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Investigating and Manipulating Immune Responses to Hepatotropic Pathogens Using Synthetic DNA

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Abstract
Hepatotropic pathogens, such as Hepatitis B virus (HBV), Hepatitis C virus (HCV) and malaria Plasmodium often escape cellular immune clearance, resulting in chronic infections. With billions at the risk of infection, the need for an immune therapy that will incite protective immune responses against these pathogens is more important now than ever. To develop effective therapies against these pathogens, it is important to understand the mechanisms by which liver-primed CD8 T cells become defective. In this report, I directly compared liver-primed CD8 T cells to secondary lymphoid tissue-primed CD8 T cells for differentiation, function, and memory programming in a highly controlled fashion. We used hydrodynamic tail vain injection of synthetic plasmids to establish liver-specific antigen expression in the P14 transgenic mouse model, and studied the priming of CD8 T cells. Intrahepatically activated CD8 T cells exhibited unique expansion, memory differentiation, polyfunctionality and cytotoxicity compared to T cells primed in the periphery. The difference in their expansion resulted in lower memory CTL frequency, which led to reduced protection against lethal viral challenge. These results demonstrated that defective liver priming of naÃ¯ve CD8 T cells contributes to the lower frequency of antigen-specific CTLs observed during liver infection in HBV and

HCV patients, which helps these pathogens to escape immune clearance.

The results from the study provide evidence that, the eradication of HBV and HCV infected hepatocytes will require both the induction of a strong antigen-specific immune

response and the subsequent deployment of that response towards the liver. We therefore assessed the ability of a synthetic DNA vaccine encoding a recombinant plasmid of the HBcAg and HBsAg to drive immunity in the liver. Intramuscular vaccination accompanied by electroporation induced both strong antigen-specific T cell and high titer antibody responses systematically and in the liver. Furthermore, immunized mice showed strong cytotoxic responses that eliminate adoptively transferred HBV-coated target cells in the spleen and liver. These data provide important insight into the generation of peripheral immune responses that are recruited to the liver; an approach that could be beneficial in the search for vaccines or immune-therapies for liver disease.

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Nyamekye Obeng-Adjei

For my daughter who is due to be born in May of 2013.
You are my angel.

I will also like to propose to the woman who made it all possible. I, Nyamekye Obeng-Adjei, will like to spend the rest of my life with you, Arionne Carroll.

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ABSTRACT

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Chapter 1 – Introduction

"A large red organ thoughtfully provided by nature to be bilious with. The sentiments and emotions, which every literary anatomist now knows to haunt the heart, were anciently believed to infest the liver; and even Gascoygne, speaking of the emotional side of human nature, calls it "our hepaticall parte." It was at one time considered the seat of life; hence its name— liver, the thing we live with." - Ambrose Bierce, editor, journalist, fabulist and satirist.

1.1 The liver

The liver is the second largest organ behind the skin in many animals. It is however the largest internal organ in the human body and weighs a little over 1, 500 grams. The organ is located between the diaphragm and the abdominal cavity. It sits on top of the right kidney, stomach and the intestines. The liver is an essential organ that is absolutely vital for survival. It performs various but connected functions including synthesizing nutrients needed for living and growing, and detoxifying the blood of harmful substances. In addition to these functions, the liver regulates the immune response to prevent hepatic damage induced by immune cells.

The liver is divided into two major lobes – the right and the left lobes. The separation of these lobes is based on two branches (right and left) of the hepatic artery and the portal vein. Both the hepatic artery and the portal vein run directly through the liver and provide the liver with 1500 mL of blood per minute. The hepatic portal vein brings in about 70% of this blood and the hepatic arteries account for the rest of the blood flow. The portal vein carries venous blood (deoxygenated blood) that contains digested nutrients from the

gastrointestinal tract and pancreas, to the liver for metabolism. Carbohydrates, peptides, lipids and other nutrients are all carried to the liver through the portal vein.

1.2 Liver infections

The liver is functionally connected to all 72 organs in the human body through 500 vital functions that have been identified. Examples of these functions are: the production of proteins that forms part of the blood plasma, the conversion of extra glucose into glycogen for storage, the production of special compounds and proteins that regulate lipid transport throughout the body, breaking down harmful substances into simple soluble substances that can be cleared out of the body, the regulation of blood peptides, lipids and carbohydrates, the processing of hemoglobin to utilize the iron, and last but not the least, the removal of pathogens and other foreign substances from the bloodstream to prevent infection.

The many functions that are performed through the regulation of the blood contents and the continuous stream of both oxygenated and deoxygenated blood through the liver make the organ readily accessible to most pathogens. Bacteria and other food pathogens from the gut have direct access to the liver through the portal vein. Other blood-borne pathogens reach the liver before they gain access to most tissues. Apart from fully assembled pathogens, the liver is also exposed to several antigens that can cause damage to the organ if the body induces immune responses towards such antigens. The liver microenvironment has a clever way of dealing with pathogens without inciting the body's defense system, which can do more harm than good. The liver has been shown to clear

many blood-borne pathogens using scavenger cells that surround the hepatocytes. These scavenger cells uptake pathogens and destroy them just like phagocytes will engulf foreign particles to kill and digest them. The two most important cells that are involved in protecting hepatocytes from infection are liver sinusoidal endothelial cells (LSECs) and Kupffer cells.

1.2.1 Liver sinusoidal endothelial cell (LSEC)

Liver or hepatic sinusoidal endothelia cells make up 50% of the non-parenchymal hepatic cells, which can be found in the "non-functional" parts of the liver. These cells separate the hepatic cells from passing blood, which is an important aspect in preventing pathogenic infections in the liver (Braet et al., 2004). LSECs express many scavenger receptors, which regulate the trade of proteins and other important materials between the liver parenchyma and blood, while protecting the parenchyma from infectious substances that pass through the blood. It is believed that LSECs preferentially eliminate denatured or manipulated proteins, extracullar matrices and some lipoproteins (Enomoto et al., 2004). The most important feature the LSEC possess are the "bristle-coated micropinocytotic vesicles" and "macropinocytotic vesicles" in their cytoplasm and lysosomal enzymes required for the degradation of foreign substances that the cell engulfs (Wise E., 1970, 1972). Although LSECs are assembled with small spaces (about 100nm in diameter) between the cells, 20nm particles are unlikely to pass through because of the presence of extracellular matrix that has been positioned between these

cells (Schlepper-Schafer, 1986). This is helpful in shielding the hepatocytes from foreign objects.

1.2.2 Kupffer cells

Kupffer cells, also referred to as stellate macrophages are currently the only identified liver resident macrophage population (Haubrich, 2004). They can be found around the walls of liver endothelial cells as part of the mononuclear phagocyte system. These cells, like many macrophages, originate from the bone marrow as monoblasts and then differentiate into monocytes. They then migrate into the blood as peripheral blood monocytes and subsequently mature into Kupffer (macrophage) cells (Naito et al., 1997). Kupffer cells have previously been confused as endothelial cells due to the difficulties involved in isolating them. These cells specialize in destroying foreign microbes, proteins and cancer cells that may infiltrate the liver. Like other macrophages, they destroy microbes by engulfment. Once a Kupffer cell engulfs a pathogen, it traps the pathogen in the phagosome, which is then linked with a lysosome to form a phagolysosome. Degradation enzymes from the phagolysosome then digest the pathogen. In order to prevent destruction by Kupffer cells, some pathogens such as *Plasmodium* spp. sporozoites infect and destroy Kupffer cells before infecting the hepatocytes.

1.3 Major liver pathogens

Despite the great deal of effort liver scavenger cells put forth to prevent hepatic cell infections, there are several pathogens that are able to use the liver as a site of infection, replication, amplification and sometimes, to escape immune response. Viruses like

Hepatitis A, Hepatitis B, Hepatitis C, Hepatitis D and Hepatitis E preferentially infect liver cells, while pathogens like malaria *Plasmodium* infect the liver among other tissues. When these pathogens are left untreated, they lead to liver damages and in extreme cases death. Liver infection by these pathogens will be described in detail in the following sections.

1.3.1 Hepatitis A virus

Hepatitis A (HAV) is a picornavirus; a non-enveloped, positive-stranded RNA virus with an icosahedral capsid. This virus is a food-borne pathogen that usually spreads when a person ingests a solid food or liquid contaminated with feces from infected individuals. The virus passes through mucosal epithelial cells after infection to enter the blood. It travels through the blood where it infects Kupffer cells before crossing the space of Dissé to infect hepatocytes. HAV replicates in the liver and has been shown to lack the ability to replicate outside the liver, *in vivo*.

There is only one known HAV serotype but as many as seven genotypes (I through VII). Almost all humans HAV strains are genotype I and III, with 80% of the infection being genotype I (Costa-Mattioli et al., 2003). Although HAV infection does not lead to chronic liver disease, infected individuals usually suffer from fever, loss of appetite, diarrhea, jaundice, malaise, fatigue and abdominal pain. These symptoms can last for a few weeks or several months, depending on the infected individual. While there is no available treatment, an effective vaccine program has been in place since the early 1990s. Although these vaccine programs have drastically reduced the infection rate in

developed countries, there are still about 1.4 million cases of HAV every year around the world. These infections are usually in countries with poor sanitation and water treatment records (Pintò et al., 2012).

There is an increase in the liver enzyme alanine transferase (ALT) in the blood during the acute infection stage, but there are no data available that show HAV-mediated cytotoxicity. It is believed that liver pathology seen after infection is immune-mediated.

1.3.2 Hepatitis B virus

Human Hepatitis B virus (HBV) is a double stranded DNA virus that is part of the *Hepadnaviridae* family. Like all *Hepadnaviridae* viruses, human HBV infects the liver. With a virion diameter of 42nm, HBV is considered to be one of the smallest human viruses. It is made up of an outer lipid envelope and a nucleocaspsid core protein. The lipid envelope contains proteins that are involved in viral attachment and entry. These proteins are known as hepatitis B surface antigen proteins (HBsAg) or Australia antigens in some old literatures. The presence of HBsAg proteins or antibodies to HBsAg in the blood is usually used to confirm an HBV infection or disease. The nucleocapsid, which is made of proteins called hepatitis B core antigen proteins (HBcAg), encloses the viral DNA polymerase and DNA. There are as many as 9 genotypes of human HBV (genotype A- I) found in different geographical locations, with 8% divergence between genotypes. There are over 24 serotypes that have been described with 8 genotypes (A-H) (Kramvis et al., 2005).

Although no particular receptor on hepatocytes has been identified to aid the entry of HBV, it is known that HBV attachment to hepatocytes requires interaction with a highly sulphated proteoglycan. After attachment and entry, the viral DNA is transcribed into mRNA, which is then translated into the core proteins and DNA polymerase.

The incubation period for the virus ranges from 30 to 180 days with an average period of 90 days. While most infected individuals do not show any symptoms during acute infection, a small population suffers from fatigue, jaundice, nausea and abdominal pains. Chronic infection on the other hand, leads to liver damage, cirrhosis of the liver and eventually, liver cancer, if left untreated. Over 2 billion people have been infected with the virus, with over 370 million chronically infected. About half a million people die each year from liver complications that arise from HBV infection (Block et al., 2007; Rodríguez-Frias F, 2008).

Most acutely HBV-infected individuals clears the virus by themselves and do not need treatment but chronic infected patients are usually placed on antiviral therapies. Currently available antiviral therapies are capable of inhibiting viral replication but unable to clear infection completely. The best method of prevention is the use of vaccines. The current vaccine induces protective antibodies in 85-95% of immunized immuno-competent individuals.

1.3.3 Hepatitis C virus

Hepatitis C virus (HCV) like HBV is a small virus (about 60nm) that infects the human liver. Unlike HBV, HCV is a positive-sense single-stranded RNA virus from the

Flaviviridae family of viruses. HCV, like most RNA viruses, contains both structural proteins and non-structural proteins. The structural proteins include one core protein and two envelope proteins (E1 and E2) while the non-structural proteins consist of 7 proteins (NS1, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The divergence within HCV genotypes are much higher (about 30-35%) than that of HBV. This great genetic diversity contributes to the difficulties that arise in the development of an effective immunotherapy for HCV (Nakano et al., 2012).

HCV uses the envelope proteins E1 and E2 to bind and initiate entry into hepatocytes. The virus attaches itself to HSPGs, and then binds to multiple receptors on the surface of hepatocytes. These receptors include low-density lipoprotein (LDL) receptor, scavenger receptor B1, claudin-1, occludin and CD81, which help the virus to endocytose after binding. HCV replication occurs on intracellular lipid membranes and virion release take place through very low-density lipoprotein secretory pathways.

The incubation period for an HCV infection is anywhere from 2 weeks to 6 months. Only 20% of acute infected individuals are symptomatic. These symptoms are similar to most liver injections; fatigue, nausea, jaundice and abdominal pains (Maheshwari et al.). Over 75% of infected patients develop chronic infection and 60-70% of the chronically infected develop liver disease. HCV accounts for 25% of all liver cancers. There are about 170 million people chronically infected and more than 350 000 die every year from HCV-related liver complications.

There is no vaccine for HCV infection. Therapies available reduce viral replication but do not result in viral clearance. Treatments, which involve antiviral and interferon therapies, respond differently to each viral genotype (Ozaras and Tahan, 2009).

1.3.4 Hepatitis D and E viruses

Hepatitis D and E (HDV and HEV) are the two remaining viruses that infect the liver and cause hepatitis, although they are less prevalent than the other hepatitis viruses. Both HDV and HEV are RNA viruses, but unlike HDV, HEV is a non-enveloped particle. HDV is enclosed in an envelope made up of the three surface antigen proteins of HBV. HDV is a subviral satellite that requires the presences of HBV to infect the human liver. While, HDV does not induce liver disease, it is able to exacerbate complications that arise from HBV infections (Riaz et al., 2011).

Currently, there are 8 known genotypes of HDV and 4 for HEV. Though multiple serotypes of these viruses have not been characterized, the different genotypes of HEV can be sub-classified into different subtypes.

Over 15 million HEV infections occur each year around the world with half a million deaths from HEV complications. The virus passes through contaminated food and water supplies and makes it way to the liver, where it spends about 40 days incubating. Symptoms following HEV infections are very similar to that of HAV; jaundice, nausea and abdominal pains (Kumar et al., 2013).

There are no specific treatments for either virus. HDV is usually prevented with HBV vaccines, since HDV infection is dependent on HBV infection. HEV vaccines are currently only available in China.

1.3.4 Plasmodium Malaria

The *Plasmodium*, which is a genus of Apicomplexen parasites, uses the liver as a site for multiplication and to escape immune responses. The plasmodium, unlike liver viruses, does not infect and reside in the liver to manifest clinical symptoms to its disease, malaria. When a female anopheles mosquito bites the human skin, it can transmit a vector form of the parasite, the sporozoite, into the blood. From there, sporozoite migrates quickly into the liver to escape detection by the innate immune system. In the liver, it uses circumsporozoite protein (CSP) and thrombospondin-related anonymous protein (TRAP) to bind to Kupffer cells, where it proceeds to infect the hepatocytes. Binding to Kupffer cells allows the parasite to overcome the scavenger receptors of the sinusoidal endothelia barrier and therefore, escapes phargocytosis. After the parasite invades the hepatocyte, it transforms into an asexually reproducing merozoite. The parasite spends a week to a month asexually and asymptomatically replicating in the liver. Then thousands of merozoites rupture the liver cells and make their way to the sinusoids where they infect red blood cells. They escape being recognized by the immune system by coating their surface with hepatocyte membrane until they reach the targeted erythrocyte. Symptoms of fever occur when merozoites replicate and bust out of erythrocytes. Other symptoms include vomiting and headaches (Nayyar et al., 2012).

It is estimated that over 200 million cases of malaria occur each year, with over a million deaths. This disease is epidemic in poor countries, especially regions of sub-Saharan Africa. There are several medications available for the treatment of malaria and

prevention. Unfortunately, there are also several undesirable effects associated with these medications. Perhaps the most disastrous aspect of malaria treatment is the parasitie's resistance to drugs, which has made prevention and treatment of malaria infection extremely difficult (Aguiar et al., 2012).

There is currently no vaccine available for malaria. There are several vaccines in different stages of clinical trials across the globe but Word Health Organization has approved none for commercial use. The best way to prevent malaria infections is to control the breeding of the vector host, mosquito (Vaughan and Kappe, 2012).

1.4 Immune defenses in the liver

Although the liver has many mechanisms in place to control pathogenic infections, some pathogens are able to overcome these immune barriers to establish chronic infections. Before pathogens such as HBV, HCV and malaria *Plasmodium* can establish infection, they have to escape both innate and adaptive immune recognition by taking advantage of the specialized tolerogenic environment of the liver.

1.4.1 The innate immune defense of the liver

The innate immune system is the first line of defense that protects a host organism from infection by foreign pathogens. In humans, it prevents the free growth of bacteria and non-specifically attacks and destroys other pathogens that may invade the body. Since the innate immune system is non-specific, there is no need for cell priming or previous engagement. There are several bone marrow-derived cells that are associated with the

innate immune system which also play a role in clearing pathogens in the liver. These are natural killer cells (NK), macrophages, neutrophils and dendritic cells. Kupffer cells, LSEC and liver stellate cells are specialized cells in the liver that operate as the first line of defense to prevent pathogenic infections.

To detect pathogens, the innate immune cells use pattern recognition receptors (PRR) such as toll-like receptors (TLRs) and cytosolic helicases to distinguish pathogen antigens from host proteins. Although there are some differences between PRR expression on splenic and hepatic innate cells, both cell populations can recognize pathogens in similar fashion (Wu et al., 2009).

Hepatotropic pathogens like most pathogens have developed mechanisms to overcome or invade the innate system. This is an important step for the hepatotropic pathogens to gain entry into the hepatic environment and to replicate after infection. The best example is Hepatitis C virus. To gain entry, HCV expresses a polyprotein precursor that induces a unique response that initiates autophagy in the host cells. This response promotes HCV RNA replication and subsequently suppresses type I interferon (IFN) responses. The virus also inhibits recognition by TLRs through the cleaving of IFNB-promoter stimulator 1, an important mitochondrial-signaling molecule that is involved in the production of IFN cytokines (Chen, 2011). HBV, on the other hand, escapes recognition sensing by synthesizing genomic materials in a capsid and suppressing TLRs on liver cells (Wieland et al., 2004).

The *Plasmodium* parasite that causes malaria uses a unique mechanism to escape detection. After infecting the Kupffer cells, the sporozoites create a 'parasitophorous vascoule' in these cells to prevent their molecules from being directly detected by

membrane-bound PRR. Sporozoites also prevent the respiratory burst in Kupffer cells and cover their surface molecules with hepatocyte membranes after bursting out of hepatocytes (Usynin et al., 2007).

It is obvious that the liver possess competent innate immune cells that are capable of clearing pathogens, food antigens and dead/cancer cells. However, some pathogens have developed illusive mechanisms to evade the immune responses induced by these cells.

1.4.2 Liver adaptive immunity and tolerance

The adaptive immune response is a highly specific and specialized system of eliminating pathogens, foreign substances, dead cells and cancer cells from an organism. The two major lymphocytes of this system are the T and B cells. When pathogens invade the body, innate immune cells do their best to clear them, but when the innate system fails, it initiates the adaptive immune system, which recognize specific, non-self antigens on the pathogens and eliminate them from the host. The most important aspect of the adaptive immune system is its ability to generate immunological memory. Immunological memory improves the specificity of the response and reduces the response period after pathogen re-invasion. The humoral response (B cells) clears pathogens by neutralizing the pathogen with antibodies produced by activated or memory B cells, while the cellular response (T cell) destroys cells infected by these pathogens.

The liver holds an enormous population of immune cells, mostly activated T cells. CD8 T cells, which are commonly cytotoxic, are more likely to be found in the liver at any particular time than CD4 T cells. Despite the huge population of CD8 T cells found in the

liver, the environment mostly exhibits tolerogenic effects towards foreign antigens. Liver transplantation within the same mammalian species is less likely to result in rejection, even when immunosuppressive drugs are not involved. This is in sharp contrast to other tissues like kidney, spleen, pancreas and skin, where rejection is inevitable (Calne, 2002). Many independent studies have suggested that the liver confers tolerance to prevent immune response towards liver antigens, which are mostly food antigens. Although the exact mechanism has not been fully elucidated, the liver is found to increase immune suppressive factors and negative immune regulators and to delete immune cells. Liver tolerance has also been attributed to professional APC (innate) antigen presentation and priming of adaptive cells within the liver (Crispe and Mehal, 1996).

Successful hepatotropic human pathogens such as HBV, HCV and *Plasmodium*, take advantage of this suboptimal liver immune response to establish chronic infection (Terilli and Cox, 2012).

1.5 Aims of this thesis

In this thesis, we tested an important hypothesis that aims to understand the defect in host cellular immunity in the liver after pathogenic invasion and explore ways to enhance immune response in the liver using a synthetic DNA vaccine platform.

Many liver immunologists consider the priming of naïve T cells in the liver renders those T cells ineffective. We believe the liver evolved this tolerogenic mechanism to prevent hepatic injuries and insults that can occur during host immune attack. This mechanism has become the liver's own 'Achilles heel' in terms of preventing some chronic infections such as HBV and HCV. Performing head to head comparison of hepatic T cell priming and lymphoid T cell priming, we detected defective effector and memory T cell differentiation after liver priming.

We hypothesized that induction of cellular and humoral immunity through the priming of immune cells in local lymph nodes using plasmid DNA vaccine would be effective in clearing pathogenic liver infections. We used different models to study how to direct immune response to the liver. We believe the generation of peripheral immune responses that can be recruited to the liver will be an important approach that will be beneficial in the search for vaccines or immune-therapies to liver diseases.

Chapter 2 – Liver Cellular immune activation

" There are at least [two] outcomes from intrahepatic T cell activation that may damage the liver rather than result in the graveyard death of cells and tolerance. First, the liver may preferentially induce low-avidity T cells that allow the persistence of viral infections resulting in antigen- specific chronic hepatitis. Second, T cell activation may result in a non-antigen-specific hepatitis through cytokine release, a not uncommon event in non-hepatotropic viral infections." - Doherty P. et al. June 2007.

2.1 Introduction to liver cellular immune priming

The key feature of adaptive immunity underpinning the basis of immunization is the ability of the immune system to mount a specific response against a particular pathogen leading to induction of long-lived memory cells that are capable of immune clearance and protection upon re-infection (Ahmed and Gray, 1996; Kalia et al., 2006). The original concept was that naïve adaptive immune cells are activated when they meet a professional antigen-presenting cell (APC) bearing their specific antigen (Ag) in the secondary lymphoid organs (Germain et al., 2012; Stremmel et al., 2006). Secondary lymphoid organs have highly organized structures and compartments allowing for the concentration of lymphocytes; which in turn enhances the access of APCs to specialized B and T cells for the initiation of adaptive immunity, involving activation, proliferation and expansion (Beltman et al., 2007; Figge et al., 2008; Linderman et al., 2010), and subsequent development of protective memory cells (in the absence of chronic infection). This theory makes lymph nodes and other lymphoid organs the most important tissues in the induction of adaptive immune responses and the generation of immune memory.

However, this view has recently been expanded in the cellular adaptive response setting. A number of independent groups have shown that T cells can not only be activated in the absence of conventional lymphoid organs by using the liver as a substitute site, but these T cells can also recognize liver cells such as Kupffer, sinusoidal endothelial cells (LSECs), hepatic stellate and even hepatocytes as APCs when they express the cognate Ag specific for that T cell (Bertolino et al., 1998; Crispe, 2011; Derkow et al., 2011; Hofmann et al., 2010; Lukens et al., 2009; Wuensch et al., 2010). Transgenic mice lacking lymph nodes and Peyer's patches showing alterations to other secondary lymphoid organs such as spleen and thymus due to point mutations in NFκ-B were immunodeficient. However, a group from Switzerland reported that the immunodeficiency exhibited by these mice was not from the modification or the total lack of the secondary lymphoid structures but rather, a defect in cellular immune activation. The group depicted an unconventional pathway from which an APC can sample antigen and present it to a newly formed lymphocyte in the liver. This process was adequate in inducing cell mediated immune (CMI) responses but not antibody mediated immune response (Greter et al., 2009). In all, these independent groups provide evidence that liver can not only act as a reservoir for the activation of certain naïve adaptive cells but also can present these antigens to the adaptive cells like an APC.

Despite this newfound knowledge, it is known that for hepatotropic infections such as Hepatitis B, Hepatitis C and malaria, there are clear impairments in cell-mediated immunity (CMI). The defect in T cell immune response has been largely credited to the impairment in pathogen resolution and T cell exhaustion, which leads to the establishment of chronic infection (Folgori et al., 2006; Guocai Lv, 2010; Struik and

Riley, 2004). Efforts directed towards the development of therapeutic vaccines against these pathogens have yet to yield fruitful results. Therefore, it is important to take a step back and evaluate the factors that are negatively affecting the induction of strong immune responses against liver-tropic pathogens, which may prove critical for the development of effective immune-therapies.

2. 2 Transfecting the liver – Hydrodynamic Injection

2.2.1 Hydrodynamic Injection model

Primary CD8 T cell activation by liver APCs has been described by several independent groups with both *in vivo* and *in vitro* models (Bertolino et al., 2001; Bertolino et al., 1998; Crispe, 2011). Until now, the fate of CD8 T cells primed in the liver has been a subject of controversy in efforts to explain the failure of immunity in chronic liver infections such as HBV, HCV and malaria. Groups using transgenic mice that express antigen in the liver believe T cells undergo rapid Bim-dependent apoptotic death immediately after activation in the liver or by liver cells. Bertolino *et al*, explains that naïve CD8 T cells which obtained antigens in the liver through liver cells up modulate a specific apoptotic marker known as Bcl-2-interacting mediator of cell death, which initiates apoptosis in the activated CD8 T cells (Bertolino et al., 1999; Holz et al., 2008; Holz et al.). Other groups, who used viral vectors as models to express antigen in the liver, immediately challenged this theory. Using viral vectors such as adenovirus (Lukens et al., 2009) and AAV (Wuensch et al., 2006), they implied that liver activated CD8 T cells undergo full differentiation with some defective anti-viral activities.

Although, these experiments were well designed, there were some caveats to the models, which encouraged a particular conclusion. Neil *et al*. believe using transgenic mice that express a specific antigen in the liver defeats the purpose of antigen processing and presentation, since the antigen exists throughout the lives of the mice and therefore pushes the responses towards tolerance instead of immunity. The group attempted to fix the issue by using transplant models and viral vectors that express de novo antigens. These models solved the tolerance issues by inducing long-lived effector cells but unfortunately, represented a chronic infection model. Expression of Ag exclusively in the liver using the transplant and viral vector models can facilitate Ag to persist for several months. Therefore, it is fair to point out that these models do not represent an acute pathogenic infection but rather represent chronic infection models.

To gain insight into effector differentiation and memory programming of CD8 T cells post liver priming, a unique model with transient Ag expression is needed. We used a hydrodynamic DNA plasmid injection approach to model an acute liver infection. Hydrodynamic injection (HI) of antigen encoded DNA has been used previously as a model for acute HBV and HCV infection (Yang et al., 2002). Depending on the type of DNA vector or promoter (Osborn, 2011), expression of the transgene can be transient or can persist for weeks. This hydrodynamic-based transfection normally results in the rapid uptake of DNA molecules into the cytosol of hepatocytes (Andrianaivo et al., 2004) with detectable Ag expression within hours. The Ag expression kinetics are comparable to what is observed in skeletal muscle after conventional intramuscular injection (Wolff, 1992). This expression was restricted to the liver (**Figure 2.1a**) and lasted for only 4 days, mimicking acute infection (**Figure 2.1b**). Unlike other models, the level of

transgene expression is likely to represent the expression of viral Ag in the liver during hepatotropic pathogenic infection. We utilized this model for expression of an LCMV-gp in mouse liver after hydrodynamically injecting the plasmid DNA vector. Expression of this transgene product was not seen in other major tissues (**Figure 2.1a).**

2.2.2 P14 transgenic model

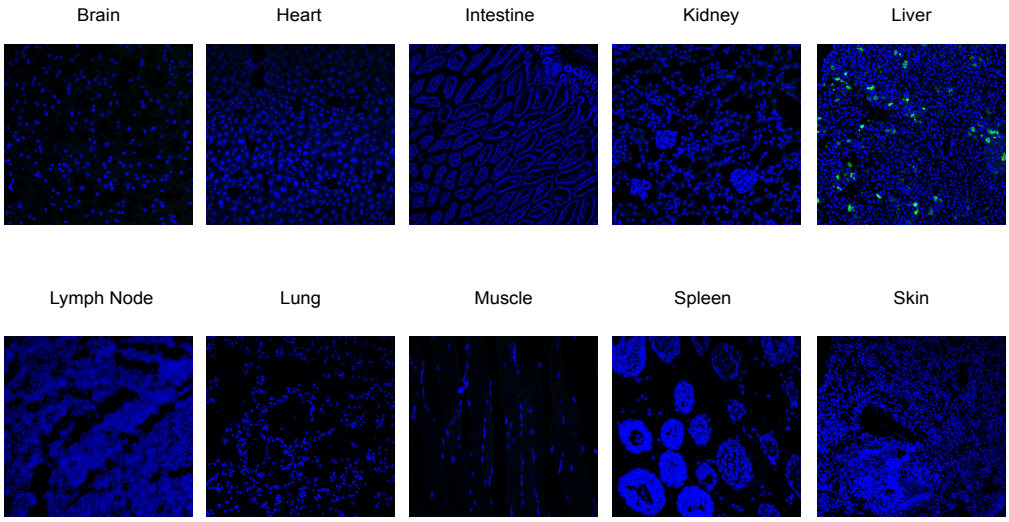
LCMV glycoprotein DNA plasmids were used instead of plasmids to antigens from liver-tropic pathogen in the hydrodynamic injection to establish infection in the liver. LCMV-gp was used because the studies were done with chimeric NOD.Cg-Tg(TcrLCMV)327Sdz/DvsJ (P14) transgenic mice. P14 mice are set of LCMV-specific CD8 T-cell clones from irradiated B10.BR/SgSn ($H\text{-}2^K$) reconstituted with T-cell depleted C57BL/10Sn (i $H\text{-}2^b$) bone marrow cells. These mice contain Tcrb-V8.1, TcrB-D, TcrB-J2.4 genes, which express under the control of H-2Kb promoters (Pircher, 1989b).

These P14 transgenic mice have many advantages, which help us understand antigen specific T cell activation. Since almost all cytotoxic T cells in these mice are specific to one epitope, there's high frequency of activated CD8 T cells after antigen expression. The high frequency of T cells facilitates phenotypic analysis on a per cell basis. It also eliminates the need for *ex vivo* stimulation of cells, which induces measurable immune cytokines after DNA or peptide vaccine immunizations. More importantly, these mice represent different congenic strains (Thy1.1 or Ly5.1) from regular black 6 mice (Thy1.2 or Ly5.2), which make it easier to stain and analyze cells without using expensive tetramer antibodies.

Figure 2-1

Hydrodynamic Injection acutely transfects the liver

a.



b.

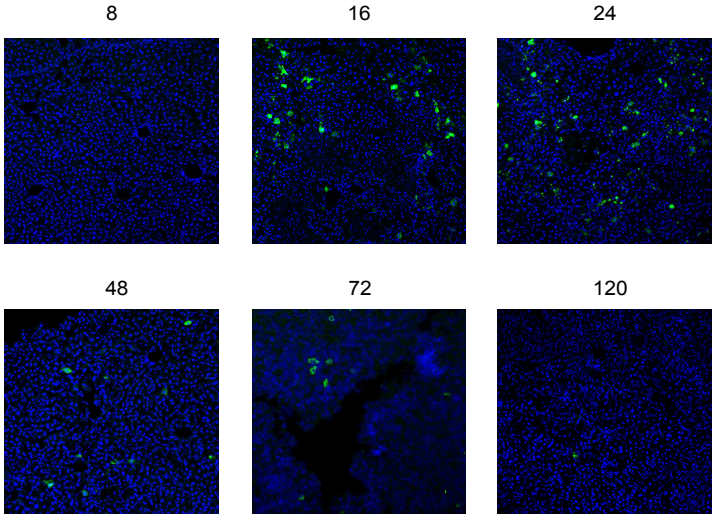


FIGURE 2-1. Hydrodynamic Injection acutely transfects the liver. Mice hydrodynamically injected with LCMV-gp mutant DNA expressed antigen exclusively in the liver. (A) Immunohistochemistry staining of the transgene product in tissue sections 24 hours post-injection with LCMV-gp mutant plasmid. Results represent brain, heart, intestine, kidney, liver, lymph node, lung, muscle, spleen, and skin. (B) Time course (in hours) of transgene transduction efficiency in mouse liver.

2.3 Intrahepatic CD8 T cell priming, proliferation, expansion, and contraction

2.3.1 Extralymphatic priming of CD8 T cells

The initial priming of CD8 T cells plays a critical role in their subsequent development into competent functional and/or memory cells (Reinicke et al., 2009; Shedlock and Shen, 2003). However, there are gaps in our understanding on the influence of the liver compartment itself on T cell activation and effector differentiation: the initial activation, clonal expansion and contraction. In this study, we qualitatively and quantitatively compared CD8 T cells primed in either the liver or in peripheral lymphoid tissue. We utilized hydrodynamic injection to transiently transfect the liver of naïve P14.Thy1.1 recipient mice with LCMV-gp transgene. Cognate antigen-expressing hepatocytes successfully induced activation of naïve P14 CD8 T cells (**Figure 2.2a**) as previously shown in transplant, adenovirus and AAV models (Bertolino et al., 1998; Lukens et al., 2009; Wuensch et al., 2010). Still, restricting Ag expression to the liver does not rule out the possibility that CD8 T cells were being primed in the lymph nodes by professional APCs that have processed liver-derived Ag. To examine if priming takes place in lymph nodes or elsewhere, we treated the mice with immunomodulating compound, (FTY720), which sequesters naïve lymphocytes in lymph nodes (**Figure 2.2b**), thus, preventing interaction with the transfected hepatocytes. The sequestering of naïve lymphocytes in lymph nodes averted the priming of transferred P14 TCR transgenic CD8 T cell induction and proliferation (**Figure 2.2c**). A group of naïve P14 recipient mice were also treated with L-selectin (CD62L) antibody prior to immunization. Consistent with previous data

(Lepault et al., 1994), anti-L-selectin treatment led to severe naïve T cell depletion in the lymph nodes (data not shown). This treatment did not change the priming of transferred P14 TCR transgenic CD8 T cells after intrahepatic (IH) expression of cognate antigen. CD8 T cell priming was documented by studies of antigen-specific proliferation and CD8 T cell expansion (**Figure 2.2c**). Together, these data suggest that liver transfection through hydrodynamic injection encourages extralymphatic priming of the transgenic CD8 T cells.

2.3.2 Expansion and contraction phases of Liver-primed CD8 T cells

Although, intrahepatic primed CD8 T cells are known to differentiate into effector cells, it is unclear whether they undergo this transition with the same dynamics and phenotypic changes that occur during the naïve-effector transition of CD8 T cells during acute viral infection (Wherry and Ahmed, 2004). Here, we compared the hydrodynamic intrahepatic injection model (IH) to intramuscular control (IM), which achieves T cell induction through Ag presentation within its associated lymph nodes.

Five days following the initial immunization, Ag-specific CD8 T cells were detectable using D^bGP33 tetramer in blood for both groups, indicating successful priming at both sites. CFSE labeled adoptively transferred cells in IH model exhibited quicker homeostatic proliferation during the first week of immunization, in peripheral lymph nodes (PLN) and spleen (SPL) as compared to the IM control model (**Figure 2.3a**). Proliferation in non-lymphoid tissues such as liver (LIV) and lung (LUN) at day 7 was

however, comparable between the two groups. Antigen specific CD8 T cell proliferation in all tissues averaged around 98% two weeks post immunization. Next, we examined the expression levels of different activation markers: CD25 and CD44, and CD62L following CD8 priming in both models. We observed a significant increase in CD25 and CD44 expression on Thy1.1 CD8 T cells of IH mice in all tissues at day 7 and 14 post infection (**Figure 2.3b**). In addition, down-regulation of CD62L, which is usually seen in effector CD8 T cells, was only visible 14 days post infection. However there was no difference in the expression levels of these activation markers between the IH model and the IM control group.

Expansion and contraction are two of the three characteristics reported when CD8 T cells are primed in a conventional setting. However, the kinetics of these two phases has not been detailed with regard to lymphoid tissue-independent activation models. We explored the frequency of CD8 T cell responses induced by IH in contrast to those induced by IM. Following immunization, T cell expansion from IH rapidly peaked on day 6 with 0.7% of Ag-specific cells detected in the blood. This early expansion was followed by a rapid contraction phase. At day 9 post-infection, 57% of the Ag-specific cells were already dead and the percent contraction increased to 82% by week 2. In the IM control group, only 0.2% Ag-specific cells were detected on day 6 (**Figure 2.3c**). However, an increase in Ag-specific CD8 T cells (to ~4.2%) was observed at day 15 post-infection. Furthermore, the Ag-specific CD8 T cells in IM control group underwent a gradual contraction over the next 3 weeks.

To evaluate the tissue distribution of Ag-specific cells in lymphoid (spleen and lymph nodes) and non-lymphoid tissues (liver and lung), we calculated the absolute numbers of

Thy1.1 cells in these tissues (**Figure 2.3d-g**). We observed similar expansion-contraction phases between groups in the tissue as observed in the blood (**Figure 2.3c**). Although IH underwent rapid and robust contraction phase after expansion, about 0.03, 0.05, 0.3 and 0.4 percent of Ag-specific CD8 T cells were left in the lymph nodes, spleen, liver and lung respectively as compared to the IM control which had 0.08, 0.17, 1.6 and 1.4 percent cells present in the respective tissues 12 weeks after immunization (**Figure 2.3g**).

