficacy trials using plasmid-based DNA vaccines.

As discussed previously, the first human clinical trial that evaluated the safety and efficacy of a plasmid-based DNA vaccine against HIV-1 was conducted in 1998. Since that time, there have been a multitude of human clinical trials examining the safety and efficacy of plasmid-based DNA vaccines against various diseases such as HIV (prophylactic and therapeutic), malaria, influenza, hepatitis B, and cancer. In addition, DNA vaccines have been successfully developed for widespread use in veterinary medicine with the recent marketing of a plasmid-based vaccine for horses against West Nile virus.

Importantly, similar to the aforementioned findings of preclinical animal studies, there have been no human studies that suggest that plasmid-based DNA vaccines pose more than minimal safety concerns. As discussed previously, the 1998 study by Weiner and
colleagues found that DNA vaccination against HIV-1 did not lead to local or systemic adverse reactions, detectable anti-dsDNA Abs, or elevated muscle enzymes. A 2000 study by Steven Hoffman also confirmed the safety of plasmid-based DNA vaccines (17). In the latter study, human volunteers received a monthly i.m. injection for three months of one of four different doses (20, 100, 500, 2500 micrograms) of plasmid DNA encoding the circumsporozoite protein from *Plasmodium falciparum* (PfCSP). The volunteers were then followed for twelve months. Similar to the findings of the Weiner lab, a small subset of both vaccine and control recipients showed mild local reactogenicity and/or systemic symptoms. None displayed clinically significant biochemical or hematologic changes, and none produced detectable anti-dsDNA Abs. In a third study, by the LoBuglio group, in which patients with colorectal carcinoma were vaccinated with plasmid encoding carcinoembryonic antigen (CEA), toxicity was limited to transient grade 1 injection site tenderness and/or fatigue and/or a slight elevation in the liver enzyme creatine kinase. Each symptom affected only a minority of patients in a non-dose-dependent manner (18).

The fact that plasmid-based DNA vaccines have been shown to be safe has spurred the effort to take them from the lab to the clinic.

While human clinical trials have highlighted the safety of plasmid-based DNA vaccines and their ability to induce both humoral (8, 17, 19-21) and cellular (19-23) immune responses against the encoded antigen, they have also, unfortunately, revealed that these vaccines are only weakly immunogenic in humans. In one study, previously mentioned in this review, LoBuglio and colleagues vaccinated patients who had metastatic colorectal carcinoma with a dual expression plasmid encoding CEA and, as an indicator of plasmid
expression, hepatitis B surface antigen (HBsAg) (18). The researchers were unable to
detect CEA-specific antibodies, but were able to detect lymphoproliferative responses to
CEA. Unfortunately, this immune response was not associated with objective tumor
regression or a sustained decline in circulating CEA. There was also no correlation
between positive lymphoproliferative responses and a stabilization of the disease (18). In
another study by the Wahren lab, asymptomatic HIV-1-infected patients were immunized
with plasmids encoding the nef, rev, or tat regulatory HIV-1 proteins (19). Researchers
found that while HIV-DNA immunization induced antigen-specific T cell proliferation
and a low-level Ab response, HIV-DNA immunization alone did not cause a significant
decrease in viral load and/or an increase in the CD4+ T lymphocyte counts. Additionally,
a study by the Hoffman lab found that while naïve individuals vaccinated with plasmid
encoding malaria antigen produced antigen-specific CD8+ CTL, the response was only
seen in 11 of the 20 study volunteers (24). In a second study by the same laboratory,
vaccination with the same plasmid did not produce a measurable antigen-specific Ab
response in any of the individuals (17).

Preclinical and clinical trials using plasmid-based DNA vaccines have clearly
demonstrated the safety of this vaccine modality. However, despite their ability to induce
both arms of the adaptive immune system, humoral and cellular, the ability of DNA-
encoded antigens to elicit immune responses in humans have been disappointing. The
reasons why humans produce such weak responses to plasmid vaccines have not yet been
elucidated. However, some possibilities include low levels of antigen production,
inefficient cellular delivery of plasmid DNA, and insufficient stimulation of the innate
immune system (25). Current efforts are now aimed at improving the immunogenicity of plasmid-based DNA vaccines so that they can be used effectively in humans. Several of these strategies have produced promising results, some of which will be discussed in the following section.

Improving the Immunogenicity of Plasmid-Based DNA Vaccines

The approaches taken to improve the immune responses elicited by plasmid-based DNA vaccines have been numerous and varied. Due to the extent of literature in this sector of plasmid-based DNA vaccine research, this review will highlight some of the major approaches taken to enhance the immunogenicity of plasmid-based DNA vaccines, including alterations of the vector, the use of adjuvants, the co-expression of immunostimulatory molecules, modifications of the delivery method, and the encapsulation of plasmid DNA into liposomes or microparticles.

Vector Alterations

The first approach taken by many labs to improve the immunogenicity of plasmid-based DNA vaccines has been to alter the plasmid itself. Modifications have included (i) altering the plasmid backbone to improve promoter efficiency; (ii) the use of alternative leader sequences to enhance mRNA stability; and (iii) the substitution of mammalian codons for those used by the pathogen (some bacterial and viral codons are rarely used by mammalian cells) (25). A 2006 study by the Wang lab evaluated the roles of these three alterations in improving the immunogenicity of a model antigen, the HIV-1 envelope glycoprotein (26). The Wang lab demonstrated that these factors were able to work
synergistically to improve the expression and subsequent immunogenicity of the DNA vaccine. In addition, they also discovered that when mammalian codons were utilized in place of viral codons, there was a substantial increase in the number of HIV-1 env-specific RNA transcripts. This observation suggests that the use of mammalian codons increased antigen expression by another mechanism in addition to increasing translation efficiency.

**Adjuvants**
Adjuvants are commonly given with subunit vaccines such as purified or recombinant protein vaccines because, unlike microbes, these pathogen-specific components lack signals to stimulate the innate immune system. Adjuvant molecules may be expressed from the plasmid or given in addition to the plasmid as purified or recombinant protein. Immunostimulatory adjuvants including various cytokines, chemokines, and co-stimulatory molecules have been tested in small animal models. In a 2005 study by the Weiner lab, researchers delivered a plasmid encoding IL-15 as a DNA vaccine adjuvant, attempting to improve the antigen-specific CD8+ T cell response to plasmid-encoded HIV-1 gag protein. They found that coadministration of plasmid-encoded IL-15 with plasmid-encoded HIV-1 gag protein resulted in increased antigen-specific CD8+ T cell proliferation and interferon gamma (IFN-γ) production (27). They also applied this method of enhancement to an influenza DNA vaccine model and found that the IL-15-encoding plasmid improved the CD8+ T cell response and protected mice against an otherwise lethal mucosal challenge with influenza virus (27). Other plasmid-encoded immunostimulatory molecules, recently tested for use with DNA vaccines, have included ligands for NKG2D, a stimulatory receptor found on natural killer (NK) cells and on
activated T cells. In a study published in 2005, researchers found that DNA vaccines encoding tumor-associated antigens and various NKG2D ligands enhanced activation of both the innate and adaptive immune responses and protected mice against two different tumors: CT-26 colon carcinoma cells, which express very low or negative levels of NKG2D ligands, and D2F2 breast carcinoma cells, which express intermediate levels of NKG2D ligands (28). Additionally, researchers have found that adjuvants, which stimulate toll-like receptors (TLRs), are able to induce a more robust immune response against plasmid-based DNA vaccines. A study by Wahren and colleagues demonstrated that a topical cream containing imiquimod, a known TLR7 and TLR8 agonist, was able to enhance the cellular immune response to a DNA vaccine against HIV (29). Importantly, conventional aluminum salt adjuvants have also been shown to enhance immune responses to DNA-encoded antigens. A 1999 study found that co-administration of aluminum salt formulations increased the Ab response 100-fold in mice and guinea pigs and 5-10-fold in non-human primates (30).

**Delivery**
The testing of various methods of plasmid-DNA delivery has been a well-studied area of DNA vaccine research. It was only a year after the proof-of-concept experiment performed by Felgner and colleagues that scientists began examining various means of delivering the plasmid DNA. Some of these techniques are designed to increase the transfection efficiency of professional APCs while others are intended to target the DNA to sites rich in APCs, such as the skin. One such method, known as “gene gun” immunization, has been shown to directly transfect dendritic cells (DCs) within the epidermal layer of the skin (9). These DCs then migrate to the draining lymph node
where they present antigen to naïve T lymphocytes. Another method of delivery involves the use of a “mucosal jet injector” to directly target the mucosa, a prime site of pathogen entry. A 2002 study by Wahren and colleagues analyzed the local and systemic responses in HIV-1 infected individuals following intraoral jet-injections of HIV-1 DNA constructs encoding the nef, rev, and tat regulatory proteins (31). Immunization via this route resulted in (i) the induction of systemic antigen-specific T cell proliferation; (ii) accumulation of CD4+ and CD8+ T lymphocytes in the vaccinated mucosa; and (iii) the generation of local and systemic, antigen-specific Abs. It is also important to note that because these methods of plasmid-based DNA delivery do not involve the use of needles, they are very appealing for use in global vaccination scenarios.

**Encapsulation or Microparticle-Based Strategies**

Encapsulation or microparticle-based strategies have been utilized to protect the DNA and/or increase its uptake by professional APCs. For example, researchers have demonstrated that particles ~1-3 μm in diameter are readily taken up by macrophages and DCs. Therefore, researchers have proposed that plasmid DNA may be taken up more efficiently if packaged into specifically sized particles. Two formulations, both based on a polymer (polyactide co-glycolide (PLG)) and a cationic lipid, have been shown to be effective at delivering DNA vaccines in this manner. A 2005 study by the Ulmer group, using a non-human primate model of HIV, showed that DNA formulated into PLG microparticles increased the antigen-specific humoral and, to a lesser extent, the antigen-specific cellular immune responses against the DNA-encoded antigen. Another study by the Hobart lab found that rabbits immunized with cationic-lipid-formulated plasmids,
encoding the protective antigen and/or the lethal factor from anthrax, were able to survive an otherwise lethal challenge with anthrax.

Another means of improving immune responses against plasmid-encoded antigens has been the selective depletion of T regulatory cells (Tregs) with an anti-CD25 antibody prior to vaccination. As discussed previously, natural Tregs (nTregs) limit the magnitude of the effector T cell response. In certain cases, the suppressive activity of these cells has been shown to prevent the host from clearing some infectious agents. Likewise, these cells have also been implicated in preventing vaccines, particularly weak vaccines, from inducing protective levels of memory B and T cells. Therefore, researchers have proposed that transient depletion of Tregs during the priming phase of the immune response, early after the initial vaccination, may lead to an increase in both the effector and memory responses against a plasmid-encoded antigen. A 2003 study by the Rouse lab found that the depletion of CD25+ T cells in mice prior to infection with HSV-1 resulted in elevated HSV-1-specific CD8+ T cell responses (32). In addition, researchers observed that the CD8+ T cells remained activated for a longer period of time, and the memory responses among the depleted animals were increased by approximately threefold as compared to non-depleted control animals. The same researchers also found that the adoptive transfer of CD4+CD25+ T cells inhibited HSV-1-specific CD8+ T cell responses, and that viral infection increased the activation of CD4+CD25+ Tregs (32). These observations led the Rouse lab to postulate that if CD25 depletion resulted in increased immunity to HSV-1 infection, then CD25 depletion might also enhance the immune response to a plasmid-based DNA vaccine. In 2004, the Rouse lab published a
study in which they measured the influence of Tregs on the immune response in mice to DNA vaccination against HSV. They found that removal of CD25+ Tregs using an anti-CD25 antibody prior to immunization with a plasmid expressing glycoprotein B significantly enhanced the resultant CD8+ T cell response to the dominant SSIEFARL peptide as determined by ELISPOT, in vitro cytotoxic T lymphocyte (CTL) assay, and intracellular IFN-γ staining. The increased CD8+ T cell response was two-fold to three-fold higher in the depleted mice as compared to the control animals and was apparent in both the acute and memory stages (33).

In conclusion, researchers have tried a multitude of methods to improve the immunogenicity of plasmid-based DNA vaccines, a few of which have been discussed in this review. In the present study, we have elaborated on the preliminary observations made by the Rouse lab regarding the use of CD25 depletion prior to plasmid immunization and have provided greater insight into the ability of CD25 depletion to enhance both the cellular and humoral immune responses to a model plasmid-based DNA vaccine.
References


CHAPTER 3: MATERIALS AND METHODS

Animals

Female C57BL/6 mice, 4 to 6 weeks of age, were purchased from Taconic (Hudson, NY). B6.PL-Thy1\(^a\)/CyJ mice, 3 to 5 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were used in compliance with institutional animal health and care regulations, and all procedures used in the experiments with animals were approved by the local Institutional Animal Care and Use Committee.

DNA vaccine preparation and validation

Genes encoding either the glycoprotein (GP) or the nucleoprotein (NP) from lymphocytic choriomeningitis virus (LCMV) were subcloned into the NotI site of plasmid pCMV (1). This plasmid contains the widely expressed immediate-early promoter from human cytomegalovirus, a splice donor-acceptor site from simian virus 40 which precedes the inserted sequence, and a transcription termination-polyadenylation site which follows the sequence. In addition, the plasmid contains an ampicillin (amp) resistance cassette. Plasmid DNA was propagated in the TOP10 strain of Escherichia coli (Invitrogen, Carlsbad, CA). Plasmid DNA was added to the E. coli, the cells were heat shocked for 30 seconds at 42°C, and then chilled for 5 minutes on ice. Luria-Bertani (LB) broth was added and cells were grown for 1 hour in a 37°C shaker and then plated on LB-amp plates (100\(\mu\)g/ml amp). Then plates were incubated overnight at 37°C. Colonies were selected and cultured overnight in LB-amp. The overnight culture was then used to
inoculate a 1L culture. The plasmid DNA was purified using a Qiagen EndoFree Plasmid Mega Kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. Plasmids were validated using diagnostic restriction enzyme digestion followed by separation on a 1% agarose gel. pCMV was digested with PstI (2,700 and 1,000 bp fragments). pCMV-GP was digested with BamHI (3,700 and 1,600 bp fragments). pCMV-NP was digested with NotI (3,700 and 1,700 bp fragments).

**DNA vaccination**

Mice were anesthetized with isoflurane and injected in each anterior tibialis muscle with 50μg plasmid DNA (1μg/μl, in saline) for a total of 100μg/mouse. For mice that were boosted, each tibialis muscle was injected with 25μg plasmid DNA (0.5μg/μl, in saline) for a total of 50μg/mouse.

**Propagation of PC 61.5.3 hybridoma**

PC61 5.3 (anti-CD25) hybridoma, a kind gift from H. Braley-Mullen (University of Missouri, Columbia, MO), was cultured in complete medium. Media was prepared as follows: 1x Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 2mM L-glutamine (Invitrogen), and 0.05mM 2-mercaptoethanol (Sigma, St. Louis, MO). Once prepared, media was stored at 4°C.
1.0ml aliquots containing 1x10^6 cells were partially thawed from liquid nitrogen in a 37°C water bath. Cells were removed from the cryogenic vials and added to 8ml of complete media in 75cm^2 0.2μm vented cap, canted neck cell culture flasks (Corning, Corning, NY). Cells were cultured in 37°C incubator with an atmosphere of 5% CO₂ and 100% humidity. Once confluent, the cells were transferred to 175cm^2 standard cap cell culture flasks (Greiner bio-one, Frickenhausen, Germany). Fresh media was added as nutrients became exhausted up to 400ml. Cells were then pelleted in 50ml conical tubes at 300g for 8min at 4°C in a Sorvall bench-top centrifuge. Media was decanted and filtered through a 500ml 0.22μm cellulose nitrate filter apparatus (Corning) and stored at 4°C until antibody purification.

**Purification of anti-CD25 (PC61) antibody**

Anti-CD25 antibody was purified from 1L of culture supernatant using a protein G column at 4°C as follows: (i) a 50ml conical tube was prepared for collection by adding 5ml 1M Tris (ii) the column was washed with 120ml of 20mM sodium phosphate buffer, pH 7.0, (iii) the supernatant was added to the column and collected for a second run in a 500ml glass beaker, (iv) the column was washed with 120ml of 20mM sodium phosphate buffer, pH 7.0, and (v) the antibody was eluted with 40ml of 0.1M glycine, pH 2.8, collecting the eluate in the 50ml collection tube. The column was then either regenerated with 20% ethanol or washed with 120ml of 20mM sodium phosphate buffer, pH 7.0 for a second run. The column and solutions were all stored at 4°C. The pH of the eluate was adjusted to 7.0 and the eluate was stored at 4°C until dialysis.
The antibody was dialyzed using a 12-30ml Pierce Slide-A-Lyzer dialysis cassette with a 10,000 molecular weight cutoff (Thermo Fisher Scientific, Waltham, MA) as follows: (i) the cassette was submerged for 30 seconds in 2L 1x phosphate buffered saline (PBS), pH 7.2, (ii) the injection site was labeled, (iii) the eluate was loaded into the cassette with a 12ml 22 gauge syringe (removing air from the membrane-bound chamber as additional liquid was added), (iv) any remaining air was removed from the membrane-bound chamber, and (v) the cassette was placed in the 2L container of PBS and placed on a stir plate at medium speed for 8 hours. The antibody was dialyzed for two additional 8 hour periods in fresh 1x PBS for a total of 24 hours of dialysis. After the dialysis, the antibody was removed from the dialysis membrane and concentrated.

Centricon Plus-20 centrifugal filters were used to concentrate the antibody as follows: (i) the antibody was inserted into the filtration apparatus, (ii) spun for 6min at 4°C and 1950g in a Sorvall bench-top centrifuge, (iii) the filtrate was disposed, (iv) steps 1-3 were repeated until the desired volume was reached, (v) the concentrated antibody was removed, (vi) to remove any remaining antibody, the concentrate cup was inverted and placed on top of the sample filter cup, and (vii) spun for 1min at 4°C at 800g in a Sorvall bench-top centrifuge.

To determine the concentration of the antibody, absorbance at 280nm was measured with a SPECTRA max spectrophotometer (Molecular Devices, Sunnyvale, CA). The antibody was aliquoted and stored at -80°C.
**In vivo depletion using anti-CD25 (PC61) antibody**

Mice were given an intraperitoneal injection of 1mg anti-CD25 antibody in 500μl PBS.

**Propagation of BHK-21 and Vero cell lines**

BHK-21 and Vero cells were grown in complete media. Media was prepared as follows:

1x Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 2mM L-glutamine (Invitrogen), penicillin G sodium (1,000 units/ml) (Invitrogen), and streptomycin sulfate (1,000 μg/ml) (Invitrogen). Once prepared, media was stored at 4°C.

1.0ml aliquots containing 1x10⁶ cells were partially thawed from liquid nitrogen in a 37°C water bath. Cells were removed from the cryogenic vials and added to 8ml of complete media in 75cm² 0.2μm vented cap, canted neck cell culture flasks (Corning). Cells were cultured in a 37°C incubator with an atmosphere of 5% CO₂ and 100% humidity. Once confluent, cells were split 1:10 into fresh media in 75cm² 0.2μm vented cap, canted neck cell culture flasks. As needed, cells were cryopreserved as follows: (i) cells were trypsinized with 3ml of trypsin-EDTA/ T75 flask (Invitrogen) for 1min in a 37°C incubator with an atmosphere of 5% CO₂ and 100% humidity, (ii) each flask was gently tapped to remove the adherent cells, (iii) cells were diluted two-fold with complete media and collected in a 50ml centrifuge tube, (iv) spun for 8min at 4°C and 300g in a Sorvall bench-top centrifuge, (v) resuspended in freezing media (filter sterilized complete growth media supplemented with 5% dimethyl sulfoxide, DMSO) 1ml/flask, (vi)
aliquoted into 1.5ml cyrotubes, (vii) placed in a rack inside a styrofoam box, (viii) stored overnight at -80°C, and (ix) the next morning transferred to liquid nitrogen.

**LCMV preparation**

Stocks of *lymphocytic choriomenigitis virus* (LCMV) were prepared as follows: (i) two confluent T75 flasks of BHK cells were trypsinized and counted by diluting 1:4 in 0.04% trypan blue to estimate the number of cells per T75 flask, (ii) a viral stock of known concentration was diluted to the appropriate multiplicity of infection (MOI) (generally 0.5 plaque forming units (pfu)/cell), (iii) media was removed from the flasks and the cells were overlayed with 4ml of diluted virus, (iv) cells were incubated for 1hr in a 37°C incubator with an atmosphere of 5% CO₂ and 100% humidity rocking the dishes at 15min intervals, and (v) after 1hr, the inoculum was removed and 10ml of fresh complete media was added. The cells were allowed to incubate for 48 hours, after which the virus stock was removed and prepared as follows: (i) media from each flask was pooled into a single T75 flask on ice and mixed gently, (ii) 10ml of complete media was added to the empty flasks and the flasks were incubated for an additional 24 hours in a 37°C incubator with an atmosphere of 5% CO₂ and 100% humidity, (iii) media from the 48hr harvest was split into 50ml conical tubes and spun for 8min at 4°C and 300g in a Sorvall bench-top centrifuge, (iv) the supernatants were removed and transferred to clean 50ml tubes and spun again for 8min at 4°C and 300g, (v) supernatants were pooled in a T75 flask on ice and mixed well, and (vi) supernatants were aliquoted into 200-500μl aliquots and stored at -80°C. The same procedure was repeated to harvest the 72hr supernatants. Duplicate aliquots of the 48hr and 72hr supernatants were used to titer the virus on Vero cells.
**LCMV plaque assay**

LCMV was titered from organs or virus stocks on 6-well plates of Vero cells. One day prior to the plaque assay, the following preparations were completed: (i) 0.5g of agarose was added to 125ml screw top bottles (1/5 plates), (ii) 2x DMEM was prepared and stored at 4°C (one packet of powered DMEM (Invitrogen) diluted in 500ml of deionized water, supplemented with 3.7g sodium bicarbonate, pH adjusted to 7.0, filtered through a 0.22μm cellulose nitrate filter, supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), 2mM L-glutamine (Invitrogen), penicillin G sodium (pen) (1,000 units/ml) (Invitrogen), and streptomycin sulfate (strep) (1,000 μg/ml) (Invitrogen)), (iii) 5ml polystyrene round-bottom dilution tubes (BD Biosciences, San Jose, CA) were prepared and stored at 4°C (1.8ml complete media/tube), and (iv) Vero cells were plated on 6-well cell culture plates (Corning)—3ml of cells per well in Roswell Park Memorial Institute media (RPMI) supplemented with 10% FBS, 2mM L-glutamine, 1,000units/ml pen, and 1,000μg/ml strep for a final concentration of 1x10^6 cells/well. The cells were incubated overnight in a 37°C incubator with an atmosphere of 5% CO₂ and 100% humidity. On day 0 the following preparation steps were completed: (i) 50ml of autoclaved deionized water was added to each bottle of 0.5g of agarose and the agarose was autoclaved for 15min (after autoclaving, the bottles were place in a 55°C water bath), (ii) the 55°C water bath was turned on, and (iii) the 2x DMEM was placed in the 37°C water bath. If virus was to be titered from organ samples, the following steps were taken: (i) the organ tubes were removed from the -80°C freezer and placed immediately on ice, (ii) the tubes were wiped down and weighed one-at-a-time (this step could be skipped if the tubes were weighed on day of organ collection), (iii) 1ml of 1x DMEM (supplemented with 10%
FBS, 2mM L-glutamine, 1,000 units/ml pen, and 1,000 μg/ml strep) was added to each tube, and (iv) the tubes were placed a bead beater (Biospec, Bartlesville, OK) for 1 min at maximum speed. For LCMV titration from both organ samples and virus stocks, dilutions were made as follows: (i) the sample tube was vortexed and 200 μl of the sample was placed in the first dilution tube (10⁻¹) (for virus stocks it may be necessary to start at 10⁻², 20 μl of sample into 1980 μl of media), (ii) 1 ml of the first dilution was then transferred to the second dilution tube, and (iii) step two was repeated until a dilution of 10⁻⁶ (for organ samples) or 10⁻⁷ (for virus stocks) was reached. Once the dilutions were prepared, the Vero cells were infected as follows: (i) plates were removed from the incubator five at a time and the media was aspirated off, (ii) 1 ml of each dilution was plated on respective wells, (iii) the cells were incubated for 1 hr in a 37°C incubator, rocking the plates every 15 min, (iii) the inoculum was aspirated off and the cells were overlayed with 3 ml nutrient rich agarose (one bottle of agarose with 50 ml of 2x DMEM—the temperature of the nutrient rich agarose should be warm enough that it remains a liquid but not so hot that destroys the monolayer), and (iv) after allowing the plates to cool for 10 min, they were placed in a 37°C incubator. On day 5, the following steps were completed: (i) the plates were removed from the incubator and 3 ml of fixative was added to each well (50 ml 10x PBS (1x final), 270 ml 37% formaldehyde (20% final) (Fisher, Pittsburgh, PA), 180 ml deionized water), (ii) incubated in the hood for 1 hr, (iii) fixative was poured off and the plugs were removed, (iv) 3 ml of crystal violet (0.1% crystal violet in 20% ethanol) was added to each well, (v) the plates were incubated for 2 hrs to 2 days at room temperature, (vi) the crystal violet was removed and the plates were inverted on an absorbent pad to dry, and (vii) the plaques were counted.
**Infection**

Mice were given an intraperitoneal injection of $5 \times 10^5$ pfu LCMV in 500μl complete 1x DMEM. For the mega challenge experiment, mice were challenged with a dose of $2.5 \times 10^6$ pfu LCMV in 500μl complete 1x DMEM.

