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Effector $CD4^+$ T lymphocyte Resolution of Acute HSV Infection at Genital and Neuronal Sites, and the Manipulation of $CD4^+$ T cell Responses via TLR Ligand-induced Proinflammatory Cytