VACCINIA AND DENGUE VIRUSES:
EXPLORING CURRENT FUNDAMENTAL ISSUES OF MEMORY T CELLS
AND UTILIZING QUANTITATIVE IMMUNOLOGY TO COMPARE
CORRELATES OF PROTECTION FOLLOWING SMALLPOX IMMUNIZATION

by

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Vaccinia and Dengue Viruses: Exploring Current Fundamental Issues of Memory T Cells and Utilizing Quantitative Immunology to Compare Correlates of Protection Following Smallpox Immunization

Abstract
by
NICHOLAS DANIEL OSTROUT

Memory T cells often perform an important role in mediating viral clearance upon secondary viral infection. Therefore, expanding the current understanding of issues surrounding memory T cell development, the long term maintenance and the functional characteristics of different memory subsets is essential. A deeper understanding of these issues surrounding memory T cells will aid in generating more successful vaccines. The unsuccessful attempts to create vaccines to protect against devastating diseases such as HIV and malaria have highlighted the flaws in traditional approaches of developing empirically derived vaccines. Data obtained from the following studies can be used and applied to rationally attain new, 21st century vaccines. The work presented here focuses on two viruses, vaccinia and Dengue. However, the questions addressed have implications beyond these two pathogens. Here we present findings on the long term maintenance and functional properties of effector and central memory T cells in humans last vaccinated with vaccinia virus over 30 years previously. These data provides a basis for a quantitative immunological comparison after administration of new smallpox vaccines. Utilizing a mouse model of vaccinia infection, we then address how antigen duration and the induction of inflammation effects memory T cell development. Finally, the four serotypes of Dengue virus provided us with an opportunity to study heterologous infection and the possible detrimental effects of partial T cell agonists on memory T cells.
Foreword

Following the events of September 11\textsuperscript{th}, 2001 and the subsequent bioterrorist attacks with anthrax, the United States Government mandated that the National Institutes of Health and other institutions across the country address epidemiological, immunological and other related issues pertaining to Category A Priority Pathogens. Pathogens in Category A of the Priority Pathogen selection listing are considered the most likely candidates for use in a bioterrorist attack. As a result of the government mandate, resources were focused on research programs involving these Category A pathogens. The increase in resources lead various institutions across the country to undertake research initiatives intended to elucidate the immunological response to many of the Category A pathogens in order to develop either preventative measures, such as vaccines, or potential treatment options.

The Midwest Regional Center of Excellence for Biodefense and Emerging Infectious Disease Research (MRCE) is an example of one initiative brought to fruition because of the United States Government mandate. The MRCE is a conglomerate of various research institutes, universities and biotechnology companies, including Case Western Reserve University, that is investigating immunological issues surrounding category A priority pathogens including \textit{Orthopox} viruses and \textit{Flavivirdae} viruses. Our lab, along with other groups within the MRCE, is responsible for developing various protocols to determine the immunological requirements necessary to protect against Variola Major virus (family member of \textit{Orthopox}), the virus that causes smallpox, with the goal of developing a safer vaccine. In addition, our lab is interested in the immunological aspects of Dengue virus (family member of \textit{Flavivirdae}) infection, that
contribute to the development of Dengue Hemorrhagic Fever (DHF) in order to provide information that can be used to develop an effective vaccine.

Moreover, this work with the MRCE, using both Vaccinia virus and Dengue virus, provided us with an opportunity to make a substantial contribution to the current understanding of fundamental issues surrounding memory T cells following acute viral infections. Some of the issues addressed included questions regarding memory T cell development and their long-term maintenance, further descriptions of the functional characteristics and phenotypes of different memory cell subsets and issues of variant peptide ligands and cross-reactivity. Scientific inquiry into these topics has not only aided our lab and other labs of the MRCE in developing a better understanding of Orthopox and Dengue virus immunogenicity, but have also had implications to the broader scientific community interested in issues of memory T cell immunology.

Specific Aims:

1) Determine the frequency and functional properties of the various memories CD8+ T cell subsets present in individuals previously vaccinated against smallpox

2) Establish the effect of enhanced stimulation through antigenic load and inflammation in directing the development of T cell effector memory and central memory after infection of mice with various preparations of vaccinia virus that differ in their replicative capacity and immunogenicity

3) Refine the mouse model of heterologous secondary Dengue Virus infection, focusing on the memory T cell’s role in causing serotype cross-reactivity leading to altered cytokine profiles and severe immunopathogenesis
CHAPTER 1

Introduction to Variola and Vaccinia Virus

Significance

Although the world was declared smallpox free in 1980 by the World Health Organization (WHO), the Variola virus, the causative agent of smallpox, still exists in two disclosed locations, not to mention the possibility that the virus may be kept in undisclosed locations by rogue states or terrorist networks (1-3). Therefore, there exists the potential of a smallpox outbreak following a deliberate release of the Variola virus in a bioterrorist attack. The release of Variola virus would require the implementation of mass vaccination. However, there have been serious side effects associated with the use of current live, ‘attenuated’ smallpox vaccines, including Dryvax, Lister and the New York City Board of Health (NYCBH) strain, particularly in individuals with a compromised immune system (4-8). Given the uncertainty of the use of smallpox virus in a bioterrorist attack and the concern with side effects associated with the current vaccines, the initiative to use Dryvax as a smallpox vaccine to vaccinate millions of Americans has effectively been terminated. Therefore, there is a demand for new smallpox vaccines that can be administered for protection against smallpox.

Accordingly, less virulent replication incompetent vaccinia virus (VV) strains, such as Modified Vaccinia Ankara (MVA), are under consideration as alternative vaccines. The new generation of smallpox vaccines must be safer than and at least as immunogenic as the historical products that utilized intradermal scarification with replication competent vaccinia virus.
Due to the successful smallpox eradication campaign, it is impossible to test the efficacy of any new smallpox vaccine in humans. In addition, it is difficult to ascertain the correlates of protection since naturally occurring smallpox was eliminated before techniques present in the modern era of immunology could be utilized (9, 10). Since the correlates of protection in human are not known, quantitative immunological approaches have been accepted as a way to compare the immunological correlates of protection in individuals vaccinated with traditional smallpox vaccines to those which develop following the administration of new vaccines. Quantitative immunology includes measuring known neutralizing antibody titers and identifying frequencies of cytokine producing T cells and determining their functional attributes. When evaluating these newer vaccines, it is important to compare their ability to induce long-term T* and B cell immunity relative to the traditional VV since the latter is the only immunogen demonstrated to protect humans against smallpox. Furthermore, for the most effective quantitative immunological comparison and understanding of protective immunity, it is important to realize the types of memory cells (i.e. effector memory and central memory T cells) present following immunization. The scientific community can then use data such as these as a basis of comparison for new smallpox vaccines.

We hypothesized that Dryvax elicits the development of both T effector (T_{EM}) and central memory (T_{CM}) cells and that these populations are maintained in remotely vaccinated individuals. In addition, we proposed that T_{EM} cells display greater cytotoxic ability than T_{CM} in response to vaccinia virus and as a result, are more important in conferring adequate protection from smallpox. Finally, utilizing a murine model of

\footnote{Our efforts at CWRU were focused on T cells, mainly CD8+ T cells, while other groups within the MRCE were focused on CD4+ T cells and antibodies. This was done in accordance with MRCE policy to prevent competition among MRCE members and minimize unnecessary experimental overlap.}
vaccinia immunization, we hypothesized that viral immunogenicity, through antigen load and inflammation, can influence the generation of different memory subsets. The data presented in these studies provide significant contributions to understanding the long term maintenance and differentiation process of memory subsets.
**Background**

The history of smallpox is described thoroughly by Donald R Hopkins and a brief history is presented here (11). The smallpox disease is thought to have first been recognized in Egypt and India over 3000 years ago. Thucydides described the first recorded account of the transmissible nature of the disease from his observations during a smallpox epidemic in ancient Greece around 430 B.C.E. Thucydides concluded from his observations that an individual who developed characteristic pox lesions and then recovered was subsequently protected from another attack of a similar illness. Primitive vaccination techniques developed from these types of observation were first described in China and India around 1000 C.E. These primitive “vaccines” used the process of variolation. Variolation consisted of taking the fluid or skin material from a pox lesion of an infected individual and using it to inoculate an individual whom had no indication of exposure to the smallpox virus. While these “vaccines” worked in some instances, the vaccinated individual often developed smallpox. This practice was introduced into Europe in the 1700’s, but physicians quickly abandoned it citing that the risk of developing smallpox was too great. An effective vaccine for smallpox that was considered to have few side effects was not developed until 1796 when Edward Jenner first reported his strategy.

Edward Jenner noticed that milk maids got sores on their hands that resembled the pox marks on individuals with smallpox (12, 13). After further study, Jenner suggested that the milkmaids were being infected with cowpox from bovines. Jenner developed his vaccine by removing material from the pox marks of milkmaids or the pox marks on bovines’ utters. He then would scratch the skin of a vaccinee and rub the material on the
As the vaccine developed over the next 150 years, the initial virus variant was consistently transferred from person to person, from person to bovine and bovine to person. When the vaccine was studied for its components in 1939, it was found that the virus in the vaccine was not cowpox, but actually a new Orthopox viral variant. It has been suggested that this new virus differentiated from cowpox although its exact origin is obscure (14). This new strain of Orthopoxvirus was called vaccinia virus (13). Vaccinia virus received its name from Jenner’s description of his vaccine, variolae vacciniae, or smallpox of the cow.
Vaccines

Before the inception of the smallpox eradication campaign by the World Health Organization (WHO) in 1967, there were many preparations of vaccinia used to vaccinate individuals around the world. These included the Copenhagen, the Temple of Heaven, the Tian Tan, and the more recognized vaccinia viral preparations, New York City Board of Health (NYCBH) and Lister. Dryvax was derived from the NYCBH preparation used in the United States and Lister was used in Great Britain. These two smallpox vaccines have been considered the main vaccines for smallpox since 1967 because of their adoption by the WHO and subsequent use during the global smallpox eradication campaign. Modified Vaccinia Ankara (MVA) was also briefly used as a vaccine against smallpox in Germany, but to a much more limited extent than Dryvax and/or Lister (15, 16). MVA was derived from a dermovaccinia virus that infected donkeys (17). This virus was passed in chicken embryo fibroblasts and after 516 passages, was renamed MVA. However, like other vaccinia vaccines, the use and therefore the study of MVA as a vaccine was terminated in the late 1970s because of the successful eradication of smallpox. Hence, the immune responses evoked by the various strains of vaccinia virus were never studied. Moreover, the protection afforded at the cellular level to vaccinia viruses is largely unknown.

Traditional smallpox vaccines, such as Dryvax, are ‘swarms’ of many different strains of vaccinia virus. In addition, these vaccines were derived from growing the virus on calf lymph without sterile techniques and many of the problems associated with these vaccines are thought to be because of contamination of the vaccine with bacteria. Therefore, ACAM1000 was generated by isolating one of the many strains of vaccinia
virus within Dryvax and mass producing it under sterile conditions so contamination is avoided (18). ACAM1000 has been tested extensively in animal models and demonstrates no neurovirulence. ACAM3000, the next generation of ACAM1000 has undergone phase III clinical trials and is currently under review by the FDA.

The problem with ACAM3000, like traditional smallpox vaccines, is that it is replication competent. Therefore, like any replication competent virus, there is a risk of the virus causing adverse events, especially in individuals that have a deficient immune system at the time of vaccination, due to dissemination. Therefore, replication incompetent viruses such as MVA have gained substantial interest. While there are other strains of replicating virus such as NYVAC, which was derived from vaccinia virus following deletion of 18 genes (including those encoding host defense factors) and ALVAC, an attenuated vector derived from canarypox, MVA has the most extensive history of safety in human beings(19, 20). Recombinant MVA has been used as a vector for the delivery of viral proteins from HIV, HPV and malaria which have reached phase I and II clinical trials(21). Therefore, it is thought that MVA is extremely safe and the best choice as a new smallpox vaccine for use in healthy, as well as immunocompromised individuals.

In order to replace traditional smallpox vaccines with ACAM3000, MVA or any other new smallpox vaccines, a greater understanding of the holistic immune response to these various vaccinia strains is required. Specifically, one must understand the development of the immune response and the establishment of effective immune correlates of protection in order to compare the protection capacity afforded by various vaccines. Recent studies have begun to suggest immunological correlates of protection
using murine and primate models, along with quantitative immunology the human
response. Results from these studies, along with this current study, will provide
confidence in a new vaccine’s ability to induce immunological protection equivalent to
that of traditional smallpox vaccines, particularly Dryvax.
Orthopox Virus Infection

Due to the complexity of Orthopox Viruses (OPV), the infection process is highly complex and not completely understood. However, some of the key mechanisms of OPV infection, replication and dissemination are described here. OPV attach to cells by binding glycoaminoglycans (GAGs) on the surface of cells. No specific receptor on the cell surface has been described for mediating attachment. However, binding is mediated by three polypeptides on the virion membrane of the virus called the 32kDa, 29kDa and the 54-58kDa (STE) polypeptides (22). Virus entry into the cell occurs via penetration of the virus through the cell membrane by a proteolytic cleavage event (23-27). Although binding of OPV to the cellular membrane does not occur through a well defined receptor, monocytes, B lymphocytes and NK cells show higher infectivity rates, while infection rates of other cell types, such as T lymphocytes, is low (28).

After OPV penetration into the cell, viral uncoating occurs in two phases (29, 30). In the first phases, about 50% of the virion membrane, including proteins and phospholipids, is lost (31). Immediately following the first phase of uncoating, simultaneous initiation of early transcription with viral associated transcriptional machinery and secondary uncoating occurs (32, 33). At this point, viral protein ‘cores’ exist in the cytoplasm of the cell and the viral DNA is accessible to DNase (31, 34). These events occur within the first 0.5 to 2.5 hours following infection.

Packaged into the infectious form of the virus are the viral RNA polymerase and other enzymes necessary for early gene transcription. During early gene transcription, about half of the viral genome is expressed including proteins necessary for DNA replication and host defense (35-37). The majority of host defense genes are expressed at
OPV DNA replication occurs at discrete areas in the cytoplasm of the infected cell called ‘factories’ or viroplasm (38). DNA replication occurs between 2 and 6 hours post-infection (p.i.) with the production of around $10^4$ genome copies by 24 hours p.i. (32). DNA replication marks the end of early gene transcription and the onset of late gene transcription.

The proteins expressed during the ‘late’ phases (>6 hours p.i.) include most of the proteins required for structural assembly and the enzymes (e.g. RNA transcription) that are packaged into mature viral particles. The events that lead to virion package and assembly are complex (22). OPV are assembled in the viroplasm of the cell into uniform, spherical, immature particles. The immature viral particles undergo extensive morphological and biochemical changes involving both the internal and external components, the mechanisms of which little is known (22). At this point, immature particles are packaged into two infectious forms of mature virus, intracellular mature virus (IMV) and extracellular envelope virus (EEV) (39).

The IMV is the most abundant virion within infected cells and is composed of a single membrane (40). The IMV can become wrapped in cell derived Golgi membrane to become an intracellular envelope virus (IEV) which is transported to the cell membrane and released from the cell (41). These released EEV infectious forms are responsible for remote cell infection. After 24 hour p.i., approximately 10,000 new infectious OPV have been generated.
Orthopox Host Defense Mechanisms

There are several mechanisms that OPV have developed to evade, shut down and alter the host’s immune response. The numerous methods that OPV use to accomplish immune evasion and defense against the host are described in detail by a recent review in Annual Reviews of Immunology published in 2003 (42). However, some of these defense mechanisms are described here briefly.

One of the predominating host defense mechanisms employed by OPV, which is especially pertinent in the context of this work, is the production of cytokine binding proteins that mimic cytokine receptors (43). These OPV produced cytokine binding proteins prevent host produced cytokine from binding their receptors and inducing proper inflammatory signaling. IFN-γ, IFN-α/β, TNF-α and IL-18 all contribute specific roles in activating immune cells, up regulating immuno-modulatory proteins (e.g. MHC) and inducing the production of anti-viral enzymes and proteases within infected cells. However, OPV encodes decoy binding proteins for all of these important cytokines (43-47). Another mechanism used by OPV to avoid clearance by the host immune response is to produce homologs to host growth factors. These growth factor homologs such as epidermal growth factor (EGF) signal infected cells to undergo cell division and proliferation, while vascular endothelial growth factor (VEGF) contributes to increased pustule formation and transmission (48).

Finally, OPV express proteins responsible for inhibiting apoptosis. Proteases called caspases induce signals within cells to undergo apoptosis. OPV produce proteins that inhibit Fas, TNF and TRAIL mediated induction of caspase-8 (49). OPV also produce serine protease inhibitors such as CrmA which prevents Granzyme B from
initiating perforin dependent apoptosis (50). Furthermore, RNA-dependent protein kinase (PKR) interrupts translation and induces apoptosis. However, OPV encodes PKR inhibitors. Lastly, OPV produce a protein capable of converting hydrogen peroxide into water (51). This is especially important in preventing macrophages and neutrophils from killing infected target cells.

Hence, it is evident that OPV encode a variety of decoy receptors and proteins to defend against host inflammatory mechanisms. The host immune response is designed to not only prevent spread of the virus or dissemination by promoting apoptosis of infected cells, but also signal cells to resist infection and promote protease generation and other inhibitors of DNA/RNA replication (e.g. DNases/RNases). Therefore, the host immune response is designed to interrupt the infection cycle at various points by preventing viral replication within an infected cell, dissemination from an infected cell and further infection of uninfected cells. However, OPV have developed several methods to evade and inhibit these immune responses. With approximately half of the proteins expressed by OPV (~100) dedicated to host evasion and defense, it is evident why OPV are difficult for the host to clear.
Current Understanding of Immunity to Orthopox Viruses

The Orthopox genus includes Variola major, the virus that causes smallpox, vaccinia virus, and related viruses that cause “pox” like lesions in various mammals, e.g. camelpox virus, monkeypox virus, Ectromelia virus (mousepox) and cowpox virus(20). Little work on the immunological correlates of protection has been done with vaccinia virus in humans because of the elimination of Variola virus as discussed previously. However, the information regarding vaccinia and MVA infection gained from murine and primate studies has indicated both cellular and humoral immunological correlates of protection (52).

Many studies using murine models have provided insight into the progression of the immune response and development of memory after vaccinia challenge (53-60). One study utilizing the mouse model of vaccinia virus immunization, followed by subsequent challenge with lethal doses of vaccinia virus, suggested that antibodies may be sufficient for protection (54). However, they did find that in the absence of antibody, T cells were necessary and sufficient for survival. Other groups utilizing similar murine models of vaccinia infection argue that both antibodies and T cells are required to confer adequate protection (55, 58, 59) and in fact, researches from two groups concluded from their data that no one specific arm of the adaptive immune response was absolutely required, but rather that there was redundancy (55, 56).

When murine models are utilized where the challenging agent is Ectromelia virus, instead of vaccinia virus, CD8+ T cells especially appear to be required in addition to antibodies, especially during primary infection (53, 60). However, doubt has even been shed on these findings in recent years when secondary infection is considered (61). The
problem with this study is that mice were primed with avirulent ETMV and then challenged with ETMV. Priming and challenging with the same virus is not equivalent to priming with vaccinia and challenging with ETMV. The most recent data on the effective protection of mice from ETMV after Dryvax immunization is from a 1985 study (62). In this study, the authors did not address immune correlates of protection.

The main problem with murine studies to identify correlates of protection after Dryvax immunization is that vaccinia virus is a human adapted virus. Therefore, vaccinia virus in mice does not emulate viral pathogenesis in humans or the human response of the immune system. Vaccinia immunization of mice followed by lethal challenge with mouse adapted vaccinia virus western reserve (VVWR) is an abstract comparison to Dryvax immunization and smallpox challenge in humans at best (61). While Dryvax administration to mice followed by ETMV would be better to identify immune correlates of protection, experiments with ETMV are difficult due to the high pathogenicity of ETMV and the problem of using vaccinia in mice is still present. Therefore, there are no murine models that are directly relevant to Dryvax immunization and smallpox challenge in humans. Moreover, no murine studies have convincingly shown that any one specific arm of the adaptive immune response is sufficient for protection from vaccinia virus. However, mice do offer the ability to address complex immunological question due to the substantial availability of different strains, low cost and accessibility to all tissue. Therefore, there are advantages for using vaccinia in mice, but the best solution to identify Dryvax mediated immune correlates of protection from smallpox is to use primate models with vaccinia priming and monkeypox challenge.
To this end, primate studies indicate that antibodies are the most important aspect of the adaptive immune response required to confer protection from death upon monkeypox virus, an Orthopox virus that effects primates in a similar manner as Variola in humans (63-66). Results from one study by Edghill-Smith et. al. provided the most convincing evidence that only antibodies are required for protection from lethal monkeypox challenge following vaccinia virus immunization (64). Treatment with α-CD4 or α-CD8 and subsequent challenge with monkeypox six months after vaccinia administration revealed that macaques still developed lesions, although they survived (Table 1-1). However, their study was done by only abolishing either CD4 or CD8 T cells and not both. Therefore, it is not clear if protection would be diminished if the entire T cell population were removed. However, another study provided further evidence for the conclusions reached by Edghill-Smith et. al. when results found that if only known neutralizing antibodies are elicited by a subunit vaccine, macaques developed moderate to severe disease upon monkeypox challenge, but survive (Table 1-2) (65). Moreover, Edghill-Smith et. al. further provided evidence that antibodies are sufficient to confer protection when they administered human vaccinia immune globulin (VIG) to unvaccinated rhesus macaques four days prior to monkeypox challenge (Table 1-3). The animals did develop skin lesions, but were protected. The interesting part is that the severity of skin lesions inversely correlated with the amount of VIG administered. These studies demonstrate that vaccinia specific antibodies are sufficient to protect from death upon subsequent challenge with another Orthopox virus. However, Dryvax immunized immunocompetent macaques are protected from disease upon monkeypox challenge (63, 64).
Results from another study utilizing Cynomolgous monkeys revealed that two doses of MVA, one dose of MVA and one dose of Dryvax, or just one dose of Dryvax were capable of protecting from a lethal monkeypox challenge (63). Moreover, antiviral antibody and T cell responses appeared to be similar among the three groups. This indicates that quantitative analysis of an immune response to vaccinia in animal models after immunization is capable of identifying the requirements necessary for protection after challenge. Hence, this study supports that comparative quantitative immunological analysis is a good method to compare correlates of protection after Dryvax or MVA human immunization.

Concerning humans, the immunological correlates of protection from smallpox are not known. Originally, Edward Jenner was able to vaccinate an individual and then challenge that vaccinated individual with smallpox. He concluded that a person who presented a lesion at the sight of vaccination or “take” was considered to be protected from smallpox. The presence of a lesion at the sight of vaccination has traditionally been the “gold standard” in measuring vaccinia vaccine efficacy. However, there are rare cases of individuals who present a “take”, but do not develop cellular or humoral immunity (67). In addition, MVA does not form a vesicle at the site of immunization. Therefore, a quantitative immunological approach, utilizing the tools available today, was required to understand vaccine efficacy. Quantitative immunology is beginning to gain acceptance as a method for determining vaccine efficacy through identifying correlates of protection when human models of infection are unavailable or impossible (9).

Most of the historical data and even some recent studies on the quantitative analyses of the adaptive immune response to vaccinia virus in humans are limited to
mainly descriptions of antibody titers (Fig. 1-1), and some basic understanding of CD4⁺ T cells and CD8⁺ T cell activity that are present in previously vaccinated individuals or present during a response following vaccination (67-74). However, only recently have studies begun to analyze the meaningful functional and phenotypic properties of the memory response in individuals previously vaccinated against smallpox (Fig. 1-2 and Table 1-4) (75-82). These reports were the first attempt to understand the critical properties of the memory cells in humans previously vaccinated with vaccinia virus from a quantitative viewpoint. Observations from these various studies included descriptions of long term memory B cells that produce neutralizing antibodies, cytokine (IFN-γ, TNF-α, IL-2) producing CD4⁺ and CD8⁺ memory T cells, as well as proliferative responses measured by 3H-thymidine assays and CFSE assays. In addition, chromium release assays have been done to observe the functional ability of vaccinia specific memory cells to lyse infected target cells. Therefore, while it is evident that there are vaccinia specific memory cell populations in individuals that have been previously vaccinated, the exact composition of the different populations of memory cells that make up the memory population has only just begun to be realized (83-86). (The concept of T cell memory and the different memory populations will be discussed under the “T cell memory” subsection of Background.) This information will be crucial for an effective quantitative comparison of the memory efficacy elicited by Dryvax to that of MVA.

One of the main issues within the smallpox community is the disagreement as to the protective capacity of vaccinia specific memory T cells, particularly CD8⁺, and their requirement in secondary challenge (52). Typically, CD8⁺ T cells are thought to play a significant role in mediating protection from intracellular pathogens during primary
infection, while antibodies are traditionally viewed as the mediator of protection during secondary challenge. It is important to realize that since the correlates of protection required for conferring immunity to smallpox are not known, the role of both antibody and T cells should not be disregarded. While many have suggested that antibodies are the only immunological requirement for protection, particularly in prevention of death from monkeypox, the role that T cells play, specifically CD8+ T cells should not be dismissed. In fact, a recent study determined that there was a correlation between CD8+ T cell memory following MVA vaccination and severity of a lesion following subsequent Dryvax immunization (87). Therefore, while vaccinia specific antibodies elicited by smallpox vaccines may be adequate to prevent death from monkeypox, CD8+ memory T cells help alleviate symptoms of infection/manifestations of disease and terminate the transmission cycle, something that is of particular importance when dealing with human beings.

Ultimately, when considering protection, it is important to decipher between protection against disease and protection against death. Up to this point, in the current discussion about vaccinia-induced immunity, the focus of vaccinia protection from lethal vaccinia viral challenge, Ectromelia virus, monkeypox and smallpox has been on protection from death. In humans, it is important to consider the other types of protection since protection against disease can prevent viral dissemination among the population and many economical side effects such as losses in productivity and strains on the medical care system. Dryvax provides protection from disease in most immunocompetent humans (52). Therefore, new smallpox vaccines are required to elicit levels of immunity for protection from disease. New vaccines that only elicit B cell
Memory may protect from death upon smallpox challenge in humans, but without
memory CD8+ T cells, it is likely that the disease would still be severe enough that
complications could arise in a significant number of individuals. Therefore, to reiterate,
it is important that the correlates of protection induced by Dryvax are identified
quantitatively and new vaccines elicit the same level of quantitative immunity as Dryvax,
including antibody and T cell responses. The examination of all aspects of the immune
response provides the best opportunity for confidence in the ability of a new smallpox
vaccine to protect against disease and not be limited to protection from death.
Groups of macaques were immunized with vaccinia virus at day 1 and challenged with monkeypox six months later. Depletion of CD8⁺ or CD4⁺ four days before challenge did not prevent disease manifestations (i.e. pox formation), but all animals survived. From Edghill-Smith et al. (64).