**Sacrificing animals and harvesting organs**

Mice were euthanized by inhalation of a lethal dose of halothane (Halocarbon Laboratories, River Edge, NJ). For intracellular cytokine staining, spleens and/or lymph nodes (superficial cervical nodes, axillary nodes, brachial nodes, and inguinal nodes) and/or livers were removed using aseptic technique and organs were placed in 15ml conical tubes containing 5ml of 1x RPMI wash media (supplemented with 5% FBS, 2mM L-glutamine, 1,000units/ml pen, and 1,000μg/ml strep). If the liver was to be removed, the organ was first perfused with PBS or saline. Additionally, after the liver was removed it was cut into pieces before placing it into the tube of wash media. For titering the virus in the spleen, one third of the spleen was placed in a pre-weighed 1.7ml microcentrifuge tube containing homogenization beads that had been placed at -80°C overnight. The tube was then placed immediately into liquid nitrogen. After the harvesting was completed, the tubes were weighed and placed at -80°C until the virus was to be titered.

**Staining for antigen-specific cells**

**Cell preparation**

Spleens and lymph nodes were prepared as follows: (i) media and organ were poured into a dounce homogenizer, (ii) organs were ground into a single cell suspension, (iii) cells
were passed through a 70 μm nylon cell strainer and collected in the original 15ml conical tube, (iv) 5ml of 1x RPMI wash media was added to the homogenizer and passed through the filter into the same collection tube, (v) the filter and homogenizer were rinsed with 1x PBS or saline and used for the next sample, (vi) after all the organs had been processed, the cells were pelleted by centrifugation at 300g for 8min at 4°C, (vii) the supernatants were discarded and the pellets resuspended with a 1ml pipette in 1ml ammonium chloride lysis buffer (500ml of 0.83% ammonium chloride and 55.6ml of 0.17M Tris pH 7.65, pH 7.2, filter sterilized through a 0.2μm filter, stored at room temperature), (viii) an additional 4ml of ammonium chloride lysis buffer was added, (ix) the cells were vortexed and allowed to incubate at room temperature for 5min, (x) 10ml of 1x RPMI wash media was added, (xi) the tubes were mixed and the cells were pelleted at 300g for 8min at 4°C, and (xii) the cells were resuspended in 5ml 1x RPMI wash media and the cells were counted with 0.04% trypan blue (typically at a dilution of 1:40 for the spleen and 1:10 to 1:40 for the lymph nodes and livers).

Livers were prepared as follows: (i) media and organ were poured into a dounce homogenizer, (ii) organs were ground into a single cell suspension, (iii) cells were passed through a 70 μm nylon cell strainer and collected in the original 15ml conical tube, (iv) 5ml of 1x RPMI wash media was added to the homogenizer and passed through the filter into the same collection tube, (v) the filter and homogenizer were rinsed with 1x PBS or saline and used for the next sample, (vi) after all the organs had been processed, the cells were pelleted by centrifugation at 300g for 8min at 4°C, (vii) the cells were resuspended in 1ml of 40% percoll (diluted in complete media) (Sigma) and an additional 9ml 40%
percoll was added, (viii) the 40% percoll/cell suspension was gently overlaid onto 10ml of 70% percoll in a 50ml conical tube, (ix) the gradient was spun for 20min at 300g with no brake at room temperature, (x) the top layer, to just slightly above the interface, was discarded, (xi) the red interface was collected and placed in a fresh 15ml conical tube, (xii) wash media was added to reach a final volume of 15ml and the tube was mixed end-over-end, (xiii) the cells were spun for 8min at 300g at room temperature, and (xiv) the cells were prepared from this point on just as the cells from the spleen and lymph node were prepared (see steps 7-12 in the previous section).

**Peptide stimulation**

Peptide stimulation was completed as follows: (i) cells were plated in 100μl 1x RPMI final media (supplemented with 10% FBS, 2mM L-glutamine, 1,000units/ml pen, and 1,000μg/ml strep) in a 96-well round bottom cell culture plate with a lid (Corning) at the desired density (typically 1x10⁶ to 3x10⁶ cells/well), (ii) 100μl of 1x RPMI final media plus brefeldin A (4μg/ml) plus/minus peptide was added (2x GP33 2.0μg/ml, 2x GP61 10μg/ml, 2x NP396 2.0μg/ml, 2x NP205 2.0μg/ml, 2x NP118 0.4μg/ml, 2x GP276 2μg/ml), and (iii) cells were incubated for 5.5hrs in a 37°C incubator with an atmosphere of 5% CO₂ and 100% humidity.

**Surface staining**

Surface staining was completed as follows: (i) cells were spun at 300g for 8min at 4°C, (ii) antibodies for surface markers were added in 50μl of staining buffer (5% FBS in 1x PBS) at given concentrations, CD8a PE-Cy5 (Ly-2, 0.0125μg/10⁶ cells) (BD Pharmingen, Franklin Lakes, NJ), CD8a PE (Ly-2, 0.0625μg/10⁶ cells) (eBioscience, San
Diego, CA), CD4 PE-Cy5 (L3T4, 0.05µg/10^6 cells) (eBioscience), and (iii) the cells were incubated overnight at 4°C protected from the light.

Intracellular cytokine staining (ICCS) for interferon gamma
Staining for interferon gamma was completed as follows: (i) without spinning, 150µl of staining buffer was added to the cell suspension, (ii) cells were spun at 300g for 8min at 4°C, (iii) supernatant was removed, (iv) plate was vortexed, (v) 200µl of staining buffer was added and steps 2-4 were repeated, (vi) cells were resuspended in 100µl fixative (2% formaldehyde PBS) and chilled on ice for 20min protected from the light, (vii) 100µl of permeabilization buffer (0.1% saponin (Sigma), 1% FBS (Atlanta Biologicals), 0.1% sodium azide (Sigma), in 1x PBS) was added, (viii) steps 2-4 were repeated, (ix) cells were washed 2x more with 200µl permeabilization buffer, (x) cells were resuspended in anti-IFN-γ antibody in 50µl permeabilization buffer (IFN-γ FITC, XMG1.2, 0.05µg/10^6 cells) (eBioscience) and incubated on ice for 30min protected from the light, (xi) cells were washed 2x with permeabilization buffer, (xii) cells were washed 1x with staining buffer, (xiii) resuspended in fixative (50-200µl/well) and transferred to 5ml polystyrene round-bottom tubes (BD Biosciences), and (xiv) stored at 4°C protected from the light until they were run on the flow cytometer. Flow cytometry was done with either a FACScan flow cytometer (Becton Dickson, Franklin Lakes, NJ) or a CyAn ADP (Dako, Ft. Collins, CO), typically collecting between 8x10^5 to 1x10^6 events. Data was analyzed with WinMDI version 2.8 or Summit version 5.0 software. All buffers were stored at 4°C. The fixative was stored at 4°C protected from light.
**Foxp3 staining**

Foxp3 staining was completed using a FITC anti-mouse/rat Foxp3 staining kit (eBioscience). Prior to staining, the fixation/permeabilization concentrate was diluted as follows: 1 part concentrate to 3 parts fixation/permeabilization diluent. Single cell suspensions from sample organs were prepared as previously described in this document and the cells were stained as follows: (i) 200μl of cells were plated in a 96-well round bottom cell culture plate with a lid (Corning) at the desired density (typically 1x10⁶ cells/well), (ii) cells were spun at 300g for 8min at 4°C in a Sorvall bench-top centrifuge, (iii) the supernatant was removed, (iv) the plate was vortexed, (v) cells were resuspended in anti-mouse CD16/CD32 Fcγ III/II receptor (2.4G2, 0.125μg/10⁶ cells) (BD Pharmingen) in 25μl of staining buffer (5% FBS in 1x PBS) and placed on ice for 15min, (vi) CD4 PE-Cy5 (L3T4, 0.05μg/10⁶ cells) (eBioscience) and biotinylated CD25/IL-2Rα (7D4, 1.0μl/10⁶ cells) (eBioscience) antibodies were added in an additional 25μl of staining buffer (vii) cells were incubated for 30min on ice protected from light, (viii) 150μl of staining buffer was added and steps 2-4 were repeated, (ix) 200μl of staining buffer was added and steps 2-4 were repeated, (x) cells were resuspended in streptavidin PE (1.0μl/10⁶ cells, eBioscience) in 50μl of staining buffer and chilled on ice for 30min protected from the light, (xi) steps 8 and 9 were repeated, (xii) 100μl of fixation/permeabilization buffer was added and the cells were incubated on ice for 30min protected from the light, (xiii) 100μl of permeabilization buffer (1x buffer made from 10x buffer) was added and steps 2-4 were repeated, (xiv) 200μl of permeabilization buffer was added and steps 2-4 were repeated, (xv) Foxp3 FITC (FJK-16s, 0.5μg/10⁶ cells) antibody was added in 50μl permeabilization buffer and incubated for 30min on ice
protected from the light, (xvi) 150μl permeabilization buffer was added and steps 2-4 were repeated, (xvii) 200μl permeabilization buffer was added and steps 2-4 were repeated, and (xviii) the cells were resuspended in an appropriate volume (~200μl) of fixative (2% formaldehyde in 1x PBS) and kept at 4°C until analyzed by flow cytometry.

**In vivo assay to measure cytotoxic T lymphocyte activity**

The *in vivo* CTL assay was completed as follows: (i) spleens from donor mice were removed (C57BL/6J donors) as described above (*Sacrificing animals and harvesting organs*), (ii) spleens were processed into single cell suspensions as described above (*Cell preparation*), (iii) after counting, an equivalent number of cells were placed in 2x 15ml conical tubes, one to be pulsed with GP33 peptide and the other to be pulsed with NP118 peptide (not exceeding 1x10^6 cells/tube), (iv) the cells were spun at 300g for 8min at 4°C, (v) resuspended in 1ml of 1x DMEM (supplemented with 10% FBS, 2mM L-glutamine, 1,000units/ml pen, and 1,000μg/ml strep), (vi) one tube was pulse with GP33 peptide (10μg/ml) and the other was pulsed with NP118 peptide (10μg/ml) for 1hr in a 37°C incubator with an atmosphere of 5% CO₂ and 100% humidity, (vii) 9ml of 1x complete DMEM was added, (viii) the tubes were spun at 300g for 8min at 4°C, (ix) cells were resuspended in 10ml Hank’s buffered salt solution (HBSS) (Bio Whittaker, Walkersville, MA) and respun, (x) the cells were washed 1x more with 10ml HBSS and resuspended in 1ml diluted 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) (Sigma), the GP33 pulsed cells were stained with 5μM CFSE and the NP118 pulsed cells were stained with 1μM CFSE, (xi) the cells were incubated for 10min in a 37°C incubator, (xii) to stop the reaction, 1ml of FBS was added to each tube, (xiii) 12ml of 1x complete DMEM was...
added to each tube, (xiv) the tubes were spun at 300g for 8min at 4°C, (xv) the cells were washed 2x more with 10ml 1x complete DMEM and resuspended in 5ml of 1x complete DMEM, and (xvi) the cells were counted and pooled such that in each dose there were 1x10^7 cells from each population—GP33 pulsed CFSE high stained (5μM) cells and NP118 pulsed CFSE low stained (1μM) cells. The cells were stored protected from the light until the time of transfer. Mice were given 1x10^7 GP33 pulsed CFSE high stained cells and 1x10^7 NP118 pulsed CFSE low stained cells by intravenous injection and the cells were harvested 6hrs after transfer when looking at the primary response and 18hrs after transfer when looking at the memory response. In order to compare the cytolytic activity among mice, percent specific lysis was calculated for individual mice, as determined using the following formula: 1-(mean control ratio/ experimental ratio) x 100. Where the control ratio equals the % CFSE low stained cells (NP118 pulsed) divided by the % CFSE high stained cells (GP33 pulsed) remaining after the incubation period in a pCMV vaccinated negative control mouse and the experimental ratio is the % CFSE low stained cells (NP118 pulsed) divided by the % CFSE high stained cells (GP33 pulsed) remaining after the incubation period in a pCMV-GP vaccinated mouse that was either sham treated or treated with anti-CD25 antibody.

Sacrificing the recipient mice and staining the cells
After the incubation, recipient mice were sacrificed, the spleens were removed as described above (Sacrificing animals and harvesting organs), and single cell suspensions were prepared as described above (Cell preparation). The cells were plated in 200μl of 1x complete DMEM in a 96-well round bottom cell culture plate with a lid (Corning) at the desired density (typically 3x10^6 cells/well) and stained for surface markers as follows:
(i) biotinylated CD90.2/Thy-1.2 (30-H12, 0.5μl/well) (Southern Biotech, Birmingham, AL) was added in 50μl staining buffer and the cells were incubated in ice for 30min protected from the light, (ii) 150μl staining buffer was added, (iii) the cells were spun at 300g for 8min at 4°C, (iv) the supernatant was removed, (v) the plate was vortexed, (vi) the cells were washed 1x more with staining buffer, (vii) PE conjugated streptavidin (0.5μl/well) (eBioscience) in 50μl of staining buffer was added and the cells were incubated in ice for 30min protected from the light, (viii) steps 2-6 were repeated, (ix) the cells were resuspended in an appropriate volume of fixative (~100μl/well) and kept at 4°C until analyzed by flow cytometry.

**Enzyme linked immunosorbent assay (ELISA)**

ELISA was used to determine the relative concentration of anti-LCMV nucleoprotein (NP) specific antibodies in the serum from immunized animals and was completed as follows: (i) one day prior to the assay, a 96-well plate (BD Falcon ref. 353279, BD Biosciences) was coated with 100μl purified LCMV (diluted in PBS, typically 1:2, to an approximate concentration of 5x10^6 pfu/ml) and stored overnight at 4°C in a humidified chamber, (ii) on the day of the assay, the virus was removed from the 96-well plate in the hood and discarded, (iii) 200μl of ELISA diluent (3% BSA, 0.05% Tween 20, 5mM Thimerosal (all from Sigma), in 1x PBS, stored at 4°C) was added to each well and the plate was incubated for 1hr at room temperature in a humidified chamber, (iv) the wells were washed 3x with 200μl of ELISA wash (0.05% Tween 20 in 1x PBS, stored at room temperature), (v) 100μl of diluted serum sample was added to each well in duplicate (diluted in ELISA diluent, typically starting at 1:100 and going to 1:3200 for measuring
IgG2a and IgG1 in immunized mice and typically starting at 1:200 and going to 1:6400 for measuring total IgG in immunized mice) and the plates were incubated for 1 hour at room temperature in a humidified chamber, (vi) the wells were washed 3x with 200μl of ELISA wash, (vii) 100μl of horseradish peroxidase linked 2° antibody (anti-mouse IgG, anti-mouse IgG2a, or anti-mouse IgG1 (1:2500) (Southern Biotech) was added to each well (diluted in 2° antibody diluent—2% FBS (Atlanta Biologicals) in 1x PBS, stored at -20°C) and the plates were incubated for 1 hr at room temperature in a humidified chamber, (viii) the wells were washed 3x with 200μl of ELISA wash, (ix) 100μl of freshly made substrate (0.5mg/ml o-Phenylenediamine Dihydrochloride (OPD) (Pierce, Rockford, IL), 0.03% H2O2 (commercial grade), in 1x citrate buffer (52mM citric acid, 989mM dibasic sodium phosphate, adjusted to pH 5.0 with 2M citric acid, autoclaved and stored at room temperature)) was added to each well and the plates were incubated for 30min at room temperature in the dark, (x) the absorbance at 450nm was measured using a SPECTRA max spectrophotometer (Molecular Devices). For each mouse, the endpoint titer was calculated as the first reciprocal serum dilution at which the mean optical density was at least 3 standard deviations above the background optical density observed in control sera derived from non-immunized mice.

Statistics

Where appropriate, significant differences were calculated with Student’s t test or Mann Whitney Rank Sum Test. P ≤ 0.05 was considered to be statistically significant.
References

CHAPTER 4: RESULTS

In vivo administration of the anti-CD25 antibody PC61 results in a decrease in the frequency of CD4+CD25+Foxp3+ T regulatory cells in the spleens of C57BL/6 mice.

C57BL/6 mice were depleted of CD4+CD25+Foxp3+ T regulatory cells by intraperitoneal (i.p.) administration of an anti-CD25 monoclonal antibody (clone PC61). In our standard immunization protocol, three days prior to immunization with plasmid DNA (day -3), mice were treated with either 1.0 mg of anti-CD25 antibody or with an equivalent volume of vehicle (1X PBS). To check the efficiency of the depletion, and examine its effect on Foxp3 expressing T regulatory cells, splenocytes were isolated from non-DNA-vaccinated control mice four days after the administration of anti-CD25 antibody (day +1). The cells were surface stained with fluorescently labeled antibodies against CD4 (PE-Cy5, clone L3T4) and CD25 (PE, clone 7D4), permeabilized, stained intracellularly for Foxp3 (FITC, clone FJK-16s) and analyzed by flow cytometry. The results of representative experiments are shown in Fig. 1 panels A and B. As shown in panel A, mice that were given anti-CD25 antibody on day -3 showed an 18-fold decrease in the percentage of CD4+CD25^{hi} cells ($P < 10^{-6}$), a 2.5-fold decrease in CD4+CD25^{int} cells ($P < 10^{-5}$), and no difference in the percentage of CD4+CD25^{lo} cells ($P = 0.40$) at 4 days post-depletion. We next wished to determine what impact the depletion of CD25^{hi} and CD25^{int} cells would have on the Treg population. Foxp3 is a lineage specific transcription factor expressed in all murine natural Treg cells, which also co-express CD25 the high affinity subunit of the interleukin-2-receptor (1). At 4 days post-antibody administration, the frequency of CD25^{hi}Foxp3^{+} splenocytes was decreased four-fold in
the anti-CD25 treated mice compared to the sham treated controls (Fig. 1, panel B, \( P < 10^{-6} \)). All p values were calculated using a two-tailed Student’s t test.

In addition, we also chose to assess the frequency of CD25+Foxp3+ Tregs in the spleen on day 14 (17 days post antibody administration) among animals which had also received an injection of plasmid DNA. Day 14 was chosen because it corresponds to the peak of the primary antigen-specific T cell response induced by the plasmid-encoded antigen and will be a point of interest in our future studies. At this timepoint, the frequency of CD25+Foxp3+ cells is still nearly two-fold lower in the depleted mice compared to the controls (Fig. 1C, \( P < 0.001 \), two-tailed Student’s t test). An analysis of the effect of antibody treatment on CD25 expression among CD4+ T cells, as shown in Fig. 1A, could not be done as these mice were vaccinated and CD4+ T effector cells induced by the vaccine also express CD25.

Thus, an anti-CD25 antibody (PC61) administered intraperitoneally depletes CD4+ T cells that express high and intermediate levels of CD25 in the spleen and presumably in other organs as well. Due to this ability, anti-CD25 antibody treatment also greatly reduces the frequency of CD25+Foxp3+ T regulatory cells. In addition, although the CD25+Foxp3+ T regulatory cell population has begun to recover by day 17 post antibody administration, the population has not reach pre-depletion levels by this time point.

**Anti-CD25 antibody administration and vaccination schedule.**
In order to study how CD4+CD25+Foxp3+ Tregs effect the generation of DNA vaccine-induced immune responses, we designed a strategy for administering anti-CD25 antibody, vaccinating with plasmid DNA, and then analyzing the vaccine-specific primary and memory immune responses (Fig. 2). As shown, each mouse received either a 1.0 mg injection of anti-CD25 antibody or vehicle three days prior to the first plasmid immunization. Select groups of mice received one dose of 100μg of pCMV-GP, while other groups received two doses of pCMV-GP, an initial priming dose of 100μg followed by a boost of 50μg three days later. Primary immune responses were analyzed 14 days after the last administration of plasmid DNA, as this time point corresponds to the peak of the vaccine-generated primary immune response. Vaccine-induced memory immune responses were analyzed at least 60 days following the last administration of DNA. For all experiments, non-antibody-treated negative control animals were vaccinated with the vector plasmid pCMV at day 0. In addition, groups of non-antibody-treated animals infected with LCMV 7 days prior were used in each experiment as positive controls for the immunological assays. In most cases T cell responses were analyzed in mice which had received the LCMV glycoprotein encoding construct pCMV-GP. In order to determine the effect of CD25 depletion on vaccine-induced antibody responses, depleted and sham treated mice were vaccinated with a plasmid encoding the nucleoprotein from LCMV (pCMV-NP). This was necessary as there is no ELISA available to measure glycoprotein-specific antibodies.
CD25 depletion prior to plasmid immunization enhances primary antigen-specific CD8+ and CD4+ T cell responses to a model DNA vaccine.

To study the in vivo role of CD4+CD25+Foxp3+ Tregs in generating plasmid-induced primary immune responses, mice were depleted of CD25+ T cells by administration of anti-CD25 antibody on day -3. Mice were then vaccinated with pCMV-GP on day 0. Negative control mice were depleted or sham treated and vaccinated with the empty vector plasmid pCMV. As positive controls, some mice were infected with LCMV 7 days prior to the assay. Fourteen days post-vaccination (no boost), primary immunodominant GP-specific CD8+ T cell (GP33-41) and CD4+ T cell (GP61-80) responses were quantitated in the spleens by intracellular cytokine staining (ICCS) for interferon gamma as described in the Materials and Methods. Fig. 3 panel A shows representative density plots of IFN-γ producing effector CD8+ T cells in the spleens of antibody treated (αCD25, upper panels) and sham treated (Sham, lower panels) DNA vaccinated mice. The percentage of antigen-specific CD8+ T cells, shown in the upper-left quadrant of each density plot, was determined using the following formula, % of interferon gamma secreting (IFN-γ+) CD8+ T cells observed with peptide stimulation – the % of IFN-γ+CD8+ T cells observed without peptide stimulation (background). Treg depleted animals had antigen-specific CD8+ T cell responses that were three to eight-fold higher than the responses observed among the sham treated mice. The percentage of antigen-specific CD8+ T cells in the spleens ranged from 1.1% to 8.2% in the depleted mice and from 0.4% to 0.9% in the sham treated mice (P = 0.03, Mann-Whitney Rank Sum Test). As expected, only background levels of interferon gamma secreting CD8+ T cells were detected in the
spleens of either depleted or sham treated mice which received the vector control pCMV (with or without peptide stimulation, data not shown).

Representative density plots of IFN-γ-producing effector CD4+ T cell responses in the same spleens of the depleted and sham treated mice used in Fig. 3 panel A are shown in Fig. 3 panel B. The percentage of antigen-specific CD4+ T cells ranged from 0.1%-1.2% in the depleted mice and from 0.06% to 0.19% in the sham treated mice ($P = 0.06$, Mann-Whitney Rank Sum Test). For the control animals, the percentage of antigen-specific CD4+ T cells in the spleens was 0.08% in the antibody treated pCMV vaccinated mouse, 0.07% in the sham treated pCMV vaccinated mouse, and 0.42% in the d7 LCMV infected mouse (data not shown). While Treg depleted animals did show evidence of elevated antigen-specific CD4+ T cell responses in their spleens at day 14 post immunization, the increase was not statistically significant.

Together, these results indicate that removal of CD25+ cells prior to DNA vaccination enhances vaccine-generated primary CD8+ and CD4+ T cell responses. Importantly, administration of anti-CD25 antibody did not result in any noticeable depletion of effector T cells generated by the vaccine-encoded antigen, despite the fact that all activated T cells are reported to express CD25. Although not directly examined in our study, we postulate that the effector T cells primed by DNA vaccination express lower levels of CD25 as compared to the T regulatory cell population, which allows them to escape deletion by the anti-CD25 antibody.
CD8+ T cells play an important role in clearing primary viral infections and protecting the host against severe disease when and if the same pathogen is encountered again. As such, they are an important component of vaccine-induced antiviral immunity. Under normal circumstances, CD4+CD25+Foxp3+ T cells act to limit the expansion of effector CD4+ and CD8+ T cells. Our initial data, and that of others, shows that pretreatment with an anti-CD25 antibody results in a transient reduction in the number of regulatory T cells and a corresponding increase in the frequency of vaccine-induced CD4+ and CD8+ effector cells. These data are consistent with a suppressive role for CD25+ regulatory T cells in vivo. Nature has apparently evolved a system of checks and balances to limit T cell activation and expansion because an overactive T cell response could lead to immunopathological consequences for the host. While modulating the number of Tregs during the initial T cell priming and expansion phase may be an effective means of increasing the overall number of antigen-specific effector cells, it could also have unanticipated and potentially detrimental consequences on the functionality of these T cells in vivo. Cytolytic CD8+ T cells express a variety of effector functions necessary to combat viral infections. They produce antiviral cytokines such as interferon gamma and recognize and destroy infected cells in an MHC class I restricted manner, via the production of perforin and granzymes. In addition, the success of any vaccine rests upon its ability to induce a protective population of long-lived memory B and T cells. To determine what, if any, effect depletion of Tregs has on vaccine-induced T cell effector functions and their ability develop into a stable population of memory T cells, we have completed a careful analysis of T cell responses induced in both anti-CD25 treated and non-treated DNA vaccinated mice.
CD25 depletion prior to DNA vaccination does not alter the level of IFN-γ secreted by antigen-specific CD8+ and CD4+ effector T cells.

As a first step in understanding the effect of CD25+ Treg depletion on the functionality of pCMV-GP-induced T cell responses, we assessed whether antigen-experienced CD4+ and CD8+ T cells generated in CD25+ depleted and non-depleted mice were able to produce similar levels of IFN-γ following a short period of *in vitro* re-stimulation with LCMV glycoprotein-specific CD8+ or CD4+ peptides. Fourteen days post-immunization, splenocytes were isolated and stimulated *in vitro* with either GP33-41 (CD8+ ligand, H-2D^b^) or GP61-80 (CD4+ ligand, I-A^b^) peptides, stained for either CD8 and IFN-γ or CD4 and IFN-γ and subjected to flow cytometry. The IFN-γ-positive CD8+ or CD4+ populations in the resulting dot plots were divided into either 4 (CD8+) or 3 (CD4+) regions based on the level of cytokine secretion (Fig. 4 panel A). The proportion of antigen-specific cells in each region was then calculated individually for each mouse (Fig. 4 panel B). During the primary immune response, antigen-specific CD8+ and CD4+ T cells generated in the depleted mice produced IFN-γ at levels similar to those observed in antigen-specific CD8+ and CD4+ T cells generated in the sham treated mice. Of the antigen-specific CD8+ T cells in the depleted mice, 2-7% were very high IFN-γ producers, 16-31% were high IFN-γ producers, 35-41% were intermediate IFN-γ producers, and 27-42% were low IFN-γ producers. Similarly, for the sham treated mice 2-8% were very high producers (*P* = 0.78), 15-30% were high producers (*P* = 0.96), 15-45% were intermediate producers (*P* = 0.48), and 23-68% were low producers (*P* = 0.62). With respect to the CD4+ T cell response, 10-36% of the antigen-specific CD4+ T cells...
in the depleted mice were high IFN-γ producers, 24-42% were intermediate IFN-γ producers and 22-66% were low IFN-γ producers. Much like the depleted mice, among sham treated mice 17-19% were high cytokine producers ($P = 0.32$), 22-67% produced intermediate levels of IFN-γ ($P = 0.93$), and 17-60% were produced low levels of cytokine ($P = 0.63$). All $p$ values were calculated using a two-tailed Student’s t test.

These observations suggest that CD25 depletion prior to plasmid immunization elevates the frequency of antigen-specific effector cells in the spleen without compromising the ability of these effector cells to produce IFN-γ. This is a significant finding, as IFN-γ secretion by antigen-experienced T cells is an important component of the immune response to intracellular pathogens.

**Treatment with anti-CD25 antibody prior to plasmid immunization does not result in the recruitment of low avidity antigen-specific CD8+ T cells into the primary response.**

One possibility we considered during these initial studies was that Tregs are acting *in vivo* as gatekeepers, preferentially limiting the expansion of low avidity T cells. Our hypothesis was that high avidity naïve T cells could maintain contact with the antigen presenting cell for prolonged periods of time and would therefore be resistant to the suppressive effects of T regulatory cells. In contrast, T cells expressing receptors with a low affinity for their peptide-MHC ligand would be unable to maintain prolonged and stable contacts with the APC and might be highly susceptible to Treg mediated
suppression. Thus, Tregs would be acting *in vivo* to ensure the development of the most effective T cell response by preventing the priming and expansion of low avidity cells.

To test this hypothesis, we determined experimentally if the removal of CD25+ cells prior to plasmid immunization would lead to the recruitment of low avidity antigen-specific CD8+ T cells into the primary immune response. Mice were depleted and vaccinated according to the *Anti-CD25 antibody administration and vaccination schedule*. In this experiment, mice received only a single dose of 100μg of pCMV-GP. At 14 days post-vaccination, isolated splenocytes were stimulated for 5.5 hours with decreasing concentrations of GP33-41 (10^{-7} M to 10^{-12} M), an immunodominant CD8+ T cell epitope peptide. The cells were then stained with fluorescently conjugated antibodies against CD8 and IFN-γ and analyzed by flow cytometry. The percentage of cytokine-secreting CD8+ T cells observed in each mouse at the highest concentration of peptide used (10^{-7} M) was defined as 100%. Responses observed in the same mouse at lower concentrations of peptide were expressed as percentages of this maximal response. The average responses in groups of 3 depleted and 3 non-depleted mice are shown in Fig. 5. The avidity profiles are similar for groups of depleted and non-depleted mice and both curves are similar to that of the two day 7 LCMV infected positive control mice. For all three groups, the half-maximal response was observed at a peptide concentration of 10^{-8} M.
Together, these results indicate that reducing the frequency of CD4+CD25+Foxp3+ Tregs before plasmid immunization does not result in the recruitment of low avidity antigen-specific CD8+ T cells into the primary immune response.

**Anti-CD25 treatment prior to plasmid vaccination results in enhanced cytolytic responses during the primary immune response.**

We wanted to assess *in vivo* whether the CD8+ T cells generated in CD25 depleted mice after vaccination were able to efficiently lyse target cells displaying the correct viral peptide. To analyze MHC class I restricted cytotoxic effector T lymphocyte responses *in vivo*, syngenic spleen cells from C57BL/6 donor mice were pulsed with the immunodominant GP-specific CD8+ T cell epitope peptide GP33-41 and labeled with a high concentration of CFSE (CFSE\textsuperscript{high}, 5.0μM). As an internal control for non-specific lysis, a second population of donor cells was pulsed with an MHC mismatched CD8+ T cell epitope peptide NP118-126 and labeled with a lower concentration of CFSE (CFSE\textsuperscript{low}, 1.0μM). Fourteen days post-boost, depleted and non-depleted pCMV-GP vaccinated B6.PL-Thy1a/CyJ recipient mice were given 1x10^7 cells of each target population via intravenous tail vein injection (a total of 2x10^7 cells per mouse). Non-depleted pCMV vaccinated mice and non-depleted day 7 LCMV infected mice also received the same cell transfer and served as negative and positive controls, respectively. Six hours post-transfer, recipient mice were sacrificed and donor T lymphocytes were identified by staining with an antibody against Thy1b. The cells were analyzed using a flow cytometer for the presence of donor cell populations, CFSE\textsuperscript{high}-Thy1b+GP33-41-pulsed cells and
CFSE\textsuperscript{low}-Thy1b+-NP\textsubscript{118-126}-pulsed cells. As shown in Fig. 6 panel A, at 6 hours post-transfer the spleen of the pCMV vaccinated mouse still contains a 1:1 ratio of CFSE\textsuperscript{high}-GP\textsubscript{33-41}-pulsed cells to CFSE\textsuperscript{low}-NP\textsubscript{118-126}-pulsed cells. Conversely, in the day 7 LCMV infected mouse nearly all of the CFSE\textsuperscript{high}-GP\textsubscript{33-41}-pulsed cells have been eliminated and the CFSE\textsuperscript{low}-NP\textsubscript{118-126}-pulsed population now accounts for 99.0% of the CFSE+Thy1b+ cells. Thus, LCMV-specific CTL present in the infected mouse are selectively killing only donor cells expressing the appropriate viral peptide. Naïve mice, which lack anti-LCMV CTL, fail to kill either cell population. Panel A also shows data from the spleens of depleted and non-depleted pCMV-GP vaccinated recipient mice. As evident after the 6 hour incubation in both depleted and non-depleted mice, the CFSE\textsuperscript{high}-GP\textsubscript{33-41}-pulsed cells account for a smaller percentage and the CFSE\textsuperscript{low}-NP\textsubscript{118-126}-pulsed cells account for a larger percentage of the total CFSE+Thy1b+ cells, as compared to the pCMV negative control mice. In order to quantify \textit{in vivo} antigen-specific cytotoxicity, the percentage of peptide-specific lysis was determined as described in the \textit{Materials and Methods}. Shown in panel B, the anti-CD25 treated pCMV-GP vaccinated mice show a trend towards enhanced CTL activity, as evident by an increase in the percentages of specific lysis during the primary response as compared to sham treated pCMV-GP vaccinated mice. However, due to the high variability in lytic responses among the mice in each group, these data are not statistically significant ($P = 0.10$, two-tailed Student’s t test).
Treatment with anti-CD25 antibody prior to DNA immunization leads to enhanced antigen-specific memory T cell responses in the lymph nodes and in the liver, but not in the spleen.

We also decided to analyze the vaccine-induced memory immune response, as the robustness of the vaccine-generated memory response is indicative of the level of protection conferred to the host. Mice were antibody or sham treated, vaccinated with pCMV-GP, and boosted according to the Anti-CD25 antibody administration and vaccination schedule. Negative control mice were vaccinated with pCMV and positive control mice were infected with LCMV 7 days prior or 60+ days prior to the day of analysis. Day 60+ post-boost, animals were sacrificed and lymphocytes were isolated from the spleens, livers, and lymph nodes as described in the Materials and Methods. Spleen-specific immune responses were analyzed in each individual mouse however, due to the small number of cells isolated from the other organs, cells from the lymph nodes and livers were pooled for each group. Lymphocytes from the spleens, lymph nodes, and livers were stimulated in vitro with GP33-41 peptide and stained with fluorescently labeled antibodies against CD8 and IFN-γ. To examine memory CD4+ T cell responses, splenocytes were also stimulated with GP61-80 and stained with fluorescently labeled antibodies against CD4 and IFN-γ. Cells from the spleen were run on a BD FACscan flow cytometer and cells from the lymph nodes and liver were run on a Dako CyAn ADP flow cytometer. Data from the FACscan was analyzed using WinMDI version 2.8 and data from the Dako CyAn ADP was analyzed using Summit version 5.0. In Fig. 7, each plot shows the antigen-specific CD8+ T cell response in the spleen (panel A), the liver (panel B), or the lymph nodes (panel C) of depleted mice or sham treated mice. For the spleens, the responses shown are of individual mice. For the livers and the lymph nodes,
the responses shown are of groups of depleted or sham treated mice. The percentage shown above the upper-right quadrant is the percentage of antigen-specific CD8+ T cells minus the background percentage of cytokine positive cells observed in the absence of peptide stimulation.

As seen in panel A, there is no detectable difference in the frequency of GP33-specific CD8+ T cells in the spleens of the two treatment groups, depleted and non-depleted. Responses in the depleted mice ranged from 0.07% to 0.29% and responses in the sham treated mice ranged from 0.08% to 0.25% ($P = 0.97$, two-tailed Student’s t test). For the control animals, the antigen-specific CD8+ T responses were 0.00% and 0.09% in the sham treated pCMV vaccinated mice, 2.14% in the d60+ LCMV infected mouse, and 7.82% in the d7 LCMV infected mouse (data not shown). The frequency of antigen-specific CD4+ T cells in the spleens of the depleted and sham treated mice were also similar ($P = 0.32$) (data not shown). However, antigen-specific CD8+ T cell responses observed in the livers (B) were slightly higher in the depleted group as compared to the sham treated group, 0.39% versus 0.26%. Most notably, antigen-specific CD8+ T cell responses were strikingly higher in the lymph nodes (C) of the depleted mice (0.34%) as compared to the sham treated mice, in which there were no detectable response (0.00%).

Overall, these data indicate that reducing the T regulatory cell population prior to plasmid immunization does not lead to an enhancement in the frequency of memory CD8+ and CD4+ T cells in the spleen, but does result in an amplified antigen-specific memory CD8+ T cell response in the lymph nodes and possibly in the liver.
Pre-treatment with anti-CD25 antibody intensifies the vaccine-induced cytolytic response during the memory phase of the immune response.

In addition to assessing the frequency of cytokine-secreting, antigen-specific memory CD8+ T cells, we also chose to determine if CD8+ memory T cells generated in the antibody treated mice were able to efficiently lyse cells displaying the correct viral peptide. To do this, we utilized an in vivo CTL assay very similar to the one described in Fig. 6. Donor splenocytes were isolated from C57BL/6 mice and divided into two populations. The first population was pulsed with the immunodominant GP-specific CD8+ T cell epitope peptide GP33-41 and stained with a high concentration of CFSE (CFSEhigh). The second population was pulsed with an MHC mismatched CD8+ T cell epitope peptide NP118-126 and stained with a lower concentration of CFSE (CFSELow). A 1:1 ratio of CFSEhigh-GP33-41-pulsed to CFSELow-NP118-126-pulsed cells (1 x 10^7 of each, 2x10^7 total) was injected intravenously into depleted or sham treated pCMV-GP immunized B6.PL-Thylf/CyJ recipient mice d60+ post-boost. In addition, the same cell transfers were made into pCMV vaccinated negative control mice and d7 LCMV infected positive control mice. After an 18 hour incubation period, the recipient mice were sacrificed and the splenocytes were stained with an antibody against Thy1b and examined using a flow cytometer for the presence of each target cell population. The data shown in Fig. 8 panel A are histograms of individual mice. The number above each CFSEhigh and CFSELow target population indicates the percentage of that population remaining in the spleen at 18 hours post-transfer. As shown, the spleen of a representative pCMV control mouse contains both target populations at approximately equivalent frequencies, 44.0% of the CFSE+ cells are CFSELow-NP118-126-pulsed and 55.8% are CFSEhigh-GP33-41-
pulsed. In contrast, in the d7 LCMV infected mouse, almost all of the CFSE\textsuperscript{high} -GP\textsubscript{33-41} pulsed cells have been eliminated and the CFSE\textsuperscript{low} -NP\textsubscript{118-126} pulsed cells now account for 99.3\% of the CFSE labeled cells in the spleen. Also shown, are histograms indicating the frequencies of both target cell populations in the spleens of antibody and sham treated recipient mice. These frequencies, and the frequencies observed in the pCMV control mice, were used to calculate the percent specific lysis for each mouse (Fig. 8 panel B). The percentage of antigen-specific lysis ranged from 27.19\% to 46.52\% in the depleted mice and from 10.57\% to 24.74\% in the sham treated mice (\textit{P} = 0.05, two-tailed Student’s t test). Thus, these data indicate that CD25 depletion preceding plasmid immunization results in elevated antigen-specific CTL activity during the memory phase of the immune response.