2.3.3 Modulation of apoptotic and survival markers on liver primed CD8 T cells

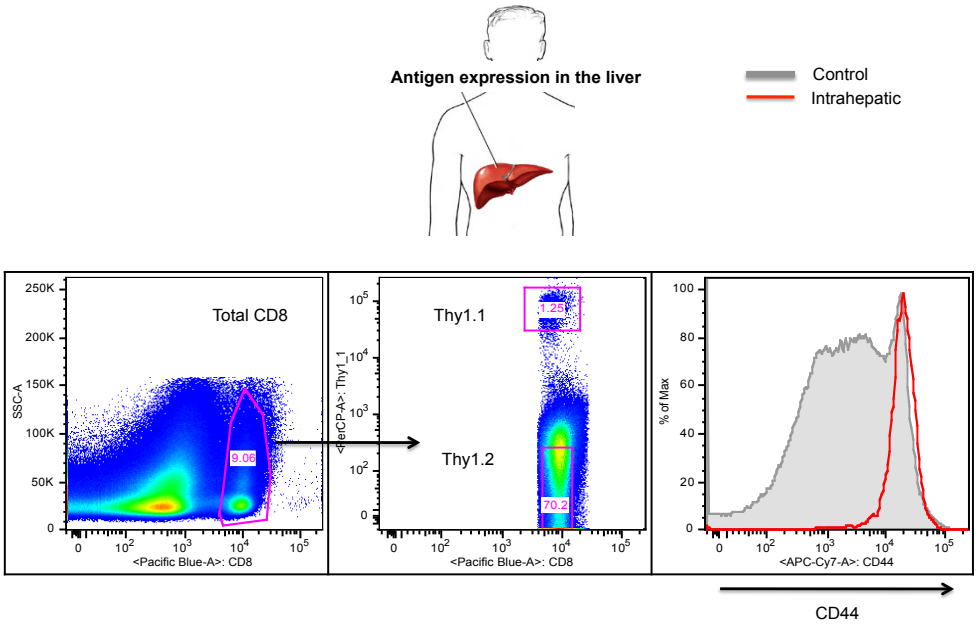
The rapid death of the primed CD8 T cells in the IH group, suggested experimentation of how pro-apoptotic and survival markers are regulated during both the expansion and contraction phase in these cells. First, we examined the modulation of Bim, a member of the Bcl-2 family that promotes apoptosis and is known to be up regulated in intrahepatic activated cells. We observed increased Bim expression in CD8 T cells from the lymph nodes, liver and lungs of the IH mice during the first week of activation (**Figure 2.4a**). However, on day 14 post-immunization, Bim expression was lower in all tissues of the IH group when compared to the IM control. Bim expression was found to be particularly high on D^bGP33-specific effector and host CD8 T cells in the liver. To determine if this increase in Bim was not due to T cell activation in the liver, Thy1.1 T cells were transferred into naïve Thy1.2 mice and the expression levels of Bim was monitored for 12 days. The expression of this death marker was highest on liver-resident CD8 T cells when compared to cells derived from the spleen and lymph nodes (**Figure 2.4b**).

We also analyzed the regulation of the survival marker, Bcl-2, to determine when these cells acquire their memory phenotypes. Peak expansion of effector CD8 T cell in the IH group coincided with the down-regulation of Bcl-2 on day 7 post-immunization. The average mean fluorescence intensity (MFI) was 422, 484, and 430 in lymph nodes, spleen and liver respectively, while the IM control mice averaged 1009, 967, and 1086 in the respective tissues. Bcl-2 expression in the IH group was higher than that of the IM group on week 2, which represents the peak of expansion for lymphoid-dependent CD8 T cell priming. The MFI of 662 in lymph nodes, 583 in spleen and 832 in liver, indicated there was increased expression of Bcl-2 in the IH group as compared to the control IM group (480 in lymph node, 493 in spleen and 349 in the liver). This enhancement was even more significant by day 21, where the average MFI was 1326, 667, 490 for IH and 509, 290, 349 for IM groups (**Figure 2.4c**). These data confirm that cells primed outside lymphoid tissues in the liver up-regulate death signals while down-regulating survival signals at earlier time points, a phenotype which is reversed at later time periods post immunization.

Figure 2-2

Intrahepatic CD8 T cell activation

a.



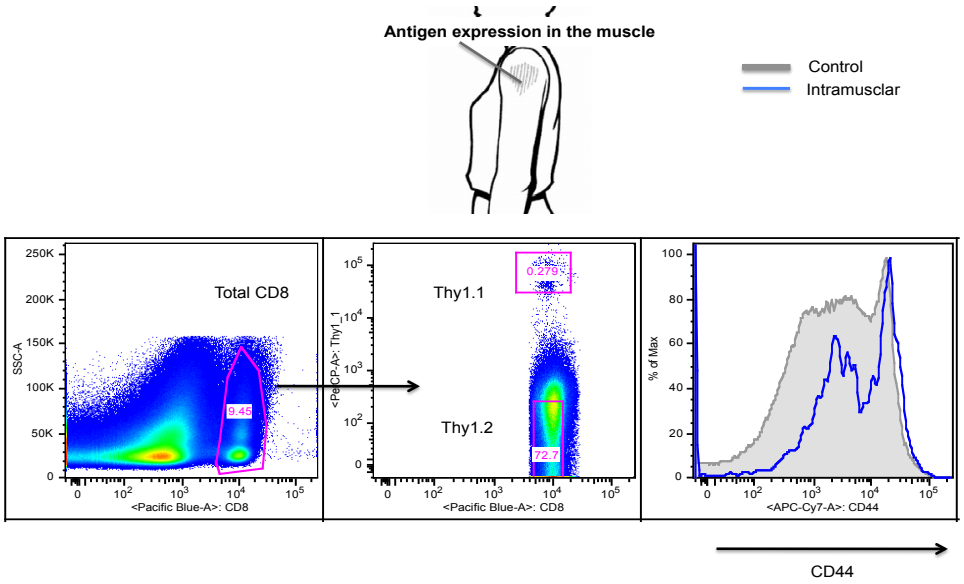
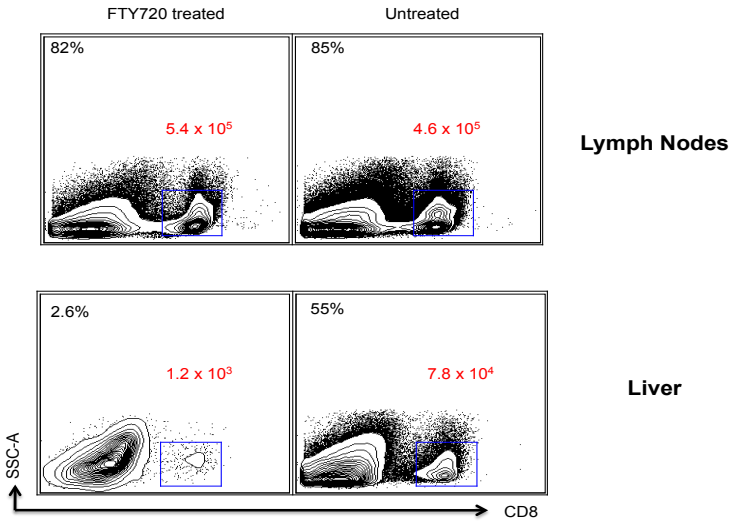


Figure 2-2 cont.

b.



c.

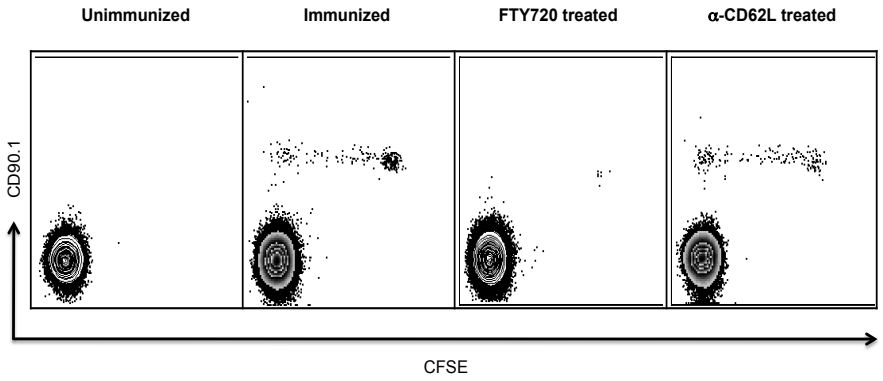
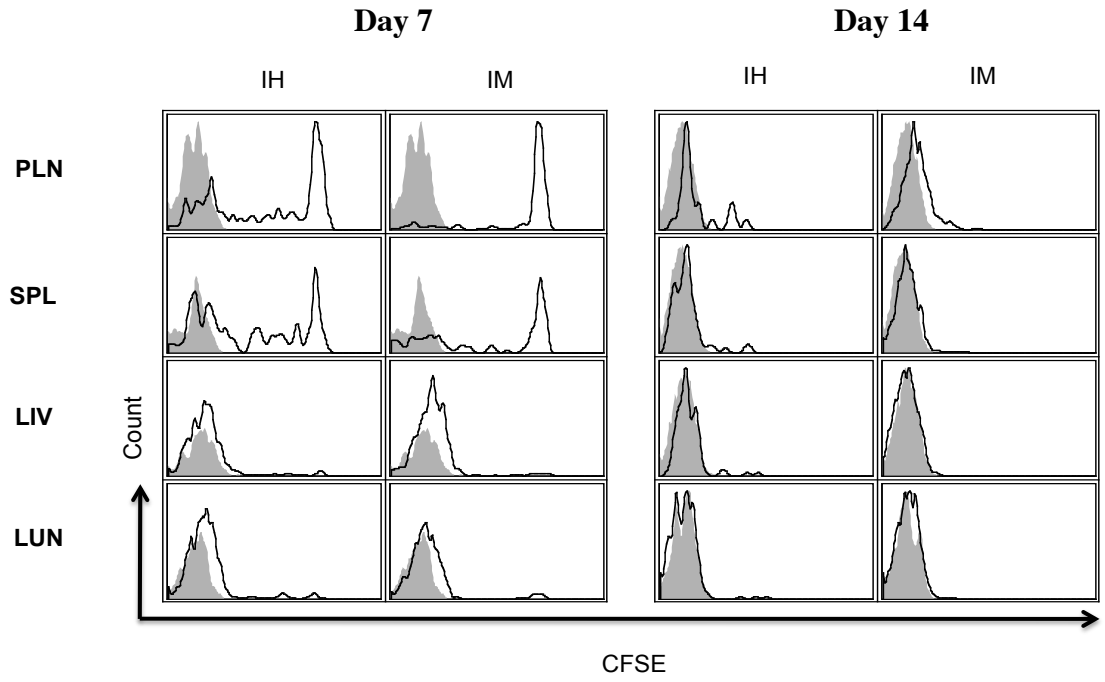


FIGURE 2-2. Intrahepatic CD8 T cell activation. Hydrodynamically injected mice expressed antigen in the liver and eventually induced CD8 T cell activation. (A) The CD8 T cell priming was evident by the expansion of Thy1.1 P14 CD8 T cells and upregulation of CD44 activation marker. The expansion and increase in CD44 in the intrahepatic group was comparable to that of intramuscular control group. (B) Treatment with FTY720, an immunosuppressive drug that sequesters circulating lymphocytes in lymph nodes. The percent lymphocytes and total naïve CD8 T cells drastically reduced in the liver after FTY720 treatment. (C) The treatment abrogated antigen specific CD8 T cell activation. However, preventing lymph node entry of naïve lymphocytes using anti-CD62L had no effect on D^bGP33^+specific CD8 T cell induction.

Figure 2-3

Expansion and contraction of liver primed D^bGP33^+specific

a.



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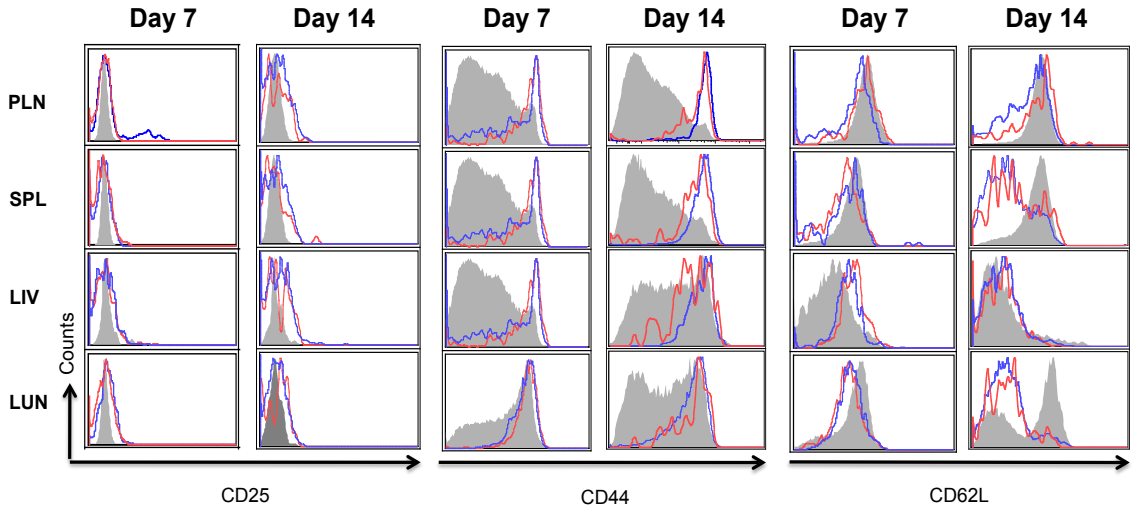
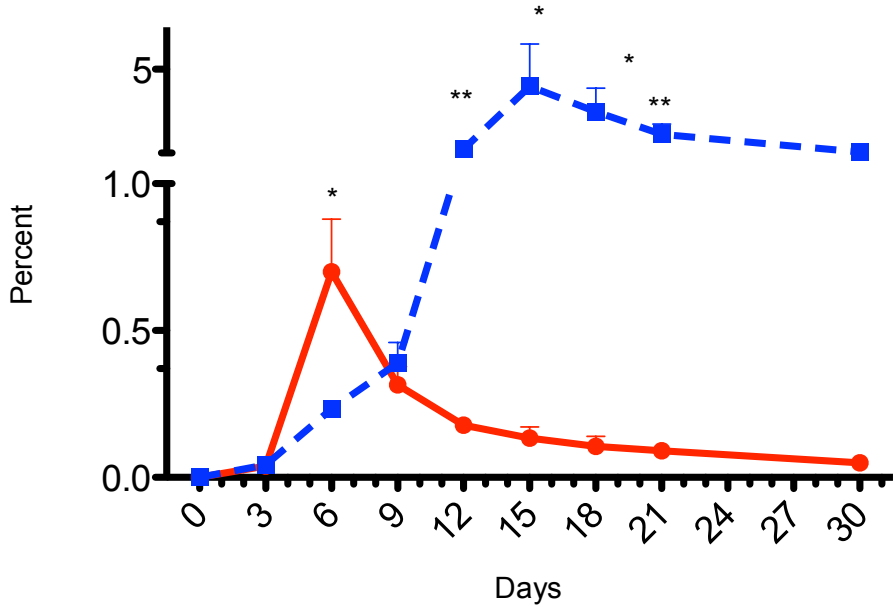


Figure 2-3 cont.

c.



d.

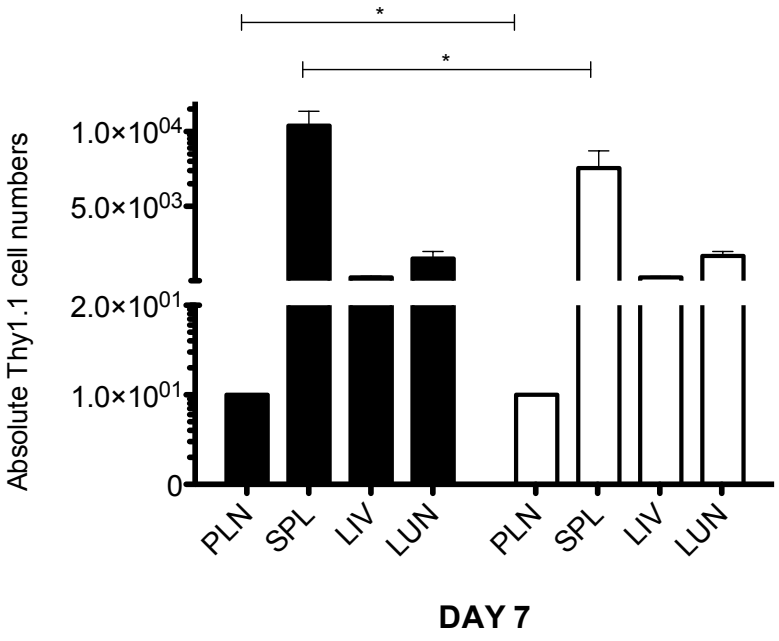
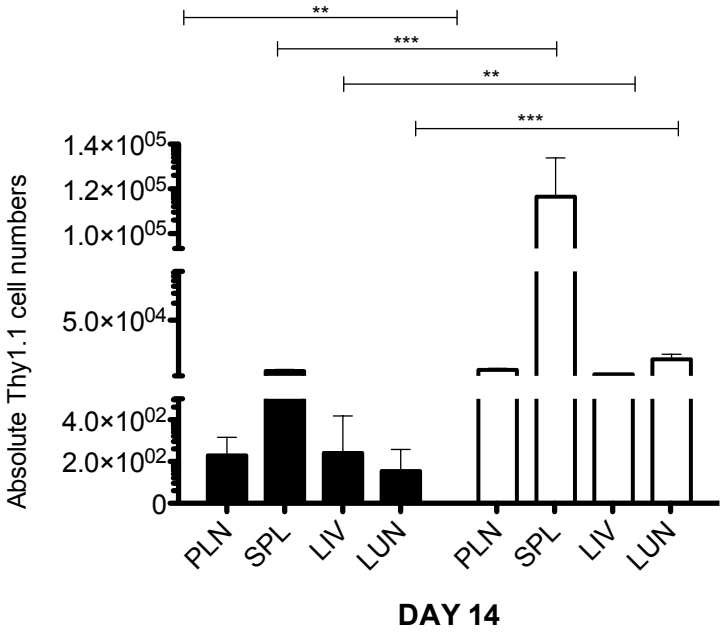


Figure 2-3 cont.

e.



f.

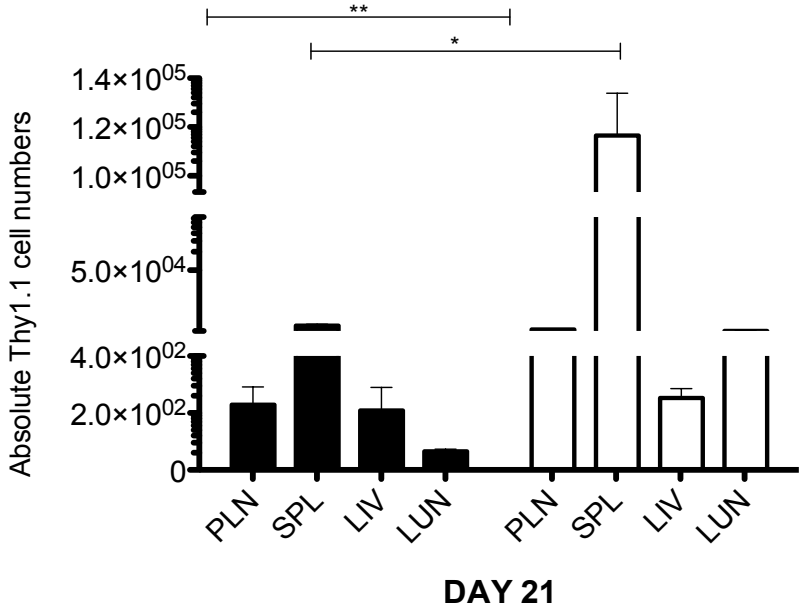


Figure 2-3 cont.

g.

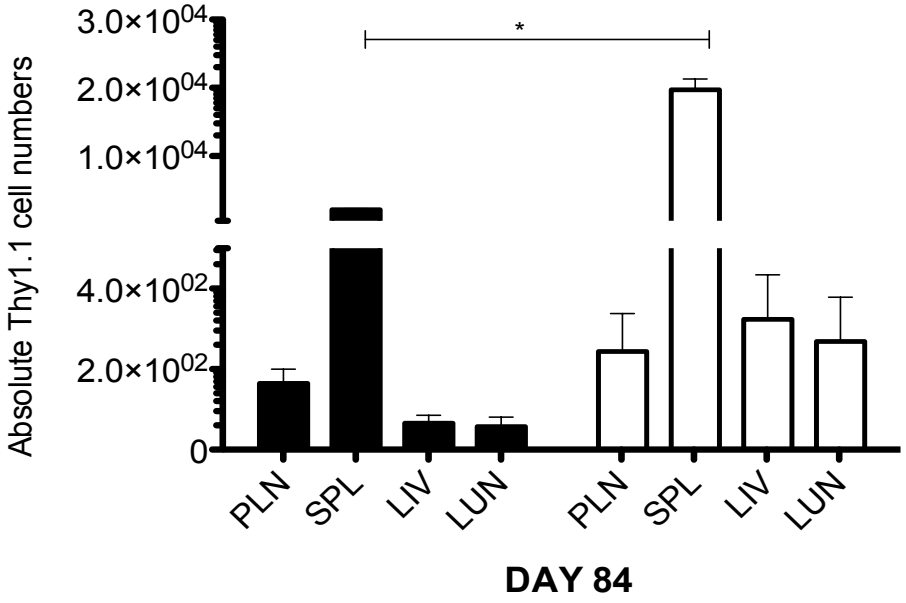


FIGURE 2-3. Early expansion and contraction of liver primed D^bGP33^+specific CD8 T cells. CFSE-labeled CD8 T cells isolated from lymphoid and non-lymphoid organs of mice either expressing either Ag intrahepatically (IH) or intramuscularly (IM) were analyzed at indicated time points. (A) Proliferative capacity and (B) expression of important activation markers on transferred D^bGP33^+specific CD8 T cells. Frequency kinetics of the of GP33-specific CD8 T cell response in (C) PBMC, (D-G) lymph nodes, spleen, liver, and lung following immunization.

Figure 2-4

Modulation of different apoptosis regulator proteins

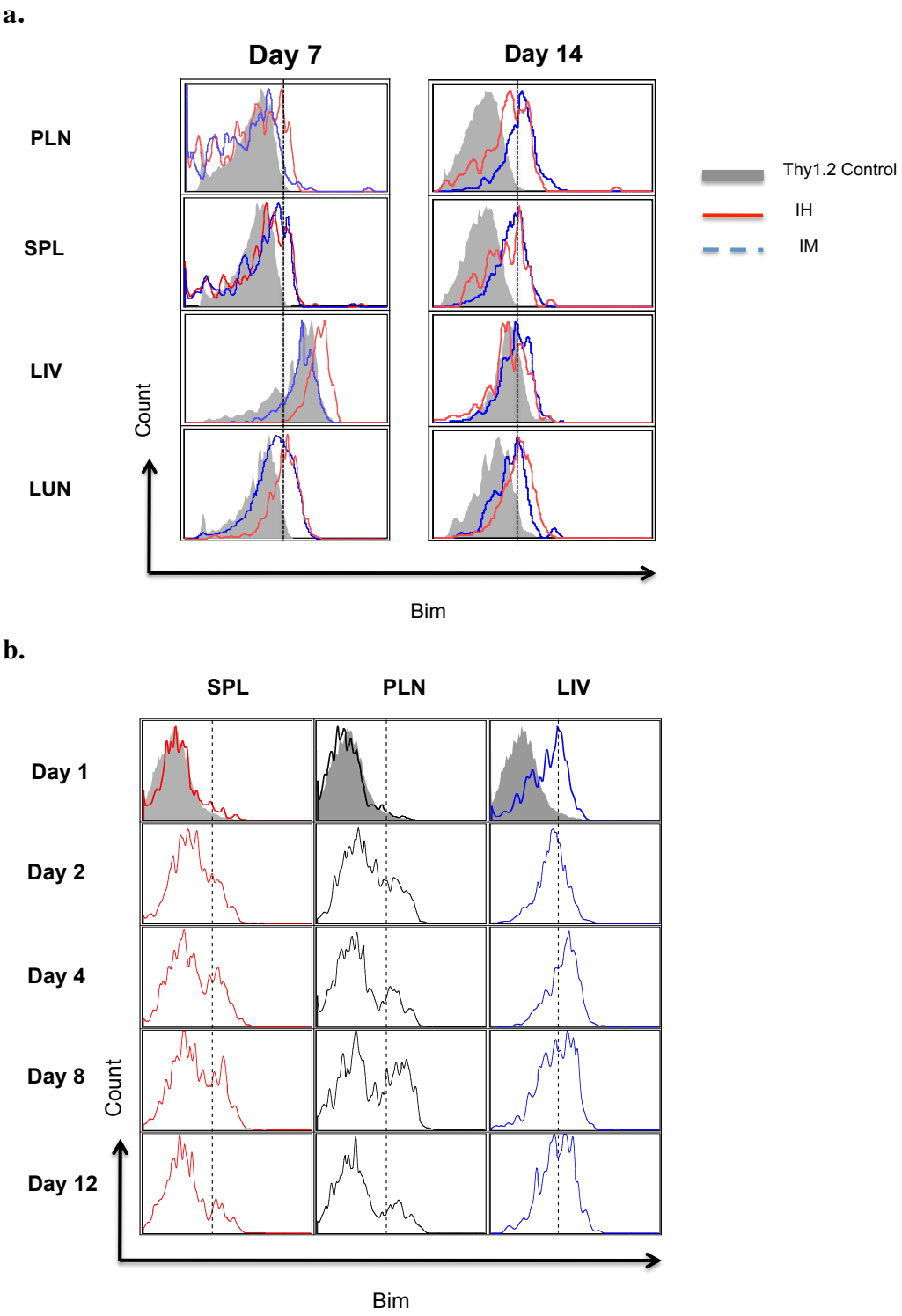


Figure 2-4 cont.

c.

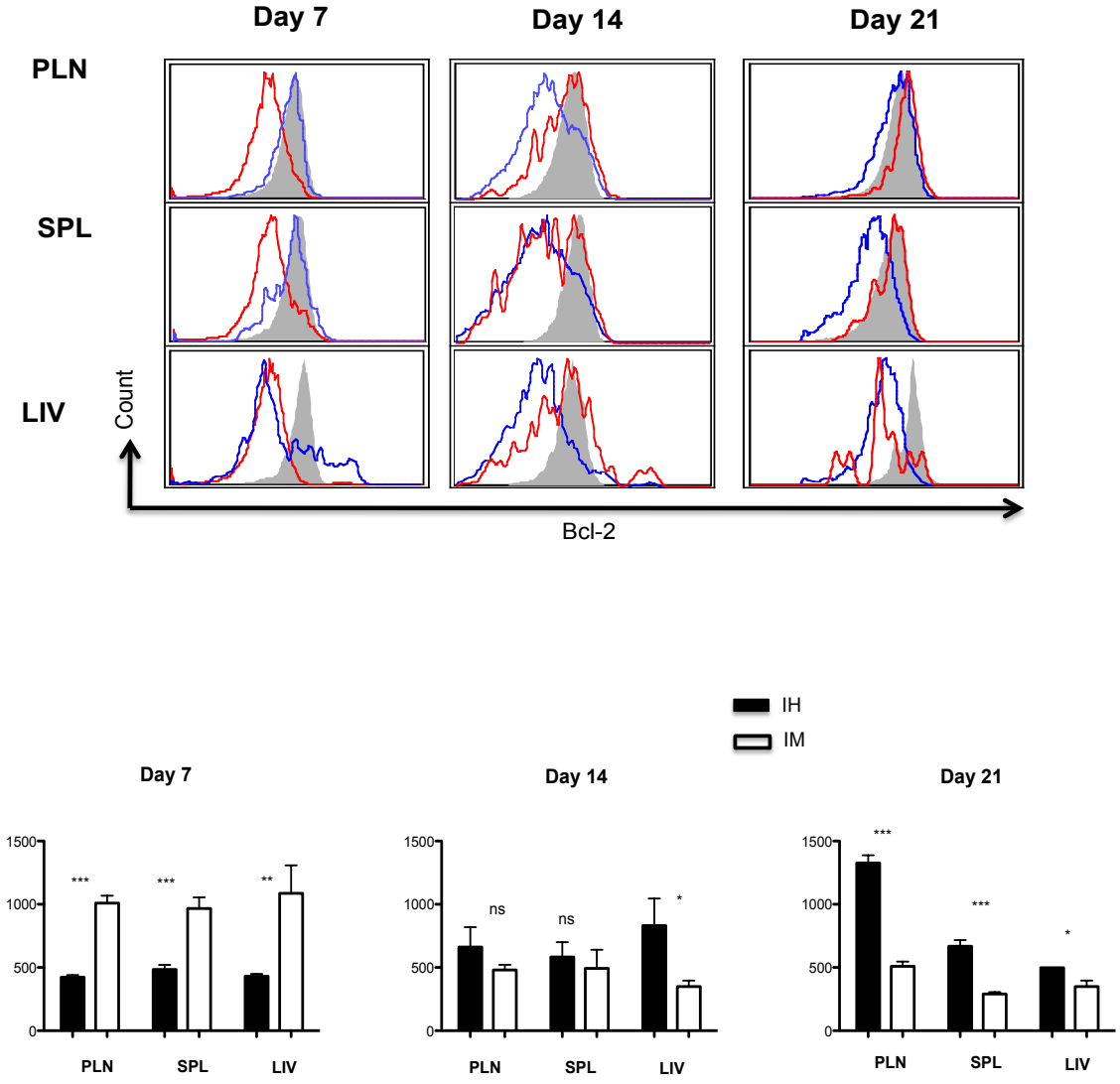


FIGURE 2-4. Modulation of different apoptosis regulator proteins during the expansion and contraction phases of liver-primed CD8 T cells. (A) Early upregulation of the pro-apoptotic marker, Bim, was observed in the activated CD8 T cells of the IH group at their peak of expansion. Increased Bim expression was seen on both activated and (B) naïve Thy1.1 cells isolated from different tissues demonstrate different levels of Bim expressions. (C) Survival signal, Bcl-2, expression was conversely correlated to that of Bim.

2.4 Effector and memory differentiation

The ultimate goal of the anti-viral immune response is to generate strong effector cells to clear infection along with long lasting memory CD8 T cells, which will quickly respond to subsequent infections. Recent studies have provided a phenotypic profile for a subset of effector CD8 T cells that will commit to become memory T cells(Amoah et al., 2012). Cells within a polyclonal response can be grouped into short-lived effector cells (SLEC) and memory precursor effector cells (MPEC) based on the expression levels of the killer cell marker, KLRG1 among many surface markers (**Figure 2.5a**). This description is well established for CD8 T cells that are primed within the lymph nodes but not for intrahepatic primed CD8 T cells. We next confirmed that CD8 T cells primed outside the SLT have the capacity to differentiate into SLEC and MPEC (**Figure 2.5a and 2.5b**). The liver-primed CD8 T cells in the blood, however, acquire more SLEC subsets early while the MPEC subset increases only 12 days post immunization, when the SLEC begin to die off. This increase of MPEC in the IH group at day 12, contrasts with that of the IM immunized group where a similar trend was seen in both lymphoid and non-lymphoid tissues. We observed early increases in $KLRG^{hi}$ cells in the IH immunized group for each individual tissue (**Figure 2.5c**). Although these markers allowed us to identify cells within the liver-primed CD8 T cell population that have the potential to become memory cells, it was not clear if they actually became memory CD8 T cells. Using CD44, IL-7Rα and L-selectin, we categorized the Thy1.1 cells into effector, effector memory and central memory CD8 T cells (**Figure 2.5d**). Flow cytometric analysis of CD8 T cells from the lymph nodes, spleen and liver on days 21 and 84, indicated similar distribution of effector and memory cells between the IH and IM groups. The intrahepatic primed CD8 T cells,

however, up-regulated both IL-7Rα and L-selectin (central memory) earlier than the lymphoid primed CD8 T cells (**Figure 2.5e**). Although liver-primed CD8 T cells differentiate into memory cells more quickly than lymphoid primed cells, the distribution of effector, effector-memory and central memory cells were similar in all tissues within the groups. Crispe *et al*, believe that direct liver priming of CD8 T cells promotes the expression of inhibitory molecules such as PD-1(Wuensch et al., 2010). We did not observe a significant increase in PD-1 expression for the IH group. The liver microenvironment seems to up-regulate PD-1 on both naïve and effector cells, regardless of the priming site (**Figure 2.6a**). Naïve Thy1.1 cells transferred into unimmunized Thy1.2 recipients maintained a higher level PD-1 expression in the liver compared to spleen and lymph node over a period of time (**Figure 2.6b**). Additionally, HBV persistence in mice has been shown to increase PD-1 expressing CD8 T cells in liver infiltrating lymphocytes(Tzeng et al., 2012). Thus, the increase expression of PD-1 on memory CD8 T cells after intrahepatic activation in their model may be a consequence of the continued Ag expression in the liver and not an undesirable effect from liver-priming. These studies support that liver-primed P14 CD8 T cells can divide and express activation and memory markers, but it is still important to examine if these CD8 T cells can induce antiviral activity in response to antigenic re-encounter. To assess this, we evaluated the ability of Ag-specific CD8 T cells elicited by hydrodynamic injection to produce antiviral cytokines including IFN-γ, TNF-α and IL-2, in response to LCMV-GP33 peptide stimulation *in vitro* (**Figure 2.7a**). About 50, 55 and 13 percent of CD8 T cells from the lymph nodes of the IH group produced IFN-γ, TNF-α or IL-2 respectively in this assay. These numbers were higher for spleen, where 77, 67 and 30 percent of CD8

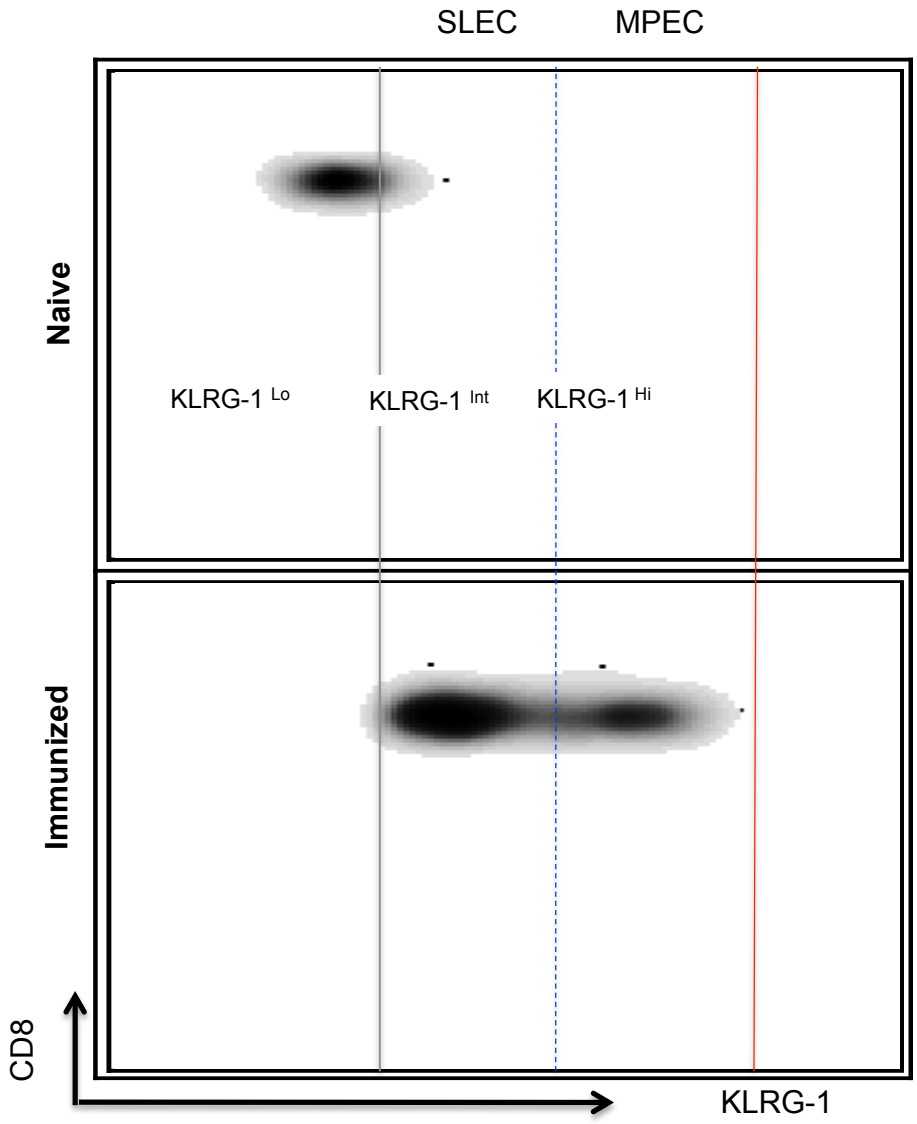
T cells produced the above-mentioned ctykines, while the liver primed showed lower cytokine production at 44, 59 and 7.8 percent respectively. However, only 44, 59 and 7.8 percent of CD8 T cells in the liver produced IFN-γ, TNF-α or IL-2, respectively. By contrast, a higher percent of lymphoid-primed CD8 T cells produced these cytokines. For example, 93, 93 and 45 percent of CD8 T cells from the spleen of the IM control group produced IFN-γ, TNF-α or IL-2 respectively.

Further, current data suggests that poly-functionality i.e, the ability of single cells to produce multiple cytokines, is one of the essential functional properties of long-lived memory cells. Indeed, similarities in poly-functionality of CD8 T cells derived from the liver and spleen were observed between the two groups. However, cells from the lymph nodes of the IH group were significantly less functional than those of the IM group (**Figure 2.7b**).

Figure 2-5

Effector and memory differentiation of liver-primed T cells.

a.



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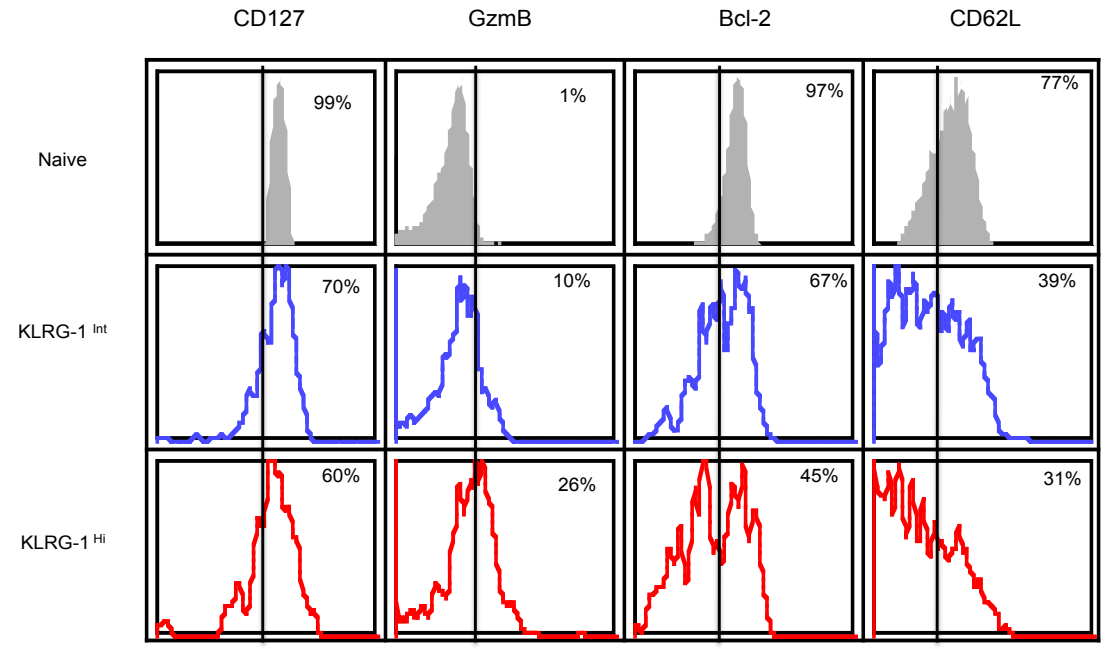
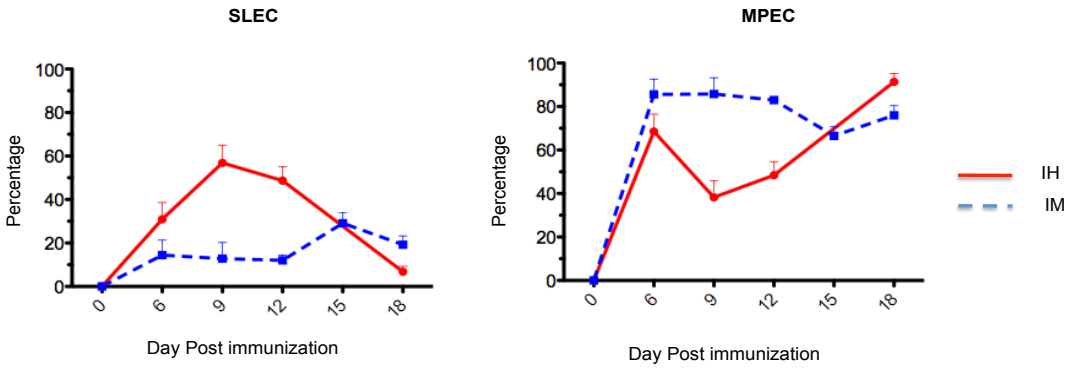


b.



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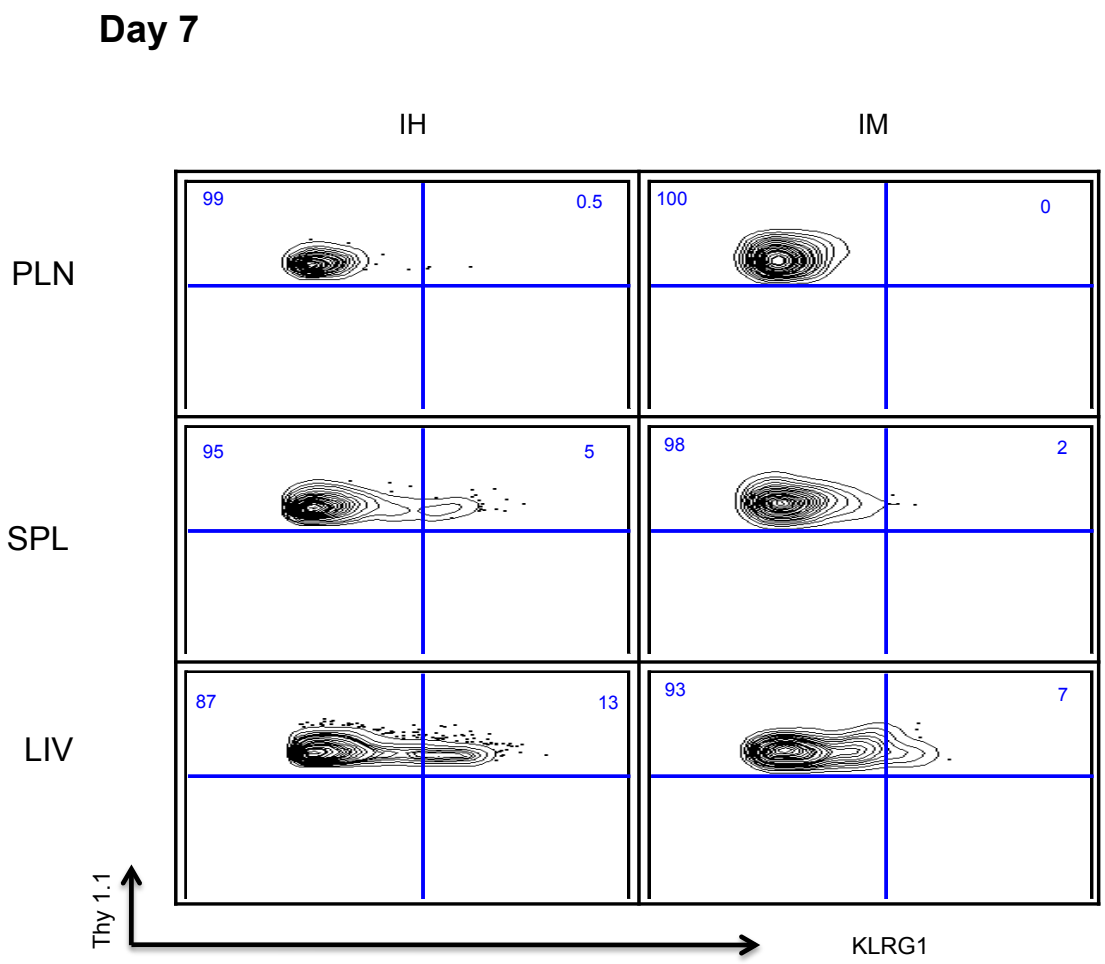
c.



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Day 14

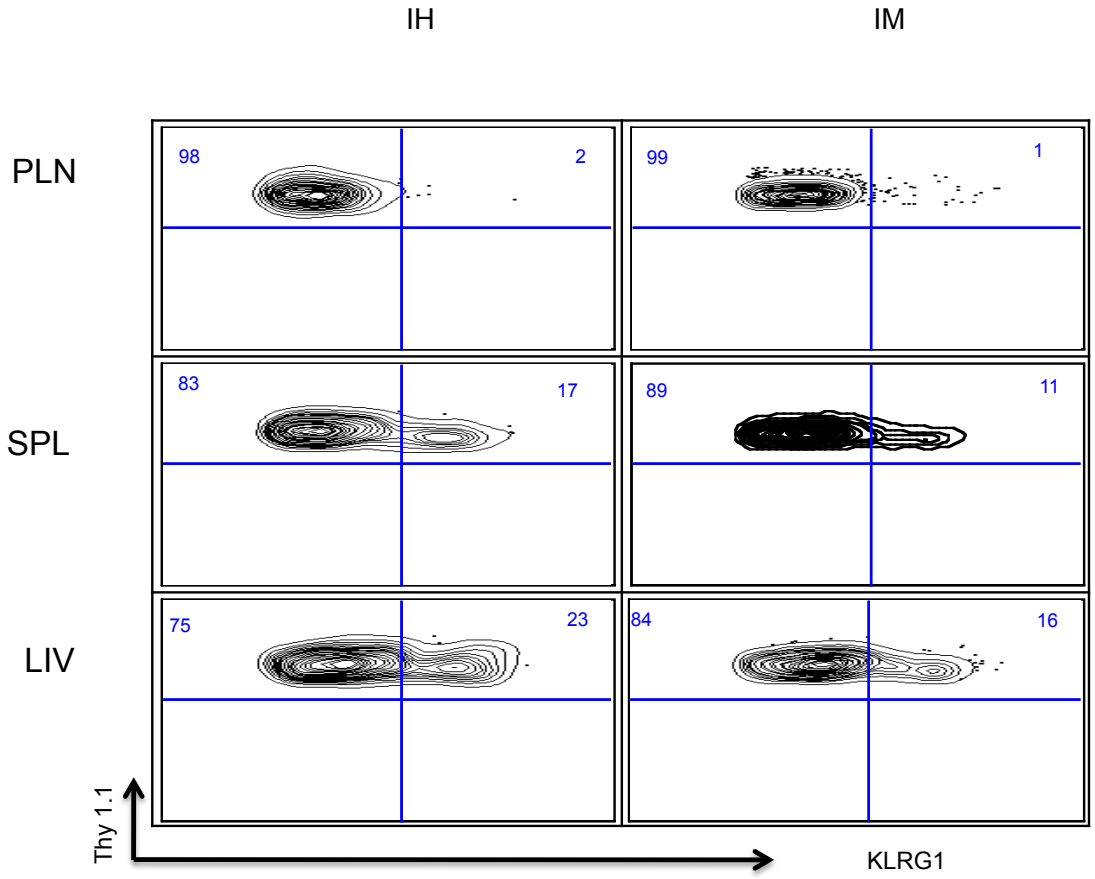


Figure 2-5 cont.

Day 21

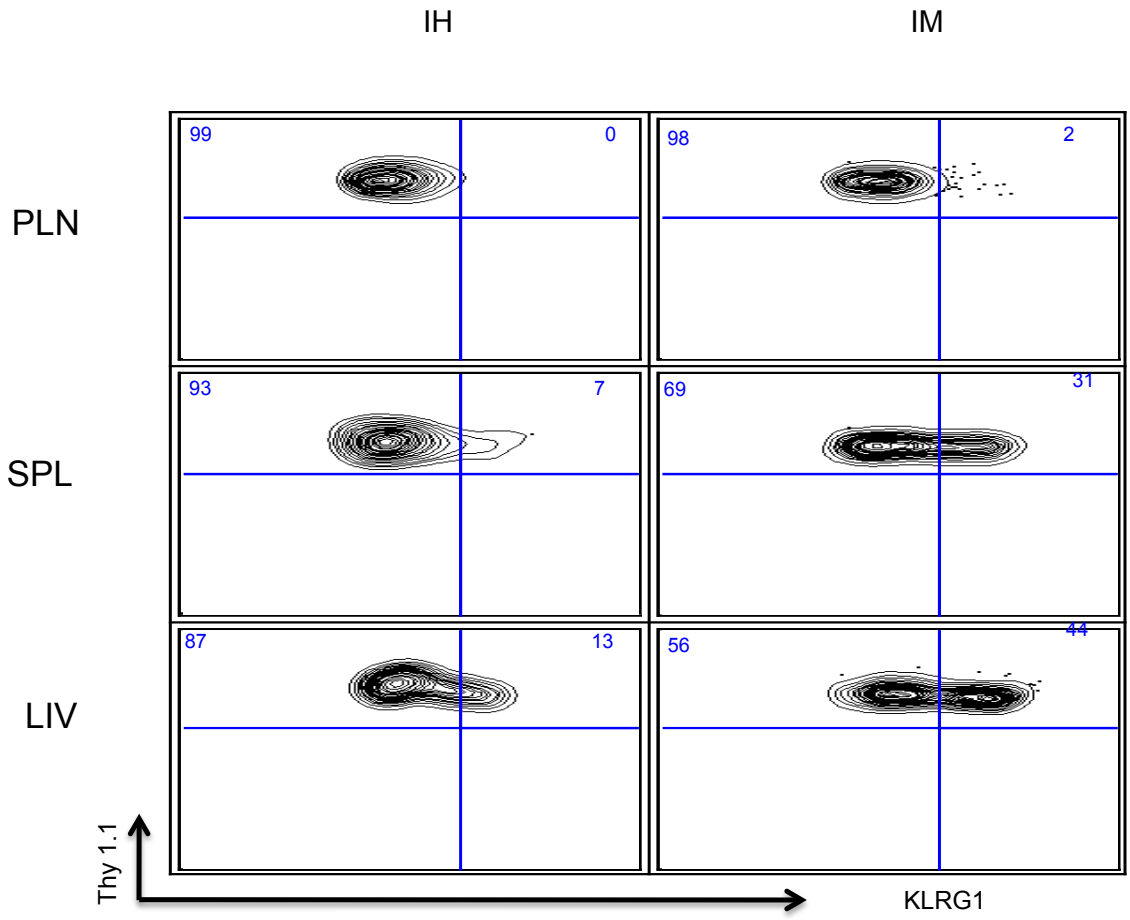
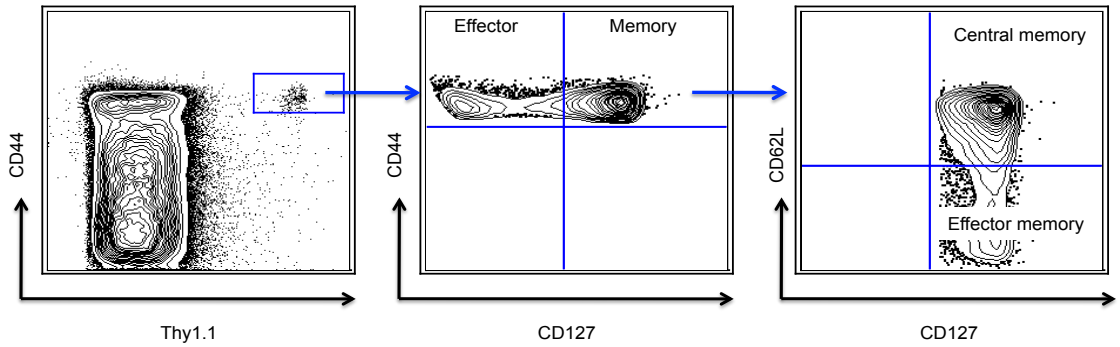


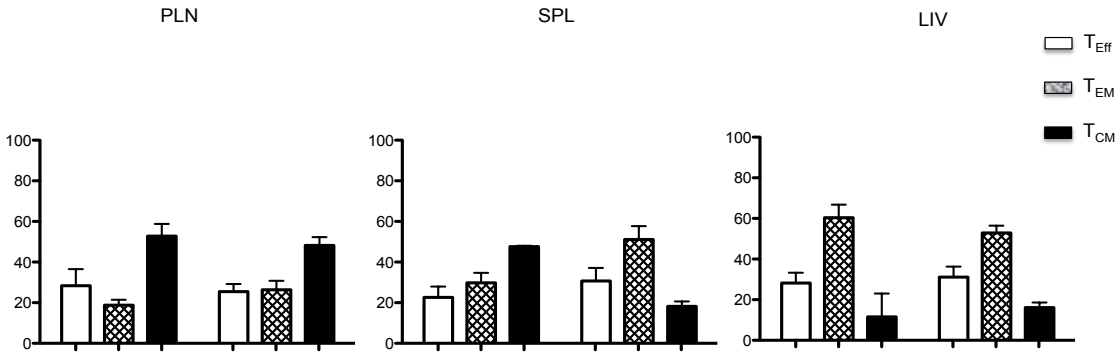
Figure 2-5 cont.

d.



e.

Day 21



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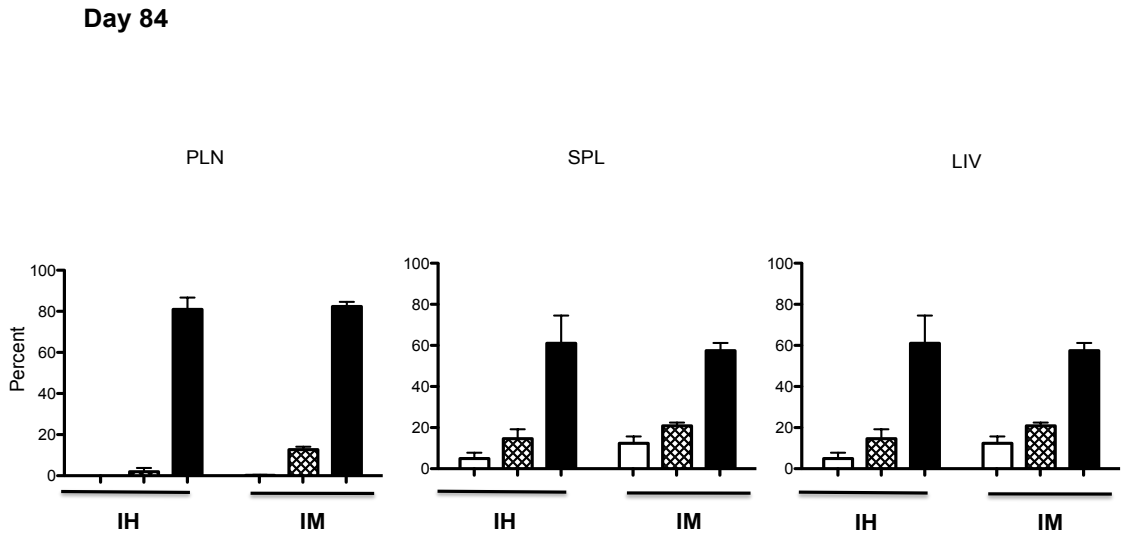
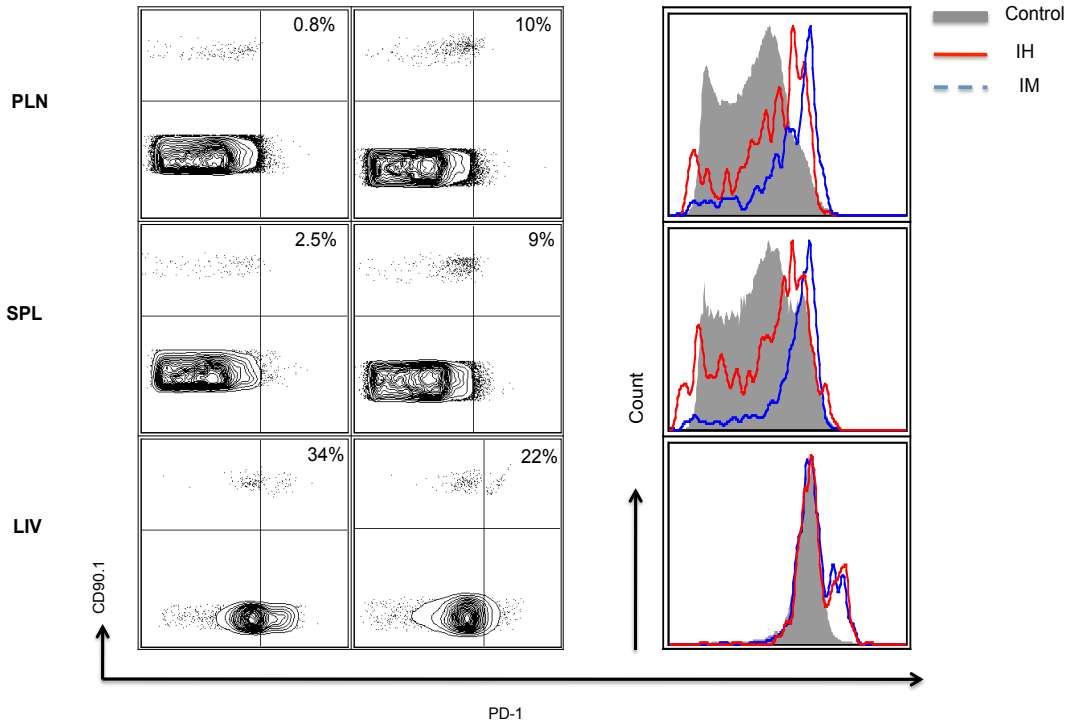


FIGURE 2.5. Effector and memory differentiation of liver-primed CD8 T cells. (A) Phenotypic profiling of effector intrahepatic-activated GP33-specific CD8 T cell subsets with distinct memory fates. Expansion and contraction of short-lived effector cells (SLEC) and memory precursor effector cells (MPEC) in (B) PBMC and (C) other lymphoid and non-lymphoid tissues. (D-E) Distribution of effector and memory CD8 T cells from IH and IM immunized mice at days 21 and 84 post-infection.

Figure 2-6

The expression of PD-1 on D^bGP33^+specific CD8 T cells

a.



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b.

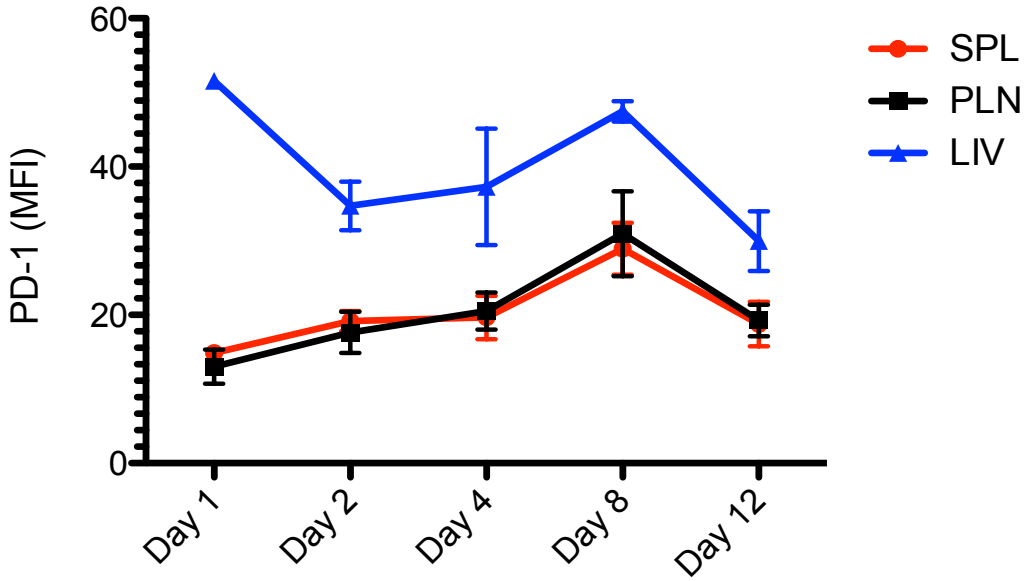


FIGURE 2.6. The expression of PD-1 on D^bGP33^+specific CD8 T cells. While there was no significant differences in the expression of the negative regulator marker (PD-1) between the (A) IH and IM immunized groups, (B) there were differences in expression on naïve CD8 T cells between the liver, lymph nodes and spleen.

Figure 2-7

Magnitude and polyfunctional profile of GP33-specific CD8 T cell responses

a.

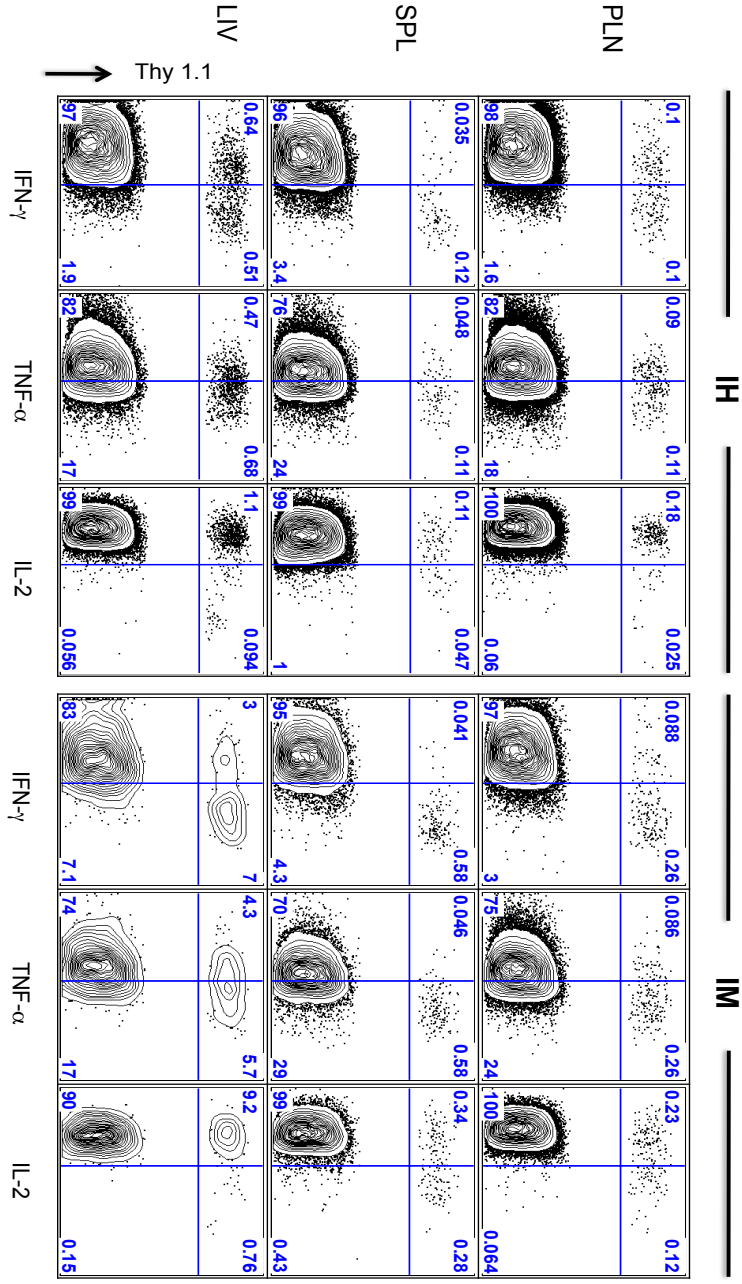


Figure 2-7 cont.

b.

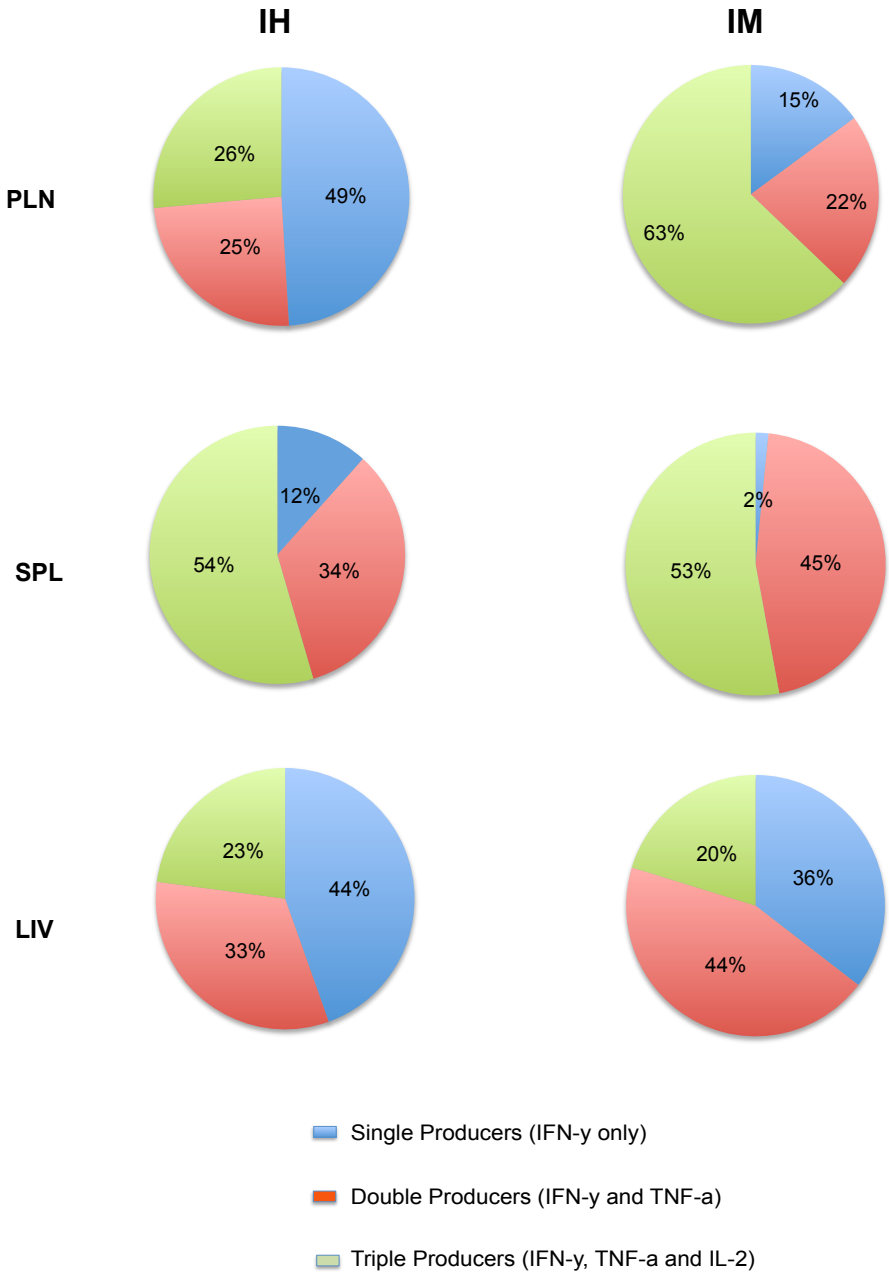


FIGURE 2.7. Magnitude and polyfunctional profile of GP33-specific CD8 T cell responses. The ability of liver-primed CD8 T cells to produce cytokines, IFN-γ, TNF-α and IL-2 following *in vivo* stimulation with synthetic peptide was analyzed. (A) A representative flow chart showing single cytokine producing cells and (B) an IFN-γ positive cells were analyzed for their ability to co-produce TNF-α and IL-2 (pie chart).

2.5 Defective viral clearance and lack of protection

In addition to antiviral cytokine production, a crucial function of effector CD8 T cells is induction of cytotoxicity. Accordingly, we used an *in vivo* cytotoxicity assay to assess the whether STL-independent primed CD8 T cells become effective CTLs. One week after immunization, during which the peak of expansion occurs, we examined the killing of transferred target cells pulsed with either LCMV-GP33 peptide or LCMV-NP (control). Twenty-four hours after transfer of target cells, about 88% of LCMV-GP33 pulsed target cells were eliminated in the blood of the IH group, while only 76% of the target cells were killed in the IM group. However, when target cells were transferred 3 weeks post immunization, where 0.15% Ag-specific CD8 T cells can be detected in the spleen of the IH group (compared to 1.5% of the IM group), only 2% of the relevant antigen pulsed target cells were eliminated. This is in sharp contrast to the IM group, which killed about 75% of the target cells (**Figure 2.8a**). We allowed the transferred target cells to reside in the recipient mice for 72 hours but did not see any improvement in killing (**Figure 2.8b**). This lack of cytolytic activity was also observed in the lymph nodes, spleen, liver and lung, indicating that the effector CD8 T cells were not selectively sequestered in any particular tissue **Figure 2.8c**). Furthermore, we stained the antigen-specific CD8 T cells for degranulation markers, granzyme B and CD107a, to verify whether they were still effective CTLs. The Ag-specific memory CD8 T cells primed in the liver were capable of inducing these degranulation markers following antigen stimulation (**Figure 2.8e**). To confirm that the lack of target cell elimination in the IH group is not due to defective liver-primed memory CD8 T cells, we transferred 3.5 x 10^4 antigen specific cells from each group into naïve mice and evaluated their cytolytic

activity in the recipient mice. Interestingly, none of the recipient groups were able to eliminate the peptide pulsed target cells (**Figure 2.8d**)

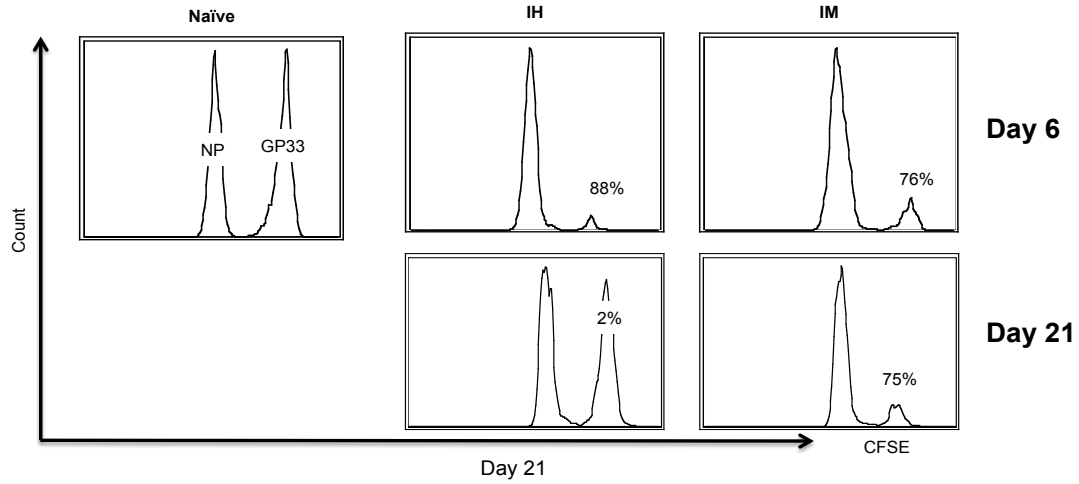
At least 4-6 weeks following the immunization, mice were challenged with 200 PFU of LCMV Arm- strong by the intracranial (I.C) route**. Figure 2.9** displays the data from the challenge study and survival for each group of mice. While the IM immunization yielded 80% protection, all naïve mice and IH vaccinated animals succumbed to infection.

This lack of killing was associated with the low frequency of effector-memory cells in the IH group. Although, the IH group's memory precursors comprise about 16% of peak effector cells in the IH group, the frequency was still very low due to its lower effector frequency. As predicted, hydrodynamic immunized mice repeatedly succumbed to lethal dose LCMV i.c challenge.

Figure 2-8

CTL-mediated cytotoxicity of liver-primed effector and memory CD8 T cells

a.



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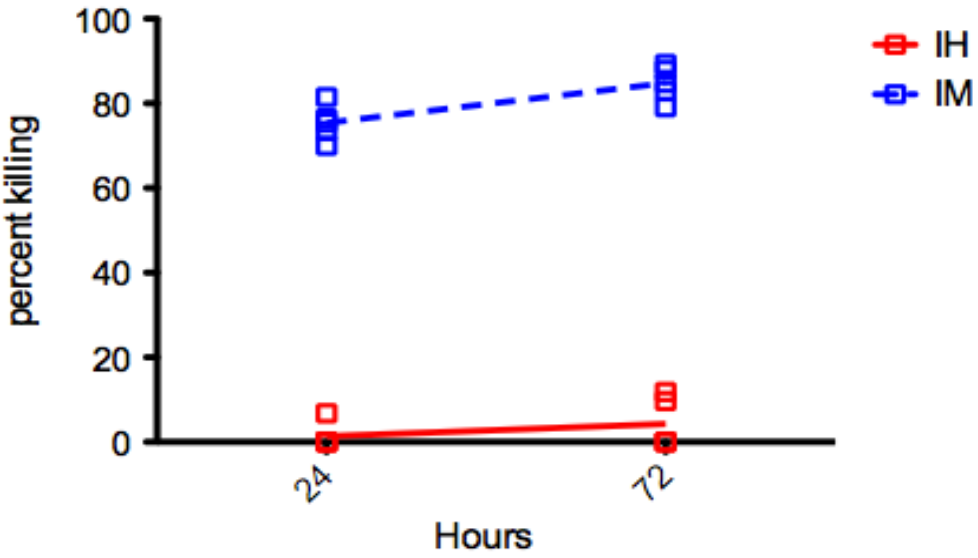

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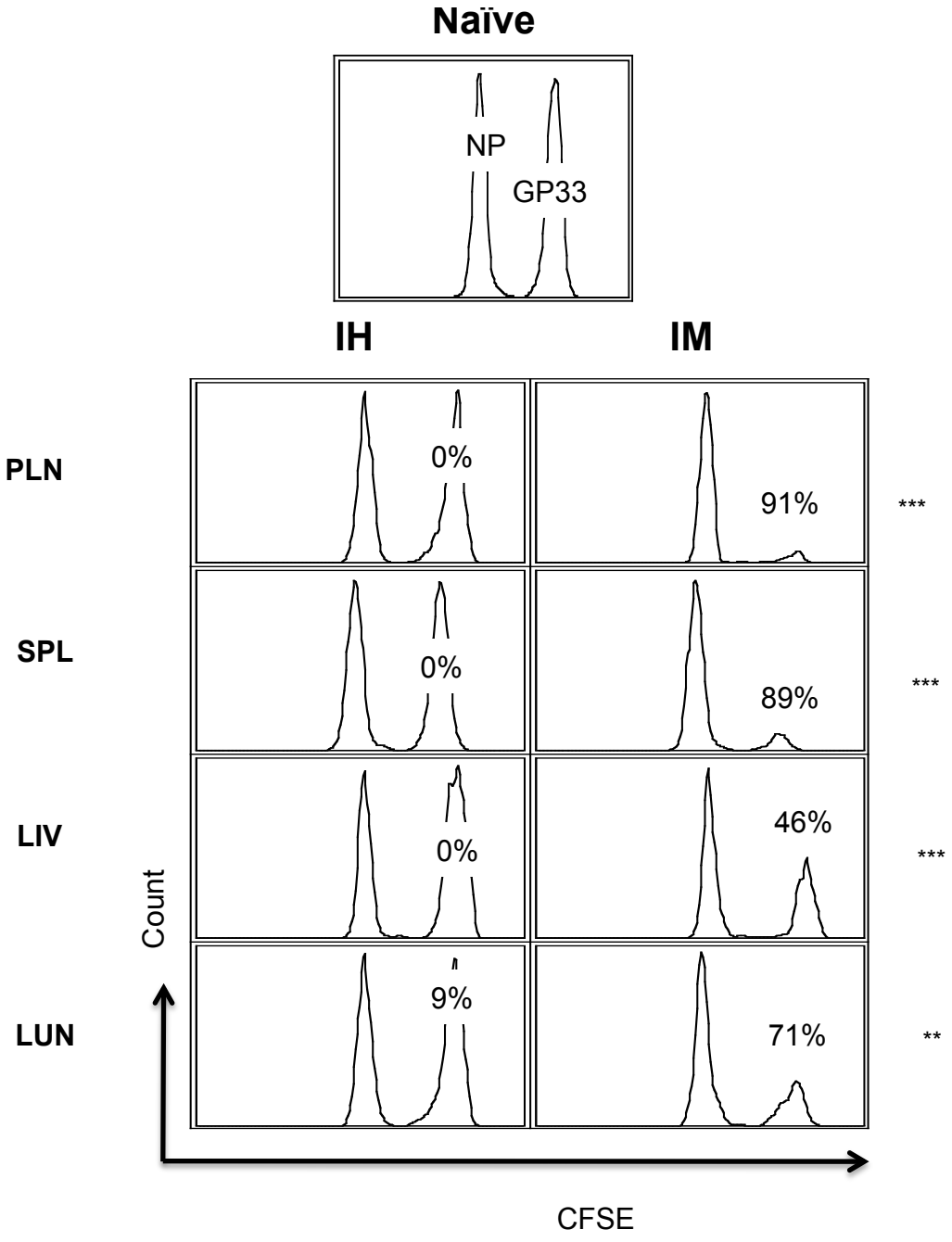
c.



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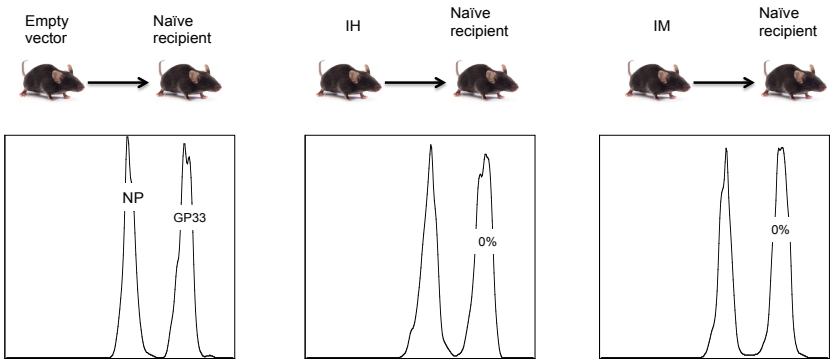
d.



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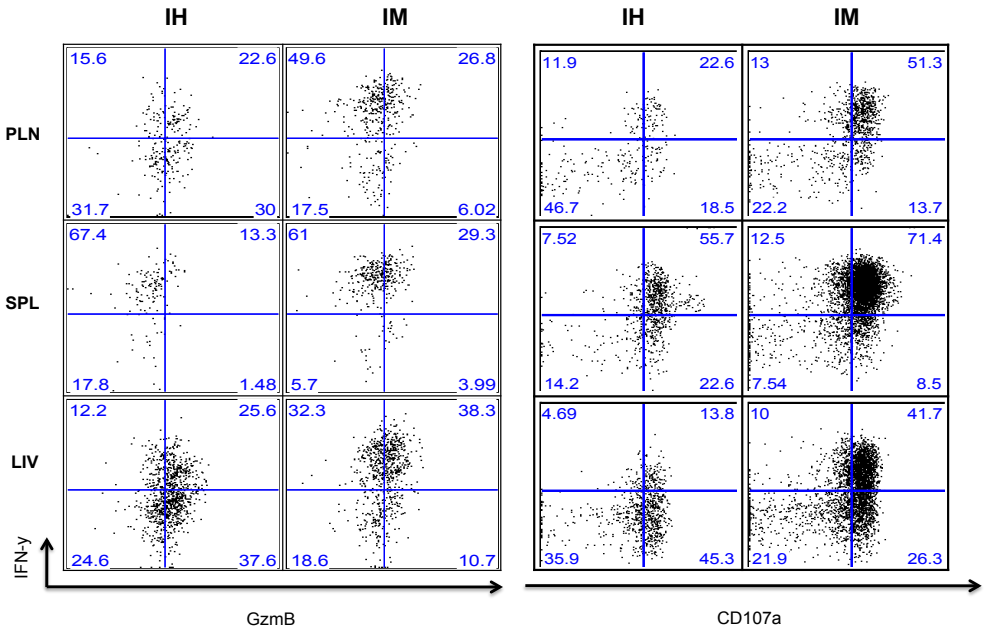


FIGURE 2.8. CTL-mediated cytotoxicity of liver-primed effector and memory CD8 T cells. (A-B) Intrahepatic activated GP33-specific CD8 T cells display rapid cytolytic activity during the first week of activation but this ability to specifically remove antigen-pulsed target cells *in vivo* diminished before day 21. (C) Percent *in vivo* killing in the spleen, lymph nodes, liver, and lung for IH and IM mice at week 3 post-immunization. (D) Cytolytic activity in the spleen of naïve mice that have respectively received equal frequencies (30,000) of activated D^bGP33^+specific CD8 T cell from IH and IM immunized mice. (E) The antigen-specific degranulation assay**.** Splenocytes from both groups (IH and IM) were stimulated ex vivo with LCMV-gp peptides in the presence of CD107a antibody. The stimulated splenocytes were subsequently stained with anti-Granzyme B (Gzm B).

Figure 2-9

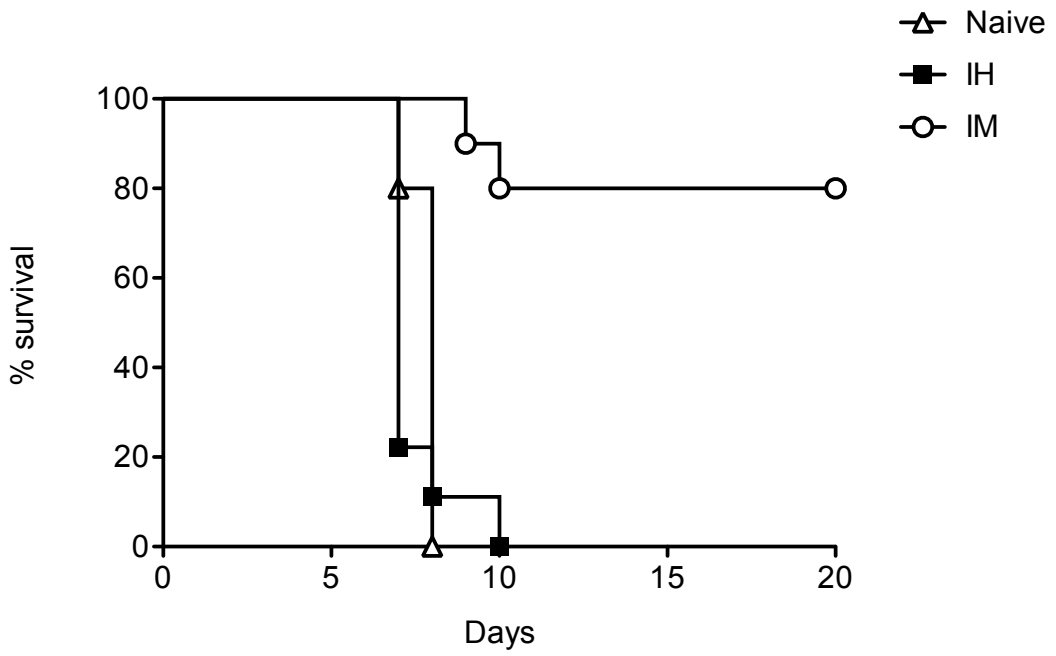


FIGURE 2.9. Protective immunity in hydrodynamically injected mice. Thirty days after immunization, groups of naïve, hydrodynamically injected and intramuscularly immunized mice were challenged with lethal dose of LCMV by i.c. infection (n= 10-15 mice/group). Following i.c. infection, mice were monitored for mortality.

2.6 Conclusions

Hepatotropic pathogens, such as HBV and HCV, often escape cellular immune clearance resulting in chronic infection. The mechanisms by which liver primed CD8 T cells become defective is not clearly understood. In this report, we directly compared liver primed CD8 T cells to secondary lymphoid tissue primed CD8 T cells for differentiation, function, and memory programming in a highly controlled fashion. We used hydrodynamic tail vain injection of plasmid to establish a liver-specific LCMV-gp antigen transient expression, and studied priming of CD8 T cells using the P14 transgenic mouse model. Intrahepatically activated CD8 T cells exhibited unique expansion, memory differentiation, polyfunctionality and cytotoxicity compared to T cells primed in the periphery. We reported an unexpected defect in the initial induction of adaptive immune response following viral antigen expression and a non-inflammatory priming of CD8 T cells in the liver. Priming of naïve CD8 T cells in the liver compromises the cells' expansion capacity, eliciting a 3 to 500-fold decrease of effector cell frequency and 2 to 9-fold decrease of memory CD8 T cell frequency in multiple tissues. Although liver priming resulted in early effector differentiation, the overall quality of the effector functions: polyfunctionality and cytotoxicity, were somewhat comparable to CD8 T cells primed in lymphoid tissues. The memory cells' poor antigenic clearance and viral control illustrate their low CTL frequency.

In summary, these results provide evidence that the lack of recall response needed to protect mice from secondary challenge after liver-priming of CD8 T cells, is directly associated with a defect in the initial clonal expansion. In human studies, the lack of viral

clearance in chronic HBV and HCV infections, correlates with the low number of HBV or HCV-specific CD8 T cells detected in the blood(Sobao et al., 2001; Sobao et al., 2002). Although, further studies are needed, we theorize that liver CD8 T cell priming during hepatotropic pathogen infection likely contributes to the low frequency of Ag-specific CTLs seen in chronic infections.

Chapter 3 – Therapeutic hepatitis B vaccine

" It was a very, very exciting time… But I really wasn't trying to develop a vaccine. Actually, all we did in our little laboratory, our little kitchen, so to speak was boil hepatitis B serum and water" – Saul Krugman, medical researcher.

3.1 Introduction to hepatitis B vaccines

The previous chapter showed that intramuscular immunization, combined with electroporation, can drive antigen-specific cell mediated immune response in the liver when mice were vaccinated with plasmids encoding parts of the LCMV genome. While the LCMV serves as an effective model for studying the dynamics of CD8 T cells in the liver, the virus is however not a natural hepatotropic pathogen. LCMV is also a rodent-borne viral infection with little clinical importance. A vaccination approach that drives immune response in the liver is important in developing therapeutic immunotherapy for hepatotropic pathogens such as hepatitis B and C virus, and malaria plasmodium. We elected HBV as a model to study this immunization approaches for reasons described below.

Hepatitis B is the only pathogen out of the three successful human liver pathogens to have an effective vaccine. The vaccine has been used to prevent the infection of HBV in billions of people across the globe and remains as one of the most successful prevention tools for any human viral infection. Despite the effectiveness of the current hepatitis B vaccine, two billion people have already been infected with the virus and 370 million of that group is currently living with chronic HBV. These chronically HBV-infected individuals are at risk of liver cirrhosis and cancer. Over a million chronically infected patients die each year from liver complications or liver cancer(Iloeje et al., 2012;

Lavanchy, 2004). The therapies available to treat HBV infection are effective for viral control but costly. The cost of treatment according to WHO can range from $20,000 to $40,000 annually, depending on the stage of the liver disease. Since current therapy only controls replication and does not clear infection, there is a major need for new approaches. These costs do not include the frequent hospitalizations or liver transplantation (over $90,000), needed at the end stage of the liver disease (Metcalf et al., 1999; Wong et al., 1995). This substantial economic burden is not only a predicament for the infected individuals but for nations around the world. Many infected individuals require assistance from government programs. Indirect costs attributable to lost workdays and lost productivity are also enormous. Most people who can afford treatment benefit very little from it, as only about 30% respond to the common interferon-α therapy. Response to treatments also differs between genotypes. There are many undesirable effects with current therapies which range from myalgia, fatigue, arthralgia, insomnia, diarrhea, depression, anorexia, erythema, dyspepsia, leucopenia, alopecia, rhinitis, vomiting, dysmenorrheal, hypertriglyceridemia and pruritus to psychological abnormalities (Sleijfer et al., 2005).

These issues support the need for therapeutic vaccines for both acute and chronic HBV infections. The ideal goal is to develop a vaccine that is capable of clearing the virus and leaving protective antibodies and T cells. The previous HBV vaccines have been effective as prophylactics but not so much as therapeutic vaccines. Since the current vaccine platform does not support the induction of antiviral CD8 T cell responses capable of clearing infected hepatocytes.

3.2 Hepatitis B vaccine history and current approaches

3.2.1 Making hepatitis B vaccine

In the late-1960s, Saul Krugman, a professor of pediatrics at the New York University School of Medicine performed what is considered one of the most unethical studies in the history of medicine. The findings from this study however became one of the biggest breakthroughs in modern medicine. The end result was the innovation of a vaccine that will over the years save billions of lives.

Baruch Blumberg (Blumberg, 2002; Das, 2002) and Alfred Prince (Ganem and Prince, 2004) initiated studies of HBV when the virus was virtually unknown to almost all virologists. Blumberg found that 1 in every 20 Americans have antibodies to a novel protein he discovered in the blood of an Australian Aborigine. He named this protein the "Australian antigen" (later found to be hepatitis B surface antigen). Although Blumberg linked this protein with different diseases, it wasn't until Alfred Prince's studies with blood transfusion patients Showed that the protein was part of the hepatitis B virus. Baruch Blumberg went on to win a Noble Price a decade later for this discovery. Following the footsteps of Blumberg and Prince, Saul Krugman made an effort to understand these particles (proteins) in the blood of the infected individuals and to see if the infectious particles (virus) were also present in the blood. He injected high-risk mentally challenged children with blood collected from infected patients. Almost all the children became sick, which led him to conclude that the hepatitis B virus is also present in the blood. With Krugman's knowledge on Blumberg's studies, which shows that some Americans have antibodies to the "Australian antigen", Krugman believed that if he

could somehow transfer the "Australian antigen" into the mentally challenged children, he might be able to generate similar and hopefully protective antibodies. And so he did. Cooking serum containing hepatitis B vaccine almost to the boiling point of water, he was able to inactivate the infectious properties of the virus and able to protect injected children from subsequent infection. This led to the development of a Hepatitis B vaccine (Krugman S, 1984; Sherlock, 1984) (Best and Neuhauser, 2010; Sherlock, 1984).

Although, Krugman did not want to call it a vaccine, Maurice Hilleman, the head of vaccine research at Merck, believed it was. Dr. Hilleman, who developed many important vaccines against deadly diseases including mumps, rubella and measles, was looking to develop a vaccine against hepatitis B when he came across Krugman's data. Hillman collected blood from homosexual men from New York (who were believed to be at risk for blood transmitted diseases like hepatitis B), filtered and concentrated the samples, and treated them with pepsin, urea and formaldehyde.to destroy potential particles. This sequence of treatments was sufficient enough to kill every known virus he tested (Human Immunodeficiency Virus (HIV) was unknown then) but it left some hepatitis B surface antigen. Hillman then filtered the product to get purified surface antigen. This was delivered into people as the first hepatitis B vaccine. Many controversies surrounded the use of blood-derived vaccines as both scientists and the public had concerns over safety. These controversies intensified when HIV was discovered. HIV was also found to be a blood-transmitted virus and was known to be prevalent in homosexuals. This led to withdrawal of Krugman/Hillman hepatitis B vaccine from the market and the development of recombinant hepatitis B vaccine, commonly known as second generation hepatitis B vaccine (Hilleman, 2003; Offit, 2008).

3.2.2 Second-generation hepatitis B vaccine

Pablo Valenzuale, a Chilean biochemist, seized the opportunity to replace the blood-derived hepatitis B vaccine with a safer option in 1986. He inserted the genes of the Australian antigen into *Saccharomyces cerevisiae* (yeast) and harvested the noninfectious protein and used it as a vaccine. This vaccine was free of blood proteins or any particles from other pathogens. Through this method, Valenzuale and his group developed the first recombinant vaccine. This recombinant hepatitis B vaccine is used globally today as part of the World Health Organization (WHO) pentavalent vaccines (diphtheria, tetanus, pertussis and Haemophius influenzae type B) routine immunization program (Santos-Lima et al.). People with levels of antibodies to the surface antigen (anti-HBs) greater than 100 IU/L after 3 dosages are considered good responders and normally are protected from HBV infection. Weak responders with anti-HBs between 10 IU/L and 100 IU/L are usually given another boost to help induce protection (Gilca et al., 2013) (Dervisoglu et al., 2011). The vaccine is believed to provide protection for a period of 10 years. However, current studies showed that the vaccine might provide life long protection. There have been a few safety concerns with this vaccine. Although some studies have suggested increased in risk of Multiple Sclerosis (MS) (Löbermann et al., 2010). CNS demyelinating (Martínez-Sernández V. Figueiras, 2012) and chronic fatigue syndrome (Nancy and Shoenfeld, 2008), many scientists and the World Health Organization have criticized most of these studies for their scientific accuracy and there remains no substantial evidence to confirm these safety concerns.

Although recombinant hepatitis B vaccine is relatively safe and induces strong protection in about 90% of the vaccinated population, there are several limitations the vaccine faces when it is used as a therapeutic vaccine.

Like most recombinant protein vaccines, the hepatitis B vaccine lacks the ability to drive strong Th1 responses. Even when it is supplemented with adjuvants that help drive Th1 responses, proteins still do not initiate the class I pathway and therefore do not generate CTLs. Simply neutralizing the virus with anti-HBs is not enough. T cell responses are needed to clear infected cells in chronically infected individuals. $CD8^+$ T cells are known to be the main effector cells responsible for acute HBV clearance through both cytolytic and non-cytolytic pathways, induced by the production of cytokines such as IFN-γ^+, TNF-α^+. This shows that a therapeutic HBV vaccine should be on a vaccine platform that drives a better cellular immune response. The current vaccine also only targets the surface antigen of the virus. Many HBV vaccinologists believe the difference in protection of the current vaccine is due to the genetic divergence that often emerges from mutations within the surface antigens. Moreover, studies characterizing the core antigen (HBcAg) as the major viral determinant of HBV persistence and showing that CTL responses to the core protein appear undetectable, as patients ability to generate IFN-γ^+ positive T cells becomes diminished following chronic infection proves that immune therapies to HBV requires the HBcAg as part of the target antigens.

Lastly, the current vaccine requires several *in vitro* molecular manipulations and production at large scale in yeast or CHO cells. This is a relatively expensive technique and therefore increases the market price for these vaccines. Stability is also an issue with recombinant protein vaccines.

With the hope of solving these shortcomings and developing a therapeutic approach, we selected the DNA vaccine platform.

3.2.3 DNA vaccines

In the early 1980's, Dubensky and his group showed that plasmid DNA can transfect cells and express the needed protein *in vivo* after injecting mice (Dubensky et al., 1984). Several groups followed up and used this technique as a vaccine platform (Fynan et al., 1993; Tang, 1992; Ulmer, 11993; Wang et al., 1993). This vaccine technology has remained relevant for over three decades, with several promising results. Studies in small animal models and non-human primates with different antigens (from different pathogens) have produced great levels of immunity and protection. Unfortunately, this level of immunity has not been well translated in human studies. Although no DNA vaccine is commercially available for human use, several licensed DNA vaccine animal products are available in the market **(Table 3.1)**

Despite the inability of this vaccine technique to yield favorable protection in clinical studies, it continues to be a suitable platform in pre-clinical studies due to the many conceptual advantages it holds over other conventional vaccines.

Safety: Unlike live attenuated vaccines, DNA vaccines are incapable of reverting to virulence. They are also free from pathogenic contamination, which is an issue with blood-derived antigen vaccines like the first generation HBV vaccine.

Antigen presentation: Proteins encoded by transfected DNA plasmids can be processed by both MHC class I and class II. This means that DNA vaccines can drive both Th1 and Th2 and CD8 responses, unlike recombinant proteins that only drives Th2 responses.

Vector neutralization: The major problem with viral vectors as antigen carriers is that the host induces neutralizing antibodies to the vectors and makes subsequent boosting very difficult. This is not the case for DNA vaccines.

Production: DNA vaccines are relatively easy to make and manipulate in large quantities compared to recombinant proteins and viral vectors.

Stability: Plasmids are very stable. They can be stored or shipped at room temperature for a period of six months. This rules out the need for a cold chain for transport, making it the ideal platform for vaccines being produced in developing countries.

In vivo translation: The expression of proteins (translation and post-translation) in multi-cell organisms with DNA plasmids guarantees an end product that is more likely to resemble the native protein. It also prevents processing and presentation of unwanted bacterial proteins.

Cost effectiveness: These vaccines are relatively cheap to produce when compared to live attenuated, recombinant protein and viral vector vaccines.

There are few disadvantages to DNA vaccination by traditional intramuscular immunization:

Low antibody response: This immunization technique is believed to induce low levels of soluble antigens, which averts the stimulation of strong humoral immune responses. Boosting with recombinant proteins seems to rectify this inadequacy (Luo et al., 2012).

Tolerance: Since genes are transcribe and translated *in vivo*, there is a possibility that the host immune cells will recognize these antigens as self-proteins. To avoid tolerance, proteins have to be expressed at high levels.

Upsetting host gene transcription: Transfecting cells with DNA plasmids can cause changes in gene regulation in host cells.

Ineffective for non-protein antigen: This technique is not very useful for pathogens that incite immune responses using non-protein antigens such as polysaccharides.

Overall, the many advantages of DNA vaccination override these shortcomings. These disadvantages can also be corrected with different immunization techniques and the use of adjuvants.

The exact mechanism by which DNA vaccination induces an immune response is not fully understood, However, it is speculated that local antigen-presenting cells are directly transfected or can acquire antigens through the secretion of antigen from transfected myocytes(Hokey and Weiner, 2006) for subsequent processing and presentation these antigens to resting lymphocytes in secondary lymphoid organs **(Figure 3.1)**

Table 3-1

Licensed DNA vaccine therapies

Company name	Vaccine name	Vaccine target	Target animal	Licensed date	Reference
Centers for Disease Control and Prevention and Fort Dodge Laboratories	West Nile Innovator	West Nile virus	Horses	2005	(Davidson AH, 2005)
Novartis	Apex-IHN	Infectious haematopoietic necrosis virus	Salmon	2005	(Greter et al., 2009)
VGX Animal Health	LifeTide-SW5	Growth hormone releasing hormone	Pig	2007	(Kutzler MA, 2008)
Merial, Memorial Sloan–Kettering Cancer Center and The Animal Medical Center of New York	Canine Melanoma Vaccine	Melanoma	Dogs	2007	(Bergman et al., 2006)

3.2.3 Improving the DNA vaccine technology

As mentioned earlier, a key challenge in DNA vaccines field at the moment is poor immunogenicity in larger animals and humans. In small animals, DNA vaccine induce less antibody responses when compared to other vaccine platforms. Numerous studies have been devoted to optimizing the DNA vaccine platform to enhance immunogenicity. Some of these strategies have been described below:

Gene modification: Different groups have used different methods to increase antigen expression by manipulating transcription elements. Codon optimization is regularly used to force the codon usage to favor the target species. This is important because differences in codon usage across species can slow down the translation of the microbial gene in eukaryotic cells. Optimizing these codons increases the transfer of RNA pools and thus enhances gene transcription (André et al., 1998; Deml et al., 2001)

Other modifications include: the inclusion of termination sites, IgE leader sequences and Constitutive Transport Element (CTE). These inclusions provide mRNA stability, proper loading of mRNA into ribosomes and increase in protein secretion. Amplifying protein secretion through these modifications increases both soluble and membrane bound antigen (Wang et al., 2006; zur Megede et al., 2000). This enhances immunogenicity in small animals and non-human primates.

Adjuvants: Traditional Th1 and Th2 cytokines have been successfully used as adjuvants to increase immune responses in DNA vaccine studies. Whereas plasmid-encoded cytokines can skew the immune response type *in vivo,* few have shown the ability to induce higher effective humoral responses. We began using chemokines, a family of small cytokines, in our laboratory as molecular adjuvants when studies revealed that

DNA vaccination induced chemokine expression on CD8+ cells. This finding quickly led to the cloning of many plasmids encoding chemokines such as MIP-1α, MCP-1, SDF-1, IL-8 and RANTES. Cytokines such as IL-2, IL-12, IL-17 and IL-28 have been able to enhance the cellular part of the immune response in both mice and monkeys. This indicated that our search for improved cellular immune responses against HBV may lie in molecular adjuvants.

Table 3-2

Summary of the advantages of DNA vaccine

Immunogenicity	They can induce both humoral and cellular immune responses at low effective dosages (micrograms) in animal models
Manufacture	Conceptually, they would be inexpensive to produce.
Engineering	Plasmid vectors are easy to manipulate and can be tested rapidly
Safety	Unlike live vaccines, they are unable to revert to virulence and unlike some killed vaccines they do not require the use of toxic treatments.
Stability	They are more temperature-stable and have a long shelf life.
Mobility	They are easy to store and transport.

Figure 3-1

Mechanism of DNA vaccine immunization

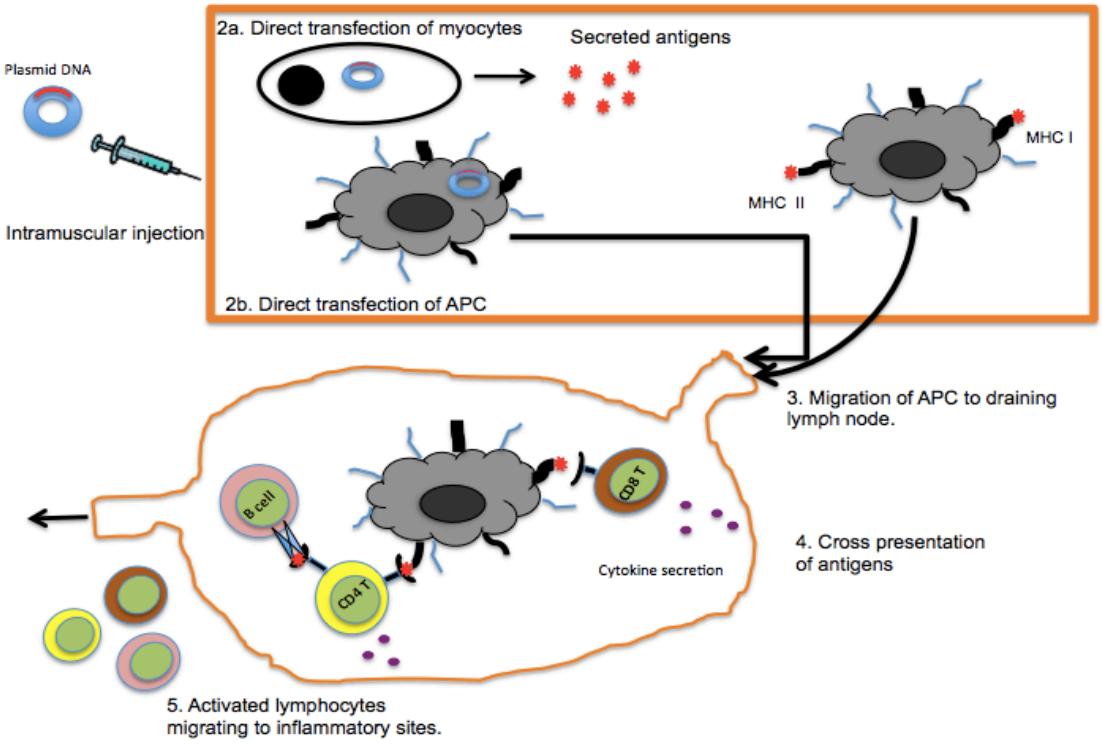


Figure 3.1 Mechanism of DNA vaccine immunizations. After intramuscular injection of DNA plasmid, myocytes and antigen presenting cells are directly transfected. The secreted proteins are processed and presented to lymphocytes in the lymph nodes.

3.3 Developing a vaccine against HBV core antigen

Resolution of acute HBV is believed to require a strong multi-specific $CD4^+$ T cell response to peptides encoded in the core antigen (Ferrari et al., 1990; Penna et al., 1996). $CD8^+$ T cells are, however, the main effector cells responsible for HBV clearance via both cytolytic and non-cytolytic pathways, induced by the production of cytokines such as IFN-γ^+, TNF-α^+(Biermer et al., 2003; Guidotti et al., 1996; Thimme et al., 2003). As such, chronically HBV-infected individuals show decreased in total HBV-specific $CD4^+$ and $CD8^+$ T cell responses compared to persons who successfully clear the virus. Moreover, CTL responses to the HBV core protein are poorly detected, as patients' ability to generate IFN-γ^+ T cells becomes diminished following chronic infection(Chang et al., 2005; Reignat et al., 2002). Studies in animals have confirmed the elimination of both IFN-γ^+ and TNF-α^+ producing $CD8^+$ cells during chronic HBV infection (Chang, 2006). These data together with studies distinguishing HBcAg as the major viral determinant of HBV persistence (Lin et al., 2010) highlight the importance of generating a strong immune response to HBcAg to resolve HBV infection.

Currently, the only therapies available for chronically infected individuals are interferon-α and nucleoside analogue treatments, which function by controlling viral replication but unfortunately do not clear infection. Interferon-α can prevent viral replication in only 30% of patients and does so with undesirable side effects. On the other hand, nucleoside analogues are much more effective at inhibiting viral replication but prolonged treatment often results in the emergence of escape mutants (Michel and Mancini-Bourgine, 2005). The failure of protection by current HBV vaccines in 15% of individuals and the difference in response to treatment in chronically infected people may be due to the

genetic divergence among different HBV genotypes (Cao, 2009; Lin and Kao, 2011; Torresi, 2002), as well as the inability to drive strong HBV-specific cytotoxic lymphocytes (CTLs) to the liver.

In the hopes of addressing these inadequacies with current approaches, we constructed a plasmid based on the HBV core (HBcAg)-specific consensus sequence from the Asian and African genotypes of HBV with genetic modifications that improve expression of the inserts. This includes codon and RNA optimization, as well as additional modifications to elicit maximum *in vivo* expression(Yan et al.). HBV core protein was chosen, as it is the major viral determinant of HBV persistence, with one of the major differences between acute and chronic human HBV infection being the detection of CTL against HBcAg in circulation (Ferrari et al., 1990; Lin et al., 2010; Tsai et al., 1992). Previous data from several groups suggest that these CTLs play a crucial role in the clearance of HBV during acute infection. In addition, recent findings suggest that the induction of both cellular and humoral immunogenicity against HBV antigens is important for controlling chronic infection (Yi-Ping Xing, 2005). Studies from our laboratory and other independent groups have shown that plasmid DNA delivery by electroporation (EP) represents an important strategy for enhancing cell or antibody mediated immune responses (Hirao et al., 2008a; Hirao et al., 2008b; Laddy et al., 2008; Yan et al., 2008), which we examined in this study.

3.4 Construction of HBcAg plasmid and Expression of pMCore

A consensus sequence of HBcAg was generated from 5 different genotype (A through E) gene sequences. The sequences were collected from different countries to avoid a sampling bias towards heavily sequenced genotypes. As shown in **Figure 3.2a**, there was an observed relative closeness of the multi-genotype consensus HBcAg sequence to all sampled sequences from different genotypes. After the consensus sequence was generated, several modifications were performed to increase the antigen expression levels from plasmid as described by our laboratory for other plasmid-based vaccines(Shedlock et al.; Yan et al.) (**Figure 3.2b)**.

The HBcAg protein was expressed by transfected pMCore DNA plasmid containing the core gene of hepatitis B (**Figure 3.2b**). An *In vitro* translation assay (a cell-free system) on lysate showed detectable HBcAg at an expected molecular weight of 28 kDa (**Figure 3.3a**). The expression was further confirmed using anti-HA tagged monoclonal antibody by immunofluorescent assay. We took advantage of confocal imaging to visualize HBcAg in the cytoplasm and around the nucleus of tranfected RD cells (muscle cells) as shown in **Figure 3.3b**. The expression pattern confirmed that a DNA plasmid carrying the core gene could be highly expressed in different cells *in vitro*.

Figure 3-2

HBV consensus core DNA construct (pMCore).

a.

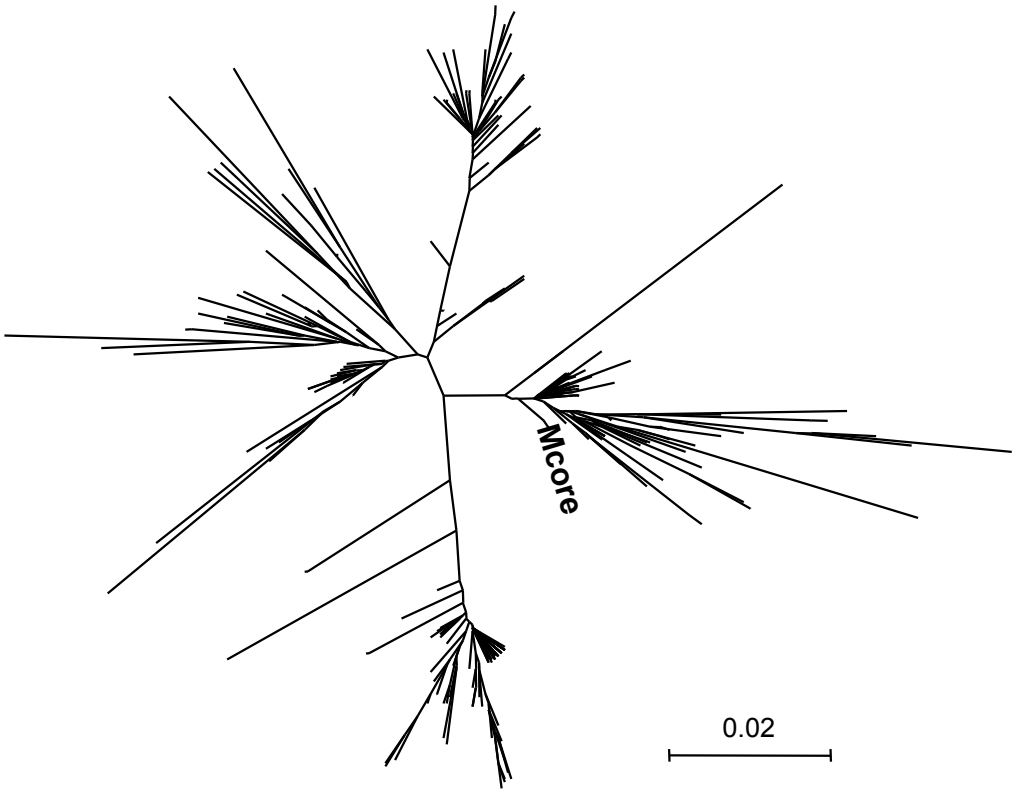


Figure 3-2 cont.

b.

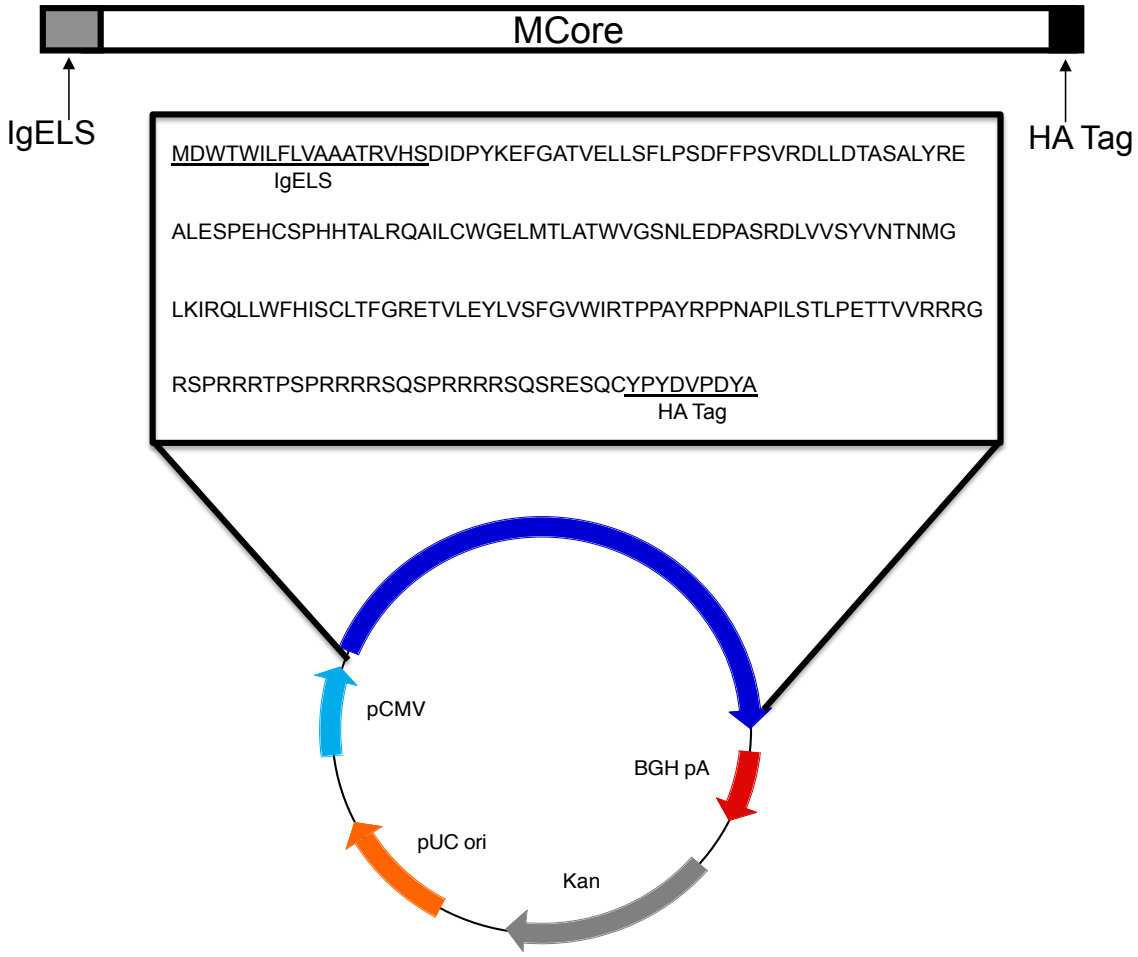
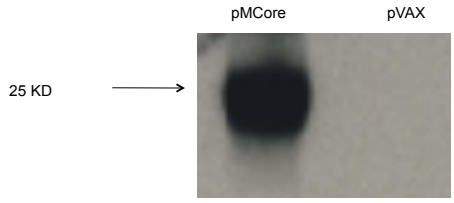


Figure 3.2 HBV consensus core DNA construct (pMCore). (A) Phylogenetic analysis of HBcAg consensus sequence as compared to individual genotypes (A through E). (B) Schematic representation of plasmid map and sequence of pMCore.

Figure 3-3

Detection of pMCore expression

a.



b.

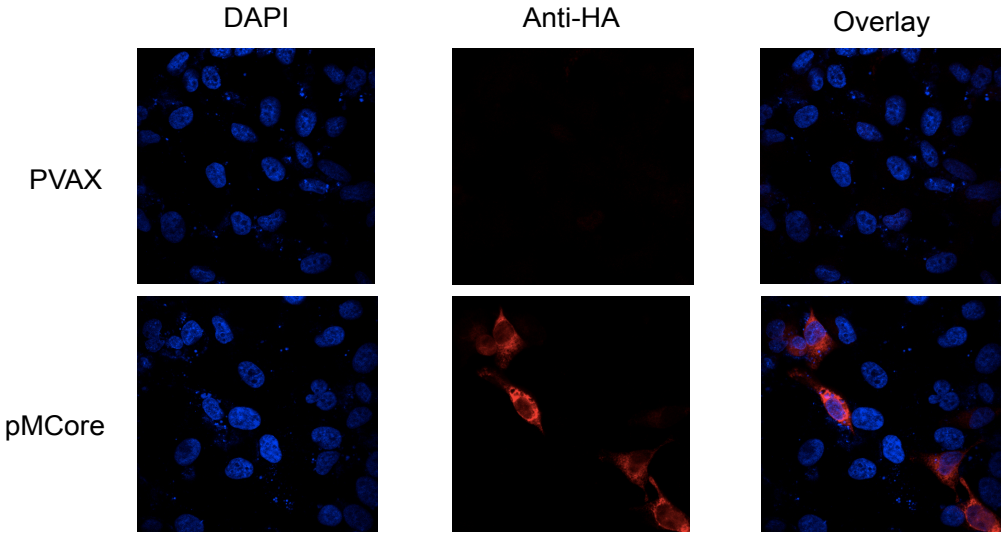


Figure 3.3 Detection of pMCore expression via *in vitro* translation and immunofluorescence. (A) Transcription/translation reaction using pMCore plasmid was immuno-precipitated with anti-HA monoclonal antibody which recognized a HA epitope encoded into the pMCore antigen. The precipitate was run on SDS-PAGE gel. (B) pMCore was detected in transiently transfected cells using a primary monoclonal HA tag antibody followed by detection with DyLight 594-labeled anti-rabbit secondary antibody (red). Hoechst stain was also used to fluorescently label cell nuclei. Expression of MCore is mostly localized to the cytoplasm as shown by the green stating patterns concentration outside the nucleus.

3.5 HBcAg-specific cellular and humoral response

3.5.1 Immunized mice exhibit immune response in the periphery

To better evaluate the generation of T and B cell immune responses, we immunized Balb/c mice and measured both responses in various peripheral tissues. Mice received three intramuscular immunizations of 30μg of pMcore or pVax followed by electroporation as depicted in the immunization scheme (**Figure 3.4a**). One week after the final immunization, pMCore immunized mice showed evidence of strong HBcAg T cell responses as identified by IFN-γ ELISPOT assay following *ex vivo* stimulation. **Figure 3.4b** clearly shows the dominant epitopes are biased towards peptide pool 2. The average HBcAg-specific IFN-γ T cell response induced was robust at 2000 (± 210) SFU per million splenocytes. Interestingly, intracellular staining of stimulated splenocytes revealed that both $CD4^+$ and $CD8^+$ cells produce almost the same amount of antigen-specific IFN-γ and TNF-α with about 0.4% of the T cells being double positive for both cytokines (**Figure 3.4c and Figure 3.4d**). The comparable cytokine production between both T cells did not predict their ultimate proliferative capacity. After 4 days of stimulation with antigen specific peptides, the $CD8^+$ T cells proliferated more than 2 fold higher than the $CD4^+$ cells (**Figure 3.4e**) showing a clear CD8 T cell bias in the response. To further explore the immune response induced in pMCore-immunized mice, we analyzed antigen-specific IgG and IgA responses by B cell ELISpot as well as in ELISA using splenocytes and sera, respectively, collected following vaccination. A high IgG and IgA titer was observed in the sera of immunized mice when compared to control animals. B cell ELISpot from immunized mice showed HBcAg-specific IgG and IgA at

approximately 200 SFU and 100 SFU per million cells respectively**. Figure 3-4b,** illustrates activation of the B cell compartment by immunization. Thus, our synthetic HBcAg plasmid effectively induced antigen-specific cellular and humoral responses after 3 immunizations.

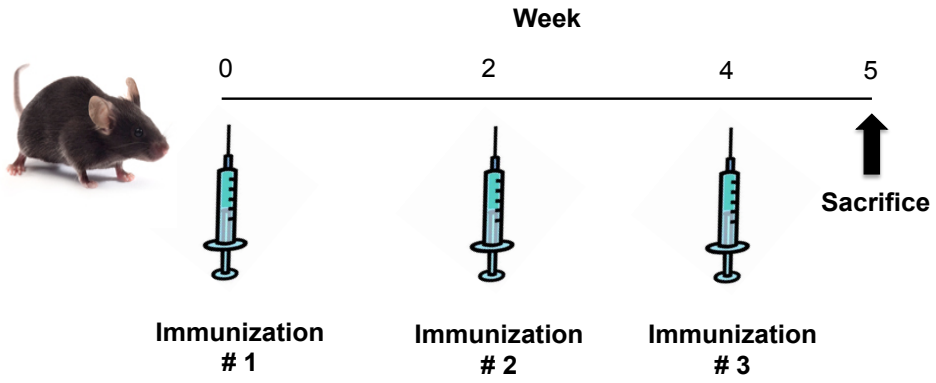
3.5.2 pMCore drives HBcAg-specific response in the liver

It is hypothesized that increasing functional anti-viral effector T cells in the liver will be important to clear chronic HBV infection. However, few studies on HBV vaccines have attempted to address this issue. Here, we examined the cytokine producing capabilities of intrahepatic antigen-specific T cells after DNA immunization. Both CD4 and CD8 T cells isolated from the liver produce IFN-γ and TNF-α when stimulated *in vitro* with HBcAg peptide (**Figure 3.5a and Figure 3.5b**). While the CD4 T cells show a high percentage of double producers of IFN-γ and TNF-α, the CD8 showed little to no IFN-γ+TNF-α+ producing cells. Instead majority of the CD8 T cells produced only IFN-γ or TNF-α. We also observed enrichment of HBcAg-specific CD4 T cells in the liver as compared to the spleen. The percent HBcAg-specific CD4 T cell producing IFN-γ and double positive of IFN-γ and TNF-α CD4 T cells in the resting liver was 4 and 2.8 fold higher than that observed in the spleen respectively. Conversely, peripheral CD8 T cells were confirmed to be better double producers than liver resident CD8 T cells. We also observed antibody-producing capabilities of liver resident B cells from immunized mice. Interestingly, the liver as a mucosal organ produced higher antigen-specific IgA than IgG (**Figure 3.5c**), an important observation that has not been previously been studied.

Figure 3-4

pMCore induces strong HBcAg-specific immunity in splenocytes of BALB/c mice

a.



b.

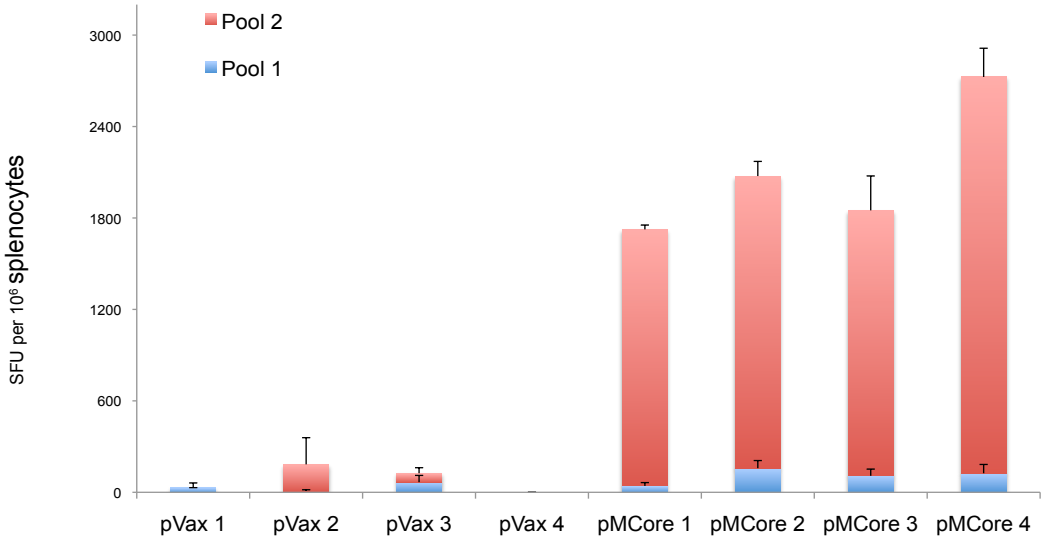
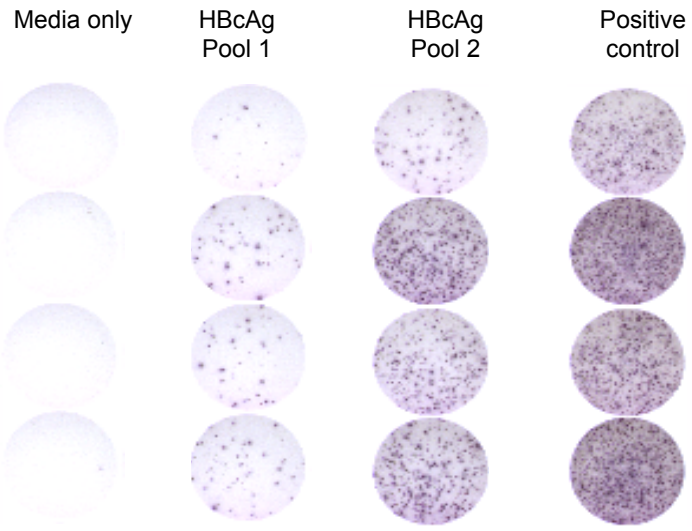


Figure 3-4 cont.



c.

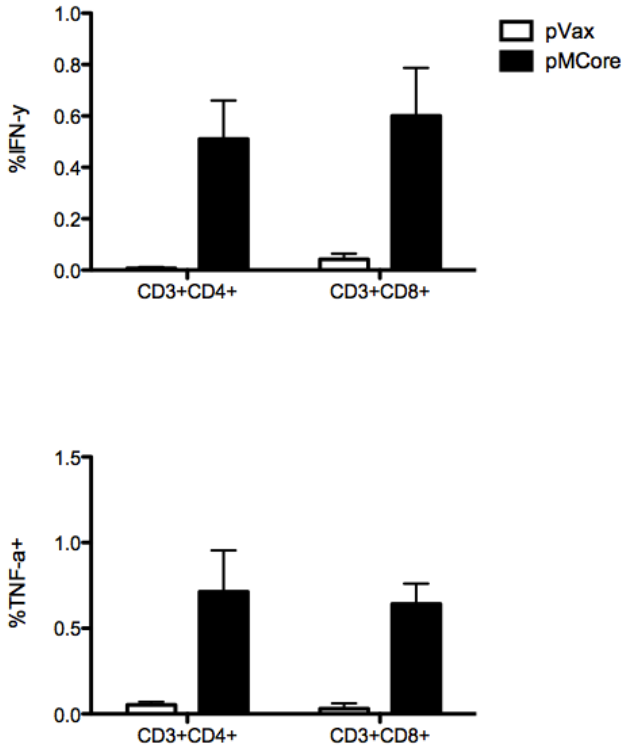


Figure 3-4 cont.

d.

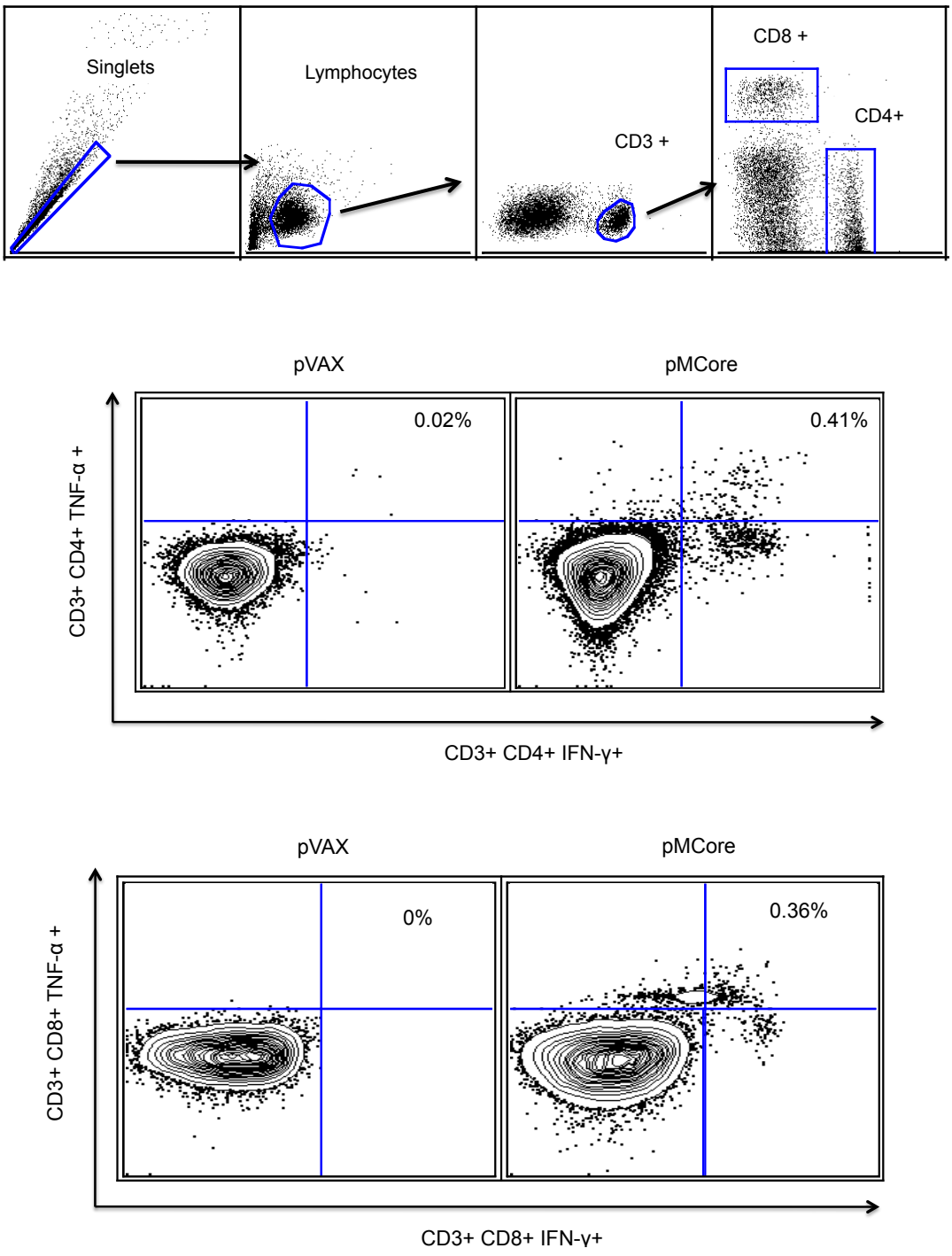
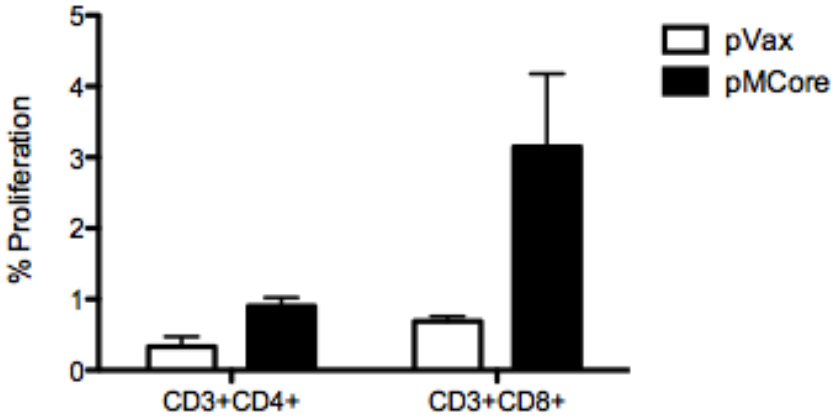


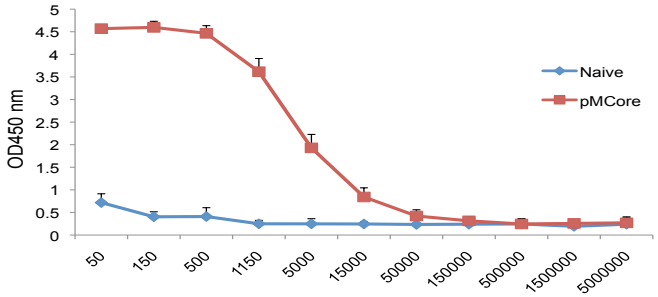
Figure 3-4 cont.

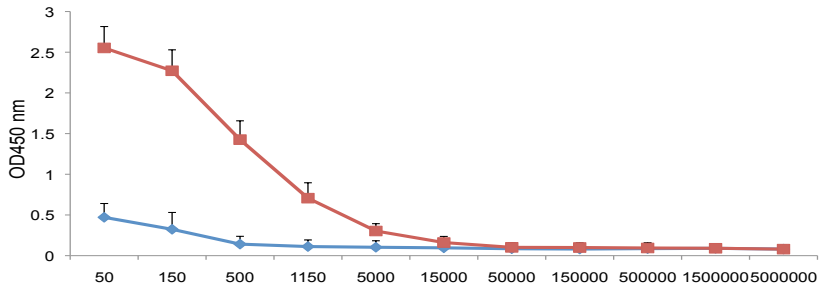
e.



f.

IgG





IgA

Figure 3-4 cont.

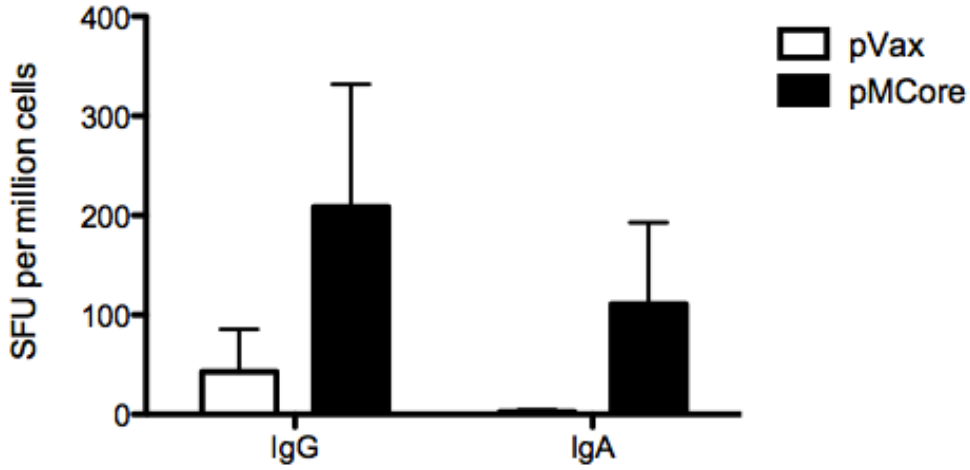


Figure 3.4 Immunization with pMCore induces strong HBcAg-specific immunity in splenocytes of Balb/c mice. (A) Immunization scheme. 4 mice were immunized intramuscularly with 30μg pMCore. (B) Frequency of HBcAg-specific IFN-γ spot forming units (SFU) per million splenocytes after stimulation of spleen cells from immunized mice. (C) Average percent HBcAg-specific CD4 or CD8 IFN-γ^+, TNF-α^+ (D) Average percent HBcAg-specific CD4 or CD8 double positive producing cells. (E) Percent proliferation of CD4 and CD8 T cells (F) HBcAg-specific humoral immune response induced by pMCore. The values are the means ± standard error of the mean. Significance was determined by Student's *t* test.

Figure 3-5

Synthetic HBcAg plasmid drives immune response in liver

a.

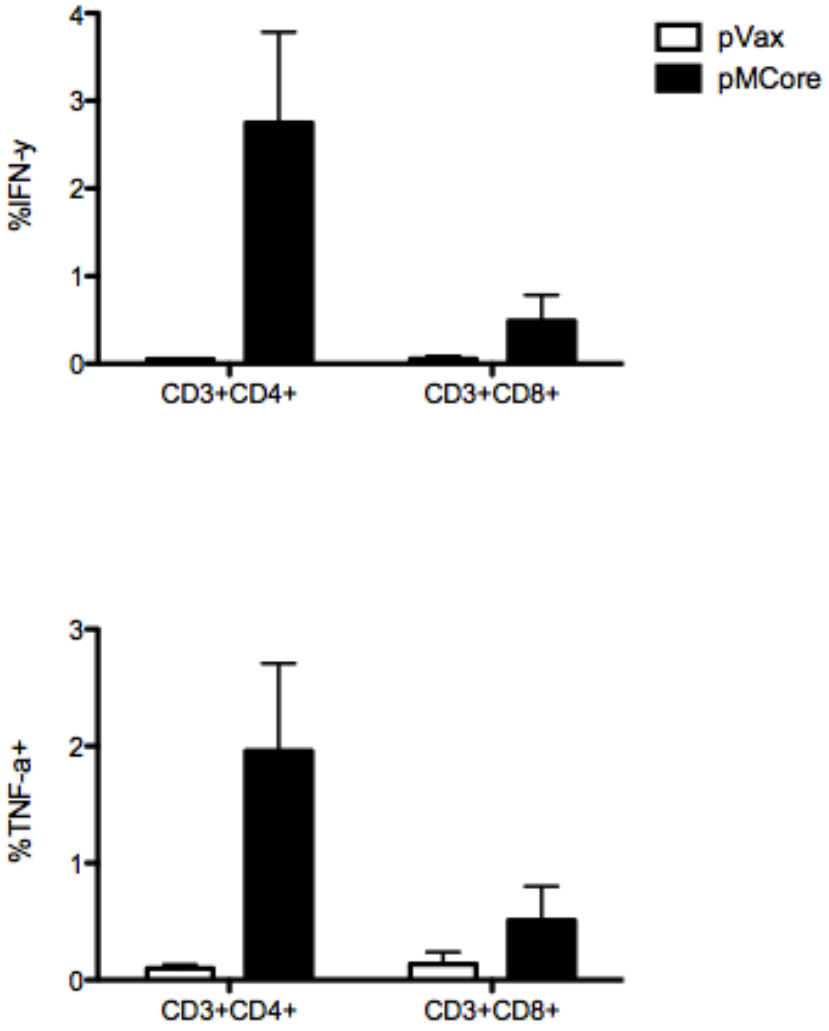
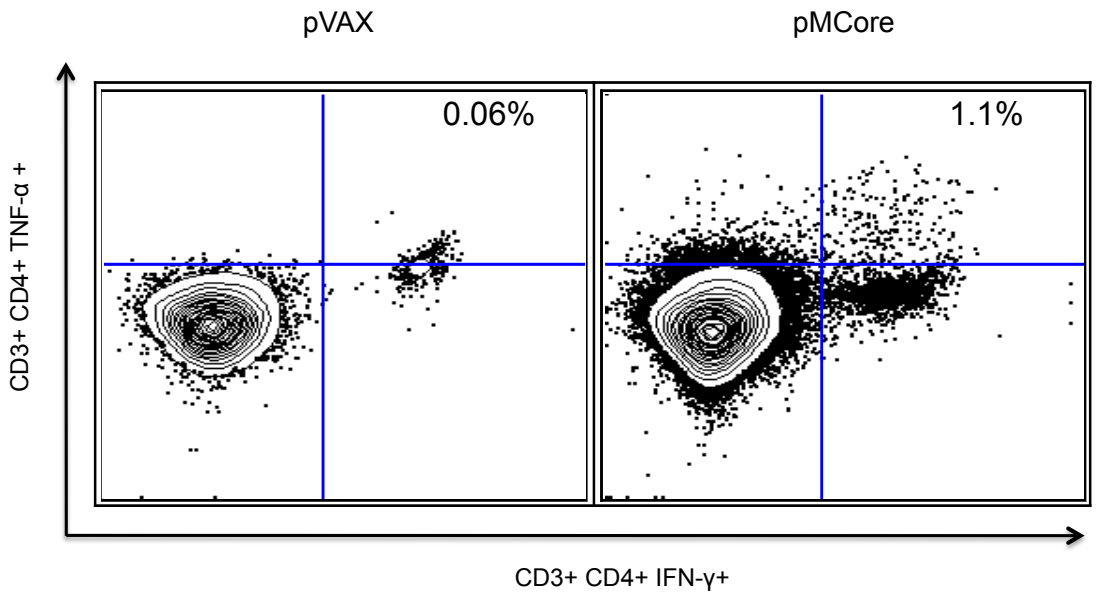


Figure 3-5 cont.

b.



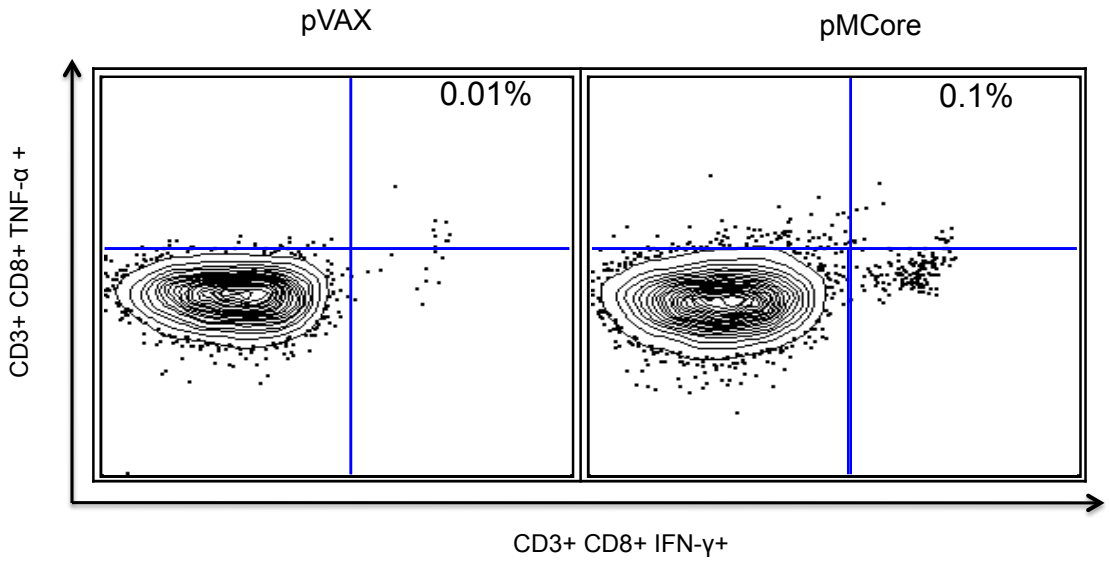


Figure 3-5 cont.

c.

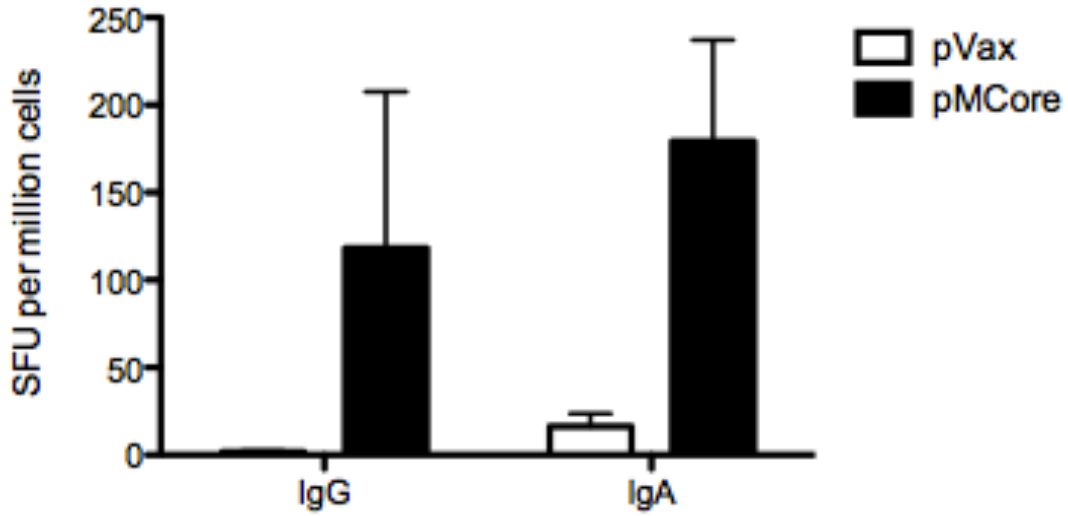


Figure 3.5 DNA immunization with pMCore drives strong antigen-specific immunity in liver of Balb/c mice. (A) Percent HBcAg-specific CD4 and CD8 IFN-γ^+, TNF-α^+ producing cells in the liver. (B) Average percent HBcAg-specific CD4 or CD8 double positive producing cells. (C) Antigen-specific antibody producing splenocytes. The values are the means ± standard error of the mean. Significance was determined by Student's *t* test.

3.6 Immune protection in pMCore immunized mice.

3. 6.1 CD8 T cells from immunized mice specifically kill target cells in vivo.

Next we assessed the ability of HBV-specific CD8 T cells induced after DNA immunization to specifically eliminate target cells *in vivo*. As previously described, human CTLs that target the core antigen are important in acute clearance of HBV versus chronic infection. One week after the final immunization, 4 mice from each of the two groups, pVax or pMCore immunized, were adoptively transferred with target splenocytes that had either been pulsed with HBcAg (relevant) or HCV-NS3/4A (irrelevant) peptides. By gating on CFSE labeled splenocytes to track killing, we observed that the pMCore vaccinated mice were able to induce strong specific killing of antigen-pulsed target cells as shown in **Figure 3.6**. Average percent killing observed in the spleen was about 83% while the average in the liver was 76%, showing that vaccine-induced CTLs that migrate to and are retained in the liver are capable of killing HBV peptide pulsed target cells. The percent killing in the spleen was comparable to previous studies that reported HBcAg-specific CTL responses using *in vitro* cytotoxicity assays (Yi-Ping Xing, 2005). To our knowledge, this is the first study to show induction of HBcAg-specific CTL responses in the liver, by any method and specifically by systemic immunization. These data provide evidence that peripheral immunization can induce effector cells that can migrate to the liver and lyse target cells.

3.6.2 Clearance of HBcAg expressing hepatocytes without any evidence of liver injury

In the absence of a small animal model for HBV to examine immune mediated clearance, we next utilized HBcAg plasmid to transiently transfect mouse liver through direct hydrodynamic injection. This model has been described in studies to acutely transfect mouse liver with different types of viral DNA (Lin et al., 2010)·(Ahlän et al., 2005; Yang et al., 2002). Here, immunized or naive mouse livers were either transfected with pMCore or an irrelevant plasmid encoding hepatitis C antigens (HCV NS3/4A). Immunohistochemistry staining three days post transfection (**Figure 3.7a**) shows clearance of HBcAg-transfected hepatocytes as compared to the NS3/4A-transfected animal livers. CD8 T cells isolated from the pMCore hydrodynamic injected mice in **Figure 3.7b** showed a higher frequency of IFN-γ^+ CD107a$^+$, a marker of degranulation, as compared to immunized animals livers transfected with the irrelevant plasmid.

Since the clearance of pMCore-transfected hepatocytes seems to involve degranulation, it was fair to assume the killing may lead to liver damage. To examine if immunized mice were able to clear the transfected hepatocytes without inducing significant liver damage, we employed a widely used assay measuring the enzyme, alanine aminotransferase (ALT) which is an indication of liver damage when enzyme levels are observed elevated in the sera, **Figure 3.7c**. These studies showed that the specific clearance of HBcAg-transfected hepatocytes did not increase ALT levels in transfected immunized animals beyond the normal range of 5-30U/L.

Figure 3-6

In vivo specific killing

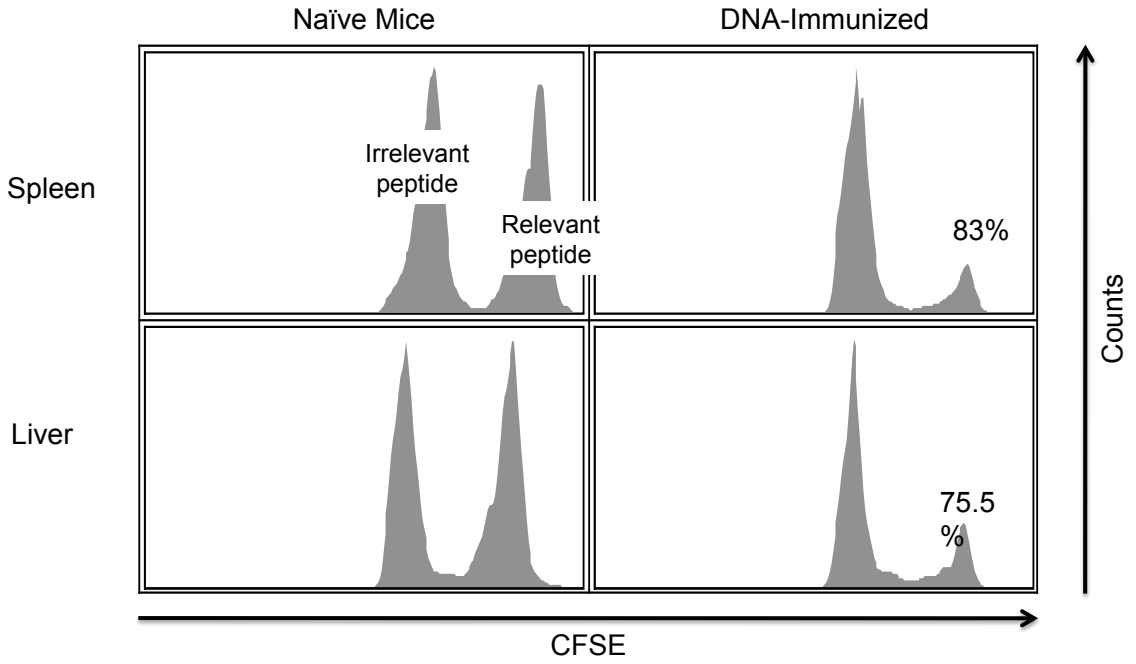


Figure 3.6 *In vivo* specific killing. Two groups of mice immunized with either pVax (control) or pMCore received CFSE-labeled target cells ($CFSE^{lo}$ pulsed with irrelevant peptide or $CFSE^{hi}$ pulsed with epitope-specific peptide) through the tail vain. CFSE-labeled cells were recovered and analysis by FACS was utilized to quantify percent killing.

Figure 3-7

Elimination of HBcAg-expressing hepatocytes

a.

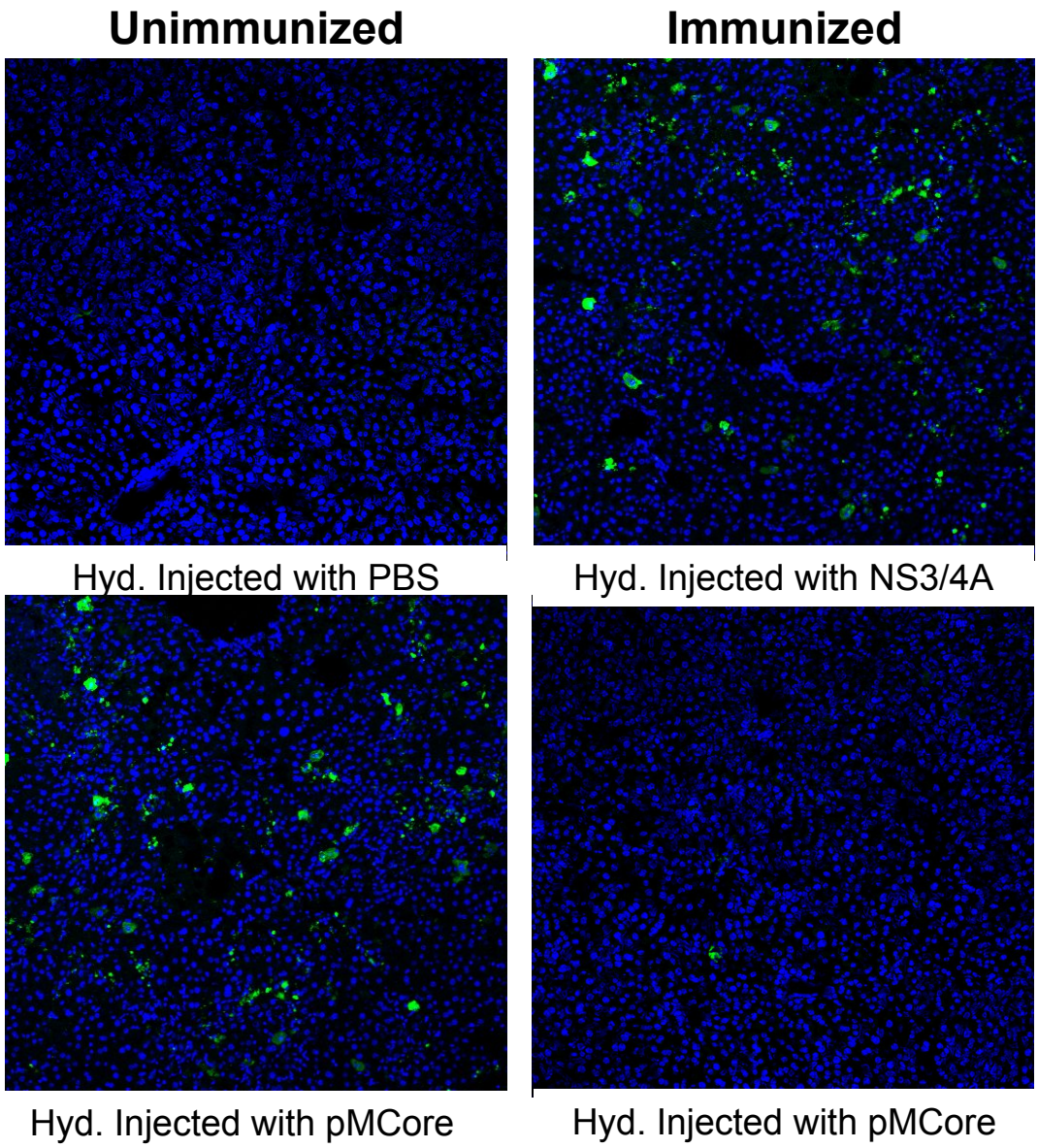
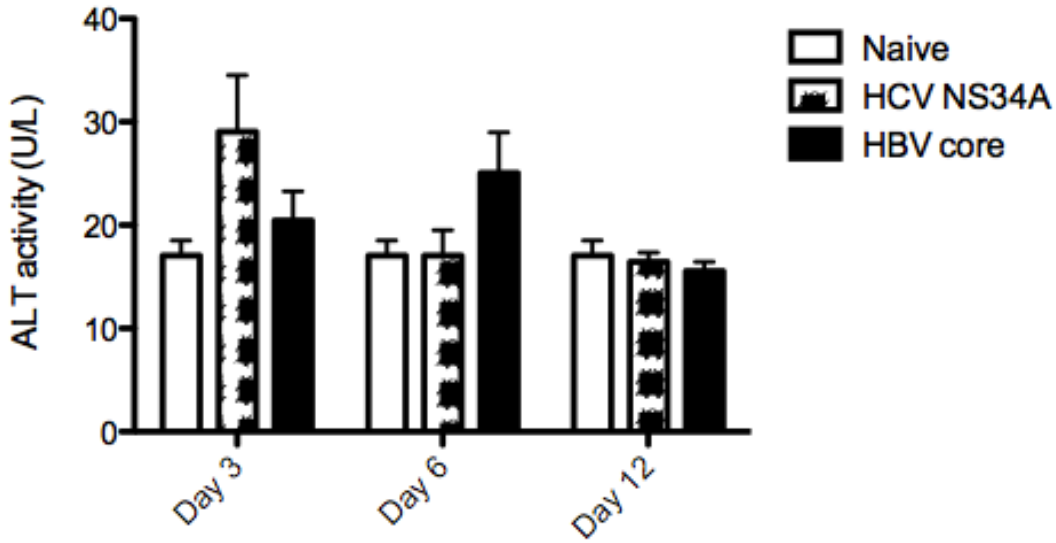


Figure 3-7 cont.

b.



c.

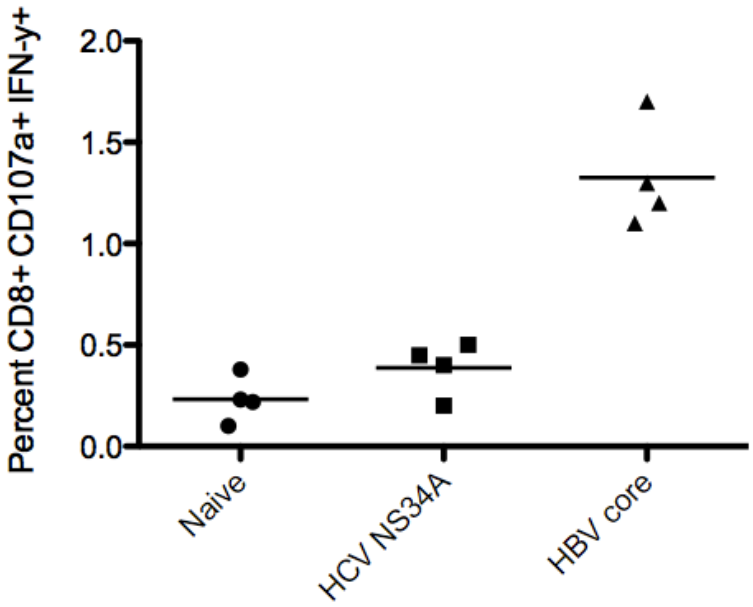


Figure 3-7 cont.

Figure 3.7 Degranulation and elimination of HBcAg-expressing hepatocytes by vaccine-primed T cells. Hepatocytes of pMCore-immunized mice were transiently transfected with HBcAg or HCV NS3/4A plasmid. (A) Immunostaining of liver sections taken three days after hydrodynamic injection of PBS, pMCore (HBcAg) or pNS3/4A (HCV NS3-4A) from naïve or mice that were immunized with pMcore is shown. Clearance is much higher for the pMCore-immunized liver as compared to the NS3/4a transfected control liver. pMcore or NS3/4A expression detected with an anti-HA antibody (green cells) (B) Serum ALT levels at day 3, 6 and 12 post transfection were measured and show no evidence of elevation in relevant vaccinated animals. (C) 3 days post transfection, cells were analyzed for degranulation marker expression, CD107a, and IFN-γ+ expression following stimulation with HBcAg peptides.