Table 1-1 Groups of macaques were immunized with vaccinia virus at day 1 and challenged with monkeypox six months later. Depletion of CD8⁺ or CD4⁺ four days before challenge did not prevent disease manifestations (i.e. pox formation), but all animals survived. From Edghill-Smith et al. (64).
Table 1-2 Clinical outcome of monkeypox challenge. Macaques were immunized with a naked DNA expression vector containing the vaccinia A33L, L1R, A27L and B5R genes and/or the same proteins expressed and purified from *E. Coli*. Immunization lowered disease pathogenesis, but all macaques still developed signs of disease (i.e. pox lesions). From Heraud *et al.* (65)
Table 1-3 Outcome of monkeypox virus challenge following CD4+ depletion or prophylactic administration of vaccinia immunoglobulin intravenously (VIGIV). Group 4 macaques were immunized with Dryvax at day 1 and treated with α-CD4 antibody at days 25, 29, 34 and 44. Group 5 macaques received human immunoglobulin prepared from plasma of recently Dryvax vaccinated humans. Group 6 macaques were given commercially available VIG that was not obtained from recently vaccinated individuals, but had a vaccinia neutralizing titer. Group 7 macaques received saline. All animals were immunized on day 1 and challenged on day 29 with monkeypox. From Edghill-smith et. al. (64)
Figure 1-1 Long term presence of vaccinia specific antibodies in previously immunized individuals. Quantification of vaccinia specific antibody responses by ELISA. International units (IU) were calibrated based on the World Health Organization International Standard for smallpox antiserum (1000 IU/ml). All serum samples from unvaccinated volunteers (n=26) scored less than 100EU, which was then used as the lowest positive titer (dotted line); 944 EU indicates the predicted antibody levels equivalent to a neutralizing titer of 1:32 (dashed line). From Hammarlund et. al. (78).
Figure 1-2 Presence of memory B cells capable of secreting vaccinia specific IgG up to 60 years following smallpox immunization. In individuals previously vaccinated against smallpox, memory B cells that secrete vaccinia specific IgG are maintained up to 60 years post vaccination. From Crotty et. al. (73).
Table 1-4 Duration of memory T cells following smallpox immunization. Vaccinia specific CD4\(^+\) and CD8\(^+\) memory T cells are maintained up to 75 years following vaccination with vaccinia virus. CD8\(^+\) memory decreases at a greater rate, but both populations are maintained long term (>50 years) at a similar frequency of individuals. Number of vaccination does not appear to effect the duration of memory T cells. From Hammarlund et. al. (78).

<table>
<thead>
<tr>
<th>Vaccinations</th>
<th>Volunteers with CD4(^+) T-cell memory(^a)</th>
<th>20–30 years(^b)</th>
<th>31–50 years</th>
<th>51–75 years</th>
<th>t(_{1/2}) of CD4(^+) T cells(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100% (16/16)</td>
<td>89% (70/79)</td>
<td>52% (23/44)</td>
<td>10.6 (0–17)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>83% (10/12)</td>
<td>78% (29/37)</td>
<td>57% (4/7)</td>
<td>8.3 (0–14.1)</td>
<td></td>
</tr>
<tr>
<td>3–14</td>
<td>82% (23/28)</td>
<td>91% (29/32)</td>
<td>ND</td>
<td>12.4 (0–20.5)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vaccinations</th>
<th>Volunteers with CD8(^+) T-cell memory</th>
<th>20–30 years</th>
<th>31–50 years</th>
<th>51–75 years</th>
<th>t(_{1/2}) of CD8(^+) T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50% (8/16)</td>
<td>49% (39/79)</td>
<td>50% (22/44)</td>
<td>15.5 (0–27.1)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>42% (5/12)</td>
<td>38% (14/37)</td>
<td>57% (4/7)</td>
<td>8.1 (0–16.9)</td>
<td></td>
</tr>
<tr>
<td>3–14</td>
<td>46% (13/28)</td>
<td>50% (16/32)</td>
<td>ND</td>
<td>9.0 (0–18.1)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Percentage of volunteers with vaccinia-specific T-cell memory is based on the proportion of immunized participants with >10 IFN-\(\gamma\)-TNF-\(\alpha\) T cells per 10\(^6\) CD4\(^+\) or CD8\(^+\) T cells, respectively. This cutoff provides 100% sensitivity at 1 month after vaccination or revaccination and 92–95% specificity, based on the vaccinia-induced IFN-\(\gamma\)-response in T cells from unvaccinated volunteers. \(^b\)Years after the last smallpox vaccination. \(^c\)Estimated half-life (t\(_{1/2}\)) in years (and 95% confidence interval in parentheses) is based on linear regression analysis using data from Figures 1 and 2. ND, not determined.
CD8+ T cell memory

There are many facets involved in the generation and maintenance of an effective memory population of CD8+ T cells. The study of these many facets is currently a rapidly growing field of investigation with numerous papers regarding the details of the differentiation and developmental process, the cell subsets involved, their phenotypes, their varying levels of cytotoxicity and their proliferation and quiescence properties. The establishment of competent and stable long term memory T cells is crucial to protect from a second infection because memory T cell have been shown to “remember” previous antigen and quickly respond upon exposure (88-90). The mechanisms required to induce a substantial response and encourage the production of memory cells are currently under review. Of particular interest is determining how CD8+ T cells progress through the immune response from initial antigen encounter until stabilizing into memory T cells; essentially the differentiation process. However, first it is important to introduce the multiple phases of the overall immune response and to describe the different subsets involved during the course of the response.

The acute CD8+ T cell immune response to an offending pathogen consists of three phases (90). Each stage is characterized by the involvement of various and different cell subsets. Memory CD8+ T cells are generated from naïve T cells through a developmental program that is initiated in response to a pathogen (90-94). Upon antigen exposure, naïve CD8+ T cells expand and proliferate into numerous antigen specific effector T cells. Effector CD8+ T cells exhibit cytotoxic ability through the production of various cytokines and cytolytic enzymes. The expansion phase is followed by the contraction phase in which 90 to 95% of the effector T cells undergo apoptosis. The third
phase of an immune response is marked by the establishment and long-term maintenance of a stable population of memory CD8$^+$ T cell. Memory T cells undergo homeostatic proliferation and can be maintained, in some instances, for life (88, 89, 95). In mice, there are two subsets of memory CD8$^+$ T cells, effector memory T cells (T$_{EM}$) and central memory T cells (T$_{CM}$). In humans, there is a third memory cell subset, effector memory CD45RA$^+$ (T$_{EMRA^+}$), which are thought to be identical to T$_{EM}$, but express CD45RA (96-98).

The proliferative properties and the reacquisition of cytotoxic functions are diverse for the different cell subsets. Generally, T$_{EM}$ home to non-lymphoid tissue, are present in the blood and are not found at high frequencies in lymphoid tissue (99, 100). T$_{CM}$ on the other hand, home to lymphoid tissue and are present at low frequencies in the blood and non-lymphoid tissue. T$_{EM}$ have inherent cytotoxicity function and can also produce cytokines such as IFN-$\gamma$ and TNF-$\alpha$ immediately upon restimulation (101-103). T$_{CM}$ do not store perforin or granzyme like T$_{EM}$ and they do not produce effector cytokines upon reactivation. However, T$_{CM}$ produce IL-2 and have an ability to proliferate upon reactivation to produce new effector cells (104, 105). It has been shown that T$_{EM}$ can also undergo proliferation in response to restimulation under some circumstances, but this is generally not the case (85, 106, 107). The availability of CD4$^+$ T cell help during priming of CD8$^+$ T cells has been suggested to be a critical determinant of T$_{EM}$ proliferation potential upon secondary infection (108, 109), although, there are arguments against this as well (110).

One crucial point of contention is the self-renewal capacity of T$_{EM}$. T$_{CM}$ are accepted as memory cells that are capable of self-renewal through their intrinsic ability to
continuously divide (102, 111). Many propose that human TEM have a capacity for self-
renewal as well. In humans, both T_{CM} and T_{EM} have been demonstrated to express anti-
apoptotic markers such as Bcl-2 and also express IL-7 and IL-15 receptors (95, 103, 105,
112-118). However, there have been suggestions that T_{EM} and TEMRA+ are not capable of
self-renewal and are rather continuously replenished by T_{CM} (102, 119, 120). In fact,
recent data compounds the issue even further by suggesting that the capacity for self
renewal is what differentiates T_{EM} from TEMRA+ cells in humans (102, 120). These
studies found that TEMRA+ T cells express low levels of Bcl-2 suggesting that they are
susceptible to apoptosis. However, others have suggested that TEMRA+ cells are long
lived and highly stable because of their high expression Bcl-2 (121, 122). While these
studies did find that TEMRA+ cells express high levels of anti-apoptotic markers, they also
found that there was no indication of current activation or cell cycle progression using
Ki-67 staining. One of the most convincing arguments for the long term maintenance of
T_{EM} and TEMRA+ cells in humans is the similarity in telomere length of T_{EM}, TEMRA+ and
T_{CM} (123, 124). Hence, it is likely that with regards to humans, T_{EM} and less certain,
TEMRA+ cells, are long lived independent of T_{CM} replenishment. However, this is a point
of debate in the literature and requires further analysis.

Three main hypothesis have been put forth to explain the progression and
differentiation of naïve to memory T cells during acute infection (Fig 1-3). The first
model is the divergent or segregation pathway that has been suggested by a few studies
(90, 125, 126). In this model, naïve T cells develop into separate lineages of either
effector T cells or memory T cells. Other studies suggest a second differentiation model,
the linear differentiation model, where T_{EM} serve as an intermediate step in the
progression of T cells from effectors to central memory T cells (96, 127-129). The third model is called the progressive differentiation model, which includes features of the decreasing potential and signal strength models (Fig 1-4). The progressive differentiation model proposes that the formation of both memory populations is much more dynamic and depends on the nature of the stimulating antigen, cytokine signaling and anatomical location (94, 97, 113, 130, 131).

The progressive differentiation model has gained much support in recent years and is now the predominating model for describing how memory CD8$^+$ T cells develop (discussed in further detail in chapter 3) (104, 119, 132-139). Hence, the discussion here will focus on this model. Some proponents of this dynamic model do not accept that TEM and TCM are static populations. Rather, these individuals propose that as these cell subsets recirculate between lymph and peripheral tissue, the cells alter phenotypic and functional properties to switch between effector and central memory (94, 97, 140, 141).

An important point to consider when thinking about this final model is that there are two slight variations combined in the progressive differentiation model with one proposition indicating that population heterogeneity is a result of multiple rounds of division (decreasing potential) and another suggestion that T$_{eff}$ (effector T cells), T$_{EM}$ and T$_{CM}$ fate is determined before initial division (signal strength). To unify these models into the progressive differentiation model, signal strength can commit cells to certain lineages during initial stimulation, but then further stimulation during subsequent rounds of replication can promote differentiation towards generation of ‘decreased potential’ T$_{EM}$ and T$_{eff}$ cells (136).
These $T_{EM}$ and $T_{eff}$ subsets are considered to have ‘decreased potential’ because of their lower ability than $T_{CM}$ and $T_N$ (naïve T cells) to undergo homeostatic proliferation (102, 142). In addition, $T_{CM}$ are more closely related to $T_N$ cells based on analysis of gene expression profiles and cytokine signaling, whereas $T_{EM}$ and, more so, $T_{eff}$ display profiles of further differentiation. Essentially, the more differentiated cell becomes during the primary response, the less capacity or ‘potential’ it has to proliferate and maintain long term. Therefore, ‘potential’ ranges from $T_N > T_{CM} > T_{EM} > T_{eff}$ and provides the basis for the progressive differentiation model.

Identification of particular memory subsets is accomplished with the use of cell surface phenotyping and the observation of functional attributes, i.e. perforin storage, cytokine production or the presence of anti-apoptotic markers such as Bcl-2. The majority of studies have been able to identify different memory subsets based on surface phenotype and have identified similar functional properties for cells of each phenotype. Cell surface phenotyping of memory $CD8^+$ T cells is often done by using a combination of antibodies against CD27, CD28, CD45RA, CD45RO, CD62L, CCR7, CCR5, and CD127(93, 105, 121, 143-146). Using these cell surface molecules allows for phenotyping of each cell at various phases of the immune response. Depending on the combination of the surface molecules expressed by each cell type, it can be determined if the cell is $T_N$ (naïve), $T_E$ (effector), $T_{EM}$ (effector memory), $T_{EMRA}^+$ (effector memory with expression of CD45RA), or $T_{CM}$ (central memory) (Figure 1-5). Although variations occur from these main subsets of memory cells, the majority of memory $CD8^+$ T cells can generally be classified as belonging to one of these major subsets.
Figure 1-3 Various models to illustrate the establishment of CD8⁺ T cell memory including the divergent model and the linear differentiation model. The divergent model proposes that effector cells and memory cells are different subsets entirely. The linear differentiation model proposes that central memory cells are the end product of a transitional process from naïve to effector to effector memory and finally to central memory. The difference between the divergent and signal strength model (presented in figure 1-4) is that in the signal strength model, effector memory cells and central memory cells are different lineages. The divergent model separates effector cells from both subsets of memory cells. Figure adopted from Kalia et al. (147)
Figure 1-4 The decreasing potential model and the fixed lineage model (signal strength) can be combined to generate the progressive differentiation model. Signal strength can introduce population heterogeneity and then stimulation during the response can generate further heterogeneity (decreasing potential). See Chapter 3 for more detail. Adopted from Kaech and Wherry(136).
Figure 1-5 Use of surface molecules to phenotype cells. Cell surface molecules used in the following studies are CD45RA, CD62L, CCR7 and CD127. Expression of each molecule on each cell subset is as follows: T_{N}(CD45RA^+, CD62L^+, CCR7^{hi}, CD127^+), T_{E} (CD45RA^+, CD62L^-, CCR7^{hi}, CD127^{lo}), T_{EM}(CD45RA^-, CD62L^+, CCR7^{hi}, CD127^{lo}), T_{EMRA}^+ (CD45RA^+, CD62L^-, CCR7^{lo}, CD127^{hi}) and T_{CM} (CD45RA^-, CD62L^+, CCR7^{hi}, CD127^{hi}). Adopted from Wherry et. al.(144)
CHAPTER 2

Specific Aim 1: Determine the frequency and functional properties of the various memory CD8+ T cell subsets present in individuals previously vaccinated against smallpox

Preface

Identification of HLA Class I restricted T cell epitopes is important to develop methods to track the evolution of T cell memory to new generation smallpox vaccines and allow comparison to older vaccinia virus preparations known to induce protection against smallpox. We evaluated the relative predictive values of four computational algorithms to identify candidate 9-mer HLA-A2 supertype epitopes that were confirmed to preferentially stimulate T cell IFN-γ responses by persons last vaccinated with Dryvax 27-54 years previously. Six peptides encoded by 14L, G1L, A8R, I8R, D12L and H3L open reading frames that were identical for Vaccinia (Copenhagen), Variola major (Bangladesh 1975) and modified vaccinia Ankara strain preferentially stimulated IFN-γ responses by healthy HLA-A2 supertype adults last given Dryvax 27-49 years earlier relative to remotely vaccinated non-HLA-A2 supertype and unvaccinated HLA-A2 supertype adults. Combining results from at least two computational algorithms that use different strategies to predict peptide binding to HLA-A2 supertype molecules was optimal for selection of candidate peptides that were confirmed to be epitopes by recall of T cell IFN-γ responses. These data will facilitate evaluation of the immunogenicity of replication incompetent smallpox vaccines such as modified vaccinia Ankara and contribute to knowledge of poxvirus epitopes that are associated with long-lived T cell memory.
**Introduction**

In order to most effectively understand the potential protection afforded by new smallpox vaccines, the immunological memory, or correlates of protection, elicited by traditional smallpox vaccines such as Dryvax must be identified. Ideally, individuals vaccinated one to ten years previously with Dryvax would be used as study participants in order to have the best chance of obtaining high frequencies of memory cells to identify characteristics of immunological memory. However, due to unforeseen political restrictions and to the eradication campaign and termination of mass vaccination in the Unites States as of the early 1970s, a large cohort of individuals recently vaccinated with Dryvax was unavailable. Therefore, the presence and long-term maintenance of memory CD8$^+$ T cells in people last vaccinated against smallpox during the eradication campaign (over 25 years ago) was examined. This methodology provided useful data to understand the immunological correlates of protection, with regards to the memory CD8$^+$ T cell populations, present in individuals that were previously vaccinated against smallpox with traditional smallpox vaccines. In addition, we were able to better realize the duration of the CD8$^+$ T cell memory subsets among previously vaccinia vaccinated individuals, which has been found to exist for 75 years and potentially even life long (77-79). Finally, the experiments described here provide data regarding the phenotypic characteristics and functional properties of the effector and central memory T cells.

Characterizing these memory cell phenotypes, cytokine profiles and cytotoxicity potential supplied us with detailed data on the likely correlates of protection provided by these vaccinia specific memory CD8$^+$ T cells. With this primary data, we were able to determine the nature of a protective memory CD8$^+$ T cell population and will be able to
use this information as a standard when comparing the memory CD8$^+$ T cells that develop following immunization with new generation smallpox vaccines, such as MVA. This is the essence of comparative quantitative immunology.

*Development of Vaccinia Specific Tetramers*

Isolated vaccinia specific memory cells were required in order to perform these studies. Therefore, vaccinia specific memory cells required a manner of identification for further analysis. In order to accomplish this task, vaccinia specific HLA-A2 (human leukocyte antigen) tetramers were constructed that were designed to identify vaccinia specific memory CD8$^+$ T cells in individuals with a MHC (major histocompatibility complex) class I locus belonging to the HLA–A2 supertype. Tetramers have been used in humans to study specific cells (148, 149). Tetramers provide the opportunity to observe the immune response at the single cell level, which is useful not only in observing immunological evolution with regards to cell phenotypes, but also in the study of T cell clones present during the various phases of the response. In addition, tetramers allow for the isolation of vaccinia specific T cells without requiring prior stimulation. Intracellular staining for cytokines and other assays such as cytotoxicity and proliferation assays require stimulation of memory cells for identification purposes. However, in order to describe the various memory CD8$^+$ T cell subpopulations accurately, it was impractical to stimulate the cells because stimulation results in activation and changes in cell surface phenotype and functional properties. Therefore, tetramers allow for direct *ex vivo* identification and analysis.

Tetramers are used to identify CD8$^+$ T cells with a particular T-cell receptor (TCR). The first step in developing tetramers is to identify peptides that are recognized
by T cells upon engagement of their TCR, with the peptide of interest complexed in the MHC. There are approximately 200 proteins expressed by Orthopox viruses that vary in length from 20 amino acids to over 100 (32). Peptides for class I tetramers are typically 8-10 amino acids in length. Hence, there are over a million potential peptides that could be used in vaccinia specific HLA-A2 restricted tetramers. Therefore, in order to have the best chance of developing successful tetramers, we established several selection criteria to narrow down the list of potential epitopes.

The first selection criterion established was to selected proteins expressed under the control of vaccinia early promoters. The reasoning for instituting this selection criterion is because it has been suggested that due to the nature of Orthopox virus infection, proteins expressed early upon infection are the most likely candidates for immunogenic peptides (150, 151). This concept is better understood if a brief review of Orthopox virus replication is examined.

The Orthopox genus of viruses invades host cells and immediately uncoats within the cytoplasm of the cell (27, 31, 152). Within the virion core is the viral machinery to undergo transcription (153). Genes that are expressed immediately after viral uncoating are called the early genes (37). About half of the viral genome is expressed during this time followed by DNA replication at about two hours post-invasion. With the initiation of DNA replication, the expression of vaccinia early genes is terminated (154). Within this early genes subsets, many proteins are expressed by the virus including proteins participating in DNA replication, nucleotide biosynthesis, and host defense (155). Since these early genes are the first to be synthesized within the host cell, they are thought to be the first to undergo MHC class I processing and presentation. Data was recently
provided in support of the notion that these early genes dominate the T cell clonotype hierarchy because of the heavy influence on the timing in antigen expression (156).

In further support for the selection of proteins expressed under the control of vaccinia early promoters are data from previous studies (150, 151, 157). These studies found that when proteins from other organisms were placed into a recombinant vaccinia vector under the control of early, intermediate and late promoters, genes expressed under the control of early promoters induced the strongest T cell immune responses. Hence, vaccinia early protein sequences were used for the derivation of epitopes as potential candidates for loading into class I tetramers. The early genes identified from various sources, including the literature and PubMed (www.pubmed.gov), are indicated in Table 2-1. After selecting early genes for potential epitopes, there were over 8000 potential peptides and more selection criteria were required.

The second selection criterion established was to identify peptides that were suspected to have a high affinity for HLA-A2 based on predictive algorithms and molecular modeling. The HLA-A2 molecule was used because it has been the most characterized of the HLA class I molecules and is the most prevalent HLA class I molecule in the American population (158-162). Protein sequences from vaccinia early proteins shared by Variola major (Bangladesh-1975 GenBank Accession Number L22579), Vaccinia virus (Copenhagen-M35027) and MVA (U94848) were then entered into various algorithms which predicted peptides suspected to have a high binding affinity to HLA-A2 (163-165). The algorithms used were BIMAS, SYFPEITHI and MULTIPRED, which are publicly available databases (166-169). Each model uses different methods to calculate binding affinity: BIMAS uses quantitative matrices,
SYFPEITHI uses binding motifs, and MULTIPRED uses both artificial neural networks (ANN) and hidden Markov models (HMM) as prediction engines. Cut off values to predict peptide binding were established for each of the models based on precedents from the literature and established standards based on known high affinity peptides. These selection thresholds were set at scores ≥1000 for BIMAS, ≥25 for SYFPEITHI, ≥4.0 for the ANN and ≥7.0 for the HMM.

During the first phase of selection, peptides that scored above the set threshold in one of the four models were selected for further analysis. These peptides selected from the first phase of analysis were then compared in the second phase and peptides that scored above the selection threshold in two or more of the models were selected for further analysis (n=34). Although a few of the peptides did not score above the selection threshold in two or more of the models, they were also included because their scores were close enough to the selection threshold that they could not be definitively excluded (n=7). In addition, three peptides that did not meet the selection threshold in any of the models were selected as negative controls. The results from the various algorithms and the 44 peptides synthesized are listed in Table 1. Following the results from these two selection criteria, we extrapolated 44 peptides expressed early upon infection by vaccinia virus and have a high affinity for HLA-A2. However, we did not yet know if one, these peptides were actually processed and presented via MHC, or two, if there is a population of CD8+ memory T cells in vaccinia immunized individuals that recognize any of these 44 peptides(170, 171). Therefore, we established one more selection criterion.

The third selection criterion established was that the peptides had to induce a recall response in only vaccinia immunized individuals. Therefore, these 44 peptides
were screened by IFN-γ ELISPOT for their ability to recall a CD8+ T cell response be
PBMC isolated from healthy adults previously given vaccinia virus. IFN-γ responses
were chosen as a screen due to the fact that this is the cytokine most consistently induced
after vaccinia infection (82).
**Materials and Methods A**

*Computational algorithms to predict epitopes*

Poxvirus ORFs predicted to be expressed early after infection and identical in sequence for VV (Copenhagen, GenBank Accession #M35027), MVA (GenBank Accession #U94848), and Variola major (Bangladesh 1975, GenBank Accession #L22579) were identified. The amino acid sequences were entered into four computational models that predict peptide binding to HLA class I molecules. BIMAS (166), SYFPEITHI (167) and MULTIPRED (172, 173) are publicly available Web servers. BIMAS uses quantitative matrices, SYFPEITHI uses binding motifs, and MULTIPRED uses both artificial neural networks (ANN) and hidden Markov models (HMM) as prediction engines. Cut off values to predict peptide binding were established for each of the models based on precedents from the literature and established standards based on known high affinity peptides. These selection thresholds were set at scores ≥1000 for BIMAS, ≥25 for SYFPEITHI, ≥4.0 for the ANN and ≥7.0 for the HMM. The primary criterion for synthesizing a peptide was a cut off value that exceeded the threshold score in two or more of algorithms.

*Human subjects*

Seventy-four healthy American adults ranging in age from 21 to 73 years were recruited. Donors completed a questionnaire describing their history of smallpox vaccination that included the time of last vaccination and number of times vaccinated. Low resolution tissue typing was performed by polymerase chain reaction using HLA class I specific primers in a 32-well format that detected HLA-A supertype (MSSP Class I locus specific; One Lambda). Members of the HLA-A2 supertype include all HLA-A*02 subtypes.
(HLA-A*0201, *0203…, HLA-A*6802, HLA-A*6901) (174). Analysis of typing results was done using DNALMT software provided by the manufacturer. Blood donors were placed into one of three study groups: 1) HLA-A2 supertype/previously vaccinated; 2) non-HLA-A2 supertype/previously vaccinated; and 3) HLA-A2 supertype/never vaccinated. The study was approved by the Human Investigations Institutional Review Board of Case Western Reserve University and University Hospitals of Cleveland.

**IFN-γ ELISPOT**

PBMC were isolated from heparin anti-coagulated blood by Ficoll-Hypaque gradient centrifugation, suspended in RPMI containing 5% fetal bovine serum, HEPES, L-glutamine and gentamicin, and placed in wells of a 96-well multiscreen plate (Millipore) at a concentration of 2.5 x 10^6 cells per ml. Peptides (synthesized by Sigma-Genosys; >95% purity) for PBMC IFN-γ recall responses were added at a final concentration of 10 µg/ml from a stock concentration of 1 mg/ml diluted in dimethyl sulfoxide and phosphate buffered saline, pH 7.4. Phytohemagglutinin (PHA) (Sigma Aldrich) at a concentration of 5 µg/ml and PBS/dimethyl sulfoxide were used as positive and negative controls, respectively. Plates were incubated at 37°C in air with 5% CO₂ for 42 hours. ELISPOT assays for IFN-γ were performed using primary non-biotinylated and secondary biotinylated antibodies (Pierce) and developed using HRP-conjugated streptavidin peroxidase (Dako) and 1% AEC (Sigma Aldrich) in 0.1M acetate buffer with 0.015% H₂O₂ (175). The plates were read using Immunospot technology (Cellular Technologies Ltd.). Results are expressed as the number of IFN-γ spot forming units (SFU) per 10^6 PBMC.
Statistics

Responses to peptides by PBMCs from previously vaccinated HLA-A2 supertype donors were compared to responses by previously vaccinated persons who did not have HLA-A alleles belonging to the A2 supertype and to responses by non-vaccinated HLA-A2 supertype donors. The number of IFN-γ SFU in peptide-containing and control wells were compared for significance of differences using a chi square test (SAS version 8.2). A positive response was scored if the number of SFU in peptide-containing wells was greater than control wells with $\alpha \leq 0.05$. Only PBMC donors with a positive response to PHA were included in the analysis.
Results A

Comparing previously vaccinated HLA-A2 supertype to previously vaccinated non-HLA-A2 supertype individuals, the range of the proportion of responders to at least one of the 34 peptides predicted to bind to HLA A2 supertype alleles by two or more the computational algorithms using the initial threshold criteria was 0 to 41 and 0 to 20 percent, respectively (Fig. 2-1; peptides A16L_259 through B19R_7 are indicated on the X axis). None of the unvaccinated HLA-A2 supertype donors - group 3 above – had PBMC that produced IFN-γ when incubated with any of these 34 peptides. The range of response rates to the seven peptides that met the selection criterion in only one algorithm (E9L_107 through F10L_209 indicated on the X axis of Figure 1) was 0 to 32 and 0 to 15 percent in previously vaccinated individuals belonging to the A2 and non-A2 supertypes, respectively. Only 1 of 17 HLA-A2 supertype donors who had never been vaccinated had an IFN-γ recall response to one of these peptides (H3L_104). The three peptides that did not meet the initial selection criteria for any of the four algorithms (C7L_74, H6R_86 and E8R_264 on the X axis) stimulated PBMC IFN-γ responses by 9 and 10 to 25 percent of previously vaccinated A2 supertype and non-A2 supertype individuals, respectively. Responses to these peptides were not observed in the group of 17 A2 supertype participants who had never been vaccinated. The strength of IFN-γ responses by previously vaccinated HLA-A2 supertype donors did not correlate with number of vaccinations or the time since last vaccination.