\textbf{Antibody treated mice show lower viral titers in the spleen 72 hours post mega-dose LCMV challenge as compared to non-treated mice.}

Previously published data by our laboratory demonstrates that immunization with plasmids expressing either the LCMV glycoprotein (pCMV-GP) or nucleoprotein (pCMV-NP) alone can protect C57BL/6 mice against peripheral challenge with a standard dose of LCMV (5 x 10\textsuperscript{5} plaque forming units (pfu)). Protection against LCMV is mediated by CD8\textsuperscript{+} CTL, and is defined in the intraperitoneal challenge model as a significant reduction in viral titer in the spleen 4 days after viral challenge. Thus, even the low numbers of CD8\textsuperscript{+} CTL primed without CD25\textsuperscript{+} depletion are capable of rapidly controlling the replication of LCMV \textit{in vivo}. However, we decided to determine if the increased frequency of antigen-specific memory CD8\textsuperscript{+} T cells observed in the lymph
nodes and livers of depleted mice, combined with the enhanced CTL activity observed in
the spleens of depleted mice, would lead to even more efficient clearance of the virus, as
assessed at four days post-challenge. Towards this end, mice were antibody or sham
treated and DNA immunized according to the Anti-CD25 antibody administration and
vaccination schedule. For this experiment, mice were immunized and boosted with
pCMV-NP, as antibody levels were also assessed in these same mice. Negative and
positive control mice were immunized with the vector plasmid pCMV or infected with
LCMV 7 days prior to the harvest, respectively. Sixty days post-boost, mice were
challenged intraperitoneally with $5 \times 10^5$ pfu of LCMV. Four days later, the animals were
sacrificed and viral titers in the spleens were determined by plaque assay on Vero cell
monolayers as described in the Materials and Methods. As shown in Fig. 9 panel A, by 7
days post infection antiviral CTL in naïve mice have effectively controlled the infection
and virus levels are below the plaque assay limit of detection (50 pfu/gram spleen). In
contrast, at 4 days post infection mice vaccinated with pCMV (null) have not yet
developed appreciable numbers of virus-specific CTL. Consequently, titers of the virus in
the spleens of the pCMV vaccinated controls are high ($4-8 \times 10^6$ pfu/ gram). Viral titers
among the anti-CD25 and sham treated pCMV-NP vaccinated mice were similar and
ranged from below the limit of detection in the assay to $7.5 \times 10^3$ pfu/gram ($P = 0.33$, two-
tailed Student’s t test). All of the mice both antibody treated and sham treated were
protected, as viral titers were reduced by at least 99.0% as compared to the pCMV
controls.
After obtaining these results, which indicated that anti-CD25 antibody treatment did not increase viral clearance and/or lead to a decrease in viral infectivity, we decided to push the system by increasing the amount of virus given during the challenge and by examining viral titers in the spleen at both three and four days post-challenge. Two groups of mice were treated according to the Anti-CD25 antibody administration and vaccination schedule, the first group to be sacrificed 72 hours post-challenge and the second to be sacrificed at 96 hours post-challenge. Negative (nondepleted, pCMV vaccinated) and positive (day 7 post LCMV) control mice for both time points were treated as previously described. Day 60+ post-boost, mice were challenged with 5x more virus than that used in the standard assay (2.5 x 10^6 pfu). Seventy-two and 96 hours post-challenge, viral titers in the spleens were measured by plaque assay. As shown in Fig. 9 panel B, at 72 hours post-challenge viral titers ranged from 6.34 x 10^5 to 6.87 x 10^6 pfu/gram among the depleted mice and from 5.33 x 10^5 to 7.64 x 10^6 pfu/gram among the sham treated mice (\(P = 0.35\), two-tailed Student’s t test). Importantly, four of the five antibody treated DNA vaccinated mice showed a reduction in titer greater than 85% as compared to the single null mouse. However, only one of the five sham treated, DNA vaccinated mice showed a similar reduction in titer. By 96 hours post-challenge, viral titers ranged from below the limit of detection to 1.79 x 10^5 pfu/gram of spleen in the depleted mice and from 9.43 x 10^4 to 4.80 x 10^5 pfu/gram in the spleens of the sham treated mice (\(P = 0.08\), two-tailed Student’s t test). At this later time point, all of the mice, antibody treated and sham treated showed a reduction in titer greater than 85% as compared to the null mouse.
Together, these data indicate that depleting CD25+ T regulatory cells prior to DNA immunization results in more rapid clearance and/or decreased infectivity of the virus as observed by three days post-challenge. However, this effect is transient as after an additional 24 hours of infection, both antibody treated and sham treated animals show similar levels of virus in the spleen.

**Anti-CD25 treated and non-treated DNA vaccinated mice develop similar CD8+ T cell recall responses following LCMV challenge.**

In addition to analyzing viral titers, we also tested if CD25 depletion prior to vaccination affects the development of secondary (specific for LCMV epitopes initially primed by vaccination) and primary (specific for viral epitopes not encoded by the vaccine) dominant and subdominant CD8+ T cell responses in the spleens of the challenged mice at 4 days post infection. For this experiment, we utilized mice immunized with pCMV-NP (Fig. 10 panel A) and pCMV-GP (Fig. 10 panel B).

Splenocytes isolated four days post-challenge from the depleted and sham treated pCMV-NP vaccinated mice challenged with $5 \times 10^5$ pfu of LCMV were restimulated *in vitro* with either a dominant or subdominant NP-specific CD8+ T cell epitope peptide (NP$_{396-404}$ or NP$_{205-212}$, respectively), or a dominant CD8+ T cell epitope not encoded by the vaccine (GP$_{33-41}$). Splenocytes isolated from depleted or sham treated pCMV-GP vaccinated mice challenged with $2.5 \times 10^6$ pfu and sacrificed at 72 or 96 hours post-challenge, were restimulated *in vitro* with an immunodominant CD8+ T cell epitope
peptide GP$_{33-41}$, a subdominant CD8$^+$ T cell epitope peptide GP$_{276-286}$, or an immunodominant CD8$^+$ T cell epitope peptide not encoded by the vaccine, NP$_{396-404}$. The cells were surface stained with fluorescently labeled antibody against CD8, permeabilized, and stained intracellularly with antibody against IFN-γ.

Among the pCMV-NP vaccinated mice, the CD8$^+$ T cell responses were very similar between the antibody treated and sham treated groups, Fig. 10 panel A. The frequency of NP$_{396-404}$-specific cells ranged from 7.99% to 12.09% in the antibody treated mice and from 8.42% to 17.39% in the sham treated mice ($P = 0.4$). Responses against the subdominant NP$_{205-212}$ peptide ranged from 0.02% to 0.90% in the antibody treated mice and from 0.01% to 0.66% in the sham treated mice ($P = 0.5$). As expected, primary responses against the non-vaccine encoded GP$_{33-41}$ epitope peptide were very low at 4 days post infection and ranged from 0.02% to 0.20% in the antibody treated mice and from below the limit of detection to 0.10% in the sham treated mice ($P = 0.6$). For the two pCMV vaccinated control mice NP$_{396-404}$-specific responses were 0.3% and 0.13%, the NP$_{205-212}$-specific responses were between 0.4% and 0.11%, and the GP$_{33-41}$-specific responses were 0.16% and 0.19% at four days post-challenge (data not shown). In the long-term LCMV immune control mouse, four days post-challenge, the NP$_{396-404}$-specific response was 18.52%, the NP$_{205-212}$-specific response was 0.82%, and the GP$_{33-41}$-specific response was 4.43% (data not shown). For the d7 LCMV infected control mouse, the NP$_{396-404}$-specific response was 8.95%, the NP$_{205-212}$-specific response was
1.97%, and the GP\textsubscript{33-41}-specific response was 8.48% (data not shown). P values were calculated using a two-tailed Student’s t test.

Epitope-specific CD8+ T cell responses in the spleens of antibody treated and sham treated pCMV-GP vaccinated mice were also very similar at three and four days post-challenge. In the pCMV-GP vaccinated mice at three days post-challenge (Fig. 10 panel B), the GP\textsubscript{33-41}-specific response ranged from an undetectable level to 1.12% in the antibody treated mice and from an undetectable level to 0.28% in the sham treated mice \((P = 0.18)\). The GP\textsubscript{276-286}-specific response was only measurable and above the response observed in the pCMV vaccinated mouse in one out of the five antibody treated mice and none of the sham treated mice and therefore, this data is not shown. As expected, primary CD8+ responses against the viral nucleoprotein were difficult to measure in all groups of mice. A primary response to the NP\textsubscript{396-404}-epitope was only detected in two of the five antibody treated mice and one of the five sham treated mice (data not shown). In contrast to what was observed at 3 days post infection, by 4 days post-challenge (Fig. 10 panel C) GP\textsubscript{33-41}-specific responses had increased significantly and ranged from 5.15% to 17.33% in the antibody treated mice and from 4.05% to 17.92% in the sham treated mice \((P = 0.6)\), the GP\textsubscript{276-286}-specific response ranged from 0.76% to 9.10% in the antibody treated mice and from 1.08% to 3.52% in the sham treated mice \((P = 0.6)\), and the NP\textsubscript{396-404}-specific response ranged from 0.12% to 0.38% in the antibody treated mice and from 0.15% to 0.53% in the sham treated mice \((P = 0.3)\).
These data together indicate that reducing the frequency of CD25+ cells before vaccination does not augment the antigen-specific CD8+ T cell response in the spleen upon LCMV challenge during the memory phase.

**Anti-CD25 treatment prior to plasmid immunization results in elevated humoral immune responses to a plasmid encoded antigen.**

Although antibodies play no role in protection against LCMV, they are extremely important in host defense against many other microbial pathogens. Thus, we felt it important to assess the effect of CD25+ T cell depletion on the induction of IgG responses to our model DNA vaccine. Groups of 5 mice were pretreated with antibody or vehicle and vaccinated with pCMV-NP according to the **Anti-CD25 antibody administration and vaccination schedule.** In this experiment, mice were not boosted. Four weeks post-immunization, anti-LCMV-specific serum IgG, IgG2a, and IgG1 endpoint titers were determined by ELISA as described in the **Materials and Methods.** In Fig. 11 panel A, where each dot represents the endpoint titer in an individual mouse, it can be observed that antibody treated mice produced greater amounts of NP-specific total IgG, IgG2a, and IgG1 as compared to the sham treated mice. The nucleoprotein-specific IgG titers ranged from 2.6 - 3.4 x10^3 among the depleted mice and from 1.97 - 2.2 x10^3 in the sham treated mice (**P < 0.001, two-tailed Student’s t test**). Vaccine induced IgG2a titers ranged from 2.0 - 2.3 x10^3 in depleted mice and from 1.7 - 1.9x10^3 in the sham treated mouse (**P < 0.001, two-tailed Student’s t test**). Antigen-specific IgG1 titers were also elevated in the antibody treated mice and ranged from 2.0 - 2.3x10^3 in the antibody
treated mice and from $1.7 - 2.2 \times 10^3$ in the sham treated mice ($P = 0.05$, two tailed Student’s $t$ test).

We also chose to determine if the depletion of CD25+ T cells would alter the Th1/Th2 balance as IgG2a is indicative of a Th1 response and IgG1 is indicative of a Th2 response. To do this we calculated the ratio of IgG2a/IgG1. The ratios for individual mice can be seen in Fig. 11 panel B. For both antibody and sham treated mice, the ratios of IgG2a to IgG1 were approximately equal to 1 in all of the mice ($P = 0.7$), indicating a balanced IgG response to the vaccine-encoded antigen. Importantly, these data suggest that anti-CD25 treatment prior to vaccination does not alter the Th1/Th2 balance. Overall, these results indicate that anti-CD25 treatment before DNA immunization enhances the antigen-specific humoral immune response without altering the Th1/Th2 balance.