3.5 Conclusions

Previous studies from other groups have shown induction of either or both cellular and humoral responses when murine (Kuhrueber et al., 1997), nonhuman primates (Sällberg et al., 1998) or humans (Livingston et al., 1999) were immunized with plasmids or retroviral (adenovirus) vectors encoding the core antigen. However, the ability of these vaccines to induce and retain antigen specific immune cells in the liver and clear infected hepatocytes was not investigated. Furthermore the magnitude of the responses induced in this study appear superior to these prior approaches. In this study, we examined the proficiency of a synthetic HBcAg encoded plasmid DNA vaccine delivered in the periphery to establish antigen-specific immune response targeting to the liver resulting in clearance of HBV transfected liver cells.

The immune phenotype induced by this vaccine was interesting. Using intracellular staining we observed that antigen specific cells from the spleen of immunized mice were double positive for both IFN-γ and TNF-α. Although the plasmid contains a core antigen, it is worth of noting the high titer HBcAg-specific IgG and IgA observed in sera. From a therapeutic point view, it is important for the activated cells to traffic to and to then be retained in the liver. Immunized mice demonstrated an increase in activated CD4 T cells in the liver as evidenced by the large percentage of HBcAg-specific IFN-γ and TNF-α double positive cells found there. Liver CD8 T cells on the other hand, were mostly single function producer cells. The importance of multiple-cytokine producing CD4 T cells in producing effector functions is well described (Kannanganat et al., 2007) but single function CD8 and more specifically intrahepatic T cells in this regard have not been previously reported. However, single function CD8 T cells are likely effector cells.

Lu *et al*. *(Yi-Ping Xing, 2005),* demonstrated that immunization with HBcAg DNA could induce antigen-specific cytotoxic activity *in vitro*. Our data using an *in vivo* killing assay extended their findings. The '*in vivo* killing' assay confirmed that antigen-specific CD8 T cells in immunized mouse spleen and liver (which were single function CD8 T cells), are able to efficiently eliminate target cells.

In order to assess the effectiveness of an HBV vaccine, a challenge model is needed. In the absence of an infectious small animal model, mouse hepatocytes can be transiently transfected *in vivo* by plasmid DNA through a process known as hydrodynamic injection to create an "infectious-like" model. Although this model mimics aspects of acute infection, it has been used by many independent groups to study viral kinetics(Yang et al., 2002), persistence (Lin et al., 2010) and clearance (Yin et al., 2011). We transfected naïve and pMCore primed mice liver with the consensus DNA through hydrodynamic injection. Three days after the transfection, degranulation in spleen was evaluated by staining CD8 T cells from both groups for CD107a. The immunized group showed higher percentage of IFN-γ and CD107a double positive CD8 T cells retained in the liver.

pMCore-Immunized mice showed complete clearance of HBcAg expressing hepatocytes but were ineffective against HCV-NS3 expressing hepatocytes, showing the level of specificity of the generated CTL. This specificity of the clearance is relevant to treatment of HBV infection, as nonspecific elimination of hepatocytes has been reported to lead to inflammation in the liver. There are concerns that cellular immune responses induced by vaccines against liver pathogens can drive hepatitis resulting in liver damage. Measuring the amount of alanine transaminase in the blood as means of evaluating liver injury, we observed no significant liver damage during or after the liver clearance process. This

important finding suggests that antigen-positive hepatocyte clearance might be through both cytolytic and non-cytolytic processes as seen during human acute HBV clearance.

In summary, a synthetic recombinant plasmid encoding HBcAg and delivered by EP can generate responses that target to liver, and such responses maybe useful in a prototype HBV immune therapy. Although further studies are needed to understand the trafficking and retention of antigen specific T cells in the liver, their ability to induce responses and clear infected hepatocytes with minimal damage by what appear to be effector T cells, as reported here, is encouraging. Further investigation of these immunization tools in HBV with relevance to liver cancer appears important.

Chapter 4 – Combined immunotherapy with HBc and HBs

" There is no cure for hepatitis B, which is why prevention is critical. Universal vaccination at birth is the most effective way to prevent infection and future consequences of the disease" – Susan Allan, director of the Oregon department of human services and public health division.

4.1 Activating both arms of the adaptive immune response

The current HBV vaccine, which is also the first ever-recombinant vaccine, like most vaccines available in the market relies on the induction of antibodies to convey protection(Averhoff et al., 1998). This is not very practical for the quarter of a billion people around the world who are already infected with the virus and are in a dying need of an effective immune therapy(Hosaka et al., 2012). Studies have shown that strong T cell responses are seen during the resolution of acute HBV infection(Guidotti and Chisari, 1996; Rehermann et al., 1996). In chronic infection however, there is a decline in the overall cellular responses and CTL to certain antigens diminishes(Chang et al., 2005; Reignat et al., 2002). This highlights the importance on why driving that part of the adaptive immune response will be useful in the therapeutic setting. While anti-HBsAg has been the hallmark of protection for the current vaccine (using antibodies), immunization against the HBcAg has shown to drive stronger cellular immunity in small animals, non-human primates and humans (Kosinska et al., 2012; Livingston et al., 1999; Zu Hu Huang, 2001). Together, these studies highlight the importance of driving both arms of the adaptive immune response to multiple antigens in the search for a successful therapeutic HBV vaccine.

The cell-mediated immune responses will help clear infected hepatocytes by inducing cytotoxic T cells (Tc). These types of lymphocytes kill infected cells by releasing cytotoxins such as granzymes and perforins. A vaccine-induced Tc will in theory be activated by recognizing processed HBc or HBs antigens on the MHC I of the infected cells. The Tc will release perforins, which will form pores in the membranes of the target (infected) cells, and then release granzymes, which will enter these target cells to initiate a caspase cascade. The activation of the various caspase domains will lead to the apoptosis of the target cell.

The humoral immune response on the other hand, does not have direct effect on the infected cells. However, antibodies can help contain infection by activating a mechanism called Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) to destroy infected cells. HBc or HBs antigen-specific antibodies will bind to infected cells expressing these antigens on their cell surfaces. This antigen-antibody complex will attract natural killer cells to destroy the infected hepatocytes in a fashion similar to the cytotoxicity induced by CTLs. Antibodies can also neutralize viruses that move out of infected hepatocytes to infect neighboring cells. Viruses that 'bud' out of infected cells have the potential to infect other cells if the immune cells do not destroy them.

Driving both arms of the immune response using different antigens may prove to be the most effective approach for a hepatitis B therapeutic vaccine. DNA vaccination seems to be the appropriate platform to develop this type of vaccine. DNA vaccines, unlike protein or peptide vaccines, can elicit robust T cell responses and CTL(Bagarazzi et al., 2012; Shoji et al., 2012). Additional manipulations during the delivery process such as the use

of electroporation has enhanced the humoral responses to a significant titer(Shoji et al., 2012). More importantly, DNA vaccines are safe and very malleable (Kutzler, 2008).

4.2 Developing synthetic DNA HBs encoded immunogens

4.2.1 Construction and expression of multiple HBsAg DNA vaccine

Previous studies from our laboratory and other independent groups have suggested that the use of consensus immunogens in the context of vaccines is important in inducing a more broad immune response as compared to immunization with one native immunogen. Here, we generated DNA constructs encoding either the major S or the preS1/S2 plus the major S of the HBsAg consensus sequence for HBV genotypes A and C in attempt to increase the breadth of the immune response against the HBsAg proteins. Clustal X (version 1.8) software was used to create multiple alignments needed to generate a single consensus sequence, **Figure 4-1a**.

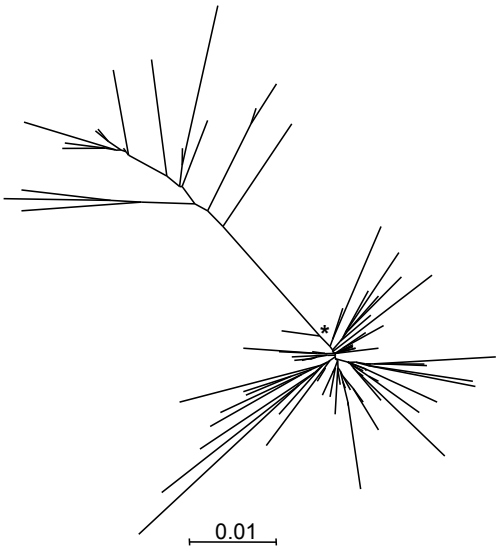
Following construction and optimization of the constructs, protein expression of each plasmid was confirmed by immunofluorescence. RD cells were transiently transfected with individual constructs or with an empty pVax vector as a control. The cells were fixed 48 later and stained with full-length HBsAg polyclonal monkey sera. Fluorochrome labeled anti-human IgG confirmed the protein expression (**not shown**). The expression was further confirmed using flow-cytometry. Transfected Hep G2 cells were intracellularly stained with sera from immunized mice and commercially available mouse monoclonal antibody (**Figure 4.1c**).

Figure 4-1

HBV surface antigen DNA constructs.

a.

HBs genotype A



HBs genotype C

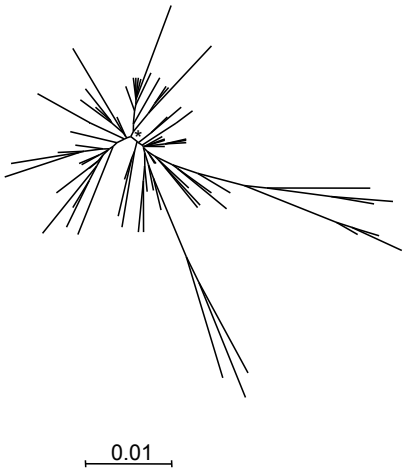


Figure 4-1 cont.

b.

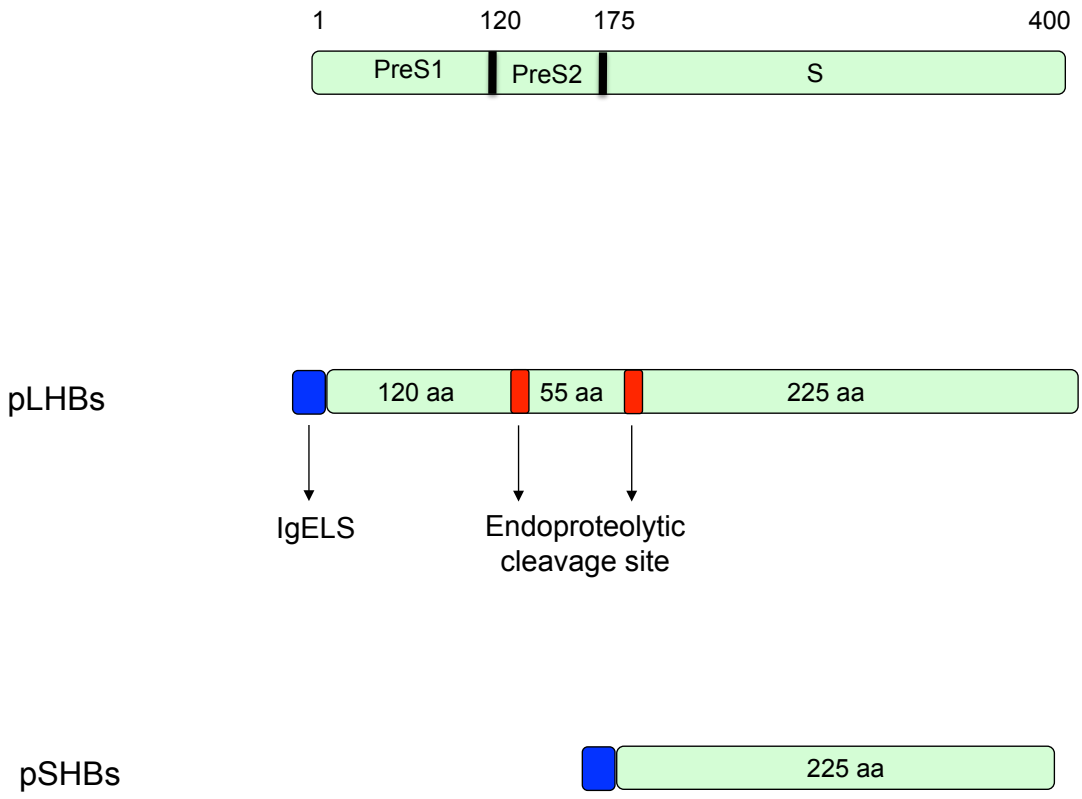


Figure 4-1 cont.

c.

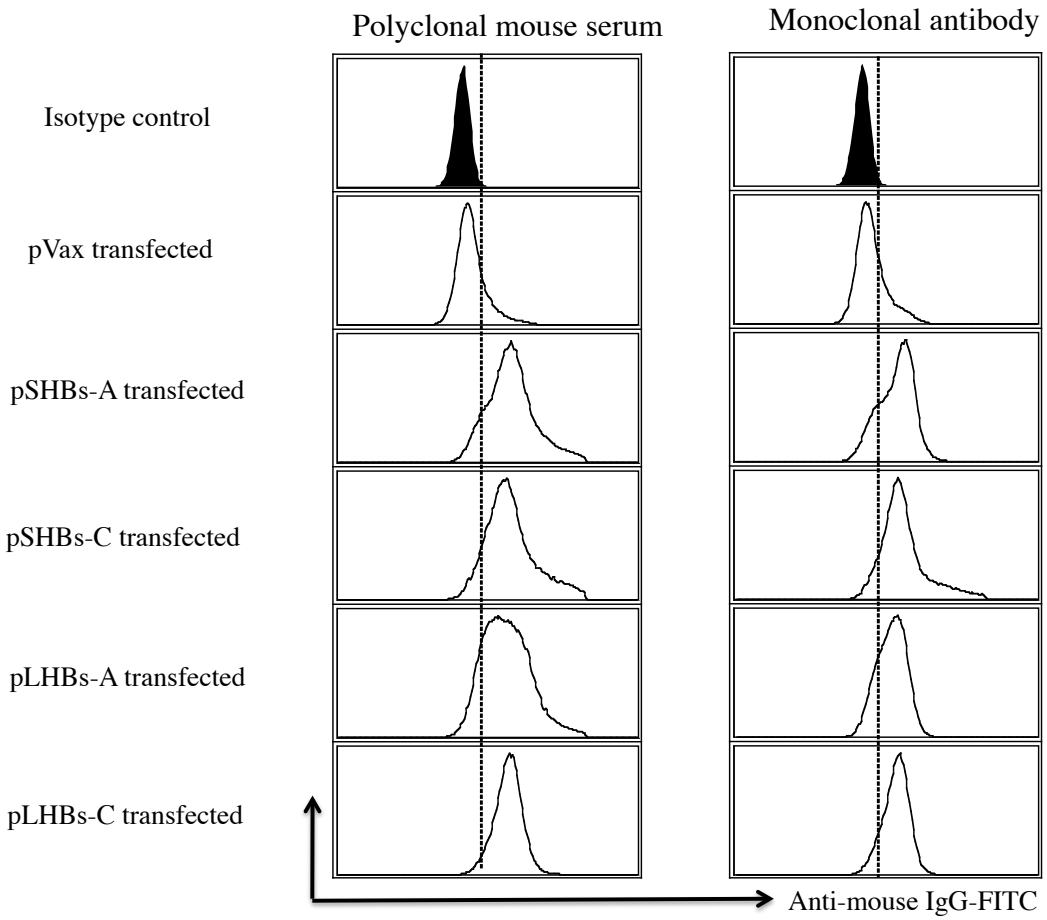


Figure 4.1 HBV Surface antigen DNA constructs. (A) Phylogenetic analysis of pHBs consensus sequences of HBS as compared to individual genotypes of A and C. (B) Plasmid map of pLHBs and pSHBs showing IgE leader sequence and endoproteolytic cleavage sites. (C) Detection of pLHBs and pSHBs via intracellular staining of transfected RD cells with monoclonal antibody and polyclonal mouse sera.

4.2.2 Immunogenicity of the individual HBsAg plasmids

After expressing the surface antigens *in vitro,* we evaluated the ability of the different HBV surface antigen constructs to individually induce antigen-specific humoral and cellular responses *in vivo.* Balb/c mice were intramuscularly immunized thrice, 2 weeks apart at week 0, 2 and 4 with 15μg of plasmids expressing either the small or large S antigen or a pVax control. The injection sites were electroporated (EP) immediately following injection. We first looked at the humoral response induced by the constructs, since DNA vaccines have not always been consistent in generating significant amount of antibodies. We performed an antibody ELISA to analyze antigen-specific IgG responses in sera collected from mice 7 days after each immunization. The ELISA plates were coated with recombinant full length HBsAg protein for this analysis. Both pLHBs and pSHBs from either genotype were greatly immunogenic. They induced significant levels of HBsAg-specific IgG antibodies following the initial immunization. Continued immunization boosted these responses, showing the greatest enhancement at week 2 after the third immunization (**Figure 4.2a**). In contrast, the mice immunized with the pVax control developed background IgG responses. Although we observed differences in response with each group, the progressive enhancement after each immunization was similar within the groups.

We next looked at the ability of pLHBs-A, pLHBs-C, pSHBs-A and pSHBs-C constructs to induce cellular immune responses as determined by IFN-γ ELISPOT. The surface antigen-specific IFN-γ secreting cells were analyzed in response to two pools of synthetic peptides from consensus large HBV surface protein. The first pool contains the N-

terminal domain of the protein (preS1) and an additional 55 amino acid preS2 domain. Although all four consensus constructs were able to induce strong T cell response (**Figure 4.2b**), there is clearly an epitope-bias towards the major S protein. We also observed strong cross immune reactivity within the two genotypes. These results indicate that our synthetic DNA immunogens encoding either the full length or only the major S portion of the HBV surface antigen exhibit immunogenicity that is diverse enough to protect against the major genotypes of HBV.

4.2.3 Breadth and magnitude of the cellular immune responses

After confirming the magnitude of cellular response of against the synthetic plasmids, we determined whether there was also an increase in the breadth of cellular immune responses against genotype-specific targets by detailing the cellular immune responses against HBsAg major S and preS1/S2 peptides in both BALB/c mice. The data suggested that there was no clear dominant epitope induced by the synthetic surface antigen plasmids in the BALB/c mouse strain. For pLHBs, there were 12 matrix pools out of 16 for genotype A and 8 out of 16 for genotype C, showing more than 50 spots. With pSHBs, there were 9 matrix pools out of 12 pools for genotype A and 12 out of 12 matrix pools demonstrating more than 50 spots in vaccinated BALB/c mice (**Figure 4-3**). These data signify that there is a significant increase in the breadth and magnitude of cross-reactive cellular responses induced by each of the 4 HBs constructs.

Figure 4-2

Immune responses induced by each HBs DNA plasmid.

a.

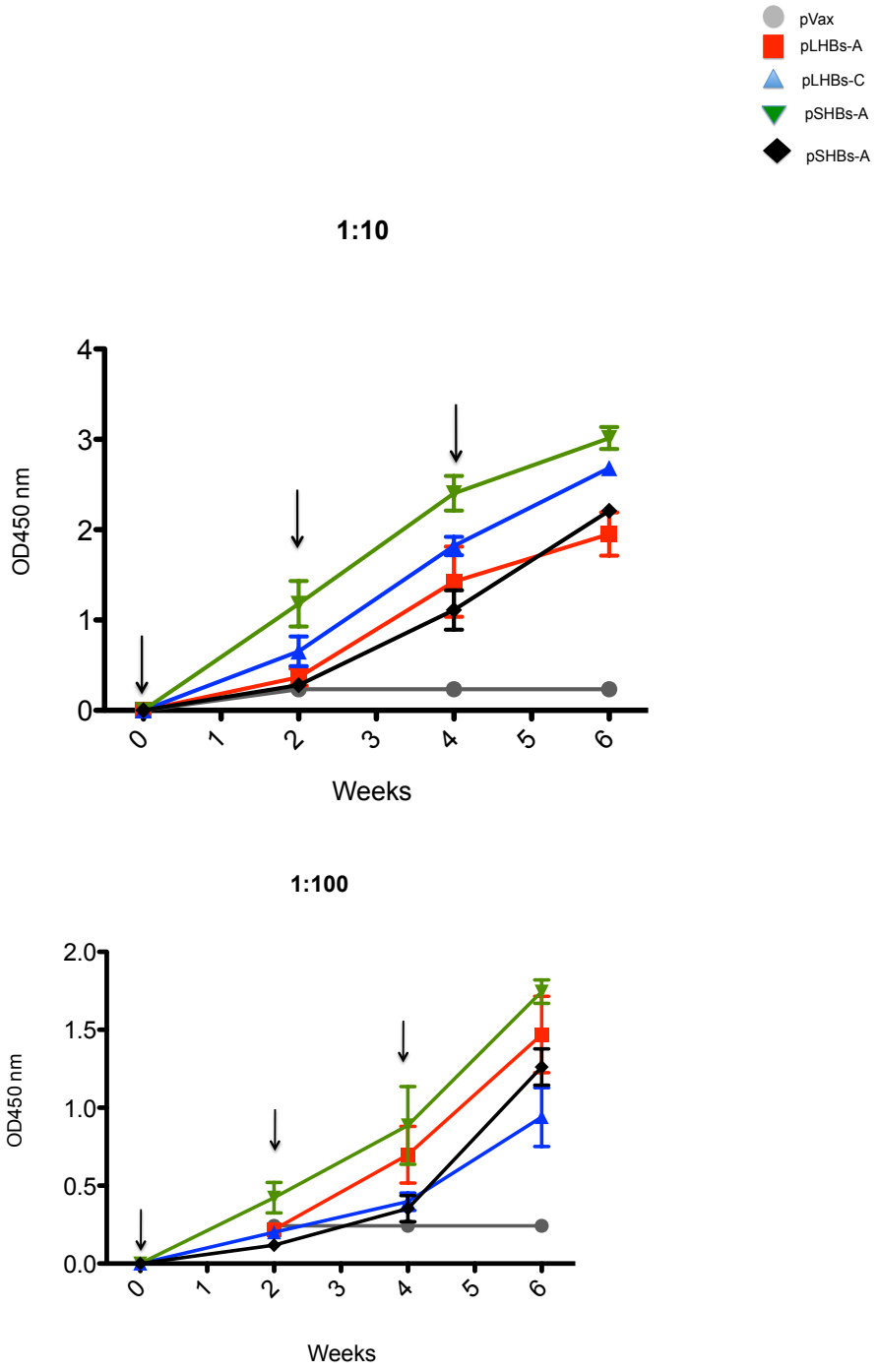


Figure 4-2 cont.

b.

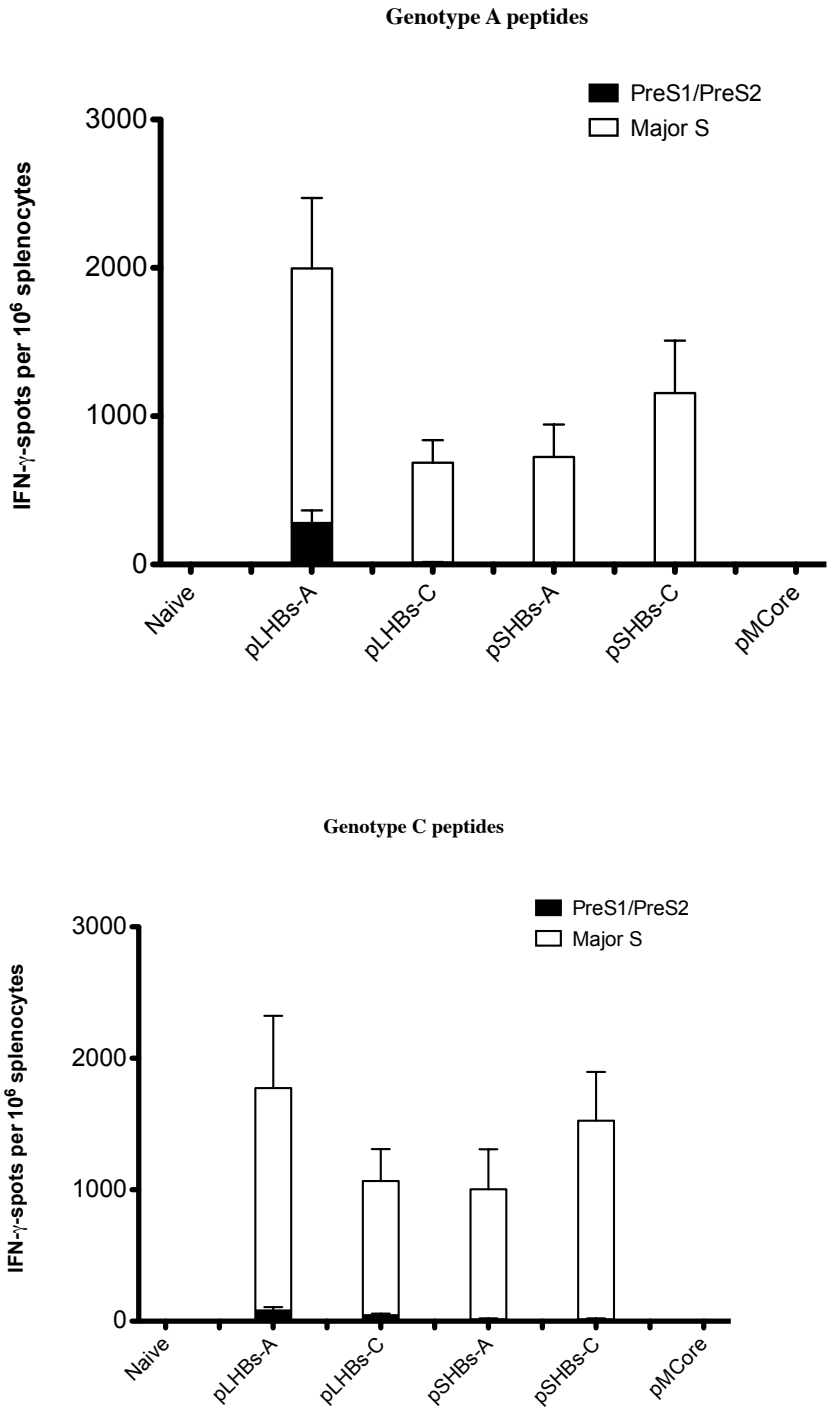


Figure 4-2 cont.

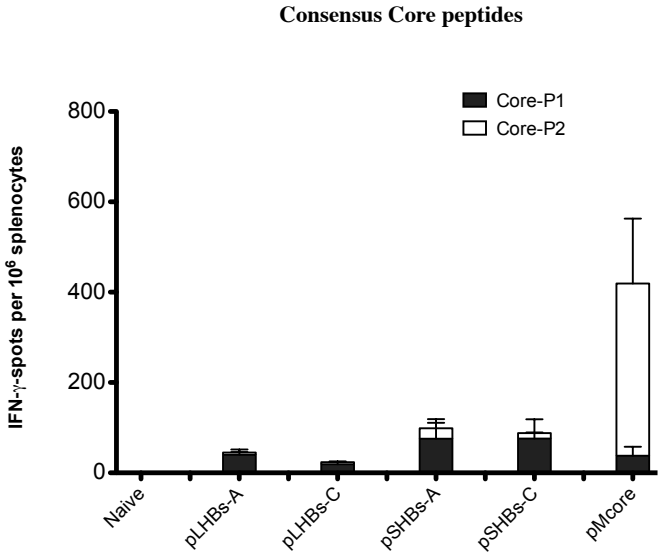
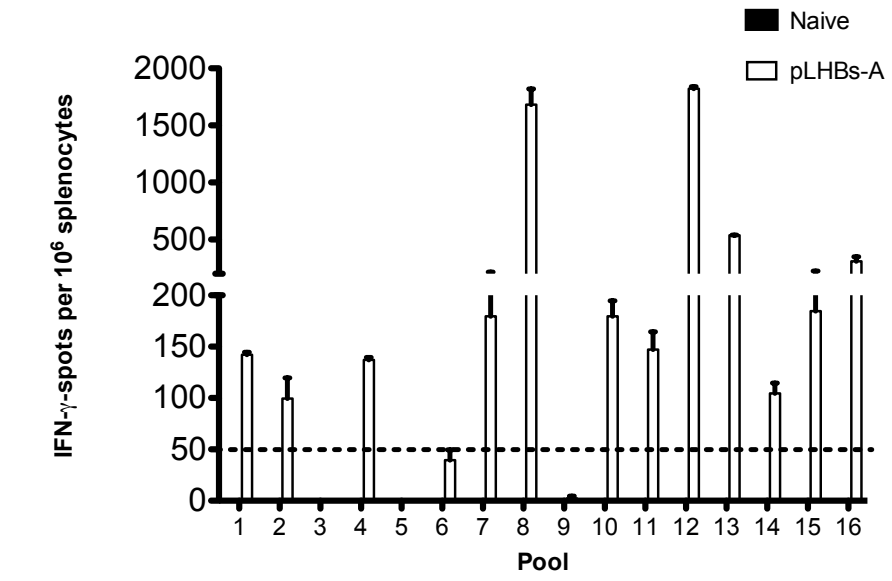


Figure 4.2 Immune responses induced by each HBs DNA plasmid. Five Balb/c mice per group were immunized and analyzed for their induction of both humoral and cellular responses (A) Antibody responses from individual HBs constructs to native HBsAg proteins one week after each immunization. (B) Stimulated splenocytes showed increased magnitude and cross reactivity of cellular responses in both genotype A and C.

Figure 4-3

Enhanced breadth of the cell-mediated immune responses

a.



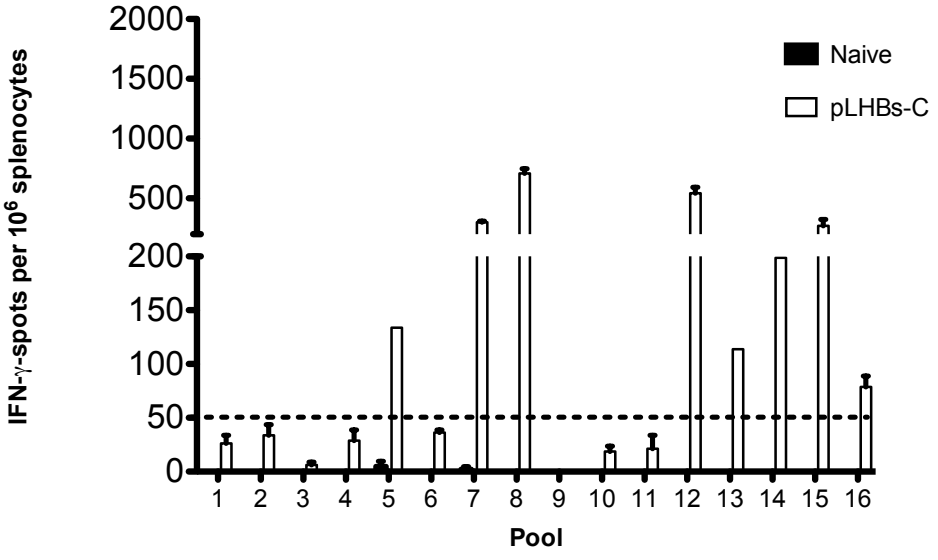


Figure 4-3 cont.

b.

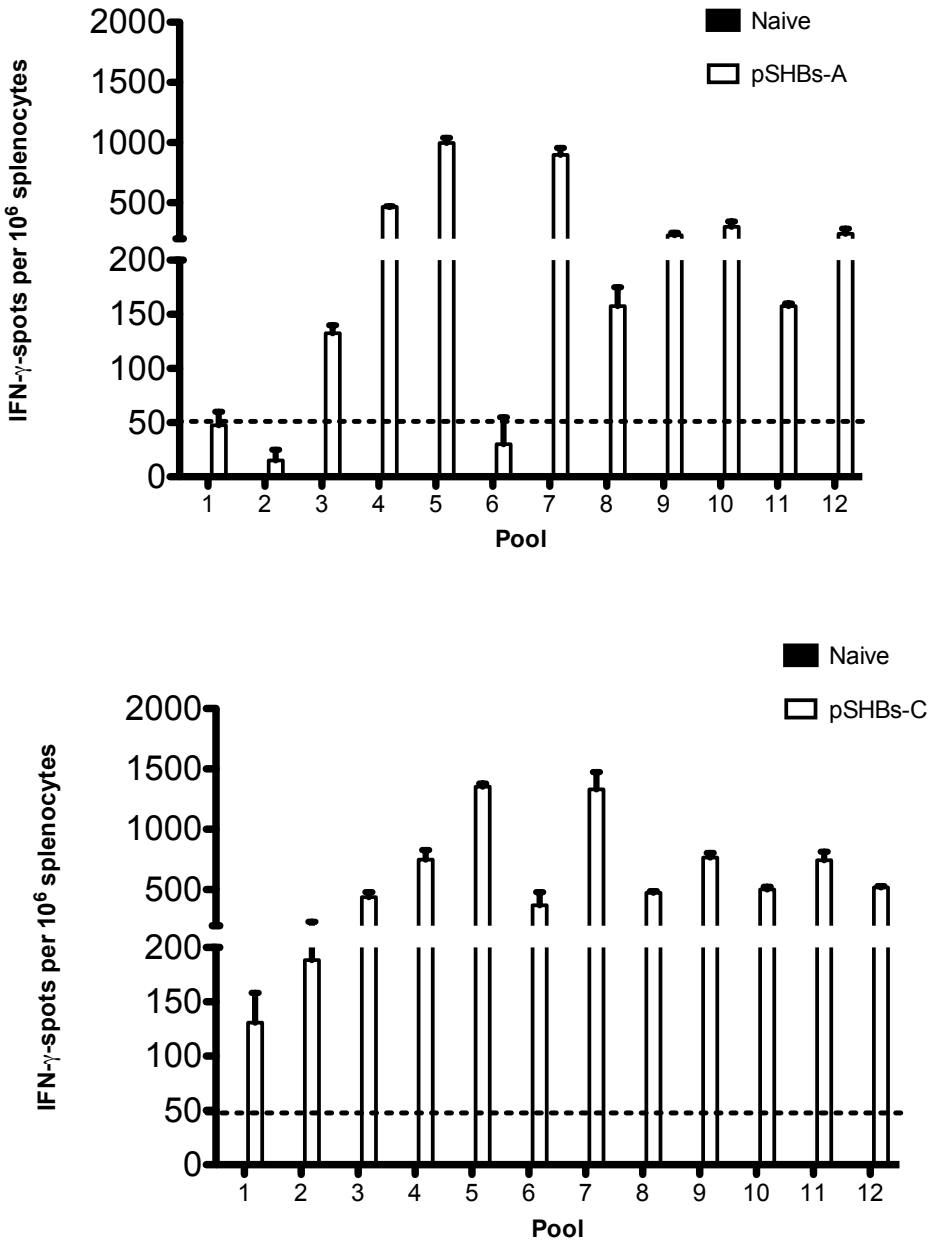


Figure 4.3 Enhanced breadth of the cell-mediated immune responses. (A-D) All four constructs showed broad T cell immunogenicity. Many dominate epitodes were observed with these constructs.

4.3 Combined therapy

4.3.1 Immunogenicity of HBsAg-HBcAg DNA cocktail

Since immunization with all four synthetic HBsAg plasmids elicited broad cross-reactive immune responses, we next combined these plasmids with HBcAg plasmids to generate a DNA vaccine cocktail for immunization. The core antigen of HBV is known to be critical for viral clearance through the induction of HBcAg-specific CTL responses. We proposed that the cocktail will enhance the previously observed cellular response by increasing the number of immune epitopes the host cell will recognize within the virus. Mice were either immunized with cocktails containing only the surface antigens (both genotypes) or with cocktails containing both the surface antigens and a consensus core antigen plasmid (pMCore) that has been previously described (**Table 4.1**). The HBsAg only groups showed high antibody titer in sera of immunized mice. Similar titers were seen in the major S plus pMCore group but not in the LHBs-pMCore cocktail group (**Figure 4.4a**).

To further explore the immune response elicited by the HBsAg-pMCore immunized mice, we evaluated the IFN-γ spots per million induced by stimulated splenocytes from each group. The average SFU per million splenocytes for groups with no pMCore were about 670 and 1200 against genotype A and genotype C peptides respectively. Importantly, the addition of pMCore on average increased the total SFU per million splenocytes by 400 spots against genotype C and doubled the response when the splenocytes were stimulated with the genotype A peptides (**Figure 4.4b**).

$CD8^+$ T cells are known to be the main effector cells responsible for HBV clearance and therefore, it is important to determine which cell type is responsible for the IFN-γ production. It is also essential to examine if these T cells can induce multiple antiviral activity in response to antigenic re-encounter by releasing other antiviral cytokines (TNF-α and IL-2) and degranulation markers such as CD107a. Intracellular staining of stimulated splenocytes revealed multi-cytokine producing activated CD8 T cells within all groups (**Figure 4.5a - f**). We also observed the induction of lymphocyte degranulation marker, CD107a in these CD8 T cells. It is noteworthy to mention that CD8 T cells from HBsAg-HBcAg groups showed significant higher poly-functional activity when stimulated with the surface antigen peptide.

Table 4-1

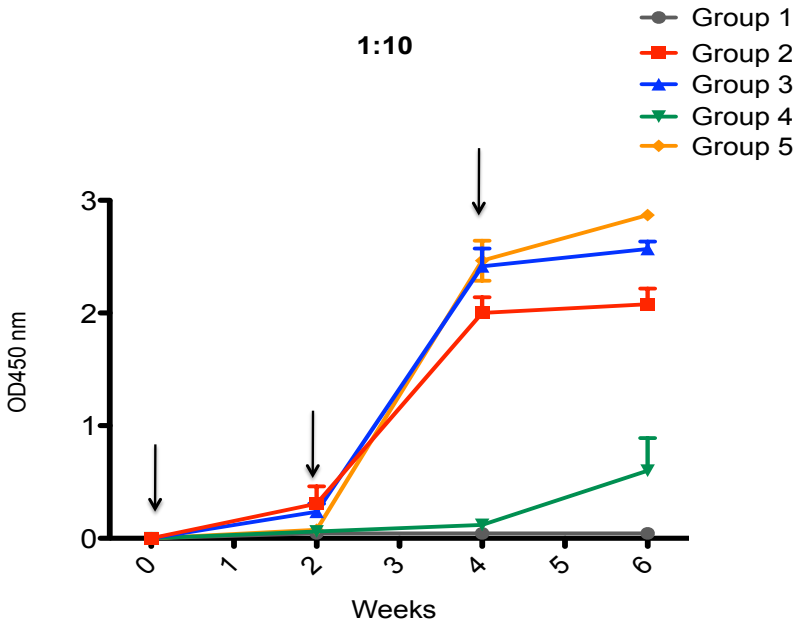
Immunization groups for combined HBV therapy

Groups	DNA cocktail
Group 1	pVax
Group 2	pLHBs-A / pLHBs-C
Group 3	pSHBs-A / pSHBs-C
Group 4	pLHBs-A / pLHBs-C / pMCore
Group 5	pSHBs-A / pSHBs-C / pMCore

Figure 4-4

HBc/HBs vaccine cocktail induces robust immune responses

a.