To evaluate further the value of the computational algorithms for predicting VV HLA-A2 restricted epitopes, we calculated the average number of individuals per group that recognized any of the 34 peptides predicted to bind to HLA-A2 by at least two of the
computational algorithms. On average, previously vaccinated HLA-A2 supertype donors recognized 2.38 (SD 1.71) peptides while vaccinated donors having a non-HLA-A2 supertype recognized 1.21 (SD 1.07) peptides (p<0.001). When IFN-γ responses to the 7 peptides selected based on predictions by a single computational model were analyzed in this way, the averages were 2.43 (SD 2.37) and 1.71 (SD 1.25) for A2 and non-A2 supertype individuals, respectively (p =0.5). We conclude that the combining results from more than one algorithm has better prediction accuracy than results from one algorithm using the initial set of cut off values. On the other hand, there was no difference in predictive value of combined versus one algorithm when responses by HLA-A2 supertype donors alone were considered.

There were eight peptides identified as showing the greatest differences in the frequency of IFN-γ responses in the group of previously vaccinated A2 supertype donors compared to unvaccinated A2 supertype donors (I4L, G1L, A8R, I8R, D12L, B19R, F10L and H3L) (Fig. 2-2). However, two of these peptides showed significant cross reactivity in non-A2 supertype individuals. Therefore, only six of these peptides encoded by the I4L, G1L, A8R, I8R, D12L and H3L VV genes were identified as being most immunogenic. The magnitude of IFN-γ responses to these peptides ranged from 32-950 SFU per 10^6 PBMC in previously vaccinated A2 supertype persons and 34-828 IFN-γ SFU per 10^6 PBMC in previously vaccinated non-A2 supertype donors (Fig. 2-3). To examine whether responses by the non-A2 supertype group were related to high peptide affinity for HLA A alleles that were not members of the A2 supertype, the BIMAS and SYFPEITHI algorithms were re-run for these peptide sequences and HLA supertypes of these individuals. These included HLA alleles that are members of the A1, A3, A19, and
A24 supertypes. None of the peptide sequences exceeded the cut-off values that predict high binding to members of these supertypes (≥1000 for BIMAS and ≥25 for SYFPEITHI). ANN and HMM were not included in this analysis.

Bioinformatics analysis by Entrez Protein indicated that the I4L gene product is a ribonucleotide reductase, G1L a metalloproteinase, A8R regulates intermediate gene transcription, I8R an RNA helicase, and D12L, an mRNA capping enzyme. H3L is a membrane or envelope protein that has been used in others studies of poxvirus immunity (176). It was the only peptide encoded by a non-early gene examined in our study. In addition, the epitope from the H3L protein was the only epitope that elicited an IFN-γ response by A2 supertype donor who had never been given VV. The C16L_29, C7L_74 and E9L_107 peptides, described by others (75, 177), showed no significant differences in their ability to preferentially stimulate PBMC IFN-γ production by HLA-2 vaccinated individuals versus unvaccinated HLA-A2 controls (data not shown).

To explore the possibility that the differing stringencies applied to each algorithm were responsible for limiting concordance, the predictive algorithms was rerun so that the threshold values would be similar. The threshold values were changed to ≥3.8 for ANN and ≥6.5 for HMM, that of BIMAS and SYFPEITHI kept constant at ≥1000 and ≥25. Defining a T cell epitope based on the ability of a peptide to elicit an IFN-γ response in at least three vaccinated HLA-A2 donors, 16 epitopes were obtained (Table 2-2). In this scenario, our new selection criteria revealed that the best individual method was SYFPEITHI (14 of 16 predictions), second best BIMAS (12 of 16), third best HMM (9 of 16) and last ANN (7 of 16 each). Combined ANN and HMM had 11 of 16 correct predictions. Combining two or more models produced 15 of 16 correct predictions. One
issue with changing threshold values post-peptide selection is that altering the selection
threshold would have provided for the addition of other peptides from the approximately
8000 in our initial screening
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<th>ANN(^c)</th>
<th>HMM(^c)</th>
<th>Concordance</th>
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\(^a\) http://bimas.cit.nih.gov/molbio/hla_bind/ (166)
\(^b\) http://www.syfpeithi.de/ (167)
\(^c\) http://research.i2r.a-star.edu.sg/multipred/ (173)
\(^d\) grey highlighting indicates a value over the set threshold value
Figure 2-1  Proportion of HLA-A2 and non-HLA-A2 vaccinees responding to 44 predicted VV peptide epitopes. Black columns indicate vaccinees belonging to the HLA-A2 supertype (n = 22) and grey columns indicate vaccines not belonging to the HLA-A2 supertype, i.e. non-HLA-A2 vaccinees (n = 20). Naïve subjects’ responses were not included because only one peptide induced a response in one naïve individual (Fig. 6). Time since last vaccination ranged from 27 to 49 years. Dashed lines separate the 34 ‘high’ binders from the ‘low’ binders and non-binders.
Figure 2-2 Selected epitopes selected from the initial 44 because of their high proportion of response from previously vaccinated HLA-A2 individuals to previously vaccinated non-HLA-A2 individuals. Only six (in bold) were selected for tetramer analysis. The low percentage of HLA-A2 unvaccinated responders was a trend for all 44 peptides tested.
Figure 2-3  Level of IFN-γ secreting T cells in response to selected peptide epitopes by PBMC from previously vaccinated individuals. Dark squares refer to HLA-A2 individuals and open squares refer to non-HLA-A2 individuals. The high responses (>400 SFU) in the non-A2 columns for peptides D12L_174 and H3L_104 are from the same non-A2 individual. All values represented here are statistically significant over background values.
Table 2-2  16 peptides that induced an IFN-γ response in at least three previously vaccinated individuals belonging to the HLA-A2 supertype with newly assigned threshold values and concordance

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*Indicates the number of peptides out of 16 that a particular model was able to predict as a high binder to HLA-A2

b grey highlighting indicates a value over the new set threshold value
c ANN and HMM were combined under Multipred (173)
Although all six immunogenic peptides could be used in tetramer studies, only three were synthesized for the initial experiments. The three selected peptides were G1L_508, D12L_174 and H3L_104. These peptides were selected because G1L is the most specific for vaccinated HLA-A2, while D12L and H3L induced recall responses in the highest percentage of vaccinated HLA-A2 as compared to the other peptides. Tetramers were synthesized with these three peptides at the National Institute of Allergy and Infectious Diseases (NIAID). The experiments with these tetramers will confirm the extent that the predicted epitopes are CD8+ specific based on the tetramers’ ability to bind to CD8+ T cells.

Various protocols were attempted in order to observe CD8+ tetramer+ T cells. PBMC were either unstimulated or stimulated with peptide or live vaccinia virus for 24 to 96 hours. However, the observation of tetramer positive CD8+ T cells required incubating PBMC from previously vaccinated individuals with live vaccinia virus for 96 hours. Then, CD8+ T cells were isolated by negative selection using magnetic coated beads (Miltenyi). 1 million CD8+ T cells were subsequently stained with tetramer (1:100 dilution), and fluorescently labeled antibodies against CD3+ and CD8+ (pretitered by manufacturer BD Biosciences). The cells were analyzed on a flow cytometry machine (LSR II BD Biosciences) and 1 million gated CD3+, CD8+ events were collected. Data were then analyzed via WinList (Verity). The number on each histogram corresponds to the number of tetramer positive CD3+ CD8+ cells per million CD3+ CD8+ cells. Pseudo-positive results were obtained when using tetramers D12L_174 and H3L_104 (Fig. 2-4). Tetramer G1L_508 consistently provided unspecific binding even at the lowest titrations.
Previously vaccinated

Unvaccinated

Tetramer

CD8

1003

463

1410

625
Figure 2-4  Tetramer positive CD8 T cells present in PBMC from previously vaccinated and unvaccinated individuals after stimulation with vaccinia virus. PBMC from two previously vaccinated individuals and two unvaccinated individuals were isolated and stimulated with vaccinia virus (Western Reserve) for 96 hours. A. Tetramer synthesized with peptide 43 (SLFKNVRLL) showed significant background in one unvaccinated control. However, this tetramer positive population was one half that of the tetramer positive population observed in the individual previously vaccinated. B. Tetramer synthesized with peptide 48 (ALWDSKFFT) showed low background.
The data from the tetramer studies are not conclusive as to whether there are tetramer\(^+\) CD8\(^+\) T cells in previously vaccinated individuals. In addition, PBMC required stimulation for 96 hours to obtain these data. Prior stimulation is not acceptable as indicated previously because the memory cells begin to activate, differentiate and alter their phenotypic and functional characteristic. Therefore, it is impossible to determine the different memory subsets, let alone, any of the functional characteristics of each distinct memory population. Therefore, further tetramer work would be required in order to develop vaccinia specific tetramers that would identify decisively vaccinia specific CD8\(^+\) T cells without stimulation.

However, developing tetramers with these characteristics is going to be extremely difficult for a couple reasons. First, finding dominant epitopes from such a large virus has proved difficult (178, 179). As stated previously, there are approximately 200 proteins expressed by vaccinia virus upon infection. Therefore, CD8\(^+\) T cells responses are directed against many different targets and not against one or two dominant peptides as seen with LCMV infection in mice or CMV and EBV infection in humans (96, 105, 180, 181). Second, since there are likely few, if any, dominant epitopes upon vaccinia infection in humans, recently vaccinated individuals are required. Individuals vaccinated recently (<1 year) will have the highest frequency of all vaccinia specific T cell clonotypes and therefore, populations of CD8\(^+\) T cells at a high enough frequency to be detected by tetramer analysis(75, 83). As the time following vaccination increases, the frequency of circulating vaccinia specific memory cells decreases(78). Therefore, after 25 years following vaccination, the frequency of circulating memory cells of one particular clonotypes is likely too low for tetramer analysis.
We had anticipated that the use of the tetramers would not only indicate vaccinia specific T cells, but also allow for further characterization of the memory phenotypes of the memory cells. Adding our tetramers in addition to fluorescent tagged antibodies such as anti-CD8, CD4, CD3, CD127, CD45RA, CCR7, CD62L IFN-γ and TNF-α to the cells would have provided us with the opportunity to observe the functional properties of the various vaccinia specific memory T cell subsets(182). In addition, if the frequency of the peptide specific CD8 T cell population were high enough, memory proliferation assays and cytotoxicity assays would have been done on effector and central memory cell populations to observe the functional properties and expansion dynamics of the two populations.
CHAPTER 2 – PART B

Specific Aim 1: Determine the frequency and functional properties of the various memory CD8⁺ T cell subsets present in individuals previously vaccinated against smallpox

Preface

The objective of this study was to define the molecular phenotypes of long-lived memory T cells in persons last given Dryvax prior to declaration of smallpox eradication. Persons last vaccinated during the eradication campaign (>25 years ago) demonstrated vaccinia-specific CD4⁺ and CD8⁺ T cells identified by increased expression of the activation marker CD69. CD8⁺ effector memory (IFN-γ⁺, CCR7⁻) and CD8⁺ effector memory CD45RA⁺ cells were detectable in remotely vaccinated individuals by intracellular cytokine staining. In addition, central memory CD8⁺ cells were detected in the peripheral blood of recently vaccinated individuals utilizing a unique proliferation assay. The long-term maintenance of CD8⁺ T cell memory cells suggests that tracking of the evolution of such populations will be helpful in evaluating the immunogenicity of new generation smallpox vaccines based on replication deficient poxviruses such as Modified Vaccinia Ankara strain.
Introduction

The development of vaccinia specific tetramers that are capable of identifying CD8+ memory T cells in individuals last vaccinated with vaccinia over 25 years ago is too time consuming and may not be as useful as originally thought in light of recent data as discussed previously (178, 183). However, vaccinia specific CD8+ memory cells can be identified by intracellular cytokine staining (ICCS), cytotoxicity assays and proliferation assays(76, 78-80, 85). These protocols require stimulation of cells with live replicating VV in order for effective identification of antigen specific memory cells. For instance, Hammarlund et. al. were able to identify memory CD8+ memory cells by ICCS with stimulation of PBMC with live vaccinia virus for only 18 hours (78). Therefore, we decided to use this technique because the time of stimulation is long enough that memory cells become activated, but short enough that these memory cells will not change phenotype. Hence, a multiple color ICCS technique was used, with antibodies against CD3, CD4, CD8, CD69, CD62L, CCR7, CD127, IFN-γ and IL-2, to reveal the presence of vaccinia specific memory cells in individuals recently vaccinated with smallpox (<4 years) and in individuals over 25 years post-vaccination. The presence of antigen specific CD8+ memory T cells capable of producing IFN-γ in numerous vaccinated individuals was demonstrated. This flow cytometric technique allowed us to explore the use of CD62L and CCR7 in surface phenotyping memory CD8+ T cells, as well as the dynamic expression of CD127. Most CD8+ IFN-γ+ cells from remotely vaccinated individuals were CCR7lo CD45RA+ (TEMRA+) with some individuals presenting a CCR7lo CD45RA− (TEM) population. Moreover, all CD8+ IFN-γ+ cells from remotely vaccinated individuals expressed CD127.
Materials and Methods B

Study Subjects
Healthy subjects were selected following standard Case Western Reserve University Internal Review Board (IRB) protocol. In addition to informed consent, individuals were asked to fill out a questionnaire regarding their vaccination history. In the case that subjects had received more than one vaccination, the last date of vaccination was used to establish years post vaccination. Eight individuals were not vaccinated (negative controls), one individual had recently received a primary vaccination within the last year (positive control), one other individual was included three years after primary vaccination (positive control) and the remaining individuals (n=30) had all been vaccinated during the eradication campaign (number of vaccines received per person ranged from 1 to 5). Only blood was obtained from the subjects via venopuncture.

PBMC Isolation
Blood drawn from the study subjects was collected in Vacutainer tubes containing heparin sulfate (BD Biosciences). PBMC were isolated from the collected blood by Ficoll-Hypaque (GE Healthcare) Gradient Centrifugation and resuspended in RPMI (Cambrex) supplemented with 5% FBS, L-glutamine, HEPES, penicillin and streptomycin.

In Vitro Activation
Sucrose gradient purified Vaccinia Virus (VV) Western Reserve Strain (WR) was provided by Dr. Mark Buller at St. Louis University following standard propagation and titration protocol (184). HeLa cells were used for propagation and BS-C-1 cells for titration (185, 186). (A brief description of the technique is found on page 100). For
stimulation of memory CD8^+ T cells, one million PBMC were placed in a well of a 96 well plate (Corning) at a concentration of 5 million cells/ml and incubated with a predetermined optimal amount of VV at a MOI of 0.2. Plates were incubated at 37°C with 5% CO_2 for 6-90 hours. For TAPI-2 experiments, cells were preincubated at 37°C with 100μM TAPI-2 (Peptides International) for 1 hour prior to addition of virus. For ICCS, stimulated and unstimulated PBMC from study subjects were incubated with Brefeldin A at a concentration of 1μg/ml for an additional 6-12 hours before proceeding with intracellular staining protocol. PBMC incubation with VV for 24 hours provided the best results for CD69 staining, while 12 hours of VV stimulation followed by Brefeldin A for 6 hours was determined to be the optimal culture condition for 18 hour ICCS.

**Flow Cytometry Staining**

PBMC were harvested from *in vitro* cultures, washed with FACS buffer and were blocked with human IgG for 30 minutes at room temperature. Cells were then labeled with various combinations of antibodies against CD4-FITC (OKT-4) and CD69-PE (FN50) from Ebiosciences, CD8β-PE (2ST8.5H7) from Beckman Coulter, and antibodies against CD8α-APC-Cy7 (SK1), CD3-Pacific Blue (UCHT1), CD127-purified (hIL-7R-M21), CD62L-APC (DREG-56), CD45RA-FITC (HI100), CCR7-AlexaFluor647 (3D12), IFN-γ-PE-Cy7 (B27) and IL-2-PE (MQ1-17H12) obtained from BD Biosciences. A secondary antibody was employed for CD127 staining (Goat α-mouse Pacific Blue: Molecular Probes). IFN-γ and IL-2 intracellular staining was performed following modified protocol established by BD Biosciences utilizing only their Cytoperm/Wash buffer after an initial 20 minute incubation at room temperature with 1% paraformaldehyde. Samples were read by an LSR II machine collecting approximately 1-
2*10^5 CD8+ T cell events per sample. Analysis was performed using FlowJo software from Treestar. Unstimulated and stimulated PBMC from unvaccinated and recently vaccinated individuals (~1 year) were included in every experimental run to ensure proper controls.

**CFSE Proliferation**

PBMC from vaccinated and unvaccinated individuals were purified and CD62L+ cells were positively selected with magnetic beads (Miltenyi). Whole PBMC, CD62L+ cells and CD62L- cells from each individual were incubated in pre-warmed 0.1% BSA/PBS solution with carboxylfluorescin diacetate succinimidyl ester (CFSE) at a concentration of 2µM per million cells for 15 minutes at 37°C (Molecular Probes). CFSE staining was quenched by the addition of ice-cold cRPMI and subsequent incubation for 5 minutes on ice. CFSE labeled cells were washed an additional two times with PBS and for some conditions, labeled CD62L+ cells were reconstituted with unlabeled CD62L- cells or vice versa. All samples were resuspended at a concentration of 1 million cells/ml. PBMC, reconstituted PBMC, purified CD62L+ and purified CD62L- were incubated with or without VV at a MOI of 1 for 7 days at 37°C. After incubation, cells were washed and stained for 30 minutes at room temperature with antibodies against CD3-APC-Cy7 (SK7) and CD8-APC (RPA-T8) from BD Biosciences. Samples were washed and then read by an LSR II machine collecting approximately 10^4 CD3+ CD8+ T cell events per sample. Analysis was performed using FlowJo software from Treestar. Unstimulated and stimulated PBMC from unvaccinated and recently vaccinated individuals (~1 year) were included in every experimental run to ensure proper controls.
Results B

Activation of VV-specific CD4+ and CD8+ T-cells detected by upregulation of CD69 expression

CD69 is a c-type lectin expressed on the surface of activated T-cells. The CD69 molecule contributes to the induction of intracellular signaling and calcium flux upon T-cell receptor triggering. In remotely vaccinated individuals, CD69+ expression increased on average by 7.0% and 9.6% for CD4+ and CD8+ T-cells comparing PBMC exposed to VV versus those not exposed to VV (Fig. 2-5). In contrast, for VV naïve donors, there was no change in CD4+ CD69+ T-cell expression and only an increase of 0.3% for CD8+ CD69+ cells. VV-specific memory CD69+ CD4+ and CD69+ CD8+ T cells were detected respectively in nine of 30 (30%) and 14 of 30 (47%) remotely vaccinated individuals.

Cytokine expression by VV memory CD8+ T cells

The data above indicate that upregulation of CD69 could be used as a marker of persisting memory T-cells after smallpox vaccination, as others have demonstrated. However, because background levels, i.e. no preincubation with VV, of CD69 expression in bulk PBMC were relatively high for both previously vaccinated and unvaccinated control subjects, it was not possible to obtain reproducible data regarding the molecular phenotypes of such cells (data not shown). We therefore evaluated the capacity of VV-exposed PBMC to produce IL-2 and IFN-γ, cytokines that respectively identify central and effector memory CD8+ T-cells upon reactivation (20). PBMC for these experiments were incubated with VV for 18 hours before phenotyping to ensure activation of memory cells without differentiation.
Figure 2-5  CD69 expression by CD4⁺ and CD8⁺ T cells in stimulated and unstimulated samples from unvaccinated and vaccinated individuals.  A) Lymphocytes were gated, followed by CD3⁺ and further by CD4⁺ and CD8⁺.  CD69 expression on both the CD3⁺ CD4⁺ and CD3⁺ CD8⁺ lymphocyte populations are shown.  Flow cytometry dot plots from one representative individual.  An isotype control for CD69 was used in each individual and the difference between positive and negative events varied depending on the individual.  The use of the isotype control in each individual indicated where the gate should be set (Isotype background was less than 0.1% in every individual).  Numbers in histograms indicate percentages of CD69⁺ CD4⁺ and CD8⁺ T cells.  B+C) Graphical representation of all individuals tested for CD69 upregulation upon VV stimulation (B: CD4⁺, C: CD8⁺).  Percentage increase of CD69 expression over background samples is shown on the y-axis and groups of individuals indicating vaccination history are displayed on the x-axis.  Bars denote the average increase in CD69 expression from unstimulated versus stimulated samples in each study group.
CD8⁺ IFN-γ⁺ cells were detectable in PBMC obtained from previously vaccinated donors whereas they were not detectable in PBMC from unvaccinated donors (Fig. 2-6). Five of 16 remotely vaccinated donors had levels of VV-induced CD8⁺ IFN-γ⁺ production that were two-fold or greater relative to parallel aliquots of PBMC not incubated with VV. After subtraction of background, the frequency of CD8⁺ IFN-γ⁺ cells ranged from 33-115 per 10⁶ CD8⁺ T cells, with a mean of 64 (0.0064% of CD8⁺ cells). There was no correlation between the frequency of IFN-γ⁺ producing CD8⁺ cells and the number of vaccinations received or the time since last vaccination. Both individuals who were given a primary vaccination with Dryvax within three years before study had higher frequencies, i.e. 633 and 2332 CD8⁺ IFN-γ⁺ per 10⁶ CD8⁺ T cells. Unvaccinated negative control individuals did not show responses, i.e. there was no difference for aliquots of PBMC’s incubated with versus without VV.

IL-2 production above background levels was not observed for any of the individuals tested. To test if the limited stimulation time with VV affected the ability to detect intracellular IL-2, VV stimulation was continued for longer durations of time for PBMC provided by the two individuals vaccinated one and three years earlier. Even after these PBMC were stimulated for 42 and 96 hours, there was still no difference in the frequencies of IL-2 producing cells for VV stimulated and unstimulated cells cultured in media alone.

The antibodies initially selected to phenotype the IFN-γ⁺ CD8⁺ T cells were CD62L, CD45RA and CD127. Depending on the expression of these molecules on the cell surface, it can be determined if the responding cells are effector memory (T_{EM}: CD62L⁺ CD45RA⁻ CD127^{hi}) or central memory (T_{CM}: CD62L⁺ CD45RA⁺ CD127^{hi}).
CD8⁺ T cells. The results indicate that many of the responding cells were actually of an effector memory CD45RA⁺ phenotype, TEMRA⁺ (CD62L⁺ CD45RA⁺ CD127hi) (Fig. 2-7). The presence of this memory cell phenotype is not well documented in mice, but has been shown to be present in humans (121, 122, 187). The molecular profile of the TEMRA⁺ cell subset indicates that it is closely related to TEM cells (142). There seems to be a protective role of TEM cells in mice due to their immediate effector acquisition (59, 96, 97). In fact, a requirement of TEM cells to facilitate protection against vaccinia challenge in mice has recently been shown (59, 134). Therefore, the presence of this cell subset in vaccinia vaccinated individuals indicates a potential correlate of protection necessary for the protection from smallpox.
**Figure 2-6** Comparison of the frequency of IFN-$\gamma^+$ CD8$^+$ T cells per million CD8$^+$ T cells in an unvaccinated individual and individuals last vaccinated at varying time points. A) Dot plots were gated from lymphocytes and further from CD3$^+$ CD8$^+$ T cells. Numbers provided represent frequencies of responding CD8$^+$ T cells over background. CD3$^+$ CD8$^-$ cells were removed from analysis because due to the large number of events collected, a more accurate representation was achieved by selecting only CD3$^+$CD8$^+$ cells. B) Graphical illustration of IFN-$\gamma^+$ CD8$^+$ T cells over background from all 25 individuals separated into three groups based on vaccination history. For a response from a previously vaccinated individual to be considered positive, the number of IFN-$\gamma^+$ CD8$^+$ T cells over background had to be at least 3 standard deviations higher than the mean of the unvaccinated control individuals. The mean was 1.4 and the standard deviation was 3.8. Therefore, the cutoff value was set at 12.8 (dotted line). In addition, all positive samples (5/5 from >25 year group and 2/2 from <4 year group) had at least twice as many IFN-$\gamma^+$ CD8$^+$ T cells upon VV stimulation versus unstimulated background control samples from the same individual.
Figure 2-7 Frequency of each phenotype of IFN-γ+ CD8+ T cells in individuals previously vaccinated against smallpox. Although there appears to be a population of responding CD62L+ CD45RA+ T cells, the frequency was not above background. In addition, this cell subset was not present in all previously vaccinated individuals and seemed to be sporadic having no correlation with time since last vaccination. Once again, these are representative histograms from each of the previously vaccinated study groups.
CD62L vs. CCR7

CD62L has been used by many studies to phenotype memory cells. However, there have been studies that have indicated that CD62L is not a good marker to use when cells are stimulated ex vivo (188, 189). This concern is limited with regards to the data presented here because most of the responding cells are IFN-γ+ CD45RA+ CD127hi, which are considered TEMRA+. These cells were all CD62L− and even if this finding was due to artificial cleavage of CD62L, expression of CD62L on this cell type would classify them as T cells with a naïve phenotype. Naïve cells should not produce IFN-γ after 18 hours of stimulation. Therefore, this classification would be inaccurate (188).

However, there was still concern that the use of CD62L to phenotype activated memory cells may be producing inaccurate results. During our initial studies with CD62L, a large population CD8+ T cells capable of producing IFN-γ+ was found in individuals previously given smallpox vaccine. These IFN-γ producing cells were identified as CD62L− CD45RA+ or TEMRA+ cells. However, CD62L is cleaved upon stimulation by an enzyme identified as TNF-α converting enzyme (TACE) (190). In order to prevent the cleavage of CD62L upon cellular activation, cultures were incubated with TNF-α proteolysis inhibitor (TAPI-2) prior to stimulation.

TAPI-2 is a TACE inhibitor and has been shown to block CD62L cleavage (191). A recent study indicated that TAPI-2 prevents CD62L cleavage without effecting cytokine production (189). Results in Figure 12 show that incubation with TAPI-2 does prevent loss of CD62L without altering cytokine production. However, preventing CD62L cleavage with TAPI-2 incubation causes inaccurate results as the main population of IFN-γ producing cells is now phenotypically CD62L+ CD45RA+. That would classify
all these cells as naïve (T_N) and this is not accurate considering the immediate production of IFN-γ (188). Hence, upon activation and subsequent cleavage of CD62L, phenotyping memory cells based on this surface molecule becomes complicated. However, initial expectations were that it would take longer before cleavage and that dual staining with CD127 and CD45RA would provide definitive answers as to the phenotype of the responding cells. The inconsistency of CD62L was not considered during the original experiments. When CCR7 was used instead of CD62L, the IFN-γ producing cells were CCR7^lo CD45RA^+ (Fig. 2-8). These data indicate once again that the vaccinia specific memory CD8^+ T cells from previously vaccinated individuals are mostly T_{EMRA}^+. Regardless, CD62L was not used in subsequent memory identification studies.
Figure 2-8  TAPI-2 influence on CD62L expression. Addition of TAPI-2 to in vitro cultures leads to the acquisition of a population of CD62L⁺ CD45RA⁺ IFN-γ⁺ CD8⁺ T cells. This population of cells is not present under the same stimulation conditions without the addition of TAPI-2. In addition, if CCR7 is used, the main population of IFN-γ⁺ CD8⁺ T cells is CCR7lo CD45RA⁺. To the best of our knowledge, CCR7 is not cleaved upon cellular activation.
Phenotype of responding cells based on CCR7 and CD45RA expression

The antibodies selected to phenotype the VV-specific IFN-γ+ CD8+ T cells were directed against CCR7 and CD45RA. Results indicate that most responding cells were of an effector memory CD45RA+ phenotype (TEMRA+); three of 18 previously vaccinated individuals also had effector memory (TEM) responses (Fig. 2-9 and Table 2-3). The frequency of TEMRA+ responses among individuals last vaccinated more than 25 years previously ranged from 33 to 86 per 10^6 CD8+ cells; for TEM, the range was 29-31. The two individuals given Dryvax within the previous 3 years had a higher ratio of TEMRA+ to TEM cells, suggesting that TEMRA+ cells dominate the peripheral memory pool immediately after vaccination and decrease within three to four years after vaccination. The frequencies of TEMRA+ and TEM cell subsets for all individuals are presented in Table 1.