**Anti-CD25 treatment prior to LCMV infection reduces the frequency of antigen-specific CD8+ T cells during both the primary and the memory phases of the immune response.**

After observing that anti-CD25 antibody, administered prior to plasmid immunization enhances both primary and memory immune responses generated by the DNA encoded antigen, we then decided to assess how antibody treatment would affect the development of primary and memory CD8+ T cell responses to a viral infection. To complete this analysis, mice were either treated with anti-CD25 antibody or treated with an equivalent volume of vehicle and infected intraperitoneally 3 days later with $5 \times 10^5$ pfu of LCMV. At five, eight, fourteen, or thirty days post-infection, mice were sacrificed and isolated
splenocytes were stimulated for 5.5hrs with either the immunodominant GP-specific CD8+ T cell epitope peptide GP33-41 or the immunosubdominant GP-specific CD8+ T cell epitope peptide GP276-286. The cells were then surface stained with fluorescently labeled anti-CD8 antibody and intracellularly stained with fluorescently labeled anti-IFN-\(\gamma\) antibody. Shown in Fig. 12 are the antigen-specific CD8+ T cell responses against both GP33-41 and GP276-286 in the sham treated and CD25+ T cell depleted mice. The immunodominant GP33-41-specific responses at day five post-infection were 4.74% and 3.58% in the sham treated mice and 1.65% and 0.20% in the antibody treated mice (\(P = 0.07\)), at day eight post-infection the responses were 10.31% and 8.48% in the sham treated mice and 3.86% and 5.55% in the antibody treated mice (\(P = 0.03\)), at day fourteen post-infection the responses were 2.56% and 6.67% in the sham treated mice and 3.37% and 3.11% in the antibody treated mice (\(P = 0.57\)), at day thirty post-infection the responses ranged from 1.30% to 3.42% in the sham treated mice and from 0.56% to 1.50% in the antibody treated mice (\(P = 0.01\)). The subdominant GP276-286-specific responses at day five post-infection were 0.16% and 0.20% in the sham treated mice and below the limit of detection in one antibody treated mouse and 0.01% in the other (\(P = 0.01\)), at day eight post-infection the responses were 2.26% and 2.07% in the sham treated mice and 0.70% and 1.7% in the antibody treated mice (\(P = 0.03\)), at day fourteen post-infection the responses were 0.92% and 1.21% in the sham treated mice and 0.63% and 0.92% in the antibody treated mice (\(P = 0.09\)), and at day thirty post-infection the responses ranged from 0.55% to 1.59% in the sham treated mice and from 0.28% to 0.93% in the antibody treated mice (\(P = 0.17\)). The p values were all calculated using a two-tailed Student’s t test.
Based on these observations, CD25+ T cell depletion prior to LCMV infection appears to result in decreased development of antigen-specific CD8+ T cell responses, directed against both immunodominant and subdominant epitopes during the initial phase of the immune response. However, during the early memory phase of the response (d30) only the antigen-specific CD8+ T cell response directed against the immunodominant epitope was decreased in the depleted mice, as the subdominant response was unchanged. Therefore, although further studies must be conducted to confirm this proposal, we postulate that effector CD8+ T cells generated in response to viral infection express high levels of CD25 and thus, are depleted by anti-CD25 antibody. As the CD8+ T cells, and for that matter the CD4+ T cells, generated by the DNA vaccine were not depleted by anti-CD25 antibody, we postulate that these cells express lower levels of CD25 as compared to those cells generated in response to the virus.
References

FIG. 1. In vivo administration of the anti-CD25 antibody PC61 results in a decrease in the frequency of CD4+CD25+Foxp3+ T regulatory cells in the spleens of C57BL/6 mice.

Mice were injected intraperitoneally with 1.0 mg of anti-CD25 monoclonal antibody (clone PC61) or sham treated with vehicle. Four (panels A and B) or seventeen (panel C) days later splenocytes were isolated and surface stained with fluorescently labeled anti-CD25 (PE, clone 7D4) and anti-CD4 (PE-Cy5, clone L3T4) antibodies. The cells were then permeabilized and stained intracellularly with anti-Foxp3 (FITC, clone FJK-16s) antibody. The data were collected with a FACScan flow cytometer (BD Biosciences) and analyzed with WinMDI software (ver 2.8, Joseph Trotter). All data shown were first gated on live lymphocytes using forward and side scatter characteristics. For panel A, each plot represents an individual mouse and for panels B and C each bar represents an individual mouse.
FIG. 2. Anti-CD25 antibody administration and vaccination schedule. Mice received 1.0 mg of anti-CD25 antibody or an equivalent volume of vehicle three days prior to the first plasmid immunization. Some groups of mice received one dose of 100μg of pCMV-GP while other groups received two doses of pCMV-GP—100μg pCMV-GP followed by a boost of 50μg of pCMV-GP three days later. The vaccine-induced primary immune response was analyzed 14 days following the last administration of plasmid DNA. The vaccine-induced memory immune response was analyzed at least 60 days following the last administration of plasmid DNA.
FIG. 3. CD25 depletion prior to plasmid immunization enhances primary antigen-specific CD8+ and CD4+ T cell responses to a model DNA vaccine. Groups of C57BL/6 mice were treated with anti-CD25 antibody or an equivalent volume of vehicle. Three days later, animals were vaccinated intramuscularly with 100 ug of pCMV-GP or the control plasmid pCMV. Immunodominant glycoprotein-specific CD8+ (GP33-41, panel A) and CD4+ (GP61-80, panel B) T cells were quantified by ICCS in the spleens 14 days post-vaccination as described in the Materials and Methods. Each dot plot represents a single mouse. The number in the upper-left quadrant denotes the percentage of peptide specific T cells minus background (the percentage of double positive T cells appearing without peptide stimulation).
FIG. 4. CD25 depletion prior to DNA vaccination does not alter the level of IFN-γ secreted by antigen-specific CD8+ and CD4+ effector T cells.

Interferon gamma levels among the population of GP61-80 and GP33-41 specific CD4+ and CD8+ T cells in the spleens was assessed by intracellular cytokine staining 14 days post-vaccination with pCMV-GP plasmid DNA. The cells were surface stained with anti-CD8 or anti-CD4 antibody and intracellularly with anti-IFN-γ antibody and (A) the IFN-γ-producing CD8+ and CD4+ T cell populations were divided into regions based upon their level of cytokine secretion. (B) Shows the percentage of antigen-specific cells for each level of cytokine secretion. Each bar represents an individual mouse.
FIG. 5. Treatment with anti-CD25 antibody prior to plasmid immunization does not result in the recruitment of low avidity antigen-specific CD8+ T cells into the primary response.

Splenocytes were obtained from individual anti-CD25 treated (n=3) or sham treated (n=3) pCMV-GP vaccinated mice 14 days post-immunization (no boost) and incubated for 5.5 hours with an immunodominant CD8+ T cell epitope peptide (GP33-41) over a 10^5-fold range of peptide concentrations, as shown. Cells were then stained for CD8 and IFN-γ expression. A total of 1 × 10^6 events were acquired on a BD FACScan flow cytometer and live cells were analyzed for expression of CD8 and IFN-γ. For each group, the response at each peptide concentration is expressed as a percentage of the maximum response (measured at 10^-7 M peptide) in that group. The half-maximal response is indicated by a horizontal dashed line. Error bars represent the standard deviation within the group. Two mice infected with LCMV 7 days prior were used as a positive control.
FIG. 6. Anti-CD25 treatment prior to plasmid vaccination results in enhanced cytolytic responses during the primary immune response.

To analyze vaccine-induced, MHC class I restricted, cytotoxic effector T lymphocyte responses in vivo, syngenic spleen cells from C57BL/6 donors were pulsed with the GP-specific CD8+ T cell epitope peptide GP33-41 and labeled with CFSE to give a high level of fluorescence intensity (CFSE\textsuperscript{high}). To control for antigen specificity, a second population of cells was pulsed with an MHC mismatched CD8+ T cell epitope peptide NP118-126 and labeled with CFSE to give a lower level of fluorescence intensity (CFSE\textsuperscript{low}). A 1:1 mixture of 1 x 10\textsuperscript{7} cells of each target cell population was injected intravenously into anti-CD25 treated or non-treated, pCMV-GP immunized B6.PL-Thy1\textsuperscript{a}/CyJ mice 14 days post-boost. Non-antibody treated mice immunized with the empty vector pCMV as well as non-antibody treated mice infected with LCMV 7 days prior to the assay were used as negative and positive controls, respectively. After 6 hours, the mice were sacrificed and splenocytes were surface stained with antibody against Thy1b and analyzed by flow cytometry for the presence of CFSE\textsuperscript{high} (GP33-41-pulsed) and CFSE\textsuperscript{low} (NP118-126-pulsed) target cell populations. To quantify in vivo cytotoxicity, the elimination of the GP33-41-pulsed CFSE\textsuperscript{high} population was monitored and the ratio between the percentage of NP118-126-pulsed and GP33-41-pulsed target cells was calculated. The percentage of specific lysis was then determined as described in the Materials and Methods. Data in panel A show histogram plots of spleen cells obtained from depleted and non-depleted mice, a pCMV vaccinated mouse, and a day 7-infected.
mouse. Panel B shows the percent specific lysis for the depleted and non-depleted mice. The horizontal bars represent the geometric mean percent specific lysis for each group.
FIG. 7. Treatment with anti-CD25 antibody prior to DNA immunization leads to enhanced antigen-specific memory T cell responses in the lymph nodes and in the liver, but not in the spleen.

Anti-CD25 treated and sham treated animals were sacrificed d60+ post-boost. Lymphocytes from the spleens, livers, and lymph nodes were isolated as described in the Materials and Methods and stimulated with the immunodominant GP-specific epitope peptide GP33-41. Following peptide stimulation, the cells were assessed for CD8 and IFN-γ expression. The antigen-specific responses in the spleens (A), livers (B), and lymph nodes (C) of antibody and sham treated mice are shown. In panel A, each dot plot represents an individual mouse. In panels B and C, each density plot shows the response for pooled lymphocytes from treated (n=5) and untreated groups (n=5). For all plots, the percentage above the upper-right quadrant denotes the percentage of GP33-specific CD8+ T cells minus the background (the percentage of double positive T cells appearing without peptide stimulation).
FIG. 8. Anti-CD25 treatment prior to plasmid vaccination results in enhanced cytolytic responses during the primary immune response.

To analyze vaccine-induced, MHC class I restricted, cytotoxic effector T lymphocyte responses in vivo, syngenic spleen cells from C57BL/6 donors were pulsed with the GP-specific CD8+ T cell epitope peptide GP33-41 and labeled with CFSE to give a high level of fluorescence intensity (CFSE_{high}). To control for antigen specificity, a second population of cells was pulsed with an MHC mismatched CD8+ T cell epitope peptide NP_{118-126} and labeled with CFSE to give a lower level of fluorescence intensity (CFSE_{low}). A 1:1 mixture of 1 x 10^7 cells of each target cell population was injected intravenously into anti-CD25 treated and non-treated, pCMV-GP immunized B6.PL-Thy1a/CyJ mice 60+ days post-boost. Non-antibody treated mice immunized with the empty vector pCMV as well as non-antibody treated mice infected with LCMV 7 days prior to the assay were used as negative and positive controls, respectively. After 18 hours, the recipient mice were sacrificed and splenocytes were surface stained with antibody against Thy1b and analyzed by flow cytometry for the presence of CFSE_{high} (GP33-41-pulsed) and CFSE_{low} (NP_{118-126}-pulsed) target cell populations. To quantify in vivo cytotoxicity, the elimination of the GP33-41-pulsed CFSE_{high} population was monitored and the ratio between the percentage of NP_{118-126}-pulsed and GP33-41-pulsed target cells was calculated. The percentage of specific lysis was then determined as

\[ \text{% Specific Lysis} = \frac{\text{NP}_{118-126} \text{CFSE}_{low} - \text{GP33-41} \text{CFSE}_{high}}{\text{NP}_{118-126} \text{CFSE}_{low}} \times 100 \]
described in the *Materials and Methods*. Data in panel A show histogram plots of spleen cells obtained from depleted and non-depleted mice, a pCMV vaccinated mouse, and a day 7-infected mouse. Panel B shows the percent specific lysis for the depleted and non-depleted mice. The horizontal bars represent the geometric mean percent specific lysis for each group.
FIG. 9. Antibody treated mice show lower viral titers in the spleen 72 hours post mega-dose LCMV challenge as compared to non-treated mice. CD25 depleted and sham treated, pCMV-NP vaccinated mice were challenged with 5 x 10^5 pfu LCMV d60+ post-boost and the virus in the spleens was titered 96 hours post-challenge (A). The solid horizontal line denotes a 99.0% reduction in viral titer as compared to the null mice. The dashed horizontal line denotes the level of detection for the plaque assay. In a separate experiment, antibody treated and non-treated pCMV-GP vaccinated mice were challenged with 2.5 x 10^6 pfu LCMV d60+ post-boost and the virus in the spleens was titered 72 hours or 96 hours post-challenge (B). The horizontal lines denote an 85% reduction in viral titer. Mice given the control plasmid pCMV (null) and mice infected with LCMV 7 days prior to the day of sacrifice were used as negative and positive controls, respectively. Each bar represents the titer in an individual mouse. Crosses denote mice for which the titer was undetectable.
FIG. 10. Anti-CD25 treated and non-treated DNA vaccinated mice develop similar CD8+ T cell recall responses following LCMV challenge.