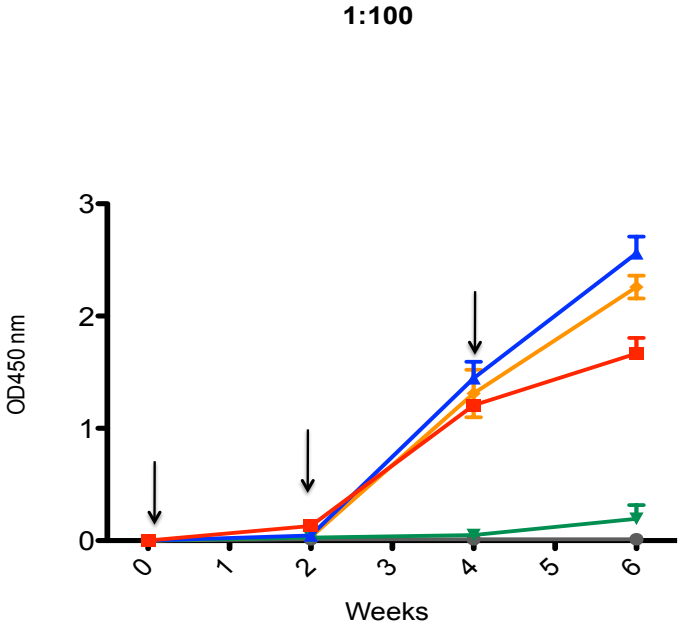


Figure 4-4 cont.

b.

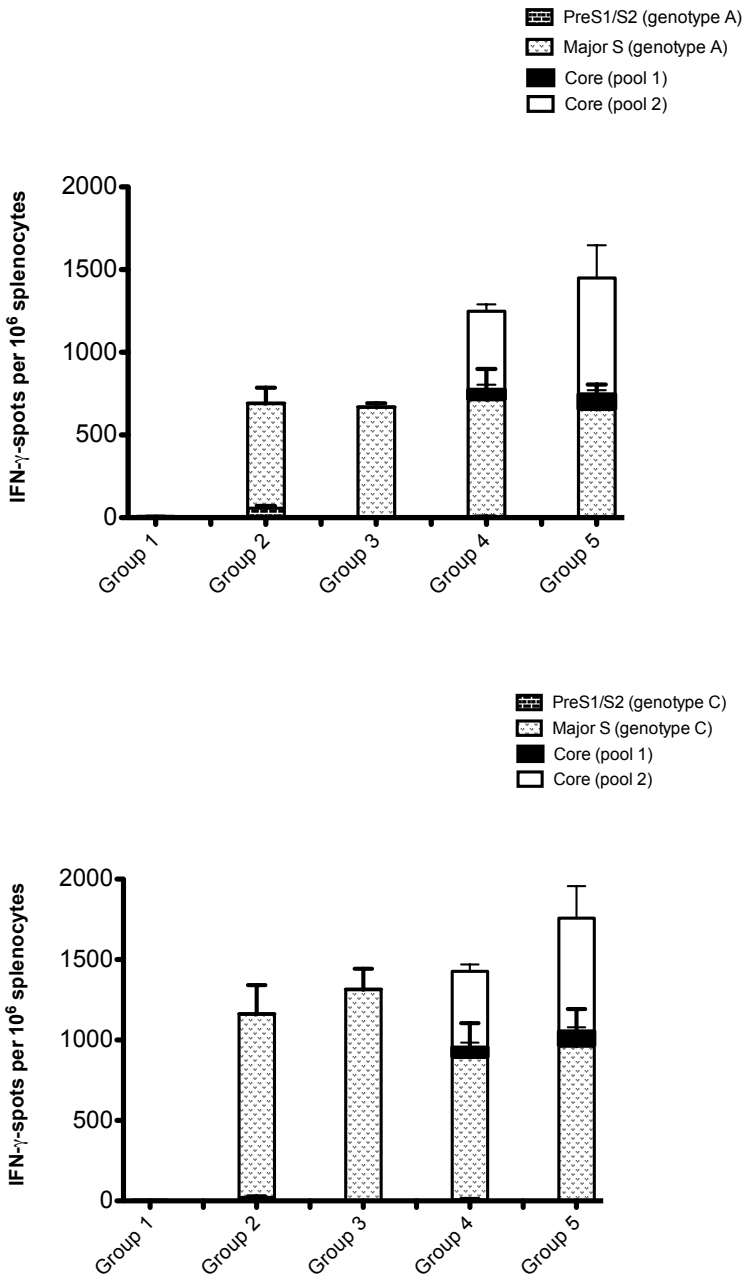
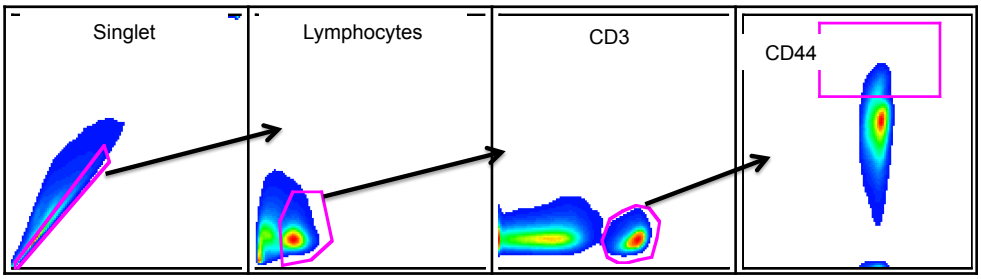


Figure 4.4 HBc/HBs vaccine cocktail induces robust immune responses. (A) Antibody responses from sera of mice immunized with HBs/HBc vaccine cocktail at different immunization time points (B) IFN-g responses after *ex vivo* stimulation of splenocytes from HBs/HBc cocktail immunized mice.

Figure 4-5

Antigen-specific CD8 T cells induce antiviral cytokines



Group 1 – pVax only

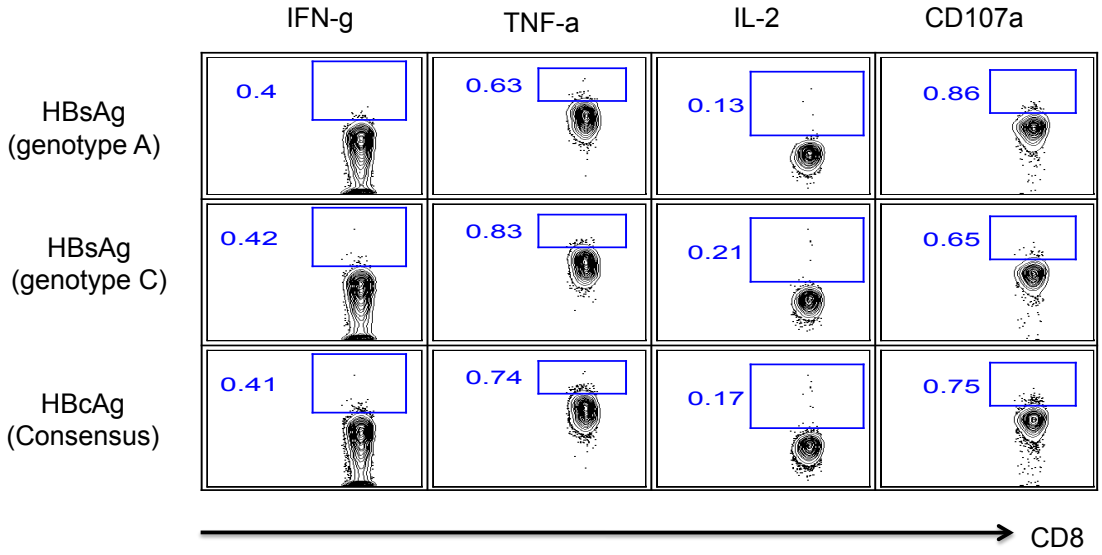
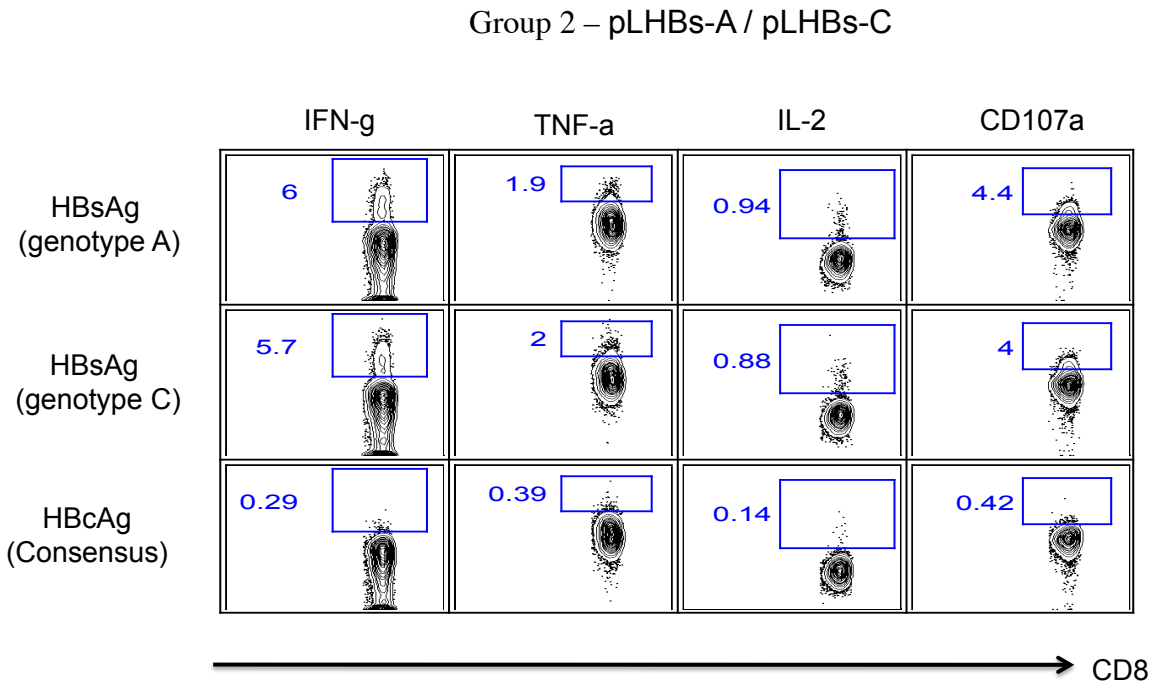


Figure 4-5 cont.



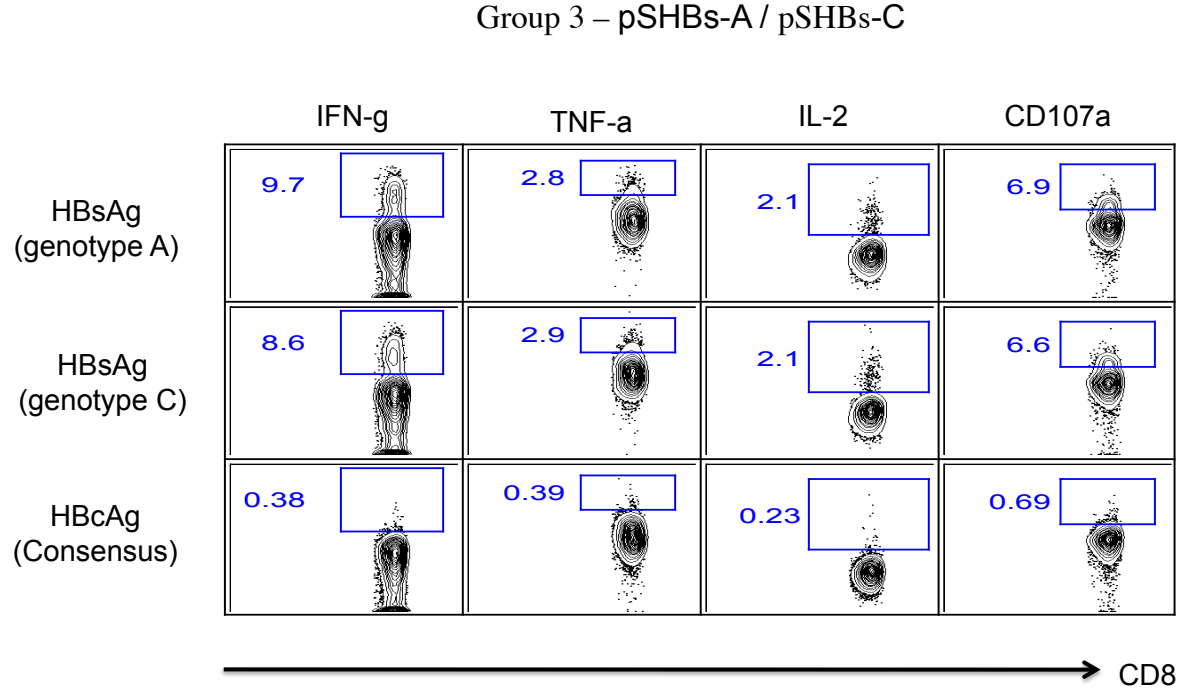


Figure 4-5 cont.

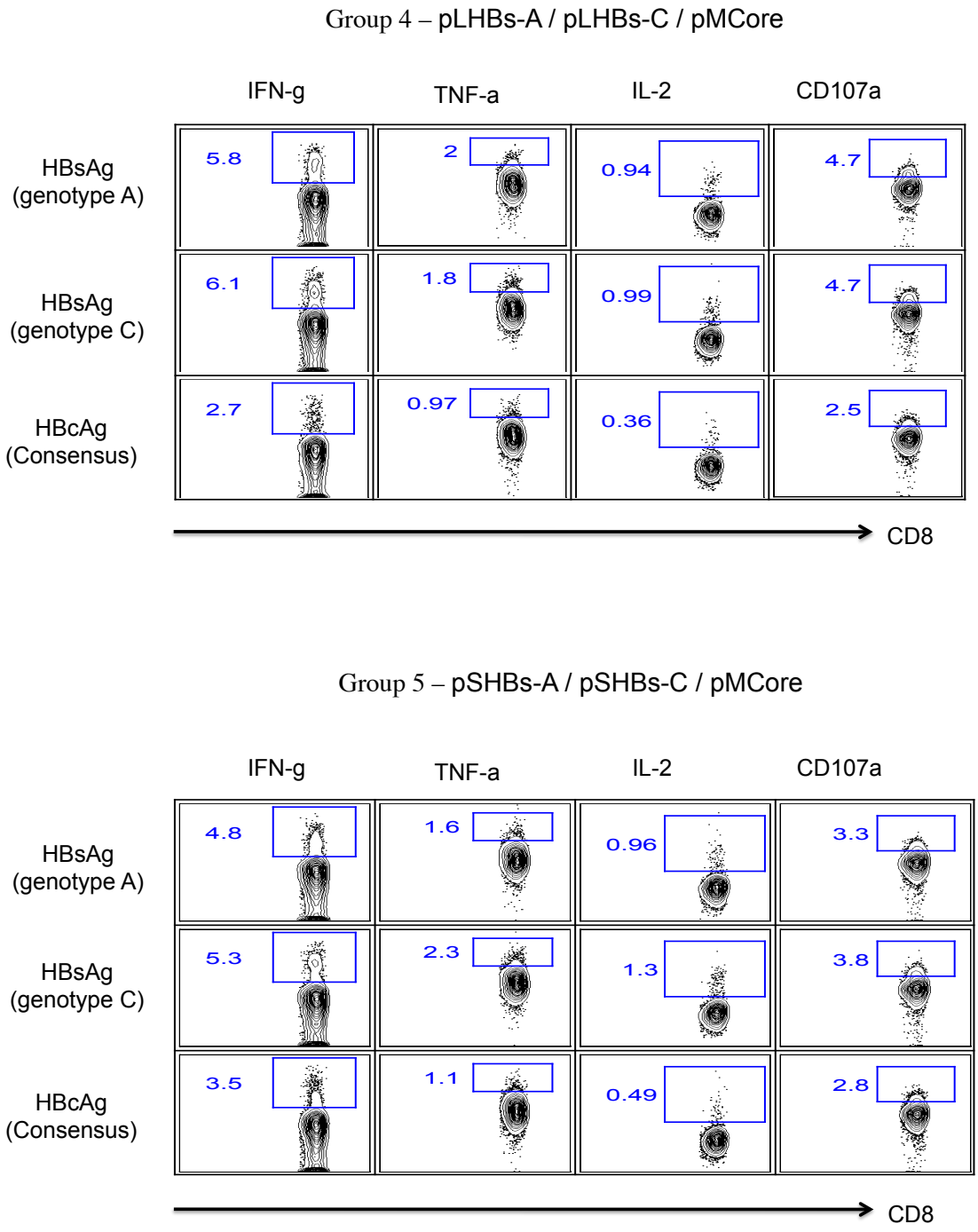


Figure 4.5 Antigen-specific CD8 T cells induce antiviral cytokines. Intracellular staining of anti-viral cytokines after ex-vivo stimulation of splenocytes from immunized balb/c mice.

4.4 Non-human primates

4.4.1 Immunization of macaques with HBsAg-HBcAg cocktail

In the past, some DNA vaccines that have been very immunogenic in small animal models such as mice. However, they typically lost effectiveness when studied in larger animals. Encouraged by the strong antibody and T cell responses incited by the combination of pLHBs or pSHBs and pMCore in mice, we decided to evaluate the immunogenicity of the combined constructs in a larger animal model. To ensure better induction of the cellular immune response, we added a molecular adjuvant. We used one of the best-described molecular adjuvant in non-human primates, IL-12. IL-12 is a Th1 polarizing cytokine that drives cellular immune responses by aiding the priming and expansion of CD8 T cells. This adjuvant has been used to enhance the immune response against different antigens from various pathogens.

Rhesus macaques were immunized IM/EP with pLHBs/pMCore, pSHBs/pMCore and pLHBs/pMCore/IL12 four times, 4 weeks apart. The animals were bled once before the first immunization and 2 weeks following each immunization. Humoral immune responses to HBV surface antigen were determined using commercial HBsAg proteins were in sera ELISA assay. High titer antibody responses were seen after two immunizations with pSHBs/pMCore but not with pLHBs/pMCore. The response against pLHBs/pMCore was boosted after the third immunization. The IL-12 adjuvant enhanced the antibody titer of the pLHBs/pMCore cocktail immunization after just two immunizations (**Figure 4.6a**)

The animals' immune responses to these cocktails were determined with IFN-gamma ELISpot assays. **Figure 4.6b** shows the sum of each monkey's response to both HBs and HBc peptide pools, as well as the average group response for each of the three time points. Little to no antigen-specific cellular immune responses were detectable following the pre-bleed and the first immunization. However, after the third immunization the responses increased significantly over 2000 SFU/10^6 PBMCs, with the IL-12 adding over 1000 SFU/10^6 PBMCs to the pLHBs/pMCore cocktail.

Intracellular staining with antibodies to antiviral cytokines reveals antigen-specific production of antiviral cytokines such as IFN-g, TNF-a and IL-2 by both CD4 and CD8 T cells from immunized monkeys. **Figure 4.6c** shows the total percent of HBV-specific CD4 and CD8 cytokine producing cells.

4.4.2 Broad cellular immune response in Rhesus macaques

Similar to the immunization in small animals, the immune response was not directed at few dominant epitopes of pHBs. The majority of immunized monkeys (four out of five) from group A were able to elicit strong cellular immune responses to at least 10 or more peptide epitopes of pLHBs. Although only two out of the five monkeys from group B showed strong immunogenicity to 10 or more peptide epitopes, their responses to the matrix peptide pools predict a diverse response. Group C had three monkeys that responded to more than 10 peptide epitodes. This suggests that the Rhesus macaques were able to elicit cellular immune responses against multiple sites within the HBV surface proteins (**Figure 4.7)**. Therefore, the DNA cocktail immunization of surface and

core HBV antigens is able to elicit both strong and broad cellular responses against the HBs proteins in Rhesus macaques.

Figure 4-6

Immunogenicity in non-human primates

Monkey Groups	DNA cocktail
Group A	pSHBs-A / pSHBs-C / pMCore
Group B	pLHBs-A / pLHBs-C / pMCore
Group C	pLHBs-A / pLHBs-C / pMCore + IL-12

a.

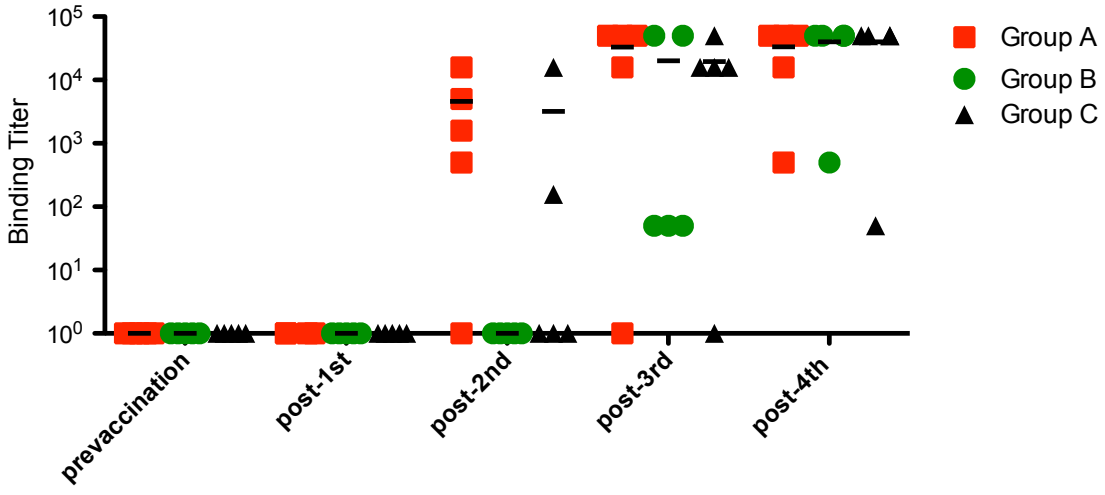
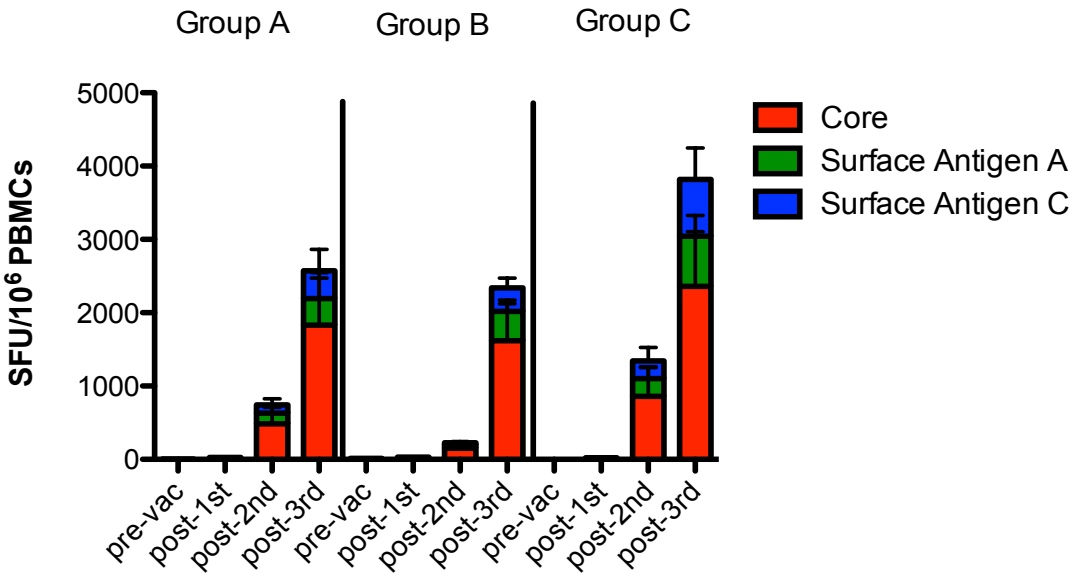


Figure 4-6 cont.

b.



c.

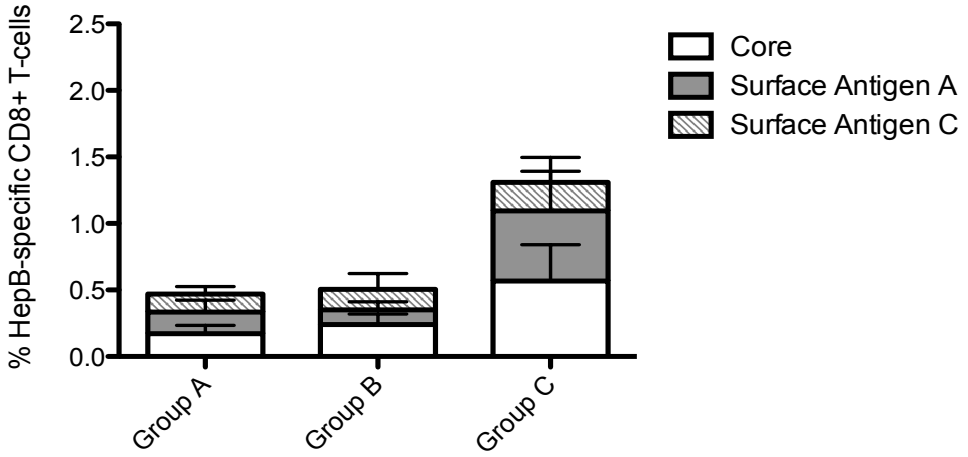


Figure 4-6 cont.

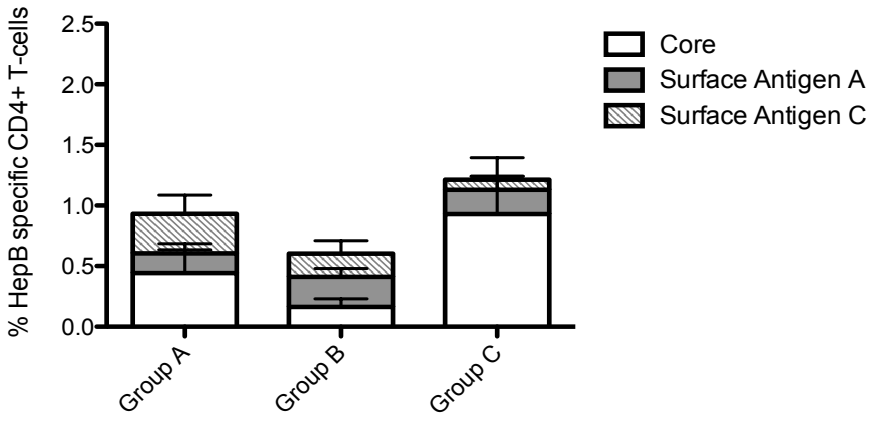
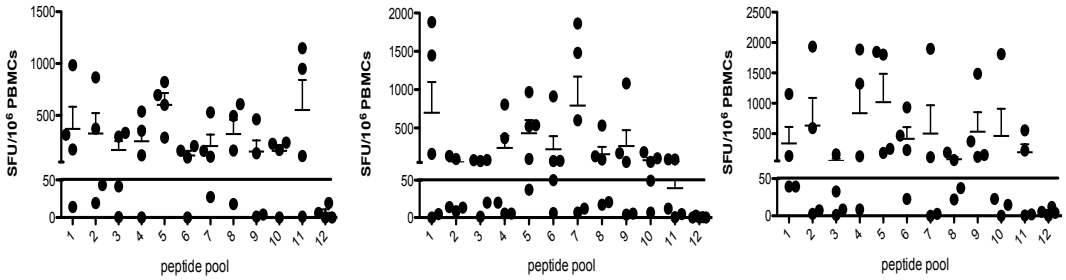


Figure 4.6 Immunogenicity in non-human primates. (A) Immunization groups. Five Rhesus Macaques were immunized intramuscularly followed by electroporation. The monkeys received three immunizations, 4 weeks apart. High antibody titers to HBs were determined after the last immunization. (B) IFN-g responses were measured once before the first immunization and 2 weeks following each immunization.

Figure 4-7

Broad T cell responses in immunized monkeys



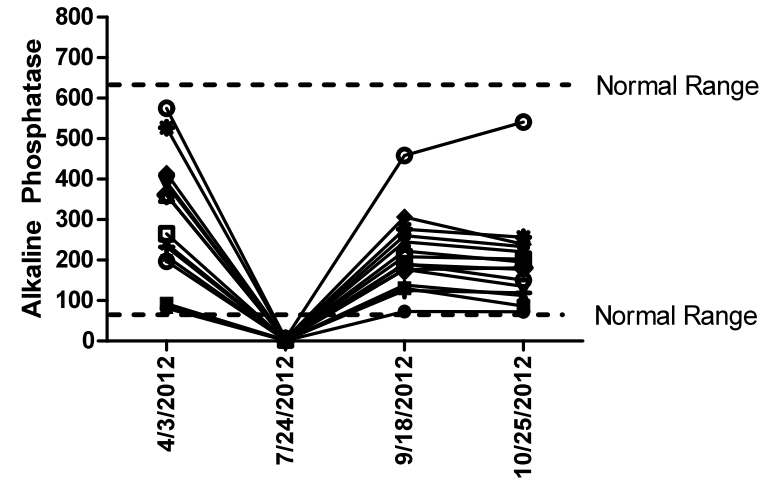
Group	Monkey ID	Positive Matrix Pools	Positive Peptide Epitopes
Group A	4634	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	30
	5022	3, 4, 5, 6, 7, 8, 9, 10	16
	4943	1, 2, 5, 6, 7, 8, 11	12
	4942	1, 2, 4, 5, 10, 11	10
	4954	2, 5, 10	2
Group B	4628	1, 4, 5, 6, 7, 8, 9, 10, 11	20
	5018	1, 2, 3, 7, 10	6
	4941	5, 6, 8, 11	4
	4938	1, 2, 3, 4, 5, 6, 8, 11	12
	4939	5, 6, 9	2
Group C	4639	1, 2, 3, 4, 8, 9, 10, 11	16
	4945	1, 2, 3, 4, 5, 6, 9, 10, 11	18
	4946	5, 6, 7, 8, 9	6
	5015	1, 2, 4, 5, 6, 7, 8, 9, 11	20
	5019	4, 5, 9	3

Figure 4.7 Broad T cell responses in immunized monkeys. All five monkeys from each group responded broadly to multiple peptide epitopes.

Figure 4-8

A hepatitis DNA vaccine appears safe in non-human primates

a – b.



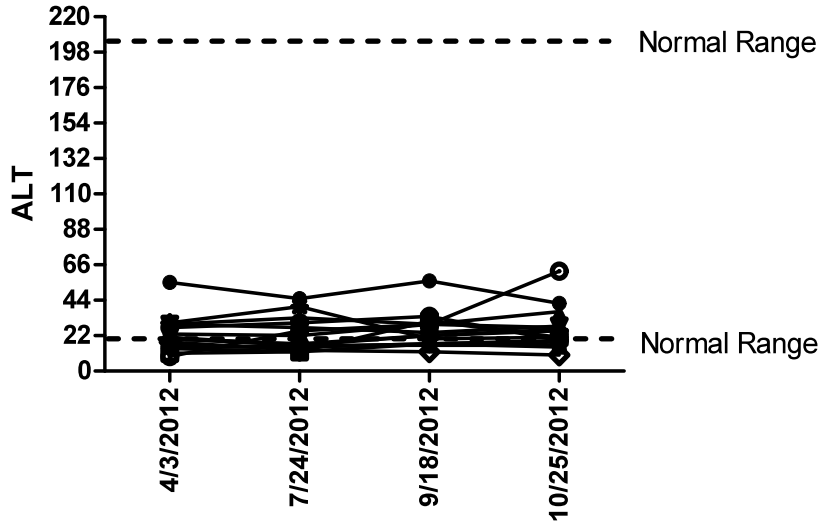
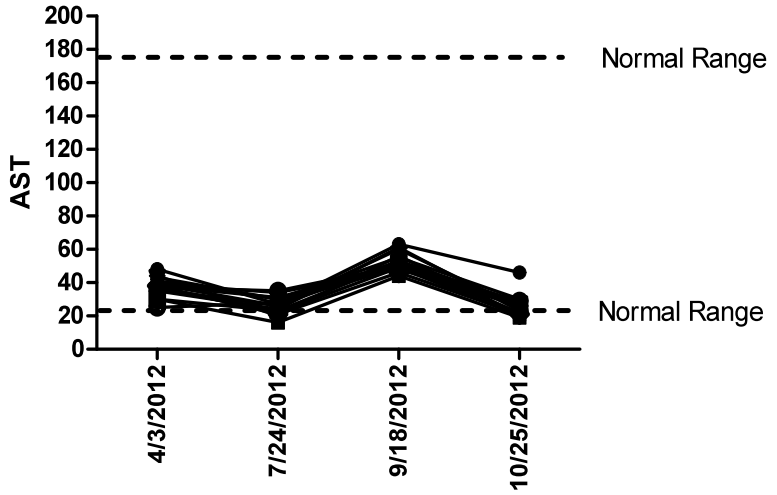


Figure 4-8 cont.



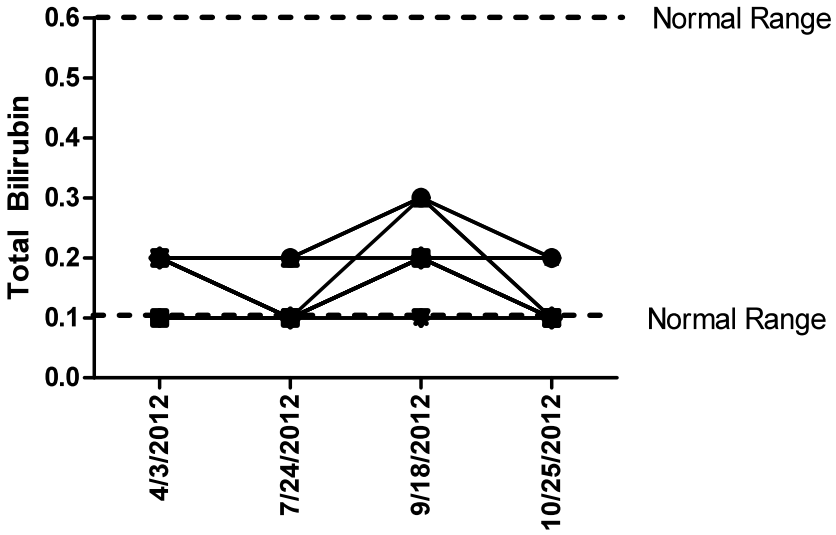


Figure 4-8 cont.

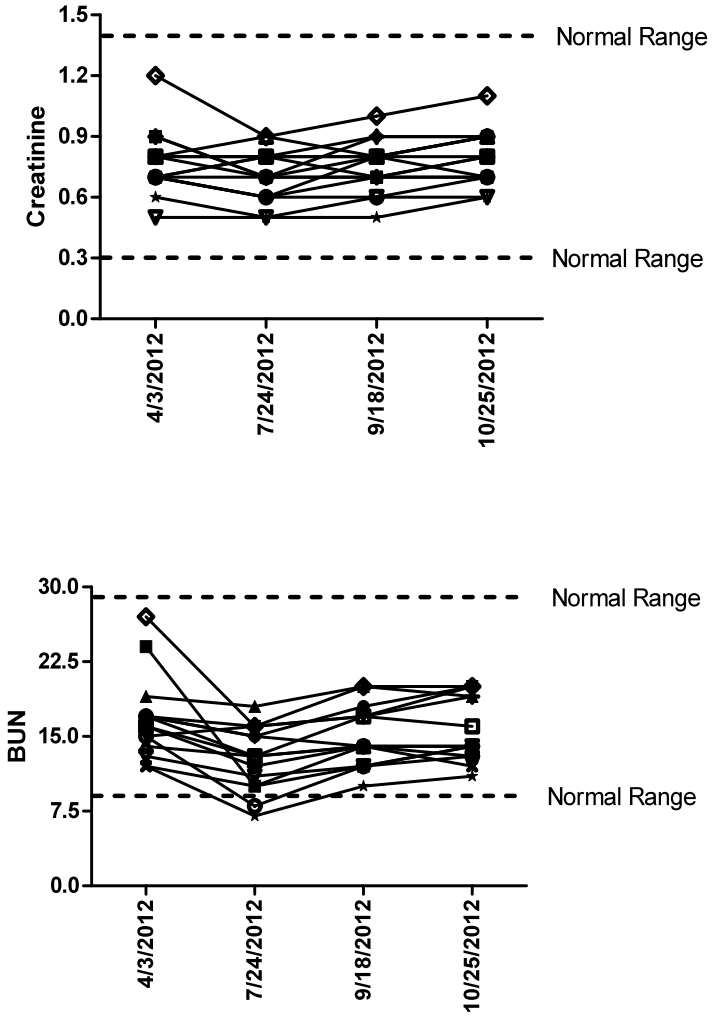


Figure 4.8 A hepatitis DNA vaccine appears safe in non-human primates. Samples were analyzed prior to vaccination (4/3/2012), after the third vaccine (7/24/2012), and after the fourth vaccination (9/18/2012 and 10/25/2012). There was an assay error for alkaline phosphatase on 7/24/2012. The normal range for all measures is depicted by dashed lines. A) Serum alkaline phosphatase. B) Serum alanine aminotransferase (ALT) levels. C) Serum aspartate aminotransferase (AST) levels. (D)Serum total billirubin levels. E) Serum Creatinine levels. F) Serum blood urea nitrogen (BUN) levels.

4.5 Conclusions

In this study we report the ability of a multivalent synthetic hepatitis B DNA immunogen cocktail to elicit both antibody and cell-mediated immunity against the surface and core antigens of this virus. Immunization of small animal models with either vaccine by itself generated the necessary immune response needed for protection. It has however, become clear over the years that both antigens are important during the resolution of acute and chronic HBV infection. We therefore believe that the combination of multiple HBV gene plasmids encoding different proteins will revolutionize the future of immunotherapy against the pathogen.

Global routine vaccination against the hepatitis B virus (HBV) has prevented both acute and chronic infection of the virus in millions of people across the world(FitzSimons et al.). The virus infects human hepatocytes and causes liver cirrhosis which can lead to hepatocellular carcinoma, a deadly form of cancer that claims over 700, 000 lives each year(El-Serag, 2004; Llovet et al., 2003). There are four major human HBV serotypes (adr, adw, ayr and ayw) and eight genotypes (A through H) that have been characterized. Genotypes A and C are most commonly found in the Americas, Asia and Africa(Cassidy et al., 2011; Shi, 2012).

Though the current HBV vaccine that targets the surface antigen (HBsAg) of the virus has demonstrated to be effective in inducing protective antibodies following three vaccination routines(Carollo et al.; Huang et al., 2011; Lemon and Thomas, 1997), 10-15 percent of individuals fail to respond and remain at risk of infection. In addition, there are about 370 million people chronically infected with virus and at the risk of developing liver cancer (WHO, 2000). Most of these chronic infected individuals have no access to

the expensive therapies available. Those who are on therapies receive drugs with undesirable side effects, which only reduce viral replication and not clearance.

The first generation of HBV vaccines became available in the 1980s when vaccinologist, Maurice Hilleman and his group treated and filtered blood sera from infected individuals. Concerns over the safety of this vaccine led to their termination and the creation of the second and third generation vaccines using the recombinant protein technology(Maupas et al., 1976; Shepard et al., 2006). The second-generation vaccine uses the major part of the HBsAg (major S), excluding several amino acids at the N-terminal of the surface protein (pre-S1 and pre-S2) known to support viral attachment and entry. The third generation vaccine, which included both the pre-S1 and pre-S2, produced a stronger immune response (Raz et al., 1996; Schumann et al., 2007). These recombinant protein-based HBV vaccines although safe, require several in vitro molecular manipulations and production at large scale in yeast or CHO cells. Like most recombinant protein-based vaccines, these vaccines lack the ability to drive strong cell mediated immunity (CMI)(Chen et al., 2011; Shen et al., 2010). It is therefore important to develop an effective immune therapy that will induce strong cellular immunity and protective antibodies in all vaccinated individuals.

Here, we developed four different HBsAg DNA vaccines to the two most common genotypes (A and C) and examined their ability to drive immune responses. We utilized the same gene optimization approach from previous studies to increase the magnitude of these responses. While all plasmids shared the major S (SHBs) amino acid segment of the HBs gene, only two had the pre-S1 and pre-S2 segments (LHBs). The amine-terminus domain of the preS1 is believed to assist viral attachment and entry while the carboxyl

terminal end of preS2 is important for infectivity. Antibodies raised against the pre-S2 are known to prevent viral infection both *in vivo* and *in vitro* (Glebe, 2007; Shen et al., 2010). We therefore, reasoned that the inclusion of both segments in our vaccine would increase immunogenicity. We saw no significant difference in HBV native protein-specific antibodies between the pLHBs and SHBs constructs. Balb/c mice immunized with either immunogen elicited broad cellular immune responses. More importantly, these responses showed cross reactivity between the two genotypes.

As stated previously, the core antigen is an important aspect of the virus in terms of prevention and clearance of the infected hepatocytes. Studies in our laboratory have shown DNA vaccine encoding the HBcAg to drive strong Th1-cell immunity and subsequent clearance of HBV in the liver (Obeng-Adjei, 2012b). We hypothesize that a vaccine cocktail that includes plasmids encoding both HBcAg and HBsAg will be advantageous in the search for an effective immune therapy. This cocktail will not only drive both cell-mediated and antibody immunity, but will also increase the breadth and magnitude of both responses. Our findings confirmed a synergistic effect in CD8 T cell antiviral cytokines like IFN-γ, TNF-α and IL-2, and degranulation marker, CD107a. More importantly, animal models immunized with these cocktails were able to clear cells that were transfected with either antigen. While there was a reduction in antibody response for the LBHs constructs, the plasmids encoding only the major S genes maintained the humoral responses achieved through the single vaccine immunization. These responses provide evidence that a therapeutic vaccine that clears infected hepatocytes and prevents continuous infection of other hepatocytes can be achieved if multiple genes and epitopes are targeted.

In all, we have shown that recombinant plasmid vaccines encoding different parts of the three co-carboxyl terminal surface proteins contained in HBV can induce strong humoral and cellular responses. Morover, the combination of these plasmids with synthetic DNA vaccines, which encode HBcAg drives multi-epitope responses and clears infected cells. Although further investigation is needed, it is apparent that this immunization tool will be useful in the search for immunotherapy for HBV.

Chapter 5 – Discussion and Future Directions

"Medical science has proven time and again that when the resources are provided, great progress in the treatment, cure, and prevention of disease can occur." – Michael J Fox, Actor.

5.1 Significance

For the liver to perform metabolic activities such as plasma protein synthesis, glycolysis, processing digested compounds and detoxifying the body of harmful unfamiliar chemicals, it requires a steady stream of blood to be delivered through the hepatic artery and the portal veins. This implies that the liver is constantly exposed to harmless food antigens and intestinal commensal bacteria. In the interest of avoiding constant inflammation that may lead to bystander damage of the liver, the liver is believe to bias immune responses towards tolerance (Yang et al., 1994). This tolerogenic effect is evident through allogeneic liver transplantation.

In contrast to other tissues such as the kidney and heart, the liver is readily accepted without immune-suppression when grafted in small animal models. In primates, less immunosuppressive therapy is required for liver transplantation than other organs (Calne and Davies, 1994; Qian et al., 1997). showing that T cell rejection by liver recipients is very low. Although this level of tolerance is beneficial in keeping the liver safe from the host immune defense system, it opens the liver up for infection by pathogens looking to escape the immune system. Pathogens such as Hepatitis B, Hepatitis C and the *Plasmodium* that causes malaria have habitually taken advantage of the liver's ability to suppress the immune response. These pathogens use liver cells as a site for infection, replication, amplification and most importantly, a site to hide from the view of any

immune surveillance. Unfortunately, these simple goals of those pathogens are not so trouble-free for the host. When Hepatitis B and Hepatitis C are left untreated, they become chronic and lead to various liver complications that can result in death. Although malaria does not cause any extensive liver problems, it is known to take many lives around the world each year through its manifested symptoms.

In spite of this well acknowledged liver tolerance, there are circumstances where there are sufficient immune responses in the liver to clear pathogenic infections. The best example is Hepatitis A, where the host more often than not clears the infection from the liver. In acute Hepatitis B and Hepatitis C, infected individuals incite enough of an immune response to clear the pathogens and mount immunological memory. In an effort to understand and utilize the immune responses induced within the liver, researchers discovered that liver cells like LSECs and hepatocytes could prime certain aspects of the adaptive immune system. These cells act like professional antigen presenting cells by taking, processing and presenting antigens to naïve T cells, specifically, CD8 T cells (Knolle, 2000). Albeit controversial, many *in vivo* and *in vitro* experiments have shown that these CD8 T cells die promptly after activation to induce tolerance in the liver (Bertolino et al., 1999; Bertolino et al., 1998). Other studies show that activated CD8 T cells stay around long enough to clear pathogens from the liver (Wuensch et al., 2010). Although the exact consequences of liver CD8 T cell activation is debatable, researchers agree that further investigations into hepatic T cell priming can be beneficial to the development of therapeutic and prophylactic immune therapies to hepatotropic pathogens.

We investigated this phenomenon by directly comparing liver primed CD8 T cells to secondary lymphoid tissue primed CD8 T cells for differentiation, function, and memory programming in a highly controlled fashion. We used hydrodynamic tail vain injection of plasmid to establish a liver-specific transient expression of LCMV-gp antigen, and studied priming of CD8 T cells using the P14 transgenic mouse model. This study was important to understand the dynamics of CD8 T cells, which play a vital role in the clearance of intracellular pathogens. We identified features that are similar between intrahepatically activated CD8 T cells and attributes that help push the liver environment into a toleragenic state, with respect to cellular immune responses.

While comparing and contrasting intrahepatic T cell activation to lymphoid activation, we observed migration of effector CD8 T cells to the liver after intramuscular immunization with synthetic DNA plasmids. Knowing the importance of intrahepatic antigen-specific T cells in the clearance of HBV and HCV, we made it our goal to develop therapeutic DNA vaccines, which will induce functional antigen-specific T cells that can clear infected cells in the liver after peripheral immunization. We selected HBV as a model because it is the only pathogen out of the three successful human liver pathogens that has an effective prophylactic vaccine. Although the HBV vaccine has been in use for over three decades, there are many chronic infected individuals that are in need of an effective therapeutic vaccine. The selective recruitment of T cells to the liver and the clearance of HBV-transfected hepatocytes by cytotoxic T cells after intramuscular DNA immunization with electroporation suggest that the DNA vaccine platform is an important candidate for HBV immune-therapy.

5.2 Intrahepatic CD8 T cell priming

In this project, we report an unexpected defect in the initial induction of adaptive immune response during LCMV expression and a non-inflammatory priming of CD8 T cells in the liver. Priming of naïve CD8 T cells in the liver compromises the cells' expansion capacity, causing a decrease in effector and memory cell frequency in the spleen, lymph nodes, liver and lungs. Although intrahepatic CD8 T cell priming resulted in early effector differentiation, the overall quality of the effector functions was somewhat comparable to CD8 T cells primed in lymphoid tissues. The memory cells' poor antigenic clearance and viral control illustrate their low CTL frequency.

An acute infection model was used to elucidate the effector differentiation and memory programming of CD8 T cells post liver priming. We utilized the hydrodynamic DNA plasmid injection approach to establish an acute liver infection. The expression of the antigen of interest was exclusive to the liver and lasted for only 4 days, mimicking acute liver infection.

With this model, LCMV D^bGP33-specific CD8 T cell priming in P14 chimera mice was confirmed by the up-regulation of effector molecules. By using models that ensure the exclusion of CD8 T cell priming in lymphoid tissues, we observed proliferation and expansion of $GP33^+$/Thy1.1 cells in P14 chimeras after intrahepatically expressing cognate viral Ag. Collectively, these data make hydrodynamic plasmid injection an acceptable model for Ag-specific, secondary lymphoid Tissue-independent CD8 T cell activation.

To clear pathogenic infection, CD8 T cell priming alone is not sufficient. The activated CD8 T cell should be able to survive long enough to differentiate into a mature effector population that possesses antiviral properties. Some groups believe that intraheptatic activated CD8 T cells upregulate pro-apoptotic markers and therefore lack the ability to survive long enough to have any impact on viral resolution. This Bim-dependent deletion of liver-primed CD8 T cells has been linked with the inability of HBV-specific CD8 T cells to control viral persistence in chronic infections (Lopes et al., 2008). We observed an early proliferative response of naïve CD8 T cells with the hydrodynamic injection method compared to the traditional IM immunization model, where T cell activation is deemed to occur in the draining lymph nodes. This early cell division of antigen specific CD8 T cells was consistent with data in the literature, which shows the kinetics of initial intrahepatic priming to be more rapid than an SLT-dependent one (Lukens et al., 2009). The early proliferation of these D^bGP33-specific CD8 T cells in intrahepatic (IH) groups was followed by untimely effector cell contraction. These CD8 T cells expanded early but the cell frequency at the peak of expansion was several folds lower than CD8 T cells primed in the draining lymph nodes. As expected the pro-apoptotic marker, Bim, was up regulated in the IH group during this contraction phase. The Bim levels in the Intramuscular (control) group eventually caught up with the IH group during its peak of expansion. Interestingly, the survival signal, Bcl-2, in the IH group was down-regulated in the first week when Bim was high but increased during the second and third weeks postimmunization. Thus, this data contradicts the idea that intrahepatic activated CD8 T cells undergo abortive activation. This is the first study to show that liver-primed CD8 T

cells, albeit hastily, experience the same death and survival phases as CD8 T cells in acute viral infection.

In addition to the phenotypic examination of liver-primed CD8 T cells' effector differentiation status, we also analyzed their functional properties. While some studies claim full functional differentiation (Wuensch et al., 2006), a more comparative analysis of these effector cells disclosed defective anti-viral activity (Lukens et al., 2009). Our findings confirm that these liver-primed effector CD8 T cells can secrete anti-viral cytokines. Though they were inferior single antiviral cytokine producers, their polyfunctionality, a property reported to correlate with protection (Betts et al., 2006), was considerably similar to the control group in all tissues except the lymph nodes. It is noteworthy to mention that lymphoid-primed effector CD8 T cells recovered from the liver were less functional than those in the spleen and lymph nodes. Therefore, we believe that the liver milieu may play a crucial role in effector suppression. Moreover, the liver activated CD8 T cells mounted a strong cell-mediated cytotoxic effect upon antigen re-encounter. This important finding thus demonstrates that effector CD8 T cells induced through liver priming can be as effective as those primed in the lymphoid tissues.

Although several independent groups have shown T cell priming and their effector status using liver cells as primary APCs (Crispe, 2011), to our knowledge prior studies have not shown if these cells can become long-lived memory cells. Our results reveal that a subset of effector cells (memory precursor effector cells) likely to become long-lived memory cells can be detected in the liver-primed effector CD8 T cell population. Increased expression of memory-markers on these cells was observed a few weeks after immunization. Although liver-primed CD8 T cells differentiate into memory cells

quicker than lymphoid primed cells, the distribution of effector, effector-memory and central memory cells were similar in all tissues within the groups. Crispe *et al*. believe that direct liver priming of CD8 T cells promotes the expression of inhibitory molecules such as PD-1 (Wuensch et al., 2010). We did not observe a significant increase in PD-1 expression for the IH group. The liver microenvironment seems to up-regulate PD-1 on both naïve and effector cells, regardless of the priming site. Naïve Thy1.1 cells transferred into unimmunized Thy1.2 recipients, maintained a higher level PD-1 expression in the liver compare to spleen and lymph node over a period of time. Additionally, HBV persistence in mice has been shown to increase PD-1 expressing CD8 T cells in liver infiltrating lymphocytes (Tzeng et al., 2012). Thus, the increased expression of PD-1 on memory CD8 T cells after intrahepatic activation in their model may be a consequence of continued antigen expression in the liver and not an undesirable effect from liver-priming.

We evaluated recall and effector differentiation of these long-lived memory CD8 T cells in both lymphoid and non-lymphoid tissues. The IM group responded and eliminated target cells from all tissues upon secondary challenge. In contrast, the hydrodynamically injected group was deficient in killing these Ag-pulsed target cells. This lack of killing was associated with a low frequency of effector-memory cells in the IH group. Although the IH group's memory precursors comprise about 16% of peak effector cells, the frequency was still very low due to its lower effector frequency. As predicted, hydrodynamic immunized mice repeatedly succumbed to lethal dose LCMV intracranial (i.c) challenge.

The results presented in this study show that the lack of recall response needed to protect mice from secondary challenge after liver-priming of CD8 T cells is directly associated with a defect in the initial clonal expansion. This is in direct comparison to human studies which have shown that lack of viral clearance in chronic HBV and HCV infections correlates with a low number of HBV or HCV-specific CD8 T cells detected in the blood(Sobao et al., 2001; Sobao et al., 2002).

5.3 Therapeutic Hepatitis B vaccine

With this project we showed that a cocktail of synthetic plasmids encoding Hepatitis B DNA immunogens is capable of eliciting robust antibody and cell-mediated immunity to the surface and core antigens of this hepatotropic virus. Both the surface and the core antigens of HBV have been shown to be essential during the resolution of both acute and chronic HBV infection. Although the current HBV prophylactic vaccine only targets the surface antigen, there are many studies that have shown that the core antigen can also drive a strong cell mediated immune response. Our studies show that synthetic DNA plasmids encoding the core antigen can drive immune responses in both spleen and liver when delivered intramuscularly with electroporation. Furthermore, immunized mice displayed strong cytotoxic responses that eliminate adoptively transferred HBV-coated target cells. Importantly, vaccine-induced immune responses provided protection from HBcAg plasmid-base liver transfection in a hydrodynamic model. This provided important insight into the generation of peripheral immune responses that are recruited to

the liver - an approach that will be beneficial in generating a potential theraupeutic HBV vaccine.

We developed two different plasmids encoding different parts of the surface antigen. While all plasmids shared the major S (SHBs) amino acid segment of the HBs gene, only the pLHBs had the pre-S1 and pre-S2 segments (LHBs). The amine-terminus domain of the preS1 is believed to assist viral attachment and entry, while the carboxyl terminal end of preS2 is important for infectivity. Antibodies raised against the pre-S2 are known to prevent viral infection both *in vivo* and *in vitro* (Glebe, 2007; Shen et al., 2010). Therefore, we reasoned that the inclusion of both segments in our vaccine would increase immunogenicity. We saw no significant difference in HBV native protein-specific antibodies between the pLHBs and SHBs constructs. Balb/c mice immunized with either immunogen elicited broad cellular immune responses. More importantly, these responses showed cross reactivity between the two different genotypes, A and C.

Immunization of small animal and non-human primate models with a cocktail of both core and surface vaccines generated a high magnitude and broad cellular and humoral responses. We observed the clearance of infected skin cells from guinea pigs after HBs-HBc vaccine cocktail immunization. We believe that the combination of multiple HBV gene plasmids encoding different proteins will revolutionize the future of immunotherapy against this pathogen in infected individuals.

5.4 Future directions

Providing CD4 help during intrahepatic priming:

Hepatocytes and Kupffer cells are believed to be the two main cells involved in the priming of naïve CD8 T cells in the liver. Their ability to cross present antigens to naïve CD8 T cells is due to the fact that they express major histocompatibility complex class I (MHC I) on their surfaces. However, their inability to express class II MHC (MHC II) means they cannot prime CD4 T cells (Crispe, 2011; Wuensch et al., 2010). CD4 T cells or T helper cells provide help to other adaptive immune cells by releasing cytokines that are essential for immune cell activation and growth. T helper cells assist with class switching and maturation, of B cells, while it helps maximize CD8 T cells' cytotoxic activity.

While the presence of CD4 help is not crucial for CD8 T cell priming, it is believed to be essential in CD8 T cell expansion and the generation of long-lasting memory cells. Recent data have also shown that lack of CD4 help results in poor recall responses and antiviral cytokine production in effector CD8 T cells (Derkow et al., 2011; Shedlock and Shen, 2003).

With this knowledge, we believe the observations made after priming naïve CD8 T cells in the liver may have been skewed due to the absence of CD4 help. Our model however lacks the ability to induce or measure CD4 T cell priming. We hope to use other models such as OT II, which recognize OVA peptides, to compare and contrast intrahepatic and lymphoid CD8 T cell priming, with and without CD4 help.

Using IL-28 cytokine as an adjuvant:

The three members of the interferon λ family, IFNλ 1 (IL-29), IFNλ 2 (IL-28A) and IFNλ 3 (IL28B) have been shown to be secreted by innate immune cells in response to viral infections. More importantly, these cytokines place immune cells in an antiviral state, which is vital in slowing down or preventing viral replication (Uzé and Monneron, 2007).

IL-28B in particular has been a subject of studies in hepatitis diseases since a significant clinical link was made between the levels of IL-28B in human sera and response to Hepatitis C therapies. Studies show that a single nucleotide polymorphism (SNP) near the IL28B gene may increase HCV patients' response to treatments with interferon and ribavirin (Ge, 2009).

Studies from our laboratory illustrate that when IL28B is used as an adjuvant in DNA vaccine immunization, it increases antigen-specific anti-viral cytokine production. This augmentation in cellular immune responses led to an increased protection. In that, mice that were co-immunized with influenza antigen and IL-28B were protected from lethal H1N1 challenge (Morrow et al., 2009; Morrow et al., 2010). These data were reproducible in non-human primate models, where we saw an increased in anti-viral cytokines when macaques were co-immunized with the IL-28B adjuvant (Morrow et al., 2010). Utilizing one of the most effective adjuvants in the DNA vaccine platform, IL-12, in HBV primate studies (chapter 4), we observed increased IFN-γ responses in monkeys that received an HBs-HBc vaccine cocktail with IL-12 adjuvant. We also observed an increase in HBs-specific IgG in the sera of the immunized monkeys.

In the future, we hope to immunize both mice and monkeys with our HBV vaccine cocktail and IL-28B adjuvant to study the effect this adjuvant will have on the induction of immune responses against HBV viral antigens.

Clinical trials

The lack of appropriate primate models to hepatitis B, such as chimpanzee, means we have to rely on the strong immunogenicity induced in rhesus macaques to move our therapeutic vaccine into clinical studies. In December of 2011, the National Institute of Health (NIH) placed a temporary moratorium on new studies using chimpanzees. Chimpanzees are currently the only known primates that have pathology that resembles human hepatitis disease after infection with HBV.

Fortunately, the strong safety record of DNA vaccines in human studies means we are likely to move the HBV DNA vaccines into clinical trials without any protection studies from higher primates. The group likely to receive this vaccine will be chronically infected individuals who are already on antiviral therapy. The patients will be monitored after receiving the vaccine for any liver damage. If any increase in ALT is observed, they will be removed from the studies and placed on steroids to suppress the immune responses.

Chapter 6 – Materials and Methods

6.1 *In vitro* Experiments

6.1.1 Media

D10 medium: Dulbecco's modified Eagle's medium plus 10% fetal bovine serum (FBS) and 1 × antibiotic-antimycotic agent.

R10 medium: Roswell Park Memorial Institute medium plus 10% FBS and 1 × antibiotic- antimycotic agent.

6.1.2 DNA plasmid preparation

Construction and modification of novel HBcAg consensus sequence

A HBV genotype A, B, C, D and E Core consensus nucleotide sequence was constructed by generating consensus sequences of core genes for each genotype and then generating a consensus sequence of all five genotype consensuses, thus avoid biasing toward heavily sequenced genotypes. The sequences were aligned using clustal X software to develop the final HBcAg consensus sequence. Once the group consensus sequence was obtained, we introduced several modifications, including the addition of a highly efficient IgE leader sequence and a C-terminal HA tag, and the construct was RNA and codon optimized. The synthesized *HBcAg* was digested with *EcoRI* and *NotI*, and cloned into the expression vector pVAX (Invitrogen) under the control of the cytomegalovirus

immediate-early promoter and this construct was then named as pMCore(Obeng-Adjei, 2012a).

Obtaining LCMV-GP mutant DNA

This plasmid construct was a gift from Dr. Rafi Ahmed of Emory University, Atlanta, GA. It contains the full length LCMV-GP with 5 mutations. Only two of the mutations are in a specific epitope sequence. One of those two mutation changes GP33 epitode ending with Methionine to Cysteine.

GP33: KAVYNFATM → KAVYNFATC

GP92: CSANNSHHY→ SANNPHHY

Synthesizing DNA plasmids encoding Hepatitis B surface antigen

Two consensus constructs were generated for hepatitis B surface antigen genotype A and genotype C. The sequences were attained from the Genebank, picked from different countries to prevent sampling errors. The sequences were then optimized for expression, including codon and RNA optimization (GeneArt, Regens- burg, Germany). These constructs were then synthesized and inserted into the pVAX1 expression vector (Invitrogen).

6.1.3 DNA transfection and Protein expression

DNA plasmid transfection

1 μg of DNA plasmid was used to transfect cell lines such as RD (muscle) cells, 293(human embryonic kidney) cells, HepG2 (hepatic) cells. Transfection reagent Turbofectin 8.0 was used as recommended by the manufacturer's guidelines.

In vitro translation Assay

Expression of DNA plasmid was detected using TNT® Quick Coupled Expression of Transcription/Translation System containing ^{35}S-methionine (Promega, Madison, WI). The synthesized gene product was immuno-precipitated using an anti-HA monoclonal antibody targeting an encoded HA epitope, Clone HA-7 (Sigma-Aldrich). The immuno-precipitated protein was electrophoresed on a 12% SDS-PAGE gel and subsequently fixed and dried. The synthesized protein with incorporation of radioactive ^{35}S was detected by autoradiography.

Western Blot Analysis

Western blotting analysis was performed according to standard protocols. 10 μg per well of lysed protein was run on a SDS-PAGE gel (Cambrex, Rockland, ME), blotted on nitrocellulose membrane, and probed with commercial antibody. The signal was amplified using an anti-mouse IgG-HRP (Zymed) and detected with ECL (GE Healthcare, Chalfont St. Giles, United Kingdom).

Immunofluorescence

Rhabdomyosarcoma (RD) cell lines were transfected with pMCore using TurboFect™ (Thermo Scientific) according to manufacturer's guidelines. The cells were first fixed with 2% formaldehyde and then assayed for protein expression. The fixed cells were incubated with rabbit monoclonal HA tag (Invitrogen) diluted in 'primary standard solution' (0.1% BSA, 0.2% saponin, 0.02% sodium azide) for an hours at room temperature. The cells were subsequently incubated with DyLight 594-labeled anti-rabbit

secondary antibody (Thermo Scientific) for 20 minuets at room temperature. Images were obtained using a Zeiss Axiovert 100 inverted confocal microscope. Analysis and quantification of florescence intensities were conducted using Image J software (NIH, Rockville, MD).

Histology.

For histology, tissues isolated from mice were fixed for 24 h in 4% paraformaldehyde. These are then place in 30% sucrose for 24 hours, frozen in OCT ((Frozen tissue matrix) and cut on a cryostat. The sectioned tissues were stained and images were obtained using a Zeiss Axiovert 100 inverted confocal microscope. Analysis and quantification of florescence intensities were conducted using Image J software (NIH, Rockville, MD).

6.2 Murine studies

6.2.1 In vivo Experiments

Mice

C57BL/6 mice (CD90.2) were purchased from Jackson Laboratories (Bar Harbor, ME). CD90.1+ P14 mice bearing the Db-GP33–specific TCR (Pircher, 1989a) were a gift from Dr. John Wherry of University of Pennsylvania. All mice were maintained in specific pathogen-free facilities at the University of Pennsylvania. The University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) approved all

protocols, and all experiments were performed according to the guidelines of the University of Pennsylvania IACUC.

P14 chimeric

Mice were generated by adoptively transferring 1 x 10^5 naive TCR transgenic T cells into naive B6 mice (referred to as P14 chimeras)(Wan et al., 2003). For FTY720 experiment, FTY720 was dissolved in sterile water at 2.5 μg/ml /ml for treated mice while control mice receive normal drinking water(Masopust et al., 2010). For L-selectin experiments, treated mice received 200μg/ml MEL-14 (anti-CD62L) blocking antibody intravenously (I.V)(Harp et al., 2010) 4 hours prior and 24 hours post adoptive transfer of P14 cells.

Intramuscular Immunization/Electroporation

For IM immunization studies, animals were divided into groups. Each animal in the immunized group received one or three intramuscular immunizations of a specific DNA plasmid with concentrations ranging from 5 to 30µg. Multiple immunizations were performed two or three weeks apart. For better generation of memory cells, immunizations were performed 1 month apart. Each immunization was accompanied by *in vivo* electroporation with the CELLECTRA® adaptive constant current electroporation device (Inovio Pharmaceuticals, Blue Bell, PA). Two 0.2 Amp constant current square-wave pulses were delivered through a triangular 3-electrode array consisting of 26-gauge solid stainless steel electrodes completely inserted into muscle. Each pulse was 52 milliseconds in length with a 1 second delay between pulses (Hutnick et al., 2011).

Hydrodynamic tailvain immunization

For hydrodynamic tail vein immunization (HDTV), mice were injected intravenously with 200μg of plasmid in 2mL (about 10% volume of the mouse weight) of PBS solution within a period of 7 seconds to transiently transfect the liver(Zhang et al., 1999).

Mouse Sacrifice and Tissue Collection

One week after final immunization, the mice were sedated with 2,2,2 tribromoethanol (Avertin), a mouse anesthetic; retro-orbital bleeding was performed before the mice were sacrificed by cervical dislocation. Spleens, lymph nodes, liver, lung, brain, skin, heart, muscle, kidney, intestine and Peyer's patches were be harvested into a 15-ml conical tube containing R10 medium. Tissues needed for histology were fixed with 2% PFA. For those needed for lymphocyte isolation, the tissues were put through a cell strainer and treated with ACK lysing buffer. Cells were then washed twice with R10 media, counted, and used for the appropriate experiments (West et al., 2011).

Splenocyte isolation and purification

Splenocytes were isolated as described elsewhere(Shedlock et al.). In brief, mice were sacrificed one week after the last immunization and spleens were harvested, placed in R10 media (RPMI media supplemented with 10% FBS and 1x Anti-Anti). The spleens were individually crushed, strained with a 40μM cell strainer and treated with 1mL ACK lysis buffer for 5 min to lysis erythrocytes. The splenocytes were resuspended in a complete R10 media and used for further immunological assays.

PBMC isolation

Less than 1 mL of blood was collected from each mouse into individual tubes containing 500μL sodium citrate. 2mL of R10 was added to the mixture and underlied with 3 mL Ficoll. The solution was then centrifuged for 20 mins at 2000 rpm with no breaks. The buffer coat was obtained and lyphocytes were isolated from it. The cells were then treated with ACK lysis buffer to lyse the red blood cells.

Lymphocyte isolation from liver and Lung

Lymphocytes from liver and lung were obtained as described elsewhere (Choo et al., 2010). Briefly, each liver was perfused by directly injecting 1mL of PBS into the hepatic vein of each mouse. A small incision was made in the heart and PBS was injected into the right ventricle of the heart to perfuse the lungs. Both liver and lung were harvested, crushed and resuspended in 5mL of 44% isotonic percoll. The lung was first treated with EDTA and collagenase. The mixtures were underlied with 3mL 66% isotonic percoll and centrifuged for 20 minutes at 2000rpm for gradient separation. Lymphocytes were collected and washed in 10 mL R10 and treated with ACK lysis buffer as necessary.

In vivo Cytotoxicity Assay

An *in vivo* cytotoxicity assay was performed as previously described (Barber et al., 2003; Durward et al., 2010). Briefly, splenocytes from naïve mice were stained with either 1μM (high) or 1nM (low) CFDA SE (Invitrogen). The labeled splenocytes were then coated with the indicated peptides (relevant or irrelevant peptides). Equal frequency of each target cell population was intravenously injected into naïve or immunized mice. 24, 48,

72 or 90 hours cells post transfer; cells from the blood, spleen or liver were isolated and analyzed by flow cytometry. The percent killing was calculated as follows: 100 - ([(% relevant peptide pulsed in infected/% irrelevant peptide pulsed in infected)/(% peptide pulsed in uninfected/% irrelevant peptide pulsed in uninfected)] x 100).

In vivo Proliferation Assay

The in vivo cell proliferation assay using CFSE labeling has been previously described. Briefly, fleshly isolated splenocytes were stained with the carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) Cell Tracer Kit (Invitrogen) in a prewarmed PBS for 10min as per the manufacturer's instructions. Stained cells were washed three times with cold saline and suspended in R10 media. The cells were washed twice in the complete R10 media and washed three times in serum free media. The cells were then resuspended at one million or appropriate frequency per mL. The cells were then transferred into naïve mice through the tail vain with a 28-gauge needle.

Viral Challenge

For lethal challenge studies, mice were challenged I.C with 200 PFU of LCMV Armstrong as previously described(Shedlock et al., 2011) in 30 μl of RPMI. Mice were observed daily for 3 weeks, a time point known to be adequate in the LCMV intracranial challenge model, and all LCMV infected animals were housed in BSL-2 facilities.

6.2.2 Ex vivo Experiments

Interferon-gamma ELISpot

IFN-γ ELISpot was performed as previously described(Kutzler et al., 2005). MultiScreenTM-IP 96-well plates (Millipore, Bedford, MA) were coated overnight at 4°C with capture antibody diluted in PBS at a concentration of 7.5 μg/mL (anti-IFN-γ clone GZ-4, Mabtech, Cincinnati, OH). Plates were washed five times with PBS and blocked with complete culture medium for two hours.Splenocytes were stimulated with two pools of 15-mer peptides spanning the entire length of pMCore and over lapping by 8 amino acids. 200,000 splenocytes in R10 media were plated in a 96 well IFN-γ capture antibody (R&D system) coated plate and stimulated overnight in the presence of a specific peptide pool at 37^{O}C in 5% CO_2. Cells were washed out and plates were washed five times. The plates were then incubated overnight with 100 μl/well of biotinylated detection antibody diluted in PBS (1 μg/mL; clone 7-B6- 1, Mabtech). Plates were washed five times with PBS and then incubated with 100 μl/well of streptavidin-alkaline phosphatase diluted in PBS (1:1000) for 1 hour at room temperature. Plates were then washed five times with PBS and developed with 100 μl/well BCIP/NBT substrate solution for 10-20 minutes at room temperature. Washing the wells three times with tap water terminated the colorimetric reaction. Plates were air- dried and the spots were counted using an automated ELISpot reader system (CTL Analyzers, OH) with the ImmunoSpot® software. The mean number of spots from triplicate wells was adjusted to 1 million splenocytes. The antigen-specific responses were calculated after subtraction of spots formed in response to culture medium alone. In addition, a response that was at several-fold higher than the medium control was considered positive.

Ex vivo Proliferation Assay

The T cell proliferation assay using CFSE labeling has been previously described(Shedlock et al., 2010). Briefly, fleshly isolated splenocytes were stained with the carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) Cell Tracer Kit (Invitrogen) in a prewarmed PBS for 10min as per the manufacturer's instructions. Stained cells were washed three times with saline and suspended in R10 media and plated in a 96-well U-bottomed plate in a total volume of 200 μL of stimulating media. The cells were incubated at 37^{O}C for 96 hours. After 48 hours, 50% of the culture media were removed and replaced with fresh R10. Following staining, cells were washed twice in PBS and fixed with 1% paraformaldehyde. For flow cytometry, cells were gated on singlets using SSC-H by SSC-A followed by gating on CD3+ T cells to examine live T-cell populations. Pure CD4+ and CD8+ T cell populations were determined by sequential CD4 and CD8 gating to account for upregulation of CD8 on activated CD4+ T cells.

Intracellular Cytokine Staining

A subset of splenocytes was resuspended in R10 media at a concentration of 10^7 per mL and 100μL was plated onto a 96 well round bottom plate. 100μL of media containing specific pooled peptides or 10 ng/ml PMA (Sigma, St. Louis, MO, USA) and 500ng/ml ionomycin (Calbiochem, Novabiochem, La Jolla, CA, USA) mix as a positive control or 0.1% dimethyl sulfoxide (Sigma, St. Louis, MO, USA) as a negative control. All wells contained 5uL/mL of two protein transport inhibitors, brefeldin A (GolgiPlug) and monensin (Golgistop) (All from BD Bioscience). The cells were incubated at 37^{O}C in 5% CO_2 for 5 hours and stained with LIVE/DEAD® Fixable Dead Cell Stain (invitrogen) for

10min at 37^{O}C. Cells were washed twice in PBS and once in PBS containing 5% FBS and 0.5% sodium azide. Extracellular staining was performed using antibodies specific to mouse CD3, CD4 and CD8 at 4^{O}C for 20 min. These antibodies were diluted in PBS containing 5% FBS and 0.5% sodium azide. Spleenocytes were then permeabilized and washed using BD Cytofix/Cytoperm^{TM} and Perm/Wash^{TM} (BD Bioscience) respectively. The cells were then washed twice with Perm/Wash^{TM} solution (BD Bioscience) and then stained intracellularly with antibodies to mouse Interferon-gamma, interleukin 2 and Tumor Necrosis Factor- alpha. The cells were incubated at 4^{O}C for 1 hour. The cells were then washed four times with Perm/Wash^{TM} solution and twice with PBS. Following staining, cells were fixed with PBS containing 2% paraformaldehyde. The cells were then stored at 4^{O}C until flow cytometry analysis.

Cells were analyzed on a modified LSR II flow cytometer (BD Immunocytometry Systems, San Jose, CA). One million events were collected per sample. Data analysis was performed using FlowJo version 9.3 (TreeStar, San Carlos, CA). Initial gating used a side scatter area (SSC-A) versus height (SSC-H) plot to remove doublets. The events were subjected to a lymphocyte gate by a SSC-A versus FSC plot. Live T cells were identified by a live/dead versus CD3+ plot. Following this, events are sequentially gated on CD8+and CD4– events versus IFN-γ to account for down- regulation. Following identification of CD8+ and CD4+ T cells, a gate was made for each respective function using combinations that provided optimal separation. After the gates for each function were created, we used the Boolean gate platform to create the full array of possible combinations, equating to 15 response patterns when testing 4 functions. Data are reported after background correction.

Conjugated anti-mouse antibodies were used during the extracellular and intracellular staining including: CD3- Phycoerythrin/Cy5 (PE/Cy5), CD4- peridinin chlorophyll protein (PerCP), CD8- allophycocyanin (APC), IFN-γ-Alexa Fluor 700, TNF-a-fluorescein isothiocyanate (FITC) and IL-2-phycoerythryin cyanine (PE) (all from BD Biosciences, San Jose, CA)(Wherry et al., 2003).

Measurements of the Sera Ag-specific IgA and IgG levels

High-binding ELISA plates (Costar, Corning, NY) were coated with 1μg/ml antigen specific protein in PBS, at 4°C for 24 hours and then were washed three times with 0.1% PBS-Tween. The plates were blocked with PBS containing 1% BSA for 2 hours at room temperature and then washed 3 times with PBS containing 0.1% Tween. Serially diluted serum samples were added to the wells and incubated for 1 hour at room temperature. Plates were then washed five times with 0.1% PBS-Tween. After washing, bound serum Antibody was revealed by HRP-labeled goat anti-mouse IgA or IgG. 100uL of HRP-conjugated IgA or IgG antibody was added to the plate and incubated at room temperature for one hour. The plates were washed three times. The peroxidase-conjugated antibodies were detected using tetramethylbenzidine (Sigma-Aldrich) as the substrate. The reaction was stopped with 2N sulfuric acid and OD values at 450 nm were measured with the Multiscan ELISA Plate Reader. IgG or IgA end-point titers were defined as the reciprocal serum dilution that resulted in OD values that were greater than twice the average OD value of the BSA wells.

6.3 Macaque studies

Vaccination:

Fifteen Indian Rhesus Macaques were housed at Bioqual (Rockville, MD) according to the standards of the American Association for Accreditation of Laboratory Animal Care. The short group consisted of five animals vaccinated with 1.0 mg each pMCore, pSHb A, pSHb C delivered IM with in vivo electroporation The long group consisted of five animals vaccinated with 1.0 mg each pMCore, pLHb A, pLHb C delivered IM with in vivo Electroporation. The Long+12 group consisted of five animals vaccinated with 1.0 mg each pMCore, pSHb A, pSHb C and 0.04mg plasmid expressed optimized rhesus IL-12 delivered IM with in vivo electroporation. DNA was delivered to a single site in the quadriceps followed by *in vivo* EP with the constant current CELLECTRA® device (Inovio Pharmaceuticals, Blue Bell, PA) with 3 pulses at 0.5 A constant current, a 52 ms pulse length and 1 s rest between pulses. Animals were vaccinated at weeks 0, 4, 12 and 30.

Sample collection:

Animals were bled 2 weeks following each immunization. Blood (20 mL at each time point) was collected in EDTA tubes and PBMCs were isolated using standard Ficoll-Hypaque procedure with Accuspin tubes (Sigma-Aldrich). An addition 5 mL blood was collected into clot tubes and sera aliquotted for analysis. Inguinal Lymph node biopsies were taken at week 0, 14 and 32. Lymphocytes were isolated by physical separation with a scalpal and passed through a 16 gauge blunt needle before being passed through a 40 um filter. Lymphocytes were spun down and frozen for later analysis.

ELISpot:

Matching HepB consensus core, surface antigen A and surface antigen C 15 mer peptides were synthesized by Genescript (Piscataway, NJ) and resuspended in DMSO and pooled at an approximate final concentration of 1 mg/mL for each peptide. Cellular responses were measured using IFN-g ELISpot (MabTech, Sweeden) following the manufacturer's instructions. Samples were run in triplicate with an R10 (RPMI 1640 containing L-glutamine with 10% heat inactivated fetal bovine serum, and 1% Penicillin/Streptomycin) and a PMA/IM (PMA 0.1 μg/mL and ionomycin 0.5 μg/mL, Sigma Aldrich, St. Louis, MO) control.

Mapping ELISpot:

To map the epitopes within the HepB core protein, a 6x5 peptide matrix was created. PBMCs collected 4 weeks following the third vaccination were stimulated with the 11-peptide pools. All responses were background subtracted and any responses over 50 SFU/106 PBMCs was considered positive. Two monkeys had high background levels and were excluded from the analysis.

ELISA: The ELISA assay was performed as previously described using 5 μg/mL HepB surface antigen type Ad (MyBioSource) in PBS-T (PBS with 0.5% Tween 20) . Endpoint titers were determined as previously reported. Briefly, the upper prediction limit of SIV-specific IgG antibodies was calculated using the Student t-distribution where the mathematical formula that defines the upper prediction limit is expressed as the standard deviation multiplied by a factor based on the number of naïve controls and a 95% confidence interval. The endpoint titer is reported as the reciprocal of the lowest dilution that remained above the upper prediction limit.

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