To confirm that VV-induced CD8+ IFN-γ producing cells were memory cells, these cells were evaluated for expression of CD127. CD127 is the IL-7α receptor, is involved in maintaining T cell survival, and may be detected on the surface of naïve and memory CD8+ T cells (105, 192, 193). Previously vaccinated and unvaccinated individuals were tested to identify CD8+ IFN-γ+ CD127^{hi/lo} populations. All of the CD8+ IFN-γ producing cells observed in PBMC from individuals >25 years post-vaccination were CD127^{hi}, whereas those from the two individuals vaccinated within the previous three years included populations that were CD127^{hi} or CD127^{lo} (Fig. 2-10).
Figure 2-9 IFN-γ+ CD8+ T cells in previously vaccinated individuals are mainly CCR7lo CD45RA+ (TEMRA+) upon 18 hours of VV stimulation. CCR7lo CD45RA- IFN-γ+ CD8+ T cells (TEM) were also detected in some individuals. These dot plots were gated from CD3+ CD8+ IFN-γ+ cells and are representative of the three groups of individuals that showed IFN-γ by CD8+ T cells.

Table 2-3 Frequencies of IFN-γ+ CD8+ TEM and TEMRA+ cells per million CD8+ T cells from PBMC of individuals previously vaccinated against smallpox.

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- 76 -
Figure 2-10  IFN-$\gamma^+$ CD8$^+$ T cells from previously immunized individuals were mainly CD127$^{hi}$. Representative dot plots from three individuals show CD127 expression profiles from gated IFN-$\gamma^+$ CD3$^+$ CD8$^+$ T cells. All IFN-$\gamma^+$ CD8$^+$ T cells from individuals 25 years post smallpox vaccination were CD127$^{hi}$ while individuals vaccinated more recently showed some CD127$^{lo}$ IFN-$\gamma^+$ CD8$^+$ T cells as well. Inset histograms show the same population of cells as in the dot plots (black solid fill) as compared to CD127$^{lo}$ control cells (black unfilled) and CD127$^{hi}$ naïve T cells (grey solid fill).
Proliferation of CD8+ T cells

No VV-specific T<sub>CM</sub> cells were detectable by intracellular staining for IL-2. However, data from experiments with CD69 staining suggested that there may be a population of reactivated memory cells that do not produce IFN-γ or IL-2 within the timeframe of our protocol. We hypothesized that these populations could be T<sub>EM</sub> or T<sub>EMRA</sub> cells that did not yet produce IFN-γ or that longer incubation periods with VV might be required to detect IL-2 production from T<sub>CM</sub>. However, as indicated previously, longer incubation times did not yield any conclusive data. Therefore, to address the presence of T<sub>CM</sub>, VV-induced proliferation by CD8+ T cells expressing CD62L were evaluated.

CD62L participates in the homing of T cells into lymph tissue, and as is the case for CCR7, its expression can be used to differentiate T<sub>CM</sub> (CD62L<sup>+</sup>) from T<sub>EM</sub> (CD62L<sup>−</sup>) populations. Therefore, in order to test for the presence of T<sub>CM</sub> cells, the proliferation potential of CFSE labeled CD62L<sup>+</sup> cells was evaluated. By labeling with anti-CD3 and anti-CD8 antibodies prior to FACS, the levels of proliferation by PBMC, CD62L<sup>+</sup> and CD62L<sup>−</sup> CD8+ T cells after exposure to VV were able to be compared.

CD8+ T cells contained in CFSE pre-labeled PBMC obtained from a donor vaccinated one year before study demonstrated high levels of proliferation (23.9% CFSE dilution with VV stimulation vs. 1.65% without VV stimulation) (Fig. 2-11A). In addition, the CD8+ T cell subset present in pre-labeled CD62L<sup>+</sup> cells contained in bulk PBMC proliferated in response to VV. In contrast, CD8+ T cells present in pre-labeled CD62L<sup>−</sup> cells contained in bulk PBMC from the same donor did not. However, the reconstitution into bulk PBMC of pre-labeled CFSE cell subsets and unlabeled CFSE cell
subsets decreased the sensitivity of the assay. Therefore, we wanted to determine if the CD8\(^+\) T cells within the CD62L\(^+\) population was able to proliferate in the absence of CD62L\(^-\) cells. Purified (>95%) CD62L\(^+\) CD8\(^+\) T cells showed substantial proliferation (48.1% CFSE dilution with VV stimulation vs. 4.1% without VV stimulation) as compared to CD8\(^+\) T cells in whole PBMC indicating that CD62L- cells are not required for CD62L\(^+\) CD8\(^+\) T cell responsiveness (Fig. 2-11B).

To assess the longevity of the CD8\(^+\) CD62L\(^+\) T cell population (T\(_{CM}\)), similar experiments were performed with cells obtained from one individual vaccinated three years before study and 16 individuals vaccinated 25 or more years earlier (Fig. 2-11C). Whereas the T\(_{CM}\) population was detectable in the individual given Dryvax three years earlier, this was not the case for any of the remotely vaccinated donors. It is important to note that while naïve and central memory CD8\(^+\) T cells both express CD62L, CD62L\(^+\) cells contained in PBMC from unvaccinated negative control subjects did not show any proliferation, indicating that the population capable of proliferation does indeed represent T\(_{CM}\) cells in vaccinated individuals.
**Figure 2-11** Proliferation of CD3⁺ CD8⁺ CD62L⁺ T cells. Histograms represent gated lymphocytes followed by gating on CD3⁺ CD8⁺ cells. A) Proliferation observed by PBMC, CFSE labeled CD62L⁺ cells with unlabeled CD62L⁻ cells and unlabeled CD62L⁺ cells with CFSE labeled CD62L⁻ cells from an individual vaccinated one year previously in response to VV. CFSE labeled populations are indicated by **. B) Purified CD62L⁺ T cells from a recently vaccinated individual (1 year) are capable of immense proliferation without any CD62L⁻ cell present. C) Proliferation of purified CD62L⁺ CD8⁺ T cells by a recently vaccinated individual (~3 years) and a representative individual vaccinated 25 years previously.
Discussion

Human T cell immunity and neutralizing antibodies induced by scarification with vaccinia virus (VV) in traditional smallpox vaccines such as Dryvax can persist for decades (73, 78, 194). Because of concerns with the safety of such vaccines, particularly in immunocompromised individuals and persons with increasingly common conditions such as atopic dermatitis and eczema, there is a need to identify VV epitopes in order to monitor the T cell immunogenicity of a newer generation of attenuated replication incompetent Orthopoxviruses such as MVA. Moreover, such information would help identify specific epitopes that induce long term immunological memory and inform the design of future protein subunit or DNA vaccines.

Using four computational algorithms to predict VV antigenic peptides that bind to HLA-A2 supertype alleles and a T cell screening assay, that does not require prior in vitro expansion of antigen-specific T cells by exposure to live VV, we identified six 9-mer epitopes that preferentially recalled IFN-γ T cell responses by HLA-A2 supertype individuals relative to previously vaccinated persons whose HLA-A alleles did not belong to the A2 supertype. These epitopes, encoded by 14L, G1L, A8R, I8R, D12L and H3L open reading frames and identical for VV, MVA, and Variola major, have a propensity to induce long-lived T cell immunity in humans since recall responses were detected in at persons last vaccinated against smallpox up to 49 years earlier.

Given the large genome of VV (~186 kbp), computational approaches to identify viral peptides that could potentially bind to HLA class I molecules were used to identify candidate amino acid sequences. While others have used this to approach with a single predictive algorithm in combination with assays of T cell responses by humans or in
HLA transgenic mice(75, 176, 177, 179, 195), four algorithms were used in the present study in order to mitigate the limitations inherent to each model, i.e. BIMAS is based on quantitative matrices which assume that each position of a peptide contributes independently in binding to HLA class I molecules (166); SYFPEITHI is based on common binding motifs for amino acids at specific positions within a peptide sequence(167); ANN capture complex relationships in data sets and avoid simplifying assumptions inherent in quantitative matrices; HMM use statistical models that capture complex relationships in data sets(173).

Our results demonstrate that the outcomes of these various computational algorithms were often not concordant. For example, with the exception of the A16L_259, J1R_102, and A46R_142 peptides described in Table 1, none of the peptides screened were predicted as a high binder by all four algorithms. In addition, H3L_104 scored extremely high in BIMAS (>8000) but poorly in the remaining models (only peptide C7L_74 scored lower in SYFPEITHI and ANN). This lack of concordance among the different models may be a result of the different strategies of the models to predict peptide binding as well as the differing stringencies of the threshold values set for each model. For example, for the two epitopes that elicited the highest percentage of responders in the current study, D12L_174 and H3L_104, D12L_174 was predicted as a high binder by two algorithms (SYFPEITHI/MULTIPRED ANN) whereas H3L_104 was predicted by only one (BIMAS). Hence, to explore the possibility that the differing stringencies limited the concordance and comparative predictive values of the four algorithms, the algorithms were re-run so that the threshold values were similar. This readjustment produced a result that showed that SYFPEITHI was the best single
method, i.e. it predicted 15 of 16 peptides that were observed to preferentially stimulate IFN-γ responses by previously vaccinated HLA-A2 supertype donors. Combining multiple prediction methods was also found to provide excellent coverage of T cell epitopes (Table 2-2). As a caveat, it is important to note that a limitation of these and other computational algorithms is that they consider only whether a peptide binds to HLA molecules without regard to whether the protein from which it is derived is processed or if the peptide engages its cognate T cell receptor (171).

Previous studies have described human T cell immunity to VV epitopes by persons given Dryvax and other VV products (75, 176, 177, 183, 195). Many of these observations included individuals who were last vaccinated within a few weeks of examination of T cell responses (75, 183, 195), a period when the frequency of VV-specific would anticipated to be high and include both effector and memory T cell population. In addition, antigen-specific T cell populations were often expanded by prior incubation with VV before assay application. Our approach differed in that only remotely vaccinated individuals (>25 years previously) were examined and PBMC were not pre-stimulate with VV in order to increase the frequencies of epitope-specific T cells. For example, Terajima and colleagues established CTL lines from PBMC of HLA-A*0201 persons given Dryvax and labeled CD8+ T cells using HLA-2 tetramers complexed with C16L and C7L 9-mer peptides (75). We found that none and only 2 of 22 remotely vaccinated HLA-A2 individuals had T cell IFN-γ responses to these peptides, respectively. The low observed response rate is likely related to the paucity of epitope-specific T cells present in PBMC that have not first been exposed to VV.
Oseroff and coworkers scanned 258 predicted VV open reading frames for peptide binding motifs to major HLA supertypes and determined the ability of 68 selected peptides to stimulate IFN-γ production following in vitro incubation of PBMC with VV (177). They described two HLA-A2 peptides that initiated a T cell response in previously vaccinated individuals that were also tested in the present study, C7L and E9L. In our study, 2 of 22 individuals whose HLA class I alleles belonged to the HLA-A2 supertype had T cell IFN-γ responses to peptides C7L and E9L. However, two and three of 20 non-HLA-A2 vaccinated donors also responded to the C7L and E9L peptides, suggesting that such responses are not highly restricted by HLA alleles exclusive to the A2 supertype. In this regard, five of the six VV HLA-A2 supertype immunogenic peptides examined also stimulated IFN-γ responses by four vaccinated individuals whose HLA class I alleles did not belong to the A2 supertype (see Fig. 2-1. HLA typing results for these four donors were: HLA-A3/A11 - A3 supertype; HLA-A1/A29 - A1 supertype; A24/A30 - A24 supertype; A23/A23 - A24 supertype). These results can be interpreted to mean that T cell responses to selected VV epitopes may bind to HLA alleles belonging to different A supertypes or to HLA-B or HLA-C alleles not examined here.

Including the six novel epitopes identified in the current report, there are to date a total of 18 VV HLA-A2 supertype epitopes shown to recall T cell IFN-γ and/or be targets of cytotoxic T cells in persons previously vaccinated against smallpox. These are summarized in Table 2-4. Given the differences in computational algorithms used to select these peptides, the vaccine history of the donors, and methods of confirming T cell immunity among the various studies, however, it is important to note that it is not yet clear which if any of these peptides is immunodominant or induces long term T cell
memory. For example, with respect to the value of computational algorithms for prescreening candidate epitopes, of the 13 HLA-A2 supertype peptides identified by Oseroff and coworkers (177) on the basis of stimulating T cell IFN-γ responses by VV pre-exposed PBMC from at least one donor given Dryvax within the previous year, 5 (38%) (F12L_FLTSVINRV, F12L_NLFDIPLLTV, VWR082_ILDDNLYKV, O1L_GLNDYLHSV, A36R_MMLVPLITV) met the selection criteria used in our computational exercise, i.e. high affinity binding to HLA-A2 in at least 2 of the 4 algorithms. However, if only the 4 peptides found to stimulate T cell immunity by at least 2 donors are considered (VWR082_ILDDNLYKV, E2L_KIDYYIPYV, O1L_GLNDYLHSV, A55R_YIYGIPLSL in Table 2-1), then 2 of 4 were identified in 2 of the 4 algorithms used, e.g. predictive accuracy of 50%. Conversely, if the criteria for defining a VV epitope based on a PBMC response by at least one HLA-A2 supertype vaccinee was altered, then our computational screening would have “correctly” predicted 40 of 44 (91%) epitopes.
### Previously Described HLA-A2 Epitopes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>BIMAS</th>
<th>SYFPEITHI</th>
<th>ANN</th>
<th>HMM</th>
<th>Citation</th>
</tr>
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<tr>
<td>H3La</td>
<td>SLSAYIIRV</td>
<td>159.970</td>
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<td>7.56</td>
<td>Drexler et. al.</td>
</tr>
<tr>
<td>A55R</td>
<td>AMLNGLIYV</td>
<td>2351.109</td>
<td>28</td>
<td>5.90</td>
<td>7.13</td>
<td>Dong et. al.</td>
</tr>
<tr>
<td>A47L</td>
<td>LLYAHINAL</td>
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</tr>
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<td>C16L</td>
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<td>27</td>
<td>5.87</td>
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<td>4.77</td>
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<td>Oseroff et. al.</td>
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</table>

- grey highlighting indicates a high score and a peptide that scored high in at least two models
- C7L was selected as an HLA-A2 restricted epitope from a previous study, but was not indicated to be a high binder by any of the models
- both F12L peptides induced responses in one previously vaccinated individual and were selected by at least two of the models as high binders. The remaining peptides that induced recall responses in less than three vaccinated individuals which were suggested by Oseroff et. al. did not meet the selection criteria in at least two of the algorithms
One further important point is that Pasquetto et. al. described one epitope that was analyzed in our study as well. A46R_142 induced a response in HLA-A201 transgenic mice according to Pasquetto et. al. (179). This peptide also induced a recall response in two HLA-A2 supertype individuals and three non-A2 supertype individuals in the present study. Hence, these results suggest that there is a certain degree of utility in using HLA-A2 transgenic mice to determine vaccinia A2 restricted peptides capable of inducing a recall response in individuals previously vaccinated against smallpox.

A goal of future work will be to broaden the search for immunodominant antigenic peptides beyond those derived from viral genes that expressed early after infection. Jing and colleagues (183) recently described the use a VV cDNA library to screen cytotoxic T lymphocyte clones derived from PBMC of 8 individuals given Dryvax 2 to 8 weeks earlier. This study revealed that there are multiple targets of acute CD8+ T cell responses after immunization with Dryvax that included not only early gene products but also structural and immune evasion proteins. Understanding whether responses to these targets are restricted according to HLA supertype and if persons remotely vaccinated against smallpox have T cell memory to them will be useful in evaluation of new generation smallpox vaccines and replication incompetent OPV that express heterologous antigens.

**Tetramer Development**

The development of tetramers was terminated because of the limitations of the tetramers, as well as the inability to acquire PBMC from recently vaccinia immunized individuals due to government restrictions. However, there is research currently underway to develop smallpox subunit vaccines(197, 198). A subunit vaccine is thought
to be capable of conferring sufficient protection from a Variola challenge, but safe
enough to allow administration to immunocompromised individuals. While our data on
vaccinia specific epitopes may not have provided for the development of tetramers, our
results, in conjunction with the results from other studies, suggest several epitopes that
could be possibly used in a smallpox subunit vaccine(75, 176, 178, 179, 183, 199-201).
In particular, one epitope identified, D12L_174, induced a substantial recall response in a
higher frequency of HLA-A2 vaccinated individuals than any other vaccinia epitope
discovered to this point.

Alternate Study to Identify Vaccinia Specific CD8^+ T Cell Memory

After considering that only samples from individuals vaccinated with Dryvax over
25 years previously would be available to us, we decided to forego tetramer identification
of vaccinia specific memory cells due to their low frequency and focus on the entire
memory population through ICCS staining for IFN-γ and IL-2. The presence of vaccinia
specific memory T and B cells in individuals remotely vaccinated against smallpox is
well documented (52, 73, 78). However, there is increasing evidence that there are
qualitative differences between types of memory cells (T_{CM} and T_{EM}) that provide for
distinctive roles in their contribution to the protection capacity against varying pathogens
(202). Therefore, it is important to understand the types of memory cells present in
individuals previously vaccinated against smallpox in order to understand completely the
correlates of protection for eventual comparative quantitative analysis.

We first wanted to identify if there were previously vaccinated individuals that
possess vaccinia specific T cells based on an upregulation of CD69 expression upon
vaccinia virus stimulation. Observing CD69 expression profiles offers a broad profile of
antigen specific cellular activation without the requirement for cytokine production. A limit of observing CD69 expression is both naïve and memory cells express CD69 upon activation (203). Therefore, this can lead to some naïve T cell activation, marked by an increase in CD69 expression, which can skew the data. However, we hypothesized that in previously vaccinated individuals, if there is memory present, the increases in CD69 expression should be greater than that observed in unvaccinated individuals. Results from these studies verify this proposition. While some unvaccinated individuals up regulated CD69 upon VV stimulation, the up regulation of CD69 in previously vaccinated individuals was greater. Unfortunately, because of the high background of CD8+ CD69+ T cells in unstimulated controls, it was difficult to determine the phenotype of CD8+ CD69+ T cells after VV stimulation. Consequently, the phenotype of the memory T cells was unable to be distinguish accurately.

Therefore, we sought to phenotype cells based on ICCS for IFN-γ and IL-2. Employing this ICCS assay provided us with the opportunity to identify individual vaccinia specific CD8+ T cells. Initial attention was focused on phenotyping memory CD8+ T cells because evidence has indicated that the phenotypic and functional differences between CD8+ TCM and TEM are significantly important in identifying and determining correlates of protection(59, 202). Data presented here indicates that the main populations of vaccinia specific memory cells capable of immediate IFN-γ in previously smallpox-immunized individuals have a CCR7lo CD45RA+ phenotype. This phenotype would classify these cells as TEMRA+. TEM IFN-γ producing cells were also identified in a few individuals tested. No IL-2 levels above background were detected in any of the individuals’ CD8+ T cells. Hence, 18 hours of stimulation with live vaccinia virus can
identify CD8+ memory T cells that are long lived over 25 years after antigenic stimulation based on cytokine expression.

It is important to note that only 31% of remotely vaccinated individuals displayed cytokine expression, while 47% displayed an upregulation of CD69. This discrepancy might be due to the fact that cells may activate and upregulate CD69 before producing cytokine or may just be due to the better availability of CD69 for detection. However, the ICCS staining assay provided more specific antigen dependent activation data, with low to no background, which was better for phenotyping vaccinia specific memory cells.

CD62L is typically used to phenotype populations of cells because of its definitive expression profile. However, in order to identify antigen specific cells without tetramers, stimulation with antigen is often required. The problem with using CD62L in these cases is that CD62L is quickly cleaved off by activated cells. Therefore, phenotyping memory cells with the use of CD62L provides inaccurate results. To circumvent this restriction on the use of CD62L as a phenotypic memory marker under these conditions, TAPI-2 has been used in mice to prevent CD62L cleavage upon cellular activation(189). In our ICCS approach, the use of CD62L as a phenotypic marker for differentiation of T cell subsets was found to be inaccurate upon ex vivo stimulation even with the use of TAPI-2. The population of IFN-γ+ CD62L− CD45RA+ CD8+ T cells (TEMRA+) became CD62L+ in the presence of TAPI-2. As suggested previously, although expression of CD62L would classify them phenotypically as T_N cells, this is functionally inaccurate(188). The observed difference in the utility of TAPI-2 between this study and the previous one is likely due to the fact that the present study was performed in humans where CD62L expression profiles and cleavage upon cellular activation might be
regulated differently then in mice. Regardless, to circumvent this issue with CD62L cleavage, staining against CCR7 was performed. There is no evidence to our knowledge that CCR7 is cleaved off upon activation.

Utilizing our ICCS approach, we were able to identify the main population of CD8+ memory T cells present in individuals vaccinated with VV as T_{EMRA+}. T_{EMRA+} memory cells has been shown by others to be present in humans with functional characteristics similar to T_{EM} (92, 121, 122, 187). In addition, the molecular profile and telomere length of the T_{EMRA+} cell subset indicates that it is closely related to T_{EM} cells (123, 142). There is an effective protective role of T_{EM} cells due to their immediate effector acquisition, particularly upon systemic peripheral viral infections (59, 97, 204, 205). In fact, a requirement of T_{EM} to facilitate protection against vaccinia challenge in mice has recently been shown (59, 134). Although the T_{EMRA+} and T_{EM} subsets share almost identical characteristics, the expression of CD45RA by T_{EMRA+} cells in humans suggests some functional distinction from T_{EM}. Perhaps the T_{EMRA+} subset indicates a further differentiated type of memory cell (102). However, recent findings indicate this is not the case (206). Therefore, more work is required to determine the significance of CD45RA expression on T_{EM} cells. Regardless, the existence of this T_{EMRA+} cell subset in vaccinia-immunized individuals indicates a potential correlate of protection necessary for protection from smallpox.

The expression of CD127 on the IFN-γ+ CD8+ T cells 25 years post vaccination indicates that these cells are long lived stable memory cells. There has been question as to the long term stability of T_{EM} cells. Murine T_{EM} have been indicated to be unstable and transition to T_{CM} or undergo apoptosis (96, 104). Although, recent studies have
contradicted these findings and observed that murine TEM can represent a stable population under physiological conditions (110, 137). With regards to humans, much of the data on the long term presence of TEM/TEMRA+ is derived from systems of chronic viral infection, such as HIV, CMV and EBV, where it is conceivable that successive reactivation and stimulation of TCM likely contribute to enhancing the TEM/TEMRA+ populations(122, 187, 207, 208). The ability to elicit a largely TEM repertoire from an initial primarily TCM repertoire upon subsequent viral challenges has been shown (209).

In the present study, it was demonstrated that upon primary antigenic exposure, as seen in the majority of VV immunizations, that TEM/ TEMRA+ are maintained concurrently with TCM as parallel memory populations over 25 years after challenge. Therefore, the observation of IFN-γ+ producing TEMRA+/TEM cells, which express CD127, provides additional supporting evidence that TEMRA+/TEM cells are independent, stable, long lived memory populations. These data highlight the importance of using CD127 to support the identification of ‘true’ memory populations.

The importance of using CD127 to accurately phenotype memory cells is further emphasized by the observation of a CD127lo population of IFN-γ+ CD8+ T cells responding to VV in recently vaccinated individuals after only 18 hours of stimulation. It is reasonable to propose that this (CCR7lo CD45RA+CD127lo) population represents ‘unfit’ effector memory CD8+ T cells that are terminally differentiated (147). These cells display immediate effector function like standard effector memory cells, but lack CD127 expression and as a result, do not receive necessary survival signals(193). Furthermore, they are not typical effector cells, which also do not express CD127, since they are maintained for an extended period of time (~1 year). Conventional understanding
maintains that traditional effector cells undergo apoptosis following antigen clearance. However, these terminally differentiated effector memory cells do eventually (25 years > 3 years) disappear from the memory pool. Additional support for this hypothesis can be found in a recent publication indicating that there are IFN-γ producing, CD127lo CD8+ T cells in individuals vaccinated with vaccinia virus one year earlier (84). However, more data is required to further substantiate that CD127lo, likely terminally differentiated effector memory CD8+ T cells, can be maintained for up to or more than 3 years following vaccinia immunization.

No evidence of VV specific CD8+ T_{CM} was found in any of the individuals tested by our ICCS technique with IFN-γ or IL-2 staining. Moreover, IL-2 ELISPOTs on purified CD8+ T cells did not show any significant IL-2 production over background levels by any of the individuals (data not shown). These ELISPOT data corroborate the data obtained from intracellular staining where IL-2 levels in VV stimulated samples were never significantly higher than levels in control samples. IL-2 levels were tested in this study because we hypothesized that any vaccinia specific T_{CM} present would produce IL-2, if not IFN-γ, in response to VV. However, no production of either cytokine by T_{CM} was measured.

CD8+ T_{CM} cells express receptors such as CCR7 and CD62L, which cause these cells to home to lymphoid tissue (92, 99). The expression of these lymph homing receptors by T_{CM} results in low frequencies of peripherally circulating CD8+ CCR7^{hi} CD45RA- cells (range 3-12% of total CD8+ T cells – average ~10%). Therefore, it is likely that reading a maximum of 2*10^5 CD8+ T cells, as was done here in our ICCS assays, resulted in missing any potentially circulating vaccinia specific CD8+ T_{CM} when
observing cytokine production alone. In addition, as time progresses after vaccinia virus immunization, VV specific CD8^+ T_{CM} decrease substantially in frequency within the blood, even after one year\(^{(83)}\). Therefore, there is a low probability of identifying T_{CM} in the peripheral blood of individuals that were vaccinated over 25 years previously.

Utilizing the ability of CD8^+ T_{CM} to undergo immense proliferation in response to antigen, a CFSE assay was developed. CFSE staining on gated CD8^+ T cells indicated a population of CD8^+ memory cells capable of proliferation upon VV stimulation in recently vaccinated individuals. However, phenotyping these expanding cells proved difficult. Post-expansion phenotyping after a certain time (~18-36 hours) is not accurate because cell phenotypes change as memory cells reactivate and expand\(^{(96)}\). Therefore, T_{CM} lose distinguishable phenotypic markers such as CD62L and CCR7 and cannot be discriminated from T_{EM}. In addition, flow cytometric sorting of the different memory populations (T_{CM}/T_{EM}/T_{EMRA^+}) before stimulation based on CD62L and CD45RA expression did not yield any conclusive data. However, CFSE labeling of positively selected CD62L^+ cells by magnetic beads before stimulation, followed by 7 day culture, did result in the detection of T_{CM} CD8^+ T cells capable of proliferation in recently vaccinated individuals.

It is important to note that although T_{CM} were not detected in the blood of remotely vaccinated individuals, this population is likely present in the lymph tissue. T_{CM} have a greater capacity to undergo homeostatic proliferation than T_{EM}. Therefore, the detection of VV specific T_{EM} in individuals vaccinated with Dryvax greater than 25 years ago indicates a strong likelihood that T_{CM} are maintained as well. Furthermore, in
individuals where no TEM were detected, populations of TCM may still exist. However, without access to lymph organs, testing this hypothesis is problematic.

The absence of TEM proliferation was not surprising and is supported by findings from previous reports using other antigenic systems (96, 107, 210). However, the data presented in the present study are in apparent contradiction with an observation that vaccinia specific effector memory cells proliferate and produce IFN-γ in response to vaccinia stimulation for 10 days (85). A caveat is that TCM generate new effector cells upon restimulation and the IFN-γ+ CFSE10 cells observed by Abate et al. were not phenotyped as TEM (99). Therefore, these vaccinia specific cells observed by Abate et al. are likely effector progeny from vaccinia specific TCM.

Cellular memory in individuals previously vaccinated against smallpox has been well documented. The data presented here identifies the memory cell subsets capable of immediate IFN-γ production and proliferation upon restimulation. However, even with the presence of this cytotoxic T cell memory in vaccinated individuals, it has yet to be determined which subsets are required for protection from smallpox. Effector memory cells were recently indicated to be more important than central memory cells in conferring protection from vaccinia virus in mice because of their immediate effector function (59, 134). A general theory that TEM are significantly important in conferring protective immunity to fast replicating peripheral viruses, such as vaccinia virus, has gained strength from recent findings (59, 106, 202). Therefore, the presence of TEMRA+ (TEM) in numerous previously vaccinated individuals is an indication that cytotoxic T cell memory may offer some level of protection, even in individuals receiving vaccination 25 years previously. However, it is evident from this study and others that CD8+ memory
wanes in the majority of people 25 years after vaccination. Therefore, many people who received smallpox vaccination during the eradication campaign are at risk of severe complications following a lethal challenge of smallpox. The extent of the risk in still unknown since it has yet to be determined the degree to which vaccinia specific antibodies correlate with protection.

Determining the correlates of protection from smallpox in humans is difficult to elucidate given the current circumstances. While animal models may provide some insights into vaccinia acquired immunity from various vaccinia vaccines, their relevance to humans is always in question. Consequently, quantitative immunological studies such as this one will allow us to begin to understand the levels of memory cells that should be induced by any new vaccine to be equivalent to those induced by traditional smallpox vaccines such as Dryvax and Lister. Fortunately and unfortunately, quantitative immunological comparison will be the only way to establish if new smallpox vaccines will protect from a smallpox infection without the natural presence of Variola.
CHAPTER 3

Specific Aim 2: Establish the effect of enhanced stimulation through antigenic load and inflammation in directing the development of T cell effector memory and central memory after infection of mice with different preparations of vaccinia virus that differ in their replicative capacity and immunogenicity

Preface

Typically, correlates of protection are identified from solely a qualitative standpoint. However, recent data have indicated that important qualitative differences exist in the memory CD8+ T cell pool. Therefore, for more effective vaccine development, it has become increasingly important to recognize the composition of the memory pool by determining the relative frequencies of the types of memory cells (effector/central) present. Depending on the pathogen, one subset may confer better protection and it would be beneficial to enhance the development of that memory subset. Using a mouse model with different strains of vaccinia virus, we present data here that indicate prolonged antigen load can enhance effector memory development and conversely, blunting antigen load prematurely leads to decreased levels of TEM. These data offer substantial evidence in favor of the progressive differentiation model of memory CD8+ T cell development and indicate that antigen load impacts lineage development more than antigen duration. Concurrently, we address the effects of TLR-2 and TLR-9 agonists on the development of memory subsets.
Introduction

The most prominent model to describe T cell memory development is the progressive differentiation model that was introduced previously. To reiterate, this model suggests that the formation of both memory population lineages ($T_{EM}$ and $T_{CM}$) is dynamic and depends on a variety of factors including the nature of the stimulating antigen, the antigenic load, inflammatory cytokine milieu, TCR engagement and costimulatory molecules (94, 97, 113, 130-132, 139, 211). The progressive differentiation model proposes that it is the level and potentially, the duration of stimulation from these various factors during primary infection which governs the frequencies of each memory subset that develop, i.e. higher inflammation leads to greater $T_{EM}$ frequencies in the memory pool, while lower inflammation leads to greater development of $T_{CM}$. Essentially, memory population heterogeneity can be introduced before initial division (signal strength) and promoted during subsequent division (decreasing potential) where stimulation during subsequent rounds of replication can promote increased frequencies of further differentiated $T_{EM}$ or terminal effector cells (136, 142). Therefore, it has been proposed that more virulent and disseminated viruses would cause higher loads and greater duration of inflammation, which would lead to an induction of greater $T_{EM}$ development (Fig. 3-1). The progressive differentiation model has gained much support over the linear differentiation model in recent years.

The linear differentiation model was the leading memory developmental model, but has lost credibility because much of the work deriving the linear differentiation model was done in a transgenic T cell receptor (TCR) LCMV system by Ahmed and colleagues (91, 96, 144). In this model, large numbers of naïve precursor cells are adoptively

* Includes minor additional background on memory differentiation models
transferred to determine memory development and it was concluded that T_{EM} are unstable and differentiate into T_{CM} over time. Hence, these data provided supporting evidence of a linear differentiation model of memory CD8^{+} T cell development. However, different model systems by recent reports have indicated a progressive differentiation model of CD8^{+} memory T cell generation (110, 131, 137, 209).

The difference between the model systems was explained by Marzo et al. in 2005 when they determined that higher numbers of precursor antigen specific T cells induced unstable T_{EM} (110). When lower precursor frequencies were adoptively transferred, the T_{EM} that developed after stimulation were stable and did not convert to T_{CM} over time. Similar data were obtained from endogenously derived levels of naïve precursors. Hence, at antigen specific naïve precursor levels that are not artificially enhanced, T_{EM} are stable and do not convert to T_{CM} over time. These data were expanded upon by Badovnic and Harty in 2007 when they found that the development of T_{EM} and T_{CM} were not only governed by precursor frequency, but also developed differently depending on the type of transgenic exogenous precursor used for the adoptive transfer (137).

Badovnic et al. argue that the avidity and affinity of the TCR may be relevant to memory development, although it is not dependent on the duration of TCR engagement (212). Based on observations of tetramer staining, CD127 and CD62L expression, as well as production of IL-2 and TNF-α, they indicate that the behavior (kinetics, proliferation, phenotype and function) of CD8^{+} T cells is progressively altered from the endogenous response as the number of naïve TCR-tg (transgenic T cell receptor) T cells transferred increases. Essentially, it has been realized that T_{CM} and T_{EM} can differentiate into stable populations that are maintained independent of each other (110, 213). Moreover, higher
stimulation stabilizes $T_{EM}$ populations. Therefore, based on this evidence for the progressive differentiation model, it is hypothetically feasible to enhance the development of a particular memory subset by altering signal strength of the stimulus.

Determining how memory cells are derived is necessary due to the fact that one subset, $T_{EM}$ or $T_{CM}$, may be more important for conferring better protection from a particular pathogen (202). Much of the initial work on memory CD8$^+$ T cell development was done with *Lymphocytic Choriomeningitis virus* (LCMV) in a murine system (96, 105). These models with LCMV have shown that murine $T_{EM}$ do not appear to be a stable population of cells and that $T_{CM}$ are the only “true” memory cells. $T_{CM}$ also respond at a greater magnitude than $T_{EM}$ in response to *Vesicular Stomatitis Virus* (VSV) (106, 205, 214). However, other studies that have utilized different antigens in mice such as *Vaccinia virus* and pulmonary viruses, have indicated that $T_{EM}$ may be more important than $T_{CM}$ in conferring protection (Fig. 3-2) (59, 104, 106, 107, 134). Studies with *Listeria monocytogenes* (L.m.) have been inconclusive in determining which memory T cell subset confers better protection from secondary infection (96, 215-217). However, it appears that both subsets contribute equally to confer protection from L.m. as well as *Leishmania* (214, 218). Therefore, the nature of the stimulating antigen seems to play a significant role in determining the composition of the memory CD8$^+$ T cell pool and the extent that either subset is required for adequate CD8$^+$ T cell protection.

The reasoning behind this ambiguity is not clear, but it seems to be due to the fact that fast replicating peripheral viruses can only be controlled efficiently by the immediate cytotoxic properties of $T_{EM}$ cells(204). Viruses of this nature will overwhelm the system before $T_{CM}$ have time to reactivate if not immediately controlled by $T_{EM}$. Conversely,
TCM may be more effective against systemic infections such as LCMV, which require significant T cell proliferation for adequate control. Therefore, if TEM are crucial to confer adequate protection from smallpox, new smallpox vaccines need to ensure that sufficient levels of TCM and particularly, TEM are induced.

Since different pathogens can elicit the development of different memory populations and different memory populations may be more important in conferring protection from a secondary challenge, it is important to understand how to enhance the development of either of these different memory populations when developing vaccines for a particular pathogen (104, 219). The idea of ‘driving’ the memory response to develop in a certain manner has recently gained much support from various studies indicating that adding an inflammatory milieu with antigen (IL-12, type-1 interferon and/or IL-21) can force further differentiation of activated cells, a requirement of TEM generation (135, 220-223). Furthermore, reducing the level of stimulation results in the development of more TCM, whereas greater stimulation impels the development of TEM (209, 217, 224, 225). Along the same line, greater antigenic load can also lead to preferential development of TEM, whereas greater naive precursor to antigen frequency preferentially leads to TCM development (104, 110, 137, 226). Therefore, replicative capacity and the immunogenicity of a particular virus can impact significantly TEM or TCM development (Fig. 3-3). Higher immunogenicity provides for greater antigenic load, a larger production of proinflammatory cytokines and a greater availability of costimulatory signals, all of which provide for a preferential generation of TEM (110, 119, 209, 212, 224, 227, 228).
A recent paper by Badovinac and Harty found that antibiotic pre-treatment of mice before *L. monocytogenes* infection results in a brief effector phase and rapid development of T\(_{CM}\) (217). However, the presence of CpG induced inflammation resulted in a prolonged effector phase and a longer period before memory development. Williams and Bevan demonstrated similar findings (226). Williams *et. al.* found that treating mice with antibiotics that had been infected with *L. monocytogenes* 24 hours post-infection (p.i.) resulted in greater effector cell numbers at seven days p.i., but diminished effector memory at day 35 p.i. when compared to mice that did not receive antibiotic treatment. Based on these findings, we hypothesize that the more virulent the vaccinia strain of virus, the greater the inflammation and the greater the T\(_{EM}\) production as opposed to T\(_{CM}\) (229).

Therefore, there may be problems with some of the new smallpox vaccines, such as MVA and other modified vaccinia viruses, since these virus strains are less virulent than current vaccines, specifically Dryvax. These new vaccines will likely not provide the same level of stimulation as Dryvax and lower levels of T\(_{EM}\) may be induced upon modified vaccinia vaccination when compared to Dryvax because of the diminished capacity to replicate in human cells. Since T\(_{EM}\) are crucial for protection from smallpox, these lower levels of T\(_{EM}\) elicited by MVA may be insufficient to confer adequate protection, at least at the T cell level, from a smallpox infection.

Further supporting evidence for the theory that a less virulent vaccinia virus will not produce T\(_{EM}\) levels equivalent to those induced by Dryvax is provided by the fact that antigen persistence for less than nine days failed to efficiently induce long-lived CD8\(^+\) T cell memory to LCMV in mice (230). Effective protection from LCMV can be conferred
by LCMV specific T_{CM}. However, since protection from VV seems to require a sizeable pool of T_{EM}, antigen persistence should be longer than 9 days according to the progressive differentiation model in order to elicit T_{EM}. Antigen load with Dryvax can last up to 21 days (67, 231). Therefore, there is likely a large population of vaccinia specific T_{EM} that develops upon Dryvax vaccination in the first few weeks, and as was demonstrated in the earlier human studies, is maintained for a long period of time (>1 year). Hence, based on our hypothesis, new modified vaccinia virus strains would not provide a level of stimulation comparable to Dryvax and therefore, the CD8^{+} T cell memory pool that develops from these modified strains would not be equivalent to that elicited by Dryvax.

In order to address the issues regarding memory CD8^{+} T cell development and the properties of the memory subsets, a murine model system of infection was developed using different strains of vaccinia virus that differ in replication competency. Hence, each deliver a different level of ‘signal strength’ to the responding T cells and provide for a quantitative variation in overall memory frequency, but also a unique qualitative profile of T_{EM} and T_{CM}. By utilizing this model, it was possible to determine the memory profile of CD8^{+} T cells following immunization with viruses that share substantial genetic homology, but differ in the antigenic load they promote and the immune response they evoke (immunogenicity).
Figure 3-1  Progressive differentiation of memory T cells based on primary signal strength and decreasing potential. This model proposes that the stronger a signal a primary T cell receives, the more differentiated its ‘fate’. The progressive differentiation model based on signal strength and decreasing potential was demonstrated in Figure 1-3. This diagram shows that primary signal can cause naïve cells to commit to ‘fate’, but that this fate is not concrete. Rather, memory precursor effector cells (MPEC) can progressively differentiate into short-lived effector cells (SLEC) if further signaling is received. Hence, MPECs are multipotent and can give rise to SLEC or TEM. Most SLECs die, but some persist as TEM as well. It has yet to be determined if populations of TEM derived from MPEC and SLEC are both stable and long-lived or if MPEC derived TEM are transitional and SLEC TEM are terminally differentiated. Germane to this model is the idea that greater stimulation of MPEC will result in more stable and long lived TEM (Fig 3-3). Adopted from Kaech and Wherry and Kalie et. al. (136, 147).
Figure 3-2  Effector (CD62L⁻ CD127⁻) and effector memory (CD62L⁻ CD127⁺) CD8⁺ T cells clear vaccinia virus more efficiently than central memory (CD62L⁺ CD127⁻) and naïve CD8⁺ T cells. $T_{\text{eff}}, T_{\text{EM}}, T_{\text{CM}}$ were generated in naïve mice by infection with 200pfu of VV. After 15 days, these subsets were removed from the spleen and FACS sorted. Sorted cells were then adoptively transferred into different groups of naïve recipients. These recipient mice were challenged with VV and 4 days later, VV titers were determined in the ovaries. It is important to note that the frequency of $T_{\text{CM}}$ in the ovary was actually higher than the frequency of $T_{\text{EM}}$ or $T_{\text{eff}}$ at the time of challenge. Hence, $T_{\text{EM}}$ are noticeably superior to $T_{\text{CM}}$ at clearing VV. Figure and text adapted from Bachmann et. al.(134)
Figure 3-3 Virulence can impact antigenic load, inflammation and costimulation, which in turn, determine development of $T_{CM}$ or $T_{EM}$. This illustration indicates that the more stimulation a system receives, the more differentiated evolving cells will become. To preferentially ‘drive’ $T_{EM}/T_{EMRA^+}$ development, a higher level of stimulation would be required than if trying to ‘drive’ $T_{CM}$. Since it is known that Dryvax elicits a strong $T_{EM}/T_{EMRA^+}$ response, then new vaccines need to elicit a similar response. Due to the inefficient ability of new modified vaccinia strains to replicate in mammalian cells, a similar dose of Dryvax and of these new strains would not elicit a response equivalent to that elicited by Dryvax because Dryvax does replicate in mammalian cells. Therefore, Dryvax would cause an increased antigenic load and based on this hypothesis, a subsequent stimulatory cascade that would lead to greater $T_{EM}/T_{EMRA^+}$ development. B) Central to this hypothesis is the fact that lower T cell precursor frequency causes longer $T_{EM}$ to $T_{CM}$ transition likely because of lower stimulation to each cell (104, 138). Higher antigenic load would cause precursor to antigen ratio to fall and result in more $T_{EM}$ stability. Adopted from Kalia et. al. (147)
**Materials and Methods**

*Viruses*

For immunizations, a stock of Dryvax obtained from the CDC was amplified and purified as previously described and graciously provided to us by Dr. Michael Cho (184). Briefly, vaccinia virus was used to infect monolayer HeLa cells at a MOI of 1 (232). Cells were incubated for three days at 37°C and then harvested. Virus was harvested from cells by light vortexing and centrifugation. The supernatant, containing the virus, was removed and washed with PBS. Virus was purified by sucrose gradient centrifugation. Purified virus was titered by standard plaque assay. For plaque assay, monolayer Vero cells were incubated overnight in six well plates in α-MEM at 37°C. Cells were washed and incubated with serial dilutions of virus starting at 1:1000 for two-three hours. Each well was then layered with α-MEM containing 1% agarose. After three days, cells were fixed with 10% formaldehyde and the agarose was removed. Staining was done with 1% Crystal Violet and plaques were counted. Purified MVA was obtained through the NIH Biodefense and Emerging Infectious Research Repository, NAIAD, NIH: Vaccinia Virus, Modified Vaccinia Ankara (MVA), from BHK-21 cells, purified, NR-727. Ectromelia virus (ETMV) and VVWR were propagated in murine L929 and HeLa cells as previously described and titered in BS-C-1 cells, similar to Dryvax, except titration was five days for ETMV (233). Dryvax was inactivated by incubation with 50ug/ml of Psoralen (Xanthotoxin; Sigma Aldrich) for 10 minutes at room temperature, followed by a 10 minute incubation under UV light (234). Titration on Vero cells revealed complete inactivation.
**Immunization Strategies and Challenges**

Groups of female Balb/c mice (Jackson Laboratories) at 10-12 weeks of age were scarified intradermally with $10^6$ pfu of Dryvax or injected intramuscularly with $10^6$ pfu of MVA. Psoralen inactivated Dryvax was also delivered intramuscularly. For scarifications, the left hind leg was shaved and a 5ul droplet of virus was placed on the skin surface. A 27 gauge needle was used to ‘scratch’ the surface of the skin and the droplet was absorbed. For intramuscular injections, MVA in 100ul was injected intramuscularly into the hamstring of the left hind leg. For intranasal delivery of VVWR, mice were restrained and 10ul of virus in PBS was injected with a pipet into one nasal.

TLR agonists peptidoglycan (Sigma Aldrich) and CpG-oligonucleotides (Invivogen) were reconstituted and mixed with MVA prior to administration to mice. Each mouse received 20ug of peptidoglycan (PGN) or CpG-Oligonucleotides (ODN) with MVA. MVA in Montanide (Seppic), an oil-in-water emulsion, was prepared following manufacturer’s instructions and injected intramuscularly. One part Montanide was combined with one part MVA and pulse vortexed for 20-30 seconds. Cidofovir (Vistide®, Gilead Sciences) was administered at all time points in 50mg/kg doses in 100ul PBS intravenously. Doses were given at d2, d3 and d4 after immunization on d0.

**Ex vivo Stimulation**

All mice were euthanized with CO$_2$, spleens were removed immediately and placed in 4ml HBSS. Splenocytes were extracted from excised spleens with 40um nylon mesh cell strainers (BD Biosciences). The cells were then purified through Ficoll centrifugation, washed with HBSS and resuspended in DMEM containing 10% FBS and penicillin/streptomycin. Splenocytes were counted and incubated with Ectromelia virus
(ETMV) at a MOI of 0.5 for 15 hours at 37°C with 5.5% CO₂. After 3 hours of incubation with virus alone, Brefeldin A (Sigma Aldrich) was added for a final concentration of 1μg/ml per well and then the incubation was continued for the additional 12 hours.

**Intracellular Staining**

Anti-CD4 antibody was purchased from BD Biosciences and all other antibodies for staining were purchased from Ebioscience. Streptavidin-Qdot 605 was purchased from Invitrogen. ETMV stimulated and unstimulated splenocytes from each mouse were washed with FACS buffer (PBS with 1% FBS and 0.09% NaN₃). Cells were blocked with polyclonal Rat-IgG for 10 minutes at RT. Anti-CCR7 (biotin) was incubated with the cells for 45 minutes at 37°C. Cells were then washed again and stained with a cocktail of streptavidin conjugated Qdot 605 and antibodies against CD3-AlexaFluor 700, CD4-PerCP, CD8-APC-AlexaFluor 750 and CD127-PE. Cells were washed and fixed with 1% formaldehyde. For permeabilization, cells were then washed twice with intracellular staining buffer (BD Biosciences). Antibodies against IFN-γ-PECy7, TNF-α-AlexaFluor 647 and IL-2-FITC were used for intracellular cytokine staining. Stained cells were run through an LSR II, acquired with FACSDiva software (BD Biosciences) and analyzed with FlowJo (Treestar).
Results

Lethal and Protective Dose

Various doses of Dryvax and MVA have been used in primates and mice to establish protective immunity from Monkeypox and Ectromelia virus (mouse pox)/Vaccinia Virus Western Reserve, respectively (55, 57, 63, 66, 235, 236). These doses range from $10^4$ pfu to $10^8$ pfu. However, consensus indicates that a dose of $10^6$ pfu of Dryvax delivered by scarification protects mice from a lethal challenge of Vaccinia Virus Western Reserve (VVWR). The same dose of $10^6$ pfu of MVA delivered intramuscularly often protects mice from death upon lethal VVWR challenge (54, 55, 57). (By comparison, the standard human dose of Dryvax given for vaccination is $2.5*10^5$ pfu.) However, since immunogenicity between the different vaccinia strains being tested varies, we wanted to keep the dose delivered constant. Therefore, a standardized dose of $10^6$ pfu was used for all our viruses being tested. The virus strains tested included traditional Dryvax (Dryvax), a single strain of VV that was plaque purified from Dryvax (Ps-ΔB8R), a recombinant strain derived from the purified Dryvax strain of VV where the B8R gene was removed and a human homolog of the IFN-γ gene was inserted (ΔB8R) and modified vaccinia Ankara (MVA). The B8R gene encodes an IFN-γ receptor homologue. In wildtype VV, the B8R protein binds IFN-γ and attenuates the human response to VV. Therefore, removing this gene prevents VV from inactivating human IFN-γ.

In order to determine if the vaccinia strains were viable and delivered at a protective dose, mice were challenged with a lethal dose ($LD_{100}$) of VVWR. The $LD_{100}$ was determined by giving groups of five 10-12 week old Balb/c female mice varying
doses of VVWR from $10^4$ pfu to $5 \times 10^6$ pfu via intranasal injection and measuring weight loss each day for 14 days (Fig. 3-4). When mice lost over 20% of their original weight, they were euthanized. A dose of $5 \times 10^5$ was found to be lethal in all mice by seven days after infection. However, to stay consistent with the literature and to ensure lethality, a dose of $10^6$ pfu of VVWR was used as a lethal dose. This dose is considered to be 10 times the LD$_{50}$ from previous reports, which is consistent with our findings(57).

Groups of 5 mice were immunized by scarification with $10^6$ pfu of Dryvax, Ps$_{-}\Delta$B8R and ΔB8R or intramuscular injection with $10^6$ pfu of MVA; control mice were given PBS (Fig. 3-5). Mice were weighed each day for 14 days. Any mice that lost more than 20% of their initial body weight were sacrificed. Data indicated that all strains of vaccinia virus delivered at $10^6$ pfu were protective in all mice. However, immunization with $10^6$ pfu of MVA did result in weight lost as compared to the other strains used for immunization. Regardless, these data indicate that $10^6$ pfu of every strain of vaccinia virus tested is viable and effective at inducing protective immunity from a lethal challenge of VVWR.
Figure 3-4 Lethal dose of Vaccinia Virus strain Western Reserve (VVWR). VVWR can induce death in 100% of mice at doses greater than $5 \times 10^5$ pfu by day seven. The LD$_{50}$ is approximately $10^5$ pfu. Although an LD$_{100}$ is achieved with $5 \times 10^5$ pfu, previous reports have used 10 times the LD$_{50}$ or $10^6$ pfu for lethal dose. This amount of VVWR delivered intranasal has been shown here and by others to be lethal in nearly 100% of mice. Therefore, $10^6$ pfu of VVWR delivered intranasal was selected for lethal challenge of mice. ‡ indicates the premature euthanization of one mouse as compared to the rest of the group. The experiment was repeated to ensure reproducibility and a similar profile was obtained.
Figure 3-5  Body weight changes following lethal challenge of VVWR in immunized and unimmunized mice. Protective doses of $10^8$ pfu of have been established from previous literature for MVA and Dryvax. The experiment was repeated to ensure reproducibility and a similar profile was obtained.
In order to identify and compare the correlates of protection, as well as to observe memory lineage development, it was important to determine the frequency and phenotype of all Orthopox virus specific CD4^+ and CD8^+ T cells in the spleen following immunization with three different strains of VV; Dryvax, ΔB8R and MVA. Therefore, to ascertain the phenotype and functional properties of VV induced memory cells from different strains of vaccinia virus, standard surface staining flow cytometry and ICCS was used. Using ICCS for the production of cytokines IFN-γ, TNF-α and IL-2 as an indicator of antigen specificity, the number of VV specific memory cells of each phenotype at seven, 14 and 28 days after vaccination was able to be determined.

The main topics we anticipated to address were the percentages of each phenotype of memory cell present at varying time points following inoculation with different strains of VV and the frequency of VV specific memory CD8^+ T cells present at day 28. Secondary to this, we sought to address the CD127 expression profiles on effector versus memory cells and determine if VV T_CM cells could express IFN-γ and TNF-α or T_EM could express IL-2 after 15 hours of stimulation. Finally, we aimed to determine how antigen load and duration effect the development of T_EM and T_CM.

Results indicated that memory CD4^+ T cells were detected consistently in MVA immunized mice at a low frequency (Fig. 3-6). In the other groups of mice, CD4^+ cytokine production was often below detectable amounts (data not shown). Therefore, memory CD4^+ T cells could not be effectively phenotyped since the frequency was too low in all groups.
Regardless, results indicate that memory CD8$^+$ T cells are present in all groups of immunized mice and are capable of producing IFN-$\gamma$, TNF-$\alpha$, and IL-2 (Fig. 3-6). Memory CD8$^+$ T cells capable of producing IL-2 were detected at a low frequency, while cells capable of producing IFN-$\gamma$ and TNF-$\alpha$ were numerous. The IL-2 producing memory cells often produced IFN-$\gamma$ and TNF-$\alpha$ as well (~85%). The frequency of memory cells that displayed dual production of IFN-$\gamma$ and TNF-$\alpha$ or single production of IFN-$\gamma$ was substantial and provides an interesting functional profile for further characterization of the memory CD8$^+$ T cells.

To determine the phenotype of the cytokine producing cells, antibodies against CCR7 and CD127 were used in our staining protocol. Based on the expression of CCR7 and CD127, cells can be divided into Teff (CCR7$^{lo}$ CD127$^-$), T$^E_M$ (CCR7$^{lo}$ CD127$^+$) and T$^C_M$ (CCR7$^{hi}$ CD127$^+$). Since the frequency of IL-2 producing cells was low in most cases and typically produced other cytokines as well, the analysis was focused on IFN-$\gamma$ and TNF-$\alpha$ producing CD8$^+$ T cells. Gating on both single producing IFN-$\gamma$ and dual producing TNF-$\alpha$/ IFN-$\gamma$ cells, the phenotype of the responding cells at each time point, d7, d14 and d28, was able to be observed (Fig. 3-7).

Considering the IFN-$\gamma$ single producing cells first, with all strains of vaccinia virus, frequencies of T$^C_M$ dominated the response over other cell types as early as day seven. The frequency of T$^C_M$ usually peaked around 0.05% of total CD8$^+$ T cells at day 28. Frequencies of T$^E_M$ were typically within 0.01% of T$^C_M$ frequencies, particularly at d28. Contrary to the profile of T$^C_M$ observed, the frequency of T$^{eff}$ peaked at day seven and declined until day 28. Overall, T$^E_M$ remained relatively constant, while T$^{eff}$ frequencies declined and T$^C_M$ frequencies increased over time since immunization.
The difference in the predominating phenotype of dual IFN-γ/TNF-α positive cells is evident. In these cells, a much higher frequency of T_{eff} and T_{EM}, instead of T_{CM}, are present from day seven. In addition, as opposed to the high frequency of single positive IFN-γ cells with a memory phenotype present at day seven, these dual IFN-γ/TNF-α are mostly T_{eff} at day seven with a lower frequency of T_{EM} present. The frequency of T_{CM} was low from the onset (<0.05%) and remains relatively constant over time. Moreover, the percentage of T_{eff} cells substantially decreases while the percentage of T_{EM} is unchanged in MVA (T_{eff}: 0.24% to 0.02%, T_{EM}: 0.10% to 0.10%) and Dryvax (T_{eff}: 0.37% to 0.06%, T_{EM}: 0.26% to 0.24%) immunized animals when comparing day seven to day 28. However, in ΔB8R immunized animals, the T_{EM} frequencies peak at day 28 (T_{eff}: 0.25% to 0.06%, T_{EM}: 0.11% to 0.35%). When all data from cytokine producing cells were considered together, by d28, T_{CM} were primarily single IFN-γ producing cells which dominate the response early (d7) following immunization, while T_{EM} were mainly dual IFN-γ/TNF-α producing cells that did not predominate the phenotype of responding cells until later (d14-d28) due to the T_{eff} dominated response early (d7).

Next, we proposed to compare the frequencies and ratios of all T_{EM} and T_{CM} in each of the vaccination groups during the memory phase of the response (d28) to observe how antigen load effects memory lineage commitment. Therefore, all the cytokine producing CD8^+ T cells were gated and the number of cytokine producing CCR7^{lo} CD127^+ and CCR7^{hi} CD127^+ cells on average per million CD8^+ T cells (Fig. 3-8) was observed. The results demonstrate that the MVA immunized animal displays the lowest ratio of T_{EM}:T_{CM} at 1.24. The ratio of T_{EM}:T_{CM} for the ΔB8R immunized mice was 2.10
and 2.01 for the Dryvax immunized animals. As presented in Figure 3-8, all vaccinia strains induced a memory population in favor of TEM. However, there were a much larger number of Ectromelia specific TEM cells in animals that received replication competent forms of vaccinia virus. TCM numbers remained relatively constant across all groups ranging from only 1273 per million CD8⁺ T cells in the MVA group of mice to 1597 and 1699 per million CD8⁺ T cells in the ΔB8R and Dryvax immunized groups of animals respectively. While the ratio of TEM:TCM was greater in the ΔB8R group versus the Dryvax group, the total number of memory cells, while not significantly different, followed the opposite trend with 3353 per one million CD8⁺ T cells on average in the ΔB8R group and 3415 per one million CD8⁺ T cells on average in Dryvax immunized mice.
CD4+ T Cell Cytokine Production in MVA Immunized Animals

A

SSC

FSC

CD3

CD4

CD8

CD4+

CD8+

VV (+)

IL-2

TNF-α

IFN-γ

VV (-)

IL-2

TNF-α

IFN-γ

B

CD4+ T Cell Cytokine Production in MVA Immunized Animals
Figure 3-6  CD4⁺ and CD8⁺ T cell cytokine production from immunized animals after 15 hours of ex vivo stimulation with Ectromelia virus.  A) Lymphocytes were gated, followed by CD3⁺ and further into CD4⁺ and CD8⁺.  Dot plots are representative from one mouse.  B) Only MVA immunized animals demonstrated CD4⁺ responses.  Even these responses were right at the detectable threshold.  C) Frequency of each subset of non-naïve CD8⁺ T cell at day seven, 14 and 28 after immunization.  Dark grey bars indicate single IFN-γ producing cells, light grey bars indicate dual IFN-γ and TNF-α producing cells and black bars denote poly functional cells that produce all three cytokines observed (IFN-γ, TNF-α and IL-2).
Figure 3-7 Frequency of each phenotype of cytokine producing CD8<sup>+</sup> T cells isolated from vaccinia immunized animals. Cytokine producing CD8<sup>+</sup> cells were gated and plotted on CCR7 versus CD127 plots. Dual and single cytokine producing cells were gated separately for characterization. Frequency is based on total number of CD8<sup>+</sup> T cells. For graphs, left column indicates frequency of each phenotype observed from IFN-γ producing cells and the right column indicates the frequency of each phenotype of cells that produced both IFN-γ and TNF-α. Each row indicated a different group of immunized animals. Grey bars, black bars and striped bars indicated percentages of effector (T<sub>eff</sub>), effector memory (T<sub>EM</sub>) and central memory (T<sub>CM</sub>) in each group of animals respectively.
Figure 3-8 Number of total cytokine producing cells per one million CD8⁺ T cells in each immunization group at day 28. CD8⁺ cells were plotted and TNF-α/IFN-γ dual positive and single positive IFN-γ were gated on CCR7 versus CD127. For graph, filled in squares indicate the average number of TₑM and open squares indicate the average number of TₑM per 1 million CD8⁺ T cells. Data in all experiments was reproducible and followed similar trends.
**Altering $T_{EM}$ Development**

The higher frequencies of $T_{EM}$ and greater ratio of $T_{EM}:T_{CM}$ in mice that were immunized with replication competent strains of vaccinia (Dryvax and $\Delta B8R$) as compared to mice that were immunized with an attenuated, replication incompetent strain (MVA) indicated that more virulent vaccinia virus strains enhanced and promoted progressive differentiation. Greater antigenic load, longer antigen persistence, increased inflammation and greater access to costimulatory molecules all likely play a role in the greater number of $T_{EM}$ that develop in response to more virulent viruses. Therefore, we decided to give multiple doses of MVA as well as combine MVA with adjuvants and different inflammatory agonists to elucidate the mechanism responsible for the generation of $T_{EM}$ over $T_{CM}$. Initial work was focused on MVA since this VV can be regulated more effectively than Dryvax derived VV.

The use of the $\Delta B8R$ strain of vaccinia in the subsequent experiments was discontinued because there was no substantial difference in the number of $T_{EM}$ and $T_{CM}$ generated as compared to Dryvax. This was unexpected considering the $\Delta B8R$ lacks the IFN-$\gamma$R homolog and contains a gene that encodes IFN-$\gamma$. IFN-$\gamma$ is highly species specific and was not expected to have any activity in mice. Regardless, we thought there might be an attenuation of the $\Delta B8R$ strain in mice considering the $\Delta B8R$ strains lacks the gene encoding for the IFN-$\gamma$R homolog. There is some evidence in the literature that the Dryvax encoded IFN-$\gamma$R can bind mouse IFN-$\gamma$ with low affinity (45). However, after data indicated that $\Delta B8R$ and Dryvax induce almost identical ratios and numbers of $T_{EM}$ and $T_{CM}$, it was realized that Dryvax generated IFN-$\gamma$R homolog binds mouse IFN-$\gamma$
with low affinity and loss of this gene does not affect viral immunogenicity in mice when compared to wildtype vaccinia (43, 45).

Adjuvants such as incomplete Freund’s, alum and Montanide can be used to prolong antigen release and extend antigen duration. Therefore, these experiments provided insight into the impact of ‘burst’ antigen load versus prolonged antigen duration on memory lineage commitment. From the initial experiments with Dryvax and MVA, results indicated that greater antigen duration did not significantly influence memory lineage commitment since memory frequencies were established at d7 and Dryvax persist for greater than seven days. Rather, the greater T\textsubscript{EM} frequencies observed in Dryvax immunized mice were likely a result of higher antigenic loads. To substantiate this finding, equivalent amounts of antigen load present at any given time were decreased by prolonging MVA duration. Concurrently, to test how enhancing antigen load effects T\textsubscript{EM} differentiation, doses of MVA were given at d0 and d7.

Pathogen associated molecular patterns or PAMPs can act as proinflammatory agonists by binding and activating pattern recognition receptors (PRR) on a variety of cell types including dendritic cells and macrophages. Toll like receptors (TLR) are a class of PRR that have gained much attention recently. By combing MVA with certain TLR agonists, it was hypothesized that an increase in inflammation and the presence of costimulatory molecules would enhance T cell differentiation into T\textsubscript{EM} (237).

Initial work on was focused on TLR2 and TLR9 ligands, peptidoglycan (PGN) and CpG oligodeoxynucleotides (CpG-ODN) respectively (238, 239). Zhu et. al. demonstrated that Western Reserve strain of Vaccinia virus (VVWR) activated murine dendritic cells through a TLR2 dependent mechanism and was TLR4 independent. In
addition, when TLR2 knockout mice were infected with VVWR, they demonstrated a much higher titer of virus in the ovaries after 3 days as compared to wildtype mice. These data suggest that TLR2 signaling plays a critical role in innate immune activation. Zhu et. al. were then able to link TLR2 signaling of the innate immune system to the development of CD8\(^+\) T cells. They found that wildtype mice had a higher number of effector CD8\(^+\) T cells capable of producing IFN-\(\gamma\) seven days after VVWR infection as compared to TLR2 knockout mice. Hence, TLR2 contributes an important role in innate immune activation upon VV infection that affects adaptive immune development.

CpG-oligodeoxynucleotides (ODN) are effective TLR9 agonists that can induce proinflammatory cytokine production (240). TLR9 has been shown to be an effective adjuvant for DNA, protein and peptide vaccines (241-243). Belyakov et. al. were able to demonstrate that the administration of CpG-ODN with \(10^5\) pfu of MVA could induce equivalent protection from VVWR in mice as MVA alone at a dose 100 times higher (238). Administration of CpG-ODN with MVA increased CD8\(^+\) T cell frequencies in the spleen by nearly 25\% at day 9 post-infection (p.i.) as compared to administration of MVA alone. The protection of mice against lethal VVWR challenge with CpG-ODN and MVA was shown to be independent of CD4 help. Whereas MVA immunization alone resulted in \(\sim\)15\% body weight loss in CD4 deficient mice upon lethal challenge, CpG-ODN/MVA immunization resulted in 0\% weight loss. Therefore, CpG-ODN, a TLR9 agonist, can influence the development of vaccinia specific memory, specifically CD8\(^+\) T cells, when administered with MVA.

We hypothesized that administration of MVA with peptidoglycan from \textit{Staphylococcus aureus} or CpG-ODN can affect the immune environment in favor of
greater inflammation and costimulation, which would result in higher signal strength and greater TEM development. In addition, greater antigen load through administration of multiple MVA doses should provide for restored inflammation and costimulation, which should lead to greater T cell differentiation and increased TEM development. Finally, the use of an oil emulsion adjuvant such as Montanide to contain MVA and release the same load of antigen over an extended period of time should lead to lower TEM frequencies in the memory pool as a result of lower ‘burst’ antigen load.

Eight groups of five 10-12 week old female Balb/c mice each were given 1) PBS in Montanide with PGN and CpG-ODN, 2) 10^6 pfu of Dryvax, 3) 10^6 pfu of psoralen inactivated Dryvax (replication incompetent), 4) 10^6 pfu of MVA, 5) 5*10^5 pfu of MVA at day 0 and at day seven, 6) 10^6 pfu of MVA in Montanide, 7) 10^6 pfu of MVA and 20µg of PGN, 8) 10^6 pfu of MVA and 20µg of CpG-ODN. As expected, results indicated that MVA in an adjuvant such as Montanide delivered that same antigen load over an extended time frame and lead to decreased frequencies of TEM and a lower TEM/TCM ratio than MVA alone (Fig. 3-9). Furthermore, greater TEM frequencies and successful enhancement of TEM:TCM ratios was achieved after augmented antigen load with two doses of MVA. However, no other vaccine regimens induced increased TEM cell frequencies. Both pro-inflammatory TLR agonists PGN and CpG-ODN actually diminished the frequency of ETMV specific MVA elicited memory cells. Finally, inactivated Dryvax did not induce a response in any of the immunized mice indicating that some level of replication competency is favorable when attempting to induce the optimal immune response. These data taken together indicate that antigen load and not duration impacts memory lineage development and commitment.
Figure 3-9  Antigen load effects memory lineage commitment.  A. Total number of ETVMV specific TEM and TCM per 1 one million CD8+ T cells present in groups of 4-5 mice 28 days after administration of initial dose of virus.  PBS controls demonstrated consistent frequencies of 100 and 500 TEM and TCM respectively per one million CD8+ T cells. Therefore, inactivated Dryvax frequencies are comparable to background levels.  B. Ratios of TEM:TCM in each vaccination group. Data was reproducible and followed similar trend.
Since CD8⁺ \( T_{EM}:T_{CM} \) ratios of MVA immunized mice could be abrogated and enhanced by prolonging antigen load and boosting antigen load respectively, we proposed that \( T_{EM}:T_{CM} \) ratios of Dryvax immunized animals could be lowered by decreasing vaccinia load after Dryvax immunization. This has been done in other models systems by administering pathogen specific immunoglobulin (Ig) or antibiotics at various time points after infection (217, 220, 226). Therefore, Dryvax immunized animals were given doses of Cidofovir two-four days after infection to determine if decreasing antigen load could lead to lower \( T_{EM}:T_{CM} \) ratios. Cidofovir negatively impacts viral DNA polymerase and causes enhanced clearance of vaccinia virus due to its diminished ability to effectively replicate (244-247). Delivery at day two to day four was selected to observe the effects of viral clearance after only a few rounds of viral replication (67, 231). We hypothesized that higher antigenic loads of Dryvax would provide for greater \( T_{EM} \) development and higher \( T_{EM}:T_{CM} \) ratios. Therefore, we expected the \( T_{EM}:T_{CM} \) ratio to increase from the group given Cidofovir at day two as compared to the group not given Cidofovir.

Results confirmed that higher antigenic loads in untreated mice resulted in greater \( T_{EM}:T_{CM} \) ratios (Fig. 3-10). Mice that were treated with Cidofovir for three days starting at day two had the lowest \( T_{EM}:T_{CM} \) ratio, while untreated mice had the highest \( T_{EM}:T_{CM} \) ratio. These findings further support our conclusions that antigen load during priming impacts memory lineage commitment and development.
Figure 3-10  Further evidence that antigen load effects memory CD8+ T cell lineage commitment. When antigen load is decreased at day 2, lower numbers of TEM cells are generated. TCM frequencies remain similar. Due to this decrease in TEM generation, the ratio of TEM:TCM is decreased when antigen load is reduced. Experiment was performed twice and results were reproducible.
Discussion

Different memory T cell subsets mediate protection more effectively depending on the nature of the stimulating pathogen (202). Whereas TCM provide more effective protection against LCMV and VSV, TEM provide better protection against vaccinia virus and pulmonary viruses (59, 96, 107, 214). Therefore, depending on the nature of the pathogen, it is important to design vaccine strategies that induce the most effective memory population required to mediate optimal protection. In order to manipulate the final memory pool of T cells to develop into greater proportions of one subset over the other, it is necessary to understand memory development and design vaccine strategies accordingly.

Based on the progressive differentiation model of memory T cells, it is hypothetically possible to influence the development of a particular subset by varying the level of stimulation (135, 147, 202, 228). Using different strains of vaccinia virus that differ in infectivity to stimulate T cells, antigen specific memory T cells that develop as a result were able to be observed. This experimental design provided an excellent model to study how antigenic load/duration and in turn, inflammation directed the development of different memory subsets. These data will also aid in the development of more effective vaccines.

ETMV, and not VVWR, was used in our studies to stimulate splenocytes from VV immunized mice because we proposed that ETMV generated responses from VV specific T cells in mice would be more relevant to likely frequencies of Variola specific T cells in humans after VV immunization. In addition, we hypothesized that frequencies of T cells that responded to VVWR after Dryvax immunization and not MVA immunization
would be incorrectly enhanced due to the significant homology between Dryvax and VVWR (~99% homologous). Therefore, in order to prevent a potentially biased response, a virus that is more distant (<95% homology) to both Dryvax and MVA was used to provide a more ‘fair’ comparison between recall responses of Dryvax and MVA T cell frequencies after VV immunization. Furthermore, preliminary experiments revealed that splenocytes isolated from Dryvax and MVA immunized animals responded to ETMV, while inferior responses to VVWR were observed in all stimulation condition.

Staining for multiple cytokines at various time points allowed the observation of mono-, dual- and polyfunctional antigen specific T cells as they evolved from the effector phase of the response to the memory phase. Single IFN-γ, dual IFN-γ/TNF-α and polyfunctional IFN-γ/TNF-α/IL-2 CD8⁺ T cells were identified at each time point. This functional profile is supported by similar findings in humans where the majority of cells produced all three cytokines, IFN-γ and TNF-α or just IFN-γ (86). For the most part, particularly with the dual positive IFN-γ/TNF-α cells, the frequency of cytokine producing T cells increased according to viral immunogenicity. For instance, on day seven, the MVA group of mice exhibited the lowest frequency of cytokine producing cells, with ΔB8R immunized animals exhibiting more and Dryvax immunized animal having the highest frequency of cytokine producing cells. This trend is in concordance with the hypothesis that viral immunogenicity should increase inflammation. The trend was continued for days 14 and 28 when the MVA immunized mice were compared to the Dryvax and ΔB8R immunized animals. However, at days 14 and 28, while the frequency of antigen specific cells was the lowest in the MVA immunized animals, the ΔB8R and Dryvax immunized animals demonstrated approximately equal frequencies of antigen
specific T cells. This indicates that these two strains do not differ significantly. Therefore, these data support that the B8R gene of Vaccinia virus does not function in wildtype mice (43, 45). Hence, removing it from the ΔB8R strain will have no effect on viral immunogenicity.

CD4⁺ T cell responses to all virus strains used for immunization were low and besides the MVA group, could often not be detected. CD4⁺ responses tend to be significantly lower than CD8⁺ T cell frequencies(58, 248). In fact, often studies that induce T cell recall responses against vaccinia virus use infected fibroblast or dendritic cell lines as target cells(55, 248). Our technique did not use pre-infected cell lines, which results in a 10 fold lower recall responses at the CD8⁺ T cells level. Since CD4⁺ T cell recall responses can be 10 fold lower than CD8⁺ recall responses when infected cell lines are used, our assay was not effective for observing CD4⁺ T cell responses in most cases, except for MVA immunized animals.

Although MVA does not replicate effectively in mammalian cells, it still infects cells and is likely processed via a MHC class I pathway for activation of CD8⁺ T cells. This is obviously the case since CD8⁺ T cell levels were above background and could easily be detected. However, the fact that CD4⁺ T cell levels were detectable in MVA immunized animals and not in ΔB8R/Dryvax immunized animals is an anomaly. Either the CD8⁺ T cell response to Dryvax and ΔB8R diminishes the CD4⁺ T cell response to below a detectable level or the mechanism for MVA processing provides more available CD4⁺ T cell epitopes. The latter proposition is supported by the fact that MVA only generates immature particles from infection that do not disseminate within the host (19).

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Therefore, there would be a large pool of non-infectious antigen available for MHC-II processing. However, there is no strong evidence in support of either proposition.

Upon phenotyping the cells, it was clear that single cytokine producing cells were mainly T<sub>CM</sub> that were present early in the response (i.e. day seven). There were also some T<sub>EM</sub> single cytokine producing cells as well. However, the majority of the T<sub>EM</sub> were dual cytokine producing cells present at high frequencies as early as day seven as well. The relatively equal frequency of T<sub>EM</sub> at days seven and 28 was surprising and indicates that T<sub>CM</sub> and T<sub>EM</sub> can arise as early as day seven and then be maintained until the memory phase. According to the progressive differentiation model, more T<sub>EM</sub> cells are generated through higher antigenic loads. Supporting data is provided in the present study because T<sub>EM</sub> frequencies were higher in Dryvax immunized animals as compared to MVA immunized animals at day seven.

The fact that memory frequencies at d7 were unchanged at d28 indicates that antigenic load and not duration is responsible for enhancing T<sub>EM</sub> development since Dryvax is present longer than seven days after intradermal administration. Longer antigen duration in Dryvax immunized animals was exemplified by the presence of effector T cells that dominated the response of IFN-γ single producing cells at day seven and of dual producing IFN-γ/TNF-α at day 14. Dryvax immunized animals were the only group of immunized animals where this was true. Hence, these data support that an active infection is present from d7-d14 in Dryvax immunized mice which causes the maintenance of T<sub>eff</sub>.

One important incongruity in the data was that Dryvax induced substantially higher frequencies IFN-γ/TNF-α dual cytokine producing T<sub>eff</sub> and T<sub>EM</sub> cells at d7
compared to those induced by the ΔB8R strains. However, the frequencies of memory cells that are induced after Dryvax immunization are equivalent to those induced by the ΔB8R strain at 28 days after infection. One possible explanation for this observed discrepancy is that the Dryvax vaccine is a ‘swarm’ of multiple clones of vaccinia. The ΔB8R strain is one single clone isolated from Dryvax. Therefore, there is a larger pool of potential epitopes for T cells responding to Dryvax as compared to the more limited pool of epitopes provided by the ΔB8R strain. This leads to a greater number of responding effector cells in Dryvax immunized animals. However, there are likely dominant clones in both strains that generate the memory populations. Although support for this hypothesis is lacking for vaccinia, the idea of dominating memory clonotypes is well established for other infection models (249-252). Therefore, while there are more effector clonotypes available in Dryvax immunized versus ΔB8R immunized animals to respond at day seven to Ectromelia virus, there are equivalent numbers of memory clonotypes available to respond in both immunization groups at day 28.

By day 28, numbers of T\textsubscript{CM} were equivalent in all immunization groups. However, the number of T\textsubscript{EM} differed significantly in the replication competent strains (ΔB8R and Dryvax) versus the incompetent strain, MVA. Based on the progressive differentiation model of memory T cell development, this was expected due to the lower immunogenicity of MVA, and as a result, antigen load and induction of inflammation (147). In other infection models, if the antigen level is blunted with administration of antibiotics or if lower antigen doses are administered, lower levels of T\textsubscript{EM} or higher levels of T\textsubscript{CM} develop (96, 224, 226). On the other hand, increasing inflammation leads to greater T\textsubscript{EM} development (217). Therefore, with greater inflammation provided by higher
antigenic loads of replication competent strains of vaccinia virus, elevated levels of MPEC and resulting $T_{EM}$ are generated. This is apparent when the relative ratios of effector, $T_{EM}$ and $T_{CM}$ in each immunization group and the final $T_{EM}:T_{CM}$ ratio at day 28 in the Dryvax versus MVA immunized animals were observed.

As previously described, Dryvax induces greater effector cell frequencies at day seven for IFN-$\gamma$ producing cells and at day 14 for dual IFN-$\gamma$/TNF-$\alpha$ producing cells. Effector cell frequencies do not dominate the response at these time points in MVA immunized animals. However, at day 28, when all cytokine positive cells are considered, $T_{CM}$ frequencies are similar in Dryvax and MVA immunized animals, whereas $T_{EM}$ levels greatly increase in Dryvax versus MVA immunized animals. Hence, the greater stimulation induced by Dryvax seems to induce greater numbers of MPEC that differentiate into $T_{EM}$ seeing as $T_{CM}$ levels do not differ significantly between the two groups. Since the responding clonotypes of T cells are likely similar to both MVA and Dryvax due to the 85% homology these two strains of vaccinia share, these data indicate that with respect to vaccinia virus infection, there is a limited number of MPECs that develop. These MPECs appear to be committed to develop into $T_{CM}$, but with increased antigen load, these responding MPECs can enhance $T_{EM}$ cell numbers by promoting further differentiation early in infection ($<d7$). The exact mechanism for this occurrence is undefined and data presented here does not address it. However, subsequent experiments with inactivated Dryvax demonstrate that the difference is not a result of immunogenicity or other factors present in the Dryvax VV that are missing from MVA, but are directly related to immune evasion and replicative ability (antigen load).
The next series of experiments were performed in order to determine how to alter memory development after MVA vaccination. These experiments were done to: 1) further define how antigen load and duration effect lineage commitment and 2) to test the decreasing potential aspect and signal strength aspect of the progressive differentiation model. Hence, antigen load was augmented (multiple dose of MVA) or prolonged over an extended period of time (MVA in Montanide). In addition, immediate primary signaling was enhanced through increased inflammation (TLR stimulators).

Containing MVA antigen load in Montanide demonstrated the expected result: decreased TEM development. This result is likely explained by the fact that MVA is slowly released from the oily adjuvant and with this slow release, the MVA viral particles are never present at a high enough frequency to fully engage the immune system and generate an effective response. This is consistent with the dose dependency requirement of multiple pathogens to elicit an effective immune response. Depending on the immunogenicity of the pathogen, different doses are required to escape innate immune control and initiate an adaptive immune response. Due to the replication incompetence of MVA, typically doses of $10^5$-$10^6$ are required to generate any memory responses (54). Therefore, $10^6$ pfu of MVA slowly released from an oil-in-water adjuvant such as Montanide is effectively controlled by the innate immune system. This effective control prevents the necessary level of stimulation factors to induce higher frequencies of T_{EM}.

By boosting antigen load at d7, results further demonstrate that antigen load influences T_{EM} development. MVA given at day zero and day seven boosted antigen exposure and led to enhanced T_{EM}:T_{CM} ratios as compared to animals immunized with one dose of MVA at day zero. These data, when taken together with the data obtained
from the initial Dryvax/MVA study, as well as the MVA in Montantide, indicate that antigen load plays a central role, more so than antigen duration, in enhancing or diminishing CD8+ memory T cells subset development. Furthermore, even though memory lineage commitment occurs by d7, boosting the antigen load after d7 can provide the necessary signaling factors to generate more T_{EM} without diminishing T_{CM} levels. Therefore, the response is still in the effector phase at d7 where MPEC are malleable. Once T_{CM} are committed in the memory phase, restimulation would lead to an increase in T_{EM} frequencies, but diminished T_{CM} frequencies.

Hence, the effect of antigen load on memory lineage development confirmed the decreasing potential aspect of the progressive differentiation model, but it was important to determine the impact of signal strength as well. Petidoglycan and CpG-ODN can enhance inflammation through TLR-2 and TLR-9 stimulation. These two TLRs are thought to perform an important role in innate immune activation upon vaccinia virus infection (238, 239). Therefore, we thought that by enhancing initial inflammation through innate immune system activation, we could enhance differentiation of CD8+ T cells into T_{EM}. However, PGN with MVA had no effect on CD8+ T cell memory generation as compared to MVA alone and CpG-ODN with MVA diminished the response.

The administration of CpG-ODN with MVA enhanced vaccinia specific memory cells in the spleen 9 days p.i., but had no effect on the CD8+ T cell response after challenge (238). If CpG-ODN is administered with MVA intranasally and not intramuscularly, it enhances CD8+ T cell mediated protection. However, the results obtained at day 9 in this study by Belyakov et. al. suggested that CD8+ T cells
frequencies could be enhanced. In our study, 28 days after MVA administration, no increase in memory CD8$^+$ T cell frequency was observed. Therefore, CpG-ODN with MVA appears to enhance effector T cell generation, but this increased frequency of vaccinia specific effector T cells does not translate into enhanced memory frequencies. In the study by Belyakov et al., they found that CpG-ODN alone could protect mice for a brief time from lethal challenge with vaccinia virus indicating that the effector cells generated by CpG are not antigen specific. Therefore, MVA with CpG-ODN induces an enhanced frequency of effector cells that are not antigen specific and by day 28, are likely absent. At this point, the antigen specific MVA cells are likely at the same frequency of animals immunized with MVA alone. This was not addressed by Belyakov et al.

Therefore, our data indicates that enhancing innate inflammation concurrently with MVA administration actually abrogates memory CD8$^+$ T cell development. One possible explanation for this occurrence is likely similar to the explanation as to why MVA in Montanide diminishes CD8$^+$ memory T cell development. Due to the MVA dose used in the present study of 10$^6$ pfu, any enhancement of innate immunity would better control the MVA virus immediately upon infection and decrease the potential to stimulate the adaptive immune response. Hence, these data and others indicate that a dose of 10$^6$ pfu of MVA is right at the threshold required to initiate adaptive immune system development. Any method which decreases the amount of virus particles below 10$^6$ will have a substantial impact on the development of adaptive vaccinia specific immune responses. Therefore, adjuvants which lead to a lower available dose of MVA available at any given time or the addition of innate system activators such as TLR
agonists will allow for effective control of MVA by the innate immune system and either lower or eliminate the CD8+ T cell adaptive immune response to MVA.

We had established that we could alter the memory population in MVA immunized animals, but we wondered if the same could be done with Dryvax immunized animals. Therefore, Dryvax immunized animals were treated with Cidofovir. The findings were significant because they provided supporting evidence to indicate that antigen load during the primary response directly contributes to TEM development and memory lineage commitment. Although similar results have been obtained in other studies, we were the first to phenotype the memory cells from the entire memory population (135, 209, 217, 220, 224, 226). More importantly, these results suggest that vaccine regimens can be adjusted to promote the desired composition of the resulting memory pool.

In summary, our data supports the fact that enhanced signaling, through greater antigenic loads during the primary response, can promote ‘progressive’ differentiation. According to the progressive differentiation model, population heterogeneity can be achieved during primary naïve cell division (signal strength) and during subsequent divisions (decreasing potential). However, this has not been definitively proven. It is impossible to determine from the experiments presented here if population heterogeneity occurs before initial division, although we do show that prolonged antigen load can increase population heterogeneity during subsequent divisions (decreasing potential). Therefore, it is difficult from our data to establish if signal strength is a supplemental mechanism responsible for introducing population heterogeneity (TEM and TCM) into the memory pool in addition to decreasing potential. Although addressing this question was
attempted by altering initial signal strength with TLR agonist and inactivated Dryvax, none of these treatments yielded conclusive data.

While we were able to demonstrate enhanced $T_{EM}$ levels with more immunogenicity and inflammation, it is important to consider that $T_{CM}$ levels remain relatively constant across groups of immunized mice. To address this issue, it is essential to decipher what is different about the $T_{CM}$ at day seven versus the $T_{CM}$ at day 28 with regards to differentiation. This idea in intriguing, especially when we consider if differences exist in the differentiation of single IFN-$\gamma$ $T_{EM}$ (potential MPEC pathway) and dual IFN-$\gamma$ /TNF-$\alpha$ $T_{EM}$ (potential SLEC pathway) cells. To further complicate the issue, we have yet to determine whether one cell can expand and differentiate into a $T_{EM}$ and a $T_{CM}$ or if each cell is limited to one ‘choice’, essentially the ‘one cell, one fate’ or ‘one cell, multiple fates’ hypotheses. Deciphering issues such as these will not only help in determining the restriction of $T_{CM}$ development in this study, but also aid in understanding memory T cell development at the single cell and population level. Ultimately, research in these areas will lead to more rational immunotherapy and informed vaccine design.
CHAPTER 4

Introduction to Relevant Material Regarding Dengue Virus

Significance

Dengue virus is the causative agent of Dengue Fever (DF) and Dengue Hemorrhagic Fever (DHF). Infection with Dengue Virus (DENV) occurs in over 50 million individuals every year (253). Most of the infections result in the mild form of disease, Dengue Fever. However, the number of cases of DHF have steadily increased over the past 50 years (253, 254). In addition, DHF cases have increased dramatically in the Western hemisphere since 1981 spreading through Central and South America and beginning to proceed into North America (255). Current estimates suggest that about 3 billion people worldwide are at risk for DHF with thousands of deaths each year due to DHF. Therefore, it is clear that Dengue Virus is spreading rapidly and represents a significant health risk to individuals around the world. Due to this fact and the thought that DENV can be easily altered to produce more virulent strains, DENV is classified as a Category A Priority Pathogen by the United State Government.

Developing a vaccine to all four DENV serotypes has proven to be difficult. Various vaccines have either been tried or are in development (256-261). However, there are four DENV serotypes and a vaccine must protect against all four DENV serotypes in order to be effective. The reasoning behind this is because there is a strong correlation between increased risk of DHF upon a secondary heterologous serotype infection (262, 263). Other factors can also contribute to DHF, which will be presented in more detail later, but the correlation of these to DHF is weaker and the mechanisms are inadequately defined (75). Therefore, if a Dengue vaccine induces immunological protection against
only one serotype and the immunized individual is subsequently exposed to a second
DENV serotype, then this individual will have a strong probability of developing DHF.

The factors contributing to the development of DHF upon secondary DENV
exposure have not been determined. Initial hypothesis suggested that cross reactive
antibodies specific for the initial DENV serotype were solely responsible for increasing
viremia upon a secondary heterologous DENV infection due to enhancing viral uptake
into monocytes (264, 265). This event is called Antibody Dependent Enhancement or
ADE. However, recently it has been suggested that T cells, specifically CD8 T cells play
an important role increasing disease severity and leading to DHF upon secondary
heterologous DENV serotype infection through a mechanism of original antigenic sin
(266).

Original antigenic sin describes a phenomenon where low affinity memory T cells
dominate the immune response over higher affinity naïve T cells. The low affinity
expansion of the memory T cells overwhems the potential high affinity response of the
naïve T cells. Compounding original antigenic sin with respect to DENV infection is the
fact that partial T cell agonists induce altered cytokine profiles and/or uncontrollable
proliferation. Therefore, while in many cases, the lower activation requirements
necessary for the expansion of memory cells is advantageous, in DENV infection, the
serotypes are similar enough to induce the activation of inefficient low affinity memory
cells that actually overwhelm the response of potential higher affinity naïve T cells. It
has been suggested that the slight difference in the serotypes provide for partial T cell
agonists that then cause the already overwhelming low affinity memory T cells to
produce altered cytokine profiles. Therefore, in the case of a second serotype DENV
infection, the subtle differences in the second serotype leads to severe cytokine storm and progression into DHF (266, 267).

Due to the fact that the development of a vaccine has been difficult and the potential ease of DENV mutagenesis to create more virulent strains, the United States Government has put many more resources towards understanding the causes of DHF and most importantly, developing an effective “tetravalent” Dengue vaccine. An effective tetravalent vaccine would induce immunological protection from all four DENV serotypes.
**Background**

Human transmission of Dengue virus is believed to have originated in southeast Asia around 300 A.D. (13, 268). However, the first recorded epidemic was described by Benjamin Rush in Philadelphia in 1780 (13). Currently, epidemics of Dengue occur around the world, particularly in South America, Asia and Australia. Dengue virus is transmitted by mosquitoes belonging to the genus, *Aedes*, and most often by the highly domesticated *Aedes aegypti*. The DENV does not require enzootic cycle for maintenance, but is rather fully adapted to humans (269).

Within the past 30 years, there has been a surge in epidemics of Dengue (269, 270). This is thought to be due mainly to urbanization practices that have occurred since World War II. First, the *Aedes aegypti* mosquito became widely distributed in urban centers. Second, there has been massive urbanization in southeast Asia and South America with a lack of mosquito control. Therefore, large population growth into urban centers, in conjunction with the increase of the vector responsible for Dengue transmission has led to a the immense population at risk for infection (254). Recent studies have indicated that this increase in transmission and resulting epidemics has resulted in an increase rate of evolution of these viruses, which in turn, has lead to a greater diversity of the viruses (271, 272). Therefore, it has been suggested that in the future, Dengue viruses will likely develop with increased immunopathogenesis and transmission potential (273).
Characteristics of Dengue Virus

Dengue viruses are single positive-stranded RNA viruses that belong to the family *Flaviviridae*. Examples of other viruses in this family are yellow fever, St. Louis Encephalitis and West Nile. The Flavivirus genome is translated as a single polyprotein, which is cleaved by host proteases into 10 proteins. The capsid (C), membrane (M) and envelope (E) proteins all make up the viral membrane. The other seven proteins are called nonstructural proteins (NS) and are responsible for viral replication and protease activity (13).

As mentioned previously, there are four serotypes of DENV. Serotypes are distinguished by their antigenic complex as measured by the plaque reduction neutralization test (274). These differences in the antigenic complex are due to sequence differences in the E protein (275). DENV-1 and DENV-2 were first isolated during World War II (49). DENV-3 and DENV-4 were isolated soon after in the 1950s (276). There are also various strains of each serotype that have been identified. Different serotypes and even selected strains of particular serotypes seem to have greater epidemic potential and virulence (150, 277-280).
Current Understanding of Immunity to Dengue

Primary DENV infection often only causes a febrile illness that last for 3-7 days resulting in headache, joint pain, malaise and in some cases, a minor maculopapular rash (281). The majority of patients who are diagnosed as cases of DF recover without complications. Therefore, the concern of researchers has been focused on determining the factors contributing to the development of DHF. As indicated previously, it has been suggested that DHF can be caused by primary infections with certain strains of DENV. Also, age and genetic factors likely participate in primary DENV infection leading to the development of DHF. However, most often, DHF is caused when an individual who was infected previously with one serotype of DENV becomes infected with a second serotype of DENV. Hence, most of the work that has been done regarding the roles of the innate and adaptive immune response to DENV deals with the pathogenic characteristics of a heterologous secondary DENV infection. DENV-2 and less frequently, DENV-3, have been most commonly associated with heterologous infections leading to DHF(255).

The causative factors for the development of DHF upon secondary heterologous DENV infection have not been characterized. Increased viral loads are most often associated with secondary cases of DENV infection and DHF(282-284). Initial studies done to determine the reasoning behind the increased viral load suggested that it was due to the inability of preexisting antibodies specific to the initial DENV serotype to neutralize the second DENV serotype (265). The non-neutralizing antibodies actually enhance viral uptake by macrophages, which leads to the increase in viremia (264, 285). As indicated previously, this phenomena is called Antibody Dependent Enhancement (286).
Enhanced viremia in patients with DHF as opposed to DF is well documented. The enhanced viremia has been suggested to be due to ADE and there are several pieces of supporting evidence for this hypothesis. It has been indicated that passive transfer of antibodies against DENV did lead to increased viremia in previous studies (264, 265, 287). In addition, DHF occurs frequently in newborns born to mothers with DENV antibody titers, indicating that transplacental acquisition of DENV antibodies can lead to DHF (288). Also, utilizing a highly sensitive flow cytometric technique, ADE has been shown to occur in several cell lines with various monoclonal anti-DENV Abs (289). ADE observed in this study was even capable of occurring in cells which lacked Fc receptors. However, in disagreement to the ADE hypothesis of increased viremia, high viremia titers can also be observed in severe cases of primary infection indicating that there are other factors involved in augmenting viral loads (290). Moreover, a recent study found that preexisting DENV antibodies are not always necessary for effective DENV enhancement (291). Hence, the increased viremia seen in patients that develop DHF as a result of a secondary heterologous DENV infection is not due solely to ADE.

Cytokine profiles have been observed in DHF patients that differ from those seen in patients with DF. An increase in soluble TNFR, IL-2 and CD8 receptors have all been shown to correlate with disease severity (292-294). In addition, IFN-γ reduction and TNF-α and IL-10 enhancement have been shown by a number of studies to correlate with disease severity, especially in DHF cases (266, 267, 287, 295, 296). As a result of these findings, it has been suggested that the cytokines affect a variety of cell types including TNF-α on endothelium cells, which enhance capillary leak, and macrophages, which use IFN-γ signaling to increase viral scavenging. Hence, it has been suggested that patients
with DHF develop plasma leakage and increased viral loads as a result of the altered cytokine profiles. While T cells, specifically CD8+ T cells, have been suggested as a source of these cytokines, the definitive proof is lacking. However, it is likely that the factors which contribute to enhanced viral loads upon secondary heterologous DENV infection are a combination of ADE and ineffective, or possibly detrimental, memory T cells. The resulting ineffective and potentially even detrimental memory T cell responses are thought to be generated from partial T cell agonists and a phenomenon termed Original Antigenic Sin.

Research to define the definitive mechanisms of DHF pathogenesis has been limited because of the inability to develop an effective small animal model due to the control of DENV in rodents (75, 297, 298). The effective control by rodents of DENV dissemination is thought to be due primarily to the inability of DENV to modulate the IFN response in these animals (299, 300). There is increasing evidence that DENV encode proteins that interfere with the host IFNα/β response, leading to greater viral dissemination in humans. However, as is the case with VV encoded IFN-γ decoy receptor, these DENV encoded IFN modulating proteins are human adapted and highly species specific. Therefore, rodent IFN responses are not affected during the innate immune response and DENV infection is effectively limited. Hence, more work is being done with mouse adopted strains of DENV to acquire one that emulates human DENV pathogenesis in mice (300).
Partial T Cell Agonists and Original Antigenic Sin

The long term maintenance of both memory CD4+ and CD8+ T cells to primary DENV is well documented (81, 282, 301-304). There have been numerous research groups that have performed studies to look at issues of cross reactivity of memory T cells among various serotypes (301, 305-308). However, these studies did not address altered cytokine profiles because of cross reactivity, but simply indicated that some clonotypes of memory T cells are cross reactive when measured by proliferation or cytotoxicity assays. While the results from these studies do not directly indicate that cross reactive memory T cells play a role in disease pathogenesis, these findings were pivotal in leading to the hypothesis that altered cytokine profiles, which do contribute to DHF, were likely a result of cross reactive memory T cells.

The result from two studies that came out in 1999 began to uncover the fact that heterologous DENV infections resulted in partial activation and altered cytokine production by cross reactive T cells (309, 310). The study by Zivny et al. indicated that even a one amino acid difference in a particular DV-NS3 derived epitope was enough to prevent IFN-γ production by memory CD8+ T cells. Gagnon et al. showed that CD4+ memory T cells specific for DENV-4 could produce high levels of TNF-α upon DENV-2 stimulation. These two studies paved the way for future studies that provided proof that variant DENV epitopes are capable of inducing altered memory cytokine responses via cross reactivity (311-313).

Mangada et al., Bashyam et al. and Mongkolsapaya et al. were able to demonstrate that stimulating memory T cells specific for one epitope derived from one DENV serotype, with a variant epitope from another DENV serotype, altered cytokine
profiles, proliferation potential and degranulation (311-313). This is true for both CD4+ memory T cells (313) and CD8+ memory T cells (311, 312, 314). However, the exact contribution of each to disease severity has not been determined. In any case, it is now clear that DENV specific memory T cells were not only cross reactive to other DENV serotypes, but upon restimulation, the cross reactivity of certain variant epitopes can cause altered cytokine responses from memory T cells (314). These altered responses likely cause a shift in the appropriate protective cytokine profile to an immunopathogenic cytokine profile. It is hypothesized that this altered cytokine profile then leads to endothelial cell damage through excess TNF-α production and a reduced ability to clear virus by macrophages as a result of decreased IFN-γ production. These phenomena lead to more severe disease and potential progression into DHF.

The conclusions from these previous studies have led to the idea that distinctive peptide T cell ligands of different DENV serotypes cause altered cytokine profiles upon secondary heterologous DENV infections, which then leads to increased disease severity and DHF. Supporting evidence for these conclusions is required and because this is a relatively new hypothesis, the data corroborating that increased cytokine production by cross reactive memory T cells is responsible for increased disease severity is limited. There has been one attempt made thus far to address this issue utilizing human studies to measure T cell cytokine profiles to a second serotype infection and the relation to the development of disease (304). They found no significant differences in T cell responses and their correlation with disease severity (304). However, an issue with this study is that only IFN-γ responses by memory T cells were sampled in individuals with a secondary heterologous DENV infection. IFN-γ responses are thought to decrease upon
a second heterologous challenge. Hence, finding substantial differences between the IFN-γ responses in a cohort of only DHF patients is going to be difficult.

Therefore, further proof is required to substantiate that a second heterologous infection of DENV changes the cytokine profiles, detrimentally, as opposed to secondary infection with the same serotype. In addition, it has yet to be determined which memory T cell pool, CD4⁺ or CD8⁺, is responsible for the greatest contribution to the altered cytokine profile. Finally, more evidence is necessary to prove that the altered cytokine profile contributes to disease pathogenesis. Understanding these issues will allow for the development of hypotheses into determining the variant peptide agonists that are responsible for observed altered cytokine profiles and potentially removing these for the development of tetravalent Dengue vaccines.

**Original Antigenic Sin**

The conceptual details of T cell Original Antigenic Sin are not well defined because Original Antigenic Sin does not frequently occur in nature and has been difficult to emulate in a laboratory setting. Dengue heterologous secondary infections may be one of the few instances where the application of the term “original antigenic sin” is appropriate (266). Original Antigenic Sin is currently described as the ability of a pathogen to cause the reactivation of low affinity memory T cells over immunodominant memory and naïve T cells. However, there are still several unanswered questions as to the reasoning for this occurrence.

Laboratory studies with mice using LCMV, Pichinde and vaccinia viruses have come close to artificially inducing original antigenic sin (315-317). One of the studies in particular showed that secondary challenge with different strains of LCMV recalled a less
effective response and decreased viral clearance (316). In addition, clones of primed
CTL were present in excess over naive precursors, which is the fundamental basis of
original antigenic sin. The response upon secondary challenge with a variant LCMV
showed CTL responses against the priming virus rather than the challenge virus.
Interestingly, the CTL response in most cases was determined by the original virus and
cross reactivity was asymmetrical. This model of LCMV infection in mice is the closest
to showing original antigenic sin outside of DENV infection models.

Other studies have shown that an infection with LCMV could actually alter the
primary response to vaccinia virus (315, 317). Cross reactive memory cells were
responsible, but the application of the term “original antigenic sin” is not completely
accurate considering that in these studies, the cross reactive, lower affinity memory T
cells did not overwhelm the responding, higher affinity naïve T cells. Hence, these
studies address the issue of heterologous immunity and the potential cross reactive effect
of memory T cells. While there was greater inflammation associated with these studies
of heterologous immunity, similar to what is seen in secondary heterologous DENV
infection, there was also increased viral clearance, the opposite of what is seen in
secondary DENV infection. Subsequent VV infection following LCMV infection led to
attrition of many LCMV memory cells, while some cells actually increased in frequency.
Hence, subsequent viral infections can decrease the pools of memory cells against
immunodominant epitopes and increase the pool of cross-reactive, less specific memory
T cells. Primary VV infection would not normally lead to the development of many of
the high frequencies of memory cells seen upon VV infection following LCMV infection.
This is likely what occurs with DENV infection, but at a much greater level. The
anomaly with DENV infection is likely a result of the DENV serotypes being nearly identical, but being different enough to provide for partial T cell agonists/ altered peptide ligands. Therefore, instead of a few cross reactive clones expanding, nearly all cross reactive memory T cells clones expand, dominate the response and overwhelm the immune system.

The current predominating hypothesis with regards to the role of memory T cells contributing to DHF upon heterologous DENV infection is the combination of original antigenic sin and altered peptide ligands leading to an exponential increase in TNF-α cytokine production, decreased viral clearance and subsequent severe immunopathology (81). Original antigenic sin provides for a ‘overwhelming’ activation of low affinity memory cells that prevent high affinity memory or naïve cell activation(266). These low affinity memory cells dominate the response and are then exposed to partial T cell agonists that result in the altered cytokine profiles observed in DHF patients. Essentially, the heterologous DENV serotypes are similar enough to induce only the low affinity memory T cells to proliferate and different enough to cause the memory T cells to be inefficient at cell lysis and produce altered cytokine profile.

It is unclear why heterologous DENV serotypes can cause increased immunopathology during secondary heterologous infection, while secondary infection with other Flaviviruses does not lead to increased immunopathology. The T cell receptors (TCR) in every human are immensely diverse due to sporadic gene rearrangements. Therefore, it would reason that there would not be any particular immunodominant cross reactive ligands. However, this is not the case. Despite the broad TCR diversity, there are definite patterns of cross reactivity to particular Flavivirus
proteins, particularly NS3 (301, 318, 319). An explanation for this may be the fact that TCRs seem to be able to recognize structural homology induced in the MHC molecules as a result of peptide binding (320). Therefore, viral peptides may cross-react independent of sequence heterology accessible by the TCR, bypassing the diversity in the TCR and allowing for cross-reactive peptide ligands (318). These data provide supporting evidence for the concept of immunodominance.

However, this reasoning still does not fully answer the question of why heterologous DENV serotypes can cause increased immunopathology during secondary heterologous infection, while secondary infection with other Flaviviruses does not lead to increased immunopathology. According to predominating hypotheses, the immunopathogenesis seen in secondary heterologous DENV infections is modulated by low affinity, cross reactive memory T cells. Therefore, all primary Flavivirus infections followed by secondary heterologous Flavivirus infections should lead to similar immunopathology seen in primary DENV infections followed by secondary heterologous DENV infections. However, while there is cross reactivity among the Flaviviruses, the relative massive increase of cytokines and marked original antigenic sin is not observed. The best explanation is that there appears to be a threshold surpassed by heterologous DENV derived peptides striking the appropriate balance between heterology and homology to cause original antigenic sin. This will be described in the subsequent paragraph. Other Flavivirus family member do not pass this threshold and are similar enough to cause only some cross reactivity among a relative few memory T cell clones. The expansion of some of these low affinity memory T cells is not enough to dominate the response. Therefore, any potentially present high affinity memory cells can respond,
but more importantly, highly specific naïve T cells can mount an effective response. However, this is only conjecture.

A possible explanation for the difference observed with heterologous DENV infections, as mentioned previously, might be due to a threshold surpassed by heterologous DENV derived peptides striking the appropriate balance between heterology and homology to cause original antigenic sin compounded by partial T cell agonists. Therefore, low affinity memory T cells dominate the response. However, these memory T cells are inefficient at lysing infected cells, which provides for greater inflammation and immunopathology. The altered peptide ligands act here because the massive expansion of low affinity memory T cells provide for the possibility of many different peptide ligands to bind and cause distorted cytokine profiles. Original antigenic sin also reasons that the higher affinity, immunodominant memory T cells do not activate because of the derivation in ligands. In addition, since the higher affinity immunodominant T cell clones present in any individual are highly peptide specific, it is likely that most of these high affinity T cells were stimulated during the primary DENV infection. Hence, the immune system must mostly rely on inefficient memory T cells during secondary heterologous DENV infection. It would be interesting if one could calculate the homology versus heterology between viruses required to pass the threshold causing cross reactive memory T cells to dominate the secondary response with enough similarities to prevent any different immunodominant peptides capable of activating naïve T cells. Data is required to support this theory.
CHAPTER 5

Specific Aim 3: Refine the mouse model of heterologous secondary Dengue Virus infection, focusing on the memory T cell’s role in causing serotype cross-reactivity leading to altered cytokine profiles and severe immunopathogenesis

Preface

The development of Dengue Hemorrhagic Fever (DHF), a debilitating disease marked by bleeding, potential shock and possible death, is most associated with secondary heterologous Dengue Virus (DENV) infection. The alteration from a typical cytokine production profile observed after primary DENV infection in cases of DHF, specifically an influx in TNF-α, has lead researches to pursue identifying the mechanisms responsible. Human studies have observed an alteration in cytokine production by cross-reactive T cells in response to DENV partial T cell agonists. However, a direct link between T cells and DHF development has not been discerned. The ability to examine further the effects of T cells in the development of DHF has been limited due to the inability to develop an effective animal model. Here we described experiments in Balb/c mice to identify the T cell contribution to cytokine alterations after secondary heterologous DENV infections. DENV3 infection followed by DENV1 infection lead to a substantial increase in systemic TNF-α levels as compared to DENV1-DENV1 infection. However, analysis of CD4+ and CD8+ T cells did not indicate that T cells were responsible. Therefore, further work is required to identify the mechanism responsible for increasing systemic TNF-α levels after DENV3-DENV1 infection.
**Introduction**

Human studies have not been able to determine the T cell contribution to DHF development. As indicated in Chapter 4, most T cell analysis has been relegated to mainly murine *in vitro* experiments with T cell lines to determine how DENV cross-reactive epitopes affect memory T cells cytokine production, although some *ex vivo* human work has recently been done (266, 284, 305, 307, 311, 312, 314, 319). It has only been proposed that the altered cytokine profiles caused by cross reactive T cells contribute to the development of DHF. However, mouse models provide the ability to determine *in vivo* the role T cell altered cytokine profiles, and potentially other mechanisms, provide for the development of DHF.

Murine studies of disease pathogenesis after DENV infection have been difficult since murine DENV infection is controlled as explained earlier. Therefore, only a limited number of studies have observed DENV pathogenesis in mice and the majority of these murine studies have focused on disease pathogenesis and viral loads after primary DENV infection in mice. In contrast, the murine response to secondary heterologous DENV infection has been unexplored (321). DHF human cases are often associated with high amounts of TNF production after secondary heterologous infections. Therefore, the goal of our research question did not focus on the induction of signs of disease and viral loads after primary DENV infection, but rather the induction of ‘DHF-like’ pathogenic serum cytokine levels and memory T cell responses after secondary heterologous DENV infection.

To determine how heterologous DENV infection affects serum cytokine levels and T cell responses in mice, wildtype virus and wildtype inbred Balb/c mice were used
in the subsequent studies. Balb/c mice are resilient to DENV infection and do not develop clinical signs of disease following primary infection (297, 298). However, non-mouse adapted DENV does disseminate to a limited extent in these mice (322). Furthermore, Balb/c mice have been most commonly used in work with DENV (289, 307, 319). Utilizing our mouse model, cytokine profile changes and cross reactive memory T cell responses, with strains of DENV derived from humans, were able to be observed in a wildtype murine system. We propose that the data derived from this model is better for human application than other, previously used mouse models.

Furthermore, using mice allowed us to observe the secondary immune response using all combinations of DENV primary and secondary infection. Human studies are restricted to naturally occurring epidemics where certain combinations of different primary and secondary DENV infection do not occur. In addition, it is difficult to determine the initial day of exposure in individuals for research purposes. Mice also provide the advantage for multiple blood draws within a short amount of time and access to all lymph organs. Therefore, the serum cytokine levels can be measured at numerous time points during secondary infection and cells can be extracted from different ‘immunological environments’ within the mouse. Most importantly, this mouse model was able to provide insight into the contribution by T cells to a DHF cytokine phenotype, (i.e. enhanced TNF-α and lower IFN-γ levels).
Materials and Methods

Viruses

All Dengue virus serotypes were obtained from the ATCC and as a generous gift from Dr. Michael Diamond at St. Louis University. C6/36 cells were grown to confluency in 75cm² flasks and infected with DENV at a MOI of 1. Cells and virus were incubated at 28°C for 7 days total. However, at four days post infection, 10ml of media was removed and replaced with fresh media. At day seven, cells and media were harvested. Cells were lysed and centrifuged at 500g for 10 minutes. All supernatant was removed and 1ml aliquots were frozen at -80°C. Titer was determined using Vero cells and serial dilutions of virus preparation. Briefly, 300,000 Vero cells per well of a six well plate were incubated in α-MEM overnight at 37°C. The next day, serial dilutions of virus were added to each well and incubated for 2.5 hours at 37°C. SeaPlaque agarose (Cambrex) was added at 1% in α-MEM to layer the cells and virus. After 5 days of incubation at 37°C, cells were fixed with 10% formaldehyde for 1 hour at room temperature. Agarose was removed and 1% crystal violet solution was added to each well. Plates were washed and plaques were counted. DENV1 titers yielded ~3-5*10⁷ pfu/ml on average, DENV2 titers yielded ~1*10⁶ pfu/ml on average, DENV3 titers yielded ~1-2*10⁷ pfu/ml on average and DENV4 titers yielded ~1-2*10⁵ pfu/ml on average.

Injection

For murine injection, the required number of aliquots of virus was thawed and placed in a centrifuge tube at the required titer of DENV. Virus was concentrated by centrifuging for 2.5 hours at 43,000g. The supernatant was removed and the pellet was resuspended in enough PBS to bring the final concentration of virus to 10⁷-10⁸ pfu/ml.
Balb/c mice obtained from Jackson Laboratories were injected at eight weeks with $10^6$ pfu of DENV intravenously in the tail veins.

**ELISAs**

IFN-γ, TNF-α and IL-10 Duoset from R&D Systems were used for all cytokine measurements by ELISA. Manufacturer’s instructions were followed. Briefly, 96 well plates were coated with capture antibody in PBS and incubated overnight at 4°C. Plates were washed and blocked with 1% BSA in PBS. Plates were washed and samples and standards were added to wells and incubated for two hours at room temperature. After washing the plates, secondary detection antibody labeled with biotin was added to every well and incubated at room temperature for two hours. After three washes, streptavidin-HRP was added to each well and incubated for 20 minutes. Plates were washed and substrate solution containing equal parts of H2O2 and Tetramethylbenzidine (BD Biosciences) was added to each well. Plates developed for 20-30 minutes and the reaction was stopped with 2N H2SO4.

**Flow Cytometry**

Blood from tail vein bleeds was suspended in Fix/Lysing solution from BD Biosciences. After ten minutes of incubation at room temperature, samples were washed and leukocytes were frozen at -80°C for batch processing. Upon thawing, cells were stained with antibodies against CD3 (Alexa 700), CD4 (PE), CD8 (PE-TR), CD69 (FITC), TNF-α (Alexa 647) and IFN-γ (PE-Cy7) from Ebiosciences. Intracellular staining was done with Permeablization solution from BD Biosciences after surface staining. After staining, cells were analyzed on the LSR II machine using FACSDiva software and analyzed using FlowJo (Treestar).
**Results**

Balb/c mice were used because they are the most frequently used wildtype strain of mice in the literature and are more susceptible to DENV than other wildtype mouse strains including C57/BL6, WT129 and Swiss (297, 300, 305, 323). Intravenous tail vein injection replicates the natural route of infection and has been shown to be the most efficient at inducing disease development in contrast to intraperitoneal or intracerebral injection (300, 323). A viral dose of 10^6 pfu was used because titers over that have been shown to cause death during the primary infection of Balb/c mice following intravenous inoculation (323). The goal of this study was to sensitize mice during primary infection and observe disease pathogenesis through cytokine alterations and/or weight loss upon secondary infection. Therefore, a titer that would induce a response without causing mortal pathogenesis, at least during initial infection, was used.

Sixteen groups of five female Balb/c mice each were injected intravenously with all the possible combinations of 10^6 pfu of DENV. Four groups received media alone followed by a primary inoculation of DENV. Two other groups of control mice received media alone with no virus. Therefore, there were 22 groups of mice or 110 mice. Mice received the first injection on day 1 and the second injection on day 28. Blood was drawn from the tail on days 28 (before heterologous challenge), 30, 32, 35, 38, 42, 45 and 49. Mice were also weighed at each time point. The serum from the blood was collected and frozen at -20°C. After all the samples were collected, they were thawed and screened in IFN-γ, TNF-α and IL-10 ELISAs.

There were no significant changes in weight measured from any of the groups (Fig. 5-1). Cytokine profiles indicated measurable levels above background of TNF-α
from a 12 of the groups, but no detection of IFN-γ or IL-10 (Fig. 5-2). No groups of mice that were challenged with a heterologous DENV infection showed altered cytokine profile as compared to the control groups of mice that were challenged with the homologous DENV serotype. There was some cross reactivity measured among the groups and these groups were selected for further analysis. Specific groups selected are listed below.
**Figure 5-1** Weights of each group of mice at various time points after secondary infection.
TNFα Cytokine Responses
Challenge with Dengue 1

TNFα Cytokine Responses
Challenge with Dengue 2

TNFα Cytokine Responses
Challenge with Dengue 3
Figure 5-2  TNF-α serum levels in each of the twenty experimental groups of mice. Media controls were not included because there was no TNF-α production in those two groups. IFN-γ and IL-10 graphs are not included because only low and sporadic levels of those cytokines were measured in a few of the groups of mice.
The groups selected to be rerun were 2-1, 3-1, 1-3, 1-4 and 3-4. These groups were selected because TNF-α levels were detectable at sustained, measurable levels. The detection of TNF-α in these groups of mice indicates that there is a level of cross reactivity among these serotypes and if a higher dose is given, then the differences in the cytokine levels might be significantly different in these experimental groups as compared to the control groups. Control groups were 1-1, M-1, 3-3, M-3, 4-4, M-4 and M-M.

Groups of five mice each were injected with 10^7 pfu of DENV 1 and 3 where appropriate and 10^6 pfu of DENV 4 where appropriate. DENV4 would not grow to a high enough titer to use at 10^7 pfu. Therefore, any groups receiving DENV4 in this subsequent study received the same dose of DENV4 as in the previous study. However, the heterologous DENV serotype of DENV1 or DENV3 given to these groups of mice was at the higher (10^7 pfu) dosage. The same experimental procedure was followed as previously described except that mice did not receive the secondary challenge until day 35. Results from the previous experiment demonstrated that some mice had elevated levels of TNF-α at day 28 after primary injection and before secondary injection. Therefore, we decided to wait an additional seven days in expectation that any potentially enduring TNF-α production after the initial injection would be resolved with an extra seven days before secondary injection.

Results from the higher dose experiments indicate that weight did not decrease significantly in any of the groups tested (Fig 5-3). IL-10 levels were not above the detection range (>3pg/ml) in any of the samples tested and IFN-γ levels were not detectable in 96% of the samples tested. Therefore, only results from TNF-α are displayed below (Fig. 5-4). TNF-α levels were substantial in one group. DENV3
primary infection followed by DENV1 infection resulted in a peak TNF-α level of 247pg/ml at day 37 (2 days after secondary challenge). Primary infection with DENV1 and homologous DENV1/DENV1 infection did not elicit a level of TNF-α response over 25pg/ml. Therefore, heterologous infection of DENV1 after DENV3 resulted in a nearly 10 fold higher level of TNF-α as compared to primary or homologous infection of DENV1.

Primary infection with DENV3 or DENV4 led to detectable levels of TNF-α production, but the levels were usually less than 50pg/ml. Homologous DENV1/DENV1, DENV3/DENV3 and DENV4/DENV4 also led to detectable levels of TNF-α, but these levels were never higher than 25pg/ml.
Percent Change of Average Weights

Day Post Primary Injection

DENV1 DENV1
DENV2 DENV1
DENV3 DENV1
C6/36 Media DENV1

DENV1 DENV3
DENV3 DENV3
C6/36 Media DENV3
Figure 5-3  Average percent weight lost upon primary, secondary homologous DENV injections and secondary heterologous DENV injections.
Figure 5-4  TNF-α levels (pg/ml) upon primary, secondary homologous DENV injections and secondary heterologous DENV injections.
Cells were saved from each mouse at every time point during the previous study. We proposed to determine if T cells were responsible for the TNF-α production detected in the serum of animals and if any IFN-γ could be detected in the cells that was not observed in the serum. To accomplish this, cells were isolated from the blood samples of each animal after obtaining serum and stained with various antibodies against cell surface and intacellular proteins.

There was no CD69 detected in any of the samples. We propose that the CD69 molecule must be unstable and upon freezing, the epitope for anti-CD69 antibodies is lost. Low levels of TNF-α and IFN-γ were detected in most samples (Fig. 5-5). However, groups of mice receiving DENV1 followed by DENV1 showed higher percentages of cytokine producing CD4^+ and CD8^+ T cells than groups of mice that received DENV3 followed by DENV1.
C

**CD4 and CD8 TNF-α**

![Graph showing TNF-α levels over time for different groups of CD4 and CD8 cells.

**CD4 and CD8 IFN-γ**

![Graph showing IFN-γ levels over time for different groups of CD4 and CD8 cells.

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Figure 5-5  TNF-α and IFN-γ production by CD4⁺ and CD8⁺ murine T cells following homologous DENV1 and heterologous DENV3-DENV1 challenge.  A. CD3⁺ cells were gated and CD4⁺/CD8⁺ cells were further gated.  B. Sample of dot plot indicating TNF-α (bottom) and IFN-γ (top) production by CD4⁺ and CD8⁺ T cells.  C. Change in cytokine level over time.  Day indicates time after primary infection.  Secondary injection was given at day 35 after blood sample taken.  Percent Cytokine Positive indicates the number of TNF-α or IFN-γ producing CD4⁺ or CD8⁺ cells of total CD4⁺ and CD8⁺ cells respectively.  Standard error bars are provided at each time point.
Discussion

Establishing a mouse model of Dengue Fever and particularly Dengue Hemorrhagic Fever has proven to be difficult (81, 282). It is hypothesized that mice are not susceptible to DENV infection because of the human specificity of DENV which results in the ineffectiveness of IFN modulating proteins in mice. Therefore, attempts to establish the relationship of DENV immunology to disease pathogenesis have failed. However, we proposed that antigenic challenge with heterologous DENV serotypes, while simply an antigenic challenge in mice, would induce a cytokine increase associated with DHF.

In all cases of homologous DENV and heterologous DENV infections, there was never a detectable level of significant weight loss. This finding is indicative of an antigenic challenge because, since there is no virus replication or spread, the innate immune system is capable of mounting an efficient response to clear the antigen before the induction of disease pathogenesis, i.e. loss of appetite which would result in weight loss.

When serum cytokine levels of TNF-α, IFN-γ and IL-10 were compared, there was rarely an increase of any cytokine during a heterologous infection that was greater than the cytokine level detected during a homologous infection. In fact, only TNF-α was detected consistently in the serum samples. When $10^6$ pfu were used of each serotype for injection, most homologous challenged groups of mice showed a detectable level of TNF-α, with the exception of DENV3-DENV3. In addition, there were a few heterologous challenged groups of mice that showed a detectable level of TNF-α. However, as mentioned previously, these levels were never greater than those of the
homologous challenged groups of mice except in the case of DENV1-DENV3 infection. Moreover, even in DENV1-DENV3 heterologous infection, increased TNF-α levels were not sustained. Therefore, several groups of heterologous challenged mice were challenged with virus titers 10 fold higher at $10^7$ pfu. TNF-α levels in the DENV3-DENV1 groups were substantially higher than the DENV1-DENV1 group and stable throughout secondary infection. These data indicated that a phenotype in mice with regards to serum cytokine levels of TNF-α was induced similar to what has been observed in human DHF cases, where TNF-α has been implicated in playing an important role in DHF (81, 267, 294). Regardless, no significant weight loss was measured.

There have been several reports that memory T cells are responsible for the massive T cell activation and cytokine storm associated with human DHF cases due to a mechanism of incomplete and suboptimal activation due to partial T cell agonists and original antigenic sin (81, 266, 309, 312, 314, 324). We hypothesized that we could address this issue of altered/partial T cell agonists initially by identifying if CD4+ and/or CD8+ T cells showed greater cytokine production upon heterologous DENV challenge when compared to homologous DENV challenge. Since administration of DENV3 followed by DENV1 resulted in higher serum TNF-α levels as compared to serum TNF-α levels in DENV1-DENV1 mice, we decided to focus our efforts on T cells in these two groups.

Over time, following secondary injection of DENV, there was fluctuation in the average frequency of TNF-α and IFN-γ producing CD4+ and CD8+ T cells in both DENV1-DENV1 and DENV3-DENV1 groups of mice. However, the DENV1-DENV1
group showed a higher increase in frequency of cytokine producing T cells when compared to the DENV3-DENV1 group. Therefore, these data indicate that T cells are not responsible for the observed increase of serum TNF-α. Hence, there must be some other cell type causing the increase of serum TNF-α in mice injected with DENV3 followed by DENV1.

TNF-α can be produced by a variety of cell types including macrophages and natural killer cells (325). Therefore, the increase of serum TNF-α observed in the current study in response to a heterologous infection of DENV3 followed by DENV1 could be due to ADE, Antibody Dependent Enhancement(289, 326). ADE suggests that antibodies against one serotype of DENV are non-neutralizing against another serotype and instead, the uptake of viral particles by macrophages augments disease severity through increased viral load. Recent data has identified that cells of the mononuclear phagocyte lineage are the primary cell target type for DENV infection (299). Therefore, macrophages are highly susceptible to DENV infection. Macrophages intake antibody-antigen complexes for degradation, but in the case of ADE, non-neutralization of the viral particle would result in enhanced macrophage infection and/or incomplete degradation. Therefore, there would be a greater viral burden on the macrophages. Increasing the number of macrophages infected with DENV would lead to greater TNF-α production. Hence, ADE is a reasonable explanation to the increased serum TNF-α observed in heterologous challenged DENV3-DENV1 mice versus homologous challenged DENV1 mice.

One other potential consequence of ADE and a possible explanation of the increased level of serum TNF-α seen in heterologous challenged DENV3-DENV1 mice
versus homologous challenged DENV1 mice is that the higher level of TNF-α observed in DENV3-DENV1 mice is a result of enhanced natural killer (NK) cell activation through a mechanism of antibody dependent cell-mediated cytotoxicity (ADCC). In this scenario, non-neutralizing antibodies specific for DENV3 cause a build up of antibody attached to DENV1 viral particles. Since these DENV3 Abs are inefficient at DENV1 specific antigen binding, there is not a significant level of neutralization or agglutination, but rather a significant increase in Ab bound DENV1 viral particles. Therefore, NK cells would bind the Fc receptor of these Abs, activate and produce significant levels of TNF-α which would then lead to systemic effects and other classical signs of DHF. 

In any case, the data presented here indicates that serum TNF-α levels can be induced by heterologous DENV3 followed by DENV1 challenge that are greater than those induced by homologous DENV1 challenge in mice. A murine model of DHF has been difficult to establish since mice can innately control DENV dissemination. However, data presented here demonstrates that a symptom similar to that seen in patients with DHF can be induced in a murine model. The source of the TNF-α production has yet to be determined, but appears not to be from CD4+ or CD8+ T cells. There is an increasing body of literature that provides evidence for supporting factors in addition to ADE that contribute to DHF (289, 291, 327). However, these factors have yet to be determined or confirmed. However, it is likely that DHF is caused by numerous factors including ADE, original antigenic sin, partial T cell agonists, genetic variability of humans and more virulent DENV strains.
CHAPTER 6

Conclusions and Future Directions

The threat of an intentional release of smallpox in a bioterrorist attack has led to a nationwide initiative to develop a safer smallpox vaccine that provides equivalent protection as traditional smallpox vaccines such as Dryvax (2, 9). During the eradication campaign, replication competent vaccinia viruses, such as Dryvax, were used with much success as vaccines to eliminate smallpox. The exact mechanisms or correlates of protection were never fully realized during the eradication campaign. Therefore, establishing the protective role of B and T cells in mediating protection from smallpox is difficult under the current circumstances. However, this must be realized in order to determine the efficacy of new smallpox vaccines without naturally occurring smallpox.

Animal models have been used to elucidate the correlates of protection from smallpox. The most relevant animal model to human Variola infection is using macaques as a host for observing protection from monkeypox (63, 64, 66). In this model system, Dryvax elicited antibodies have been shown to protect nearly 100% of animals from death. However, while vaccinia specific antibodies alone can protect macaques from death upon monkeypox challenge, these animals still develop disease as indicated by observed pox lesions (65). When a holistic immune response to Dryvax is evoked, macaques do not develop pox lesions, an indication that T cells play a significant role in mediating protection from disease if not from death.

Preventing transmission is crucial following a deliberate release of wildtype smallpox or a genetically modified, highly contagious form of Variola virus. Antibodies alone may protect individuals from death. However, antibodies alone cannot prevent
these infected individuals from spreading the disease. Therefore, T cell immunity is crucial, specifically, effector memory CD8+ T cells since they can immediately kill virally infected cells which will prevent viral dissemination. Effector memory CD8+ T cells have been shown to be more important than central memory T cells in mediating protection from vaccinia virus and other peripheral viruses in mice (106, 107, 134, 205, 215). Therefore, it is important that the most effective TEM response be elicited for effective protection from disease following infection with smallpox.

The long term persistence (>75 years) of memory B and T cells in humans following traditional smallpox immunization is well documented (52, 67, 71, 73, 77, 78, 80). However, since the type of memory T cells present is important in determining vaccine efficacy, it is necessary to phenotype the memory CD8+ T cells present in individuals vaccinated during the eradication campaign in order to obtain a better indication of possible protection these individuals are still afforded, as well as supporting evidence that substantial levels of TEM cells are elicited following smallpox immunization. Using these data, the community trying to develop newer smallpox vaccines can have a “gold” standard of immune correlates of protection in order to compare to the immunity elicited by new smallpox vaccines (9, 10).

We have demonstrated from these data that traditional smallpox vaccines such as Dryvax elicited substantial numbers of TEM cells that are capable of persisting for over 25 years following vaccination. The presence of TCM in the peripheral blood of these individuals was not observed. However, the presence of vaccinia specific CD8+ TEM cells in previously vaccinated individuals likely indicates a correlate of protection and an indication that TEM are important to consider when developing new smallpox vaccines.
The problem with many new smallpox vaccines in development, such as MVA and subunit vaccines, is that they are no longer replication competent. Therefore, the immune response they elicit is significantly attenuated as compared to that elicited by Dryvax. Hence, murine and primate studies have been done to determine how to ‘enhance’ the response to new smallpox vaccines to ensure the same level of immunity is elected as Dryvax (54, 63). The studies done to date have disregarded the significance of identifying the different memory T cells subsets that develop. Therefore, we describe the phenotype of memory T cells that develop following Dryvax immunization and two other strains of VV with varying degrees of attenuation. We found that Dryvax can induce a significantly higher ratio of $T_{EM}:T_{CM}$ than MVA. Therefore, in order to increase the protective efficacy of MVA, we enhanced antigen exposure and added inflammatory stimulus in order to alter the $T_{EM}:T_{CM}$ ratio.

By prolonging antigen load over an extended period of time with Montanide, boosting antigen load in MVA immunized mice with two doses of MVA and by adding Cidofovir to Dryvax immunized animals to decrease antigen load, we were able to provide substantial supporting evidence in favor of the progressive differentiation model. Hence, antigen load significantly influences CD8$^+$ T cell lineage development. This is exhibited in a human model of T cell memory development presented in Figure 6-1. While this model is continuing gaining greater support and a major piece of the memory T cell development ‘puzzle’ appears to be in place, there are several addition pieces of crucial information required. Some of these are discussed below.
Figure 6-1 Progressive differentiation model of memory CD8⁺ T cell development including signal strength and decreasing potential. Initial signal strength initiates short lived effector cell (SLEC) and memory precursor effector cell (MPEC) formation. Depending on the amount of initial signal strength, the cell will express varying amounts of KLRG1 and regulate CD127 expression. Higher KLRG1 expression is associated with the inability of cells to express CD127, although these molecules do not interact (135). Once a cell passes the threshold into SLEC, that cell’s fate is determined as terminal. These terminal cells respond to IL-15 for survival for a limited amount of time, but eventually undergo apoptosis(135). MPEC have the ability to differentiate into MPEC of ‘decreasing potential’ as more stimulation/antigen load is received. The differentiation status of a memory precursor effector cell (MPEC) determines which type of memory CD8⁺ T cell will develop. CD127 expression is integral for MPEC cell survival and development to TCM and TEM. By increasing the antigenic load, we were able to provide supporting evidence for this model through the observed enhancement of TEM.
One major point of contention within the field trying to explain how memory T cells develop is determining if the one cell, one fate model or one cell, multiple fates model is correct (227). The data presented here does not directly deal with this issue. However, using this model system and exploring the clonotypes in the effector memory and central memory populations could easily address if naïve cells can become both effector and central memory cells (the one cell, multiple fates). There has been much supporting evidence for this model when using different transgenic T cells specific for one ligand systems by various, separate research groups (96, 104, 110, 134, 137). The only evidence for the one cell, one fate hypothesis is that $T_{EM}$ and $T_{CM}$ repertoires were found to be largely distinct in humans (328). Although, this same group later found that one naïve cell could give rise to both $T_{EM}$ and $T_{CM}$ in mice (104).

It is clear that environment plays a significant role in the evolution of memory based on the availability of costimulatory molecules, cytokine milieu and antigen load. In addition, an argument can be made that the TCR plays a role because higher affinity/avidity increases length of time and numbers of times a clonotype is held in signaling rich environment (137). Therefore, one clonotype will expand into several progeny and many will likely become the same memory subset due to the TCR affinity/avidity ($T_{EM}$ or $T_{CM}$). That is why the clonotypes of the subsets tend to be distinct (328). However, there is the possibility of a certain clonotype’s progeny becoming both $T_{EM}$ and $T_{CM}$ if the one cell, multiple fates model is accepted. These issues will require further exploration before memory T cell development is truly understood.
Another point that is debated is the idea of the broad classification of memory T cells into $T_{EM}$ and $T_{CM}$ subsets. Many present arguments that the memory pool is significantly more complex and all these variances need to be taken into account (213, 329-331). In fact, there are several individuals that present data completely against this classification system and argue that memory is completely dependent on tissue factors, not cell intrinsic factors(332). One recent study that was the most profound indicated that activation status dictates memory responsiveness upon secondary challenge, not surface homing markers such as CCR7 and CD62L (331). In addition, they demonstrate that the most differentiated cells induce the strongest response at one month post infection, but are absent one year after infection. These data indicate that less differentiated cell types may be more important in protective memory. In the current study, we found that greater inflammation drives greater differentiation. However, it is important to impede differentiation past the point of ‘no return’ in order to prevent memory loss.

While many of these reports that differ from the current classification system have tended to be relatively ignored by most of the field, it is important to recognize the possibility that the general classification system is not perfect. Recent studies are beginning to better develop the memory classification system and understand the properties that provide for the development and long term maintenance of memory cells beyond cell surface phenotypes (135, 211, 213, 331). However, it is typical for the scientific community to disregard data outside of the ‘status quo’.

For instance, people initially suggested that T-regulatory cells were one group of cells with a set of distinct functional properties. However, now it is realized that T regulatory cells are a complex cell type that possess many unique and different functional
properties depending on a variety of factors (333). In most cases, the functionality is
significantly different among “T-regulatory cells” to warrant separate nomenclature.
Therefore, it is important to observe the poly-functionality of cells in addition to minor
nuisances in phenotypes for many different antigen systems in order to determine if cell
functionality of memory cells is similar enough across systems to be defined as $T_{EM}$ and
$T_{CM}$ or if different classification logic is required.

Studies such as the ones just proposed and the data presented in the current study
present significant implications beyond smallpox vaccines and can be utilized by
vaccinologists in general to enhance or ‘skew’ memory T cells in favor of one type over
another (99, 131, 207, 334, 335). As more information becomes available regarding T
cell memory development and the qualitative differences that exist between memory
populations are realized, designer vaccines based on the antigen of interest can be
developed to target the development of a unique immune response tailored toward
protection from a particular pathogen.

Concerning Dengue virus, the causes of Dengue Hemorrhagic Fever continue to
elude researchers. Before a better understanding of how partial T cell agonists, Original
Antigenic Sin and Antibody Dependent Enhancement lead to DHF is gained, it will
continue to be difficult to develop a successful vaccine for protection against all four
Dengue virus serotypes. Murine models such as the one presented here have not had
much success because the ability of mice to innately control DENV infection with
relative ease.

We were able to demonstrate that we could induce an increase in TNF-α
production upon heterologous DENV infection. However, a disease phenotype was not
obtained. From the data presented here, it is evident that the TNF-α increase is not
dependent on T cells. Hence, it appears that from our data that altered T cell ligands do
not play as significant of a role in inducing DHF as previously hypothesized (81, 266,
312). It is also possible that the innate ability of mice to protect against DENV prevented
a large enough increase of cytokine (i.e. TNF-α) to ensure a dramatic change and cause
immunopathogenesis.

It is important to note that although altered peptide ligands after subsequent
heterologous infections have been implicated in causing DHF due to altered cytokine
profiles, no direct relation to human disease or in vivo animal model of infection has been
found (311, 313). However, there have been data presented that indicate alterations in T
cell reactivity, through a mechanism of original antigenic sin, are responsible for causing
changes in the cytokine profile of patients that developed DHF (266). It is possible that
these altered cytokine profiles from T cells are insignificant in their contribution to DHF,
and rather it is the increased viremia due to ADE that overwhelms the systems and
generates the large increase of TNF-α. Therefore, it has yet to be definitely determined
that T cells contribute to DHF after DENV serotype heterologous infection.

The groups working on Dengue virus are relatively few and because of this
reason, the progression of knowledge regarding DENV pathogenesis has been slow. To
best understand how heterologous infections of DENV contribute to DHF development,
an animal model is required. Although primate models have been used and potentially
offer more significant value, the focus seems to be on the development of an effective
mouse model (285, 297, 299, 300). Only recently have mouse models been developed
that emulate human pathogenesis or DHF(299, 300). These models will be crucial in
fully understanding the development of DHF and the contributions of original antigenic
sin and ADE to DHF.

As time since September 11th, 2001 passes and the political climate changes, it is likely that resources will begin to shift away from research on Category A pathogens and focus back to ‘hot’ topics like cancer and HIV research. Therefore, it is important to develop research that is applicable to the entire field of immunology when working with these pathogens. Addressing issues of memory T cell development and cross reactivity have implications for designing better vaccines for the majority of pathogens. Hence, as focus begins to shift away from understanding how to develop a Dengue vaccine or a safer smallpox vaccine, the research presented here provides the basis to use these pathogens as tools in addressing other scientific inquiries.
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