Anti-CD25 treated and sham treated, pCMV-NP vaccinated mice were challenged with 5x10^5 pfu LCMV d60+ after the final immunization and the frequency of NP_{396-404}-specific, NP_{205-212}-specific, and GP_{33-41}-specific CD8+ T cells were assessed in the spleens at 4 days post-infection (A) by ICCS as described in the Materials and Methods. In panels B and C, anti-CD25 treated and sham treated, pCMV-GP vaccinated mice were challenged with 2.5x10^6 pfu LCMV d60+ after the final immunization and the frequency of GP_{33-41}-specific CD+ T cells were assessed in the spleens 3 days (B) and 4 days (C) post-infection. The frequency of GP_{276-286}-specific and NP_{396-404}-specific CD8+ T cells was assessed in the spleens 4 days post-infection (C). Each bar represents the response in an individual mouse. Crosses denote mice for which the response was undetectable.
FIG. 11. Anti-CD25 treatment prior to plasmid immunization results in elevated humoral immune responses to a plasmid encoded antigen.
Groups of C57BL/6 mice were treated with anti-CD25 antibody or vehicle and then vaccinated 3 days later with 100 μg of either pCMV-NP or the negative control plasmid pCMV. For this experiment, mice were not boosted. Four weeks post-vaccination anti-LCMV specific serum IgG, IgG2a, and IgG1 endpoint titers were determined by ELISA as described in the Materials and Methods. (A) Each symbol represents the endpoint titer in an individual mouse. The horizontal lines represent the geometric mean titer for each group. (B) Shows the ratio of IgG2a to IgG1 for an individual mouse.
FIG. 12. Anti-CD25 treatment prior to LCMV infection reduces the frequency of antigen-specific CD8+ T cells during both the primary and the memory phases of the immune response.

Groups of C57BL/6 mice were treated with anti-CD25 antibody or vehicle and then infected 3 days later with LCMV. The frequency of GP33-41 specific CD8+ T cells was measured at 5, 8, 14, and 30 days post-infection in the spleens of individual mice by ICCS as described in the Materials and Methods. Each bar represents the response in an individual mouse. Crosses represent mice for which the response was undetectable.
CHAPTER 5: DISCUSSION

T regulatory cells are a unique population of immune cells that benefit the host by maintaining peripheral tolerance (1-3) and by preventing immune-mediated tissue damage caused by an overly exuberant immune response (4, 5). However, recently it has also become apparent that by dampening the immune response these cells may also inadvertently promote the survival and long-term persistence of certain pathogens, thereby increasing pathogen-induced tissue damage (6, 7). The observation that T regulatory cells are able to actively suppress effector T cell responses, prompted a 2004 study by Barry Rouse and colleagues in which they demonstrated that depletion of CD25+ T cells prior to immunization with a plasmid DNA encoding the glycoprotein B gene of HSV-1 (gB-DNA) resulted in an increase in the frequency of both primary and memory CD8+ T cell responses against an immunodominant HSV-1 peptide encoded by the vaccine (8). This initial report spurred our present study in which we have provided new insight into the effect of T regulatory cells on the generation of plasmid-vaccine-induced immune responses, both primary and memory responses and cellular and humoral responses.

Prior to initiating our studies, in which we examined the role of Tregs in plasmid-based vaccination, we assessed the efficiency of Treg depletion after administration of anti-CD25 monoclonal antibody (PC61). Data shown here indicate that anti-CD25 antibody specifically depletes CD4+ T cells expressing high and, to a lesser extent, intermediate levels of CD25 protein. In the spleen at four days post antibody administration, we
observed a four-fold reduction in CD25+Foxp3+ T regulatory cells. Seventeen days post antibody administration, the Treg population had begun to recover, but still remained at levels at least two-fold lower than those observed in non-depleted mice. Further experiments must be conducted to determine when the T regulatory cell population returns to pre-depletion levels.

The data presented here show that reducing T regulatory cells prior to plasmid vaccination results in a measurable increase in the frequency of CD8+ and CD4+ T cells which respond to the plasmid-encoded antigen during a primary immune response. As compared to mice that received DNA alone, CD25 depleted mice showed CD8+ T cell responses that were elevated two to eight-fold in the spleen. Among CD4+ T cells, responses were elevated two to six-fold in the spleens of mice which had been treated with PC61. Many vaccinologists are of the opinion that “more is always better” so that if you directly compare two vaccine regimens and one results in the priming of a greater number of effector cells, then that vaccine will also provide better protection to the host. This rather simplistic view is not always correct. In many instances, the quality of the immune response is often as important as the quantity. For example, protection against LCMV is mediated by CD8+ T cells expressing the cytolytic pore forming molecule perforin. These cytotoxic T lymphocytes recognize and lyse cells expressing viral peptides presented by virus-infected cells in association with major histocompatibility class I molecules. If the “better” vaccine primes CD8+ cells which fail to upregulate perforin or are unable to traffic efficiently to the site of viral replication or recognize the viral peptide efficiently then the host will be unable to clear the infection. Thus, both
quantitative and qualitative aspects of the acquired immune response need to be optimized for effective vaccine design. Importantly, it was also demonstrated that the CD8+ T effector cells generated in the vaccinated anti-CD25 treated mice were functionally equivalent to the CD8+ cells generated in the vaccinated non-depleted controls. The populations of CD8+ T effector cells primed by plasmid vaccination were equivalent in both their ability to produce IFN-\(\gamma\) and in their avidity, as measured directly \textit{ex vivo}. Additionally, data assessing antigen-specific \textit{in vivo} cytolytic activity indicated that the elimination of cells displaying a viral peptide encoded by the vaccine was enhanced in antibody treated mice. Together, these data suggest that T regulatory cells dampen plasmid-vaccine-generated primary immune responses and that reducing this population of cells prior to vaccination thereby enhances the primary immune responses to the vaccine.

Data generated during the course of this study also emphasize the effect of T-regulatory-cell-mediated suppression on vaccine-induced memory responses. Unlike what was observed during the effector phase of the immune response (day 14), it was observed that antigen-specific memory CD8+ T cell responses (day 60+) were not elevated in the spleens of mice which had received the anti-CD25 antibody. Although no effect was seen in the spleens, antigen-specific CD8+ T cell responses were surprisingly elevated in the lymph nodes and livers of the antibody treated mice. These data are not in agreement with data presented in the 2004 paper by Barry Rouse and colleagues, in which they demonstrated a two-fold increase in the antigen-specific memory CD8+ T cell responses in the spleens of PC61 treated mice (8). The difference in experimental findings might be
explained by the fact that in our studies we boosted at three days post primary immunization whereas the Rouse group boosted 21 days post primary immunization. After priming by a professional antigen presenting cells, recently activated effector T cells undergo a rapid period of expansion. As the antigen or infection is cleared from the body, approximately 90% of these effector cells will die by apoptosis, leaving behind a stable population of long-lived memory T cells. The expansion phase generally occurs within the first week to ten days and contraction has been observed to occur within the second to the fourth week after antigen exposure. We boosted during the initial period of the expansion phase whereas the Rouse group boosted during the contraction phase. Boosting during or after contraction may serve to enhance the pool of antigen-specific memory cells, whereas boosting prior to contraction may not have the same effect. The discrepancy between our results and those of Rouse might also be explained by the fact that the Rouse lab gave slightly more anti-CD25 antibody, the antibody was given 24 hours earlier, and the antibody was prepared in a different manner—ammonium sulfate precipitate of hybridoma culture supernatant or as ascites produced in \textit{nu/nu} mice and purified using a Prosep G immunoglobulin purification kit. In our studies, antibody was purified from PC61 culture supernatant using a Protein G column. In agreement with the Rouse lab, we also observed an enhanced antigen-specific memory CD8+ T cell response in the lymph nodes and, as was not examined by the Rouse lab, we observed an enhanced antigen-specific CD8+ T cell response in the liver. The elevated response in the lymph nodes was striking, as grouped lymphocytes from the lymph nodes of depleted mice showed an immunodominant antigen-specific CD8+ T cell response of 0.34%, whereas the response was not measurable in the lymph nodes of sham treated mice.
While we were unable to detect a difference in the frequency of antigen-specific memory CD8+ T cells in the spleens of depleted mice, we did observe a significant enhancement in peptide-specific cytolysis among the depleted mice during the memory phase of the response as assessed in the spleen. This increase in antigen-specific lysis measured in the spleen in the absence of an observed increase in the frequency of antigen-specific memory CD8+ T cells in the same organ, might be explained by the antigen-specific target cells having been lysed in a different organ(s), such as the lymph node and/or liver. Thus, target cells displaying the correct viral peptide may never have made it to the spleen. Additionally, it is possible that after the injection of viral-peptide–coated target cells, CTL from other sites were stimulated to home to and lyse the antigen-specific target cells in the spleen. Importantly, it was observed, although not shown here, that target cells transferred intravenously via tail vein injection do travel to the lymph nodes and spleen. Further studies examining cytolytic activity and antigen-specific T cell frequency within these sites after the transfer of target cells may provide insight into the location of antigen-specific cell lysis by the CD8+ T cells.

After observing amplified antigen-specific memory CD8+ T cell frequencies in the lymph nodes and livers of depleted mice, along with elevated CTL activity, we suspected that after LCMV challenge the antibody treated mice might also exhibit increased clearance of the virus and/or decreased infectivity of the virus. Interestingly, mice given a mega-dose challenge with LCMV showed reduced viral titers in the spleens at 72 hours post-infection, as four of the five antibody treated mice showed a reduction in viral titer.
greater than 85% compared to the pCMV vaccinated (null) control mice. Conversely, only one of the five sham treated mice showed a similar reduction in viral titer. However, given an additional 24 hours, all mice, both antibody and sham treated, displayed viral titers in the spleen that were reduced by greater than 85% as compared to the null controls. This finding that upon challenge antibody treated DNA vaccinated mice cleared the virus more rapidly may be very important in the generation of vaccines against infections where infectivity and damage to the host occurs rapidly.

This data not only demonstrates that anti-CD25 treatment before plasmid vaccination leads to enhanced antigen-specific primary and memory cellular immune responses, but that CD25 depletion also results in an increase in antigen-specific humoral responses. At 4 weeks post-vaccination a statistically significant enhancement in anti-LCMV specific IgG, IgG2a and IgG1 was observed in the depleted compared to the sham treated mice. Additionally, DNA vaccination without antibody treatment resulted in a mixed Th1/Th2 response and, when antibody was given prior to vaccination the Th1/Th2 balance was not altered. These findings may be very crucial in the generation of vaccines against infections where antibody responses are essential for pathogen clearance and protection. Additionally, because anti-CD25 pre-treatment did not alter the Th1/Th2 balance this therapy may be used in conjunction with vaccines specifically engineered to generate either a Th1 or Th2 response.

Our studies also examined the effect of anti-CD25 treatment prior to LCMV infection. Interestingly, antibody administered before infection reduced rather than enhanced the
frequency of primary and memory CD8+ T cells in the spleen. This data is in disagreement with data presented in a 2003 paper by Barry Rouse and colleagues, in which they reported that anti-CD25 antibody administered before infection with HSV elevated the immunodominant antigen-specific CD8+ T cell response two to three-fold in the spleen during both the primary and memory immune responses (9). We would hypothesize that the differences between our studies and that of Rouse et al. are most likely due to differences in the level of CD25 expression on the effector T lymphocytes. CD8+ T effector cells generated in response to the LCMV infection likely express very high levels of CD25, and are therefore susceptible to depletion by the anti-CD25 antibody. Conversely, effector cells generated in response to the HSV-1 infection likely express lower levels of CD25, and are therefore not depleted by the antibody. A difference in CD25 expression on the effector cells induced by HSV and those induced by LCMV would not be surprising as these viruses differ greatly in their pathogenesis. It is also important to note that the effector cells generated in response to the anti-LCMV plasmid-vaccine were not depleted by the antibody, and therefore likely express lower levels of CD25 than those induced by the virus.

As demonstrated in these studies, CD25 depletion prior to plasmid immunization greatly enhances the primary and memory antigen-specific cellular immune responses as well as antigen-specific humoral immune responses. These data provide important insights into the possible therapeutic use of Treg curtailing therapies prior to vaccination. These types of therapies may be useful for both plasmid-vaccines, which, as mentioned previously,
have been shown to be only weakly immunogenic in human clinical trials, and other
types of weakly immunogenic vaccines.
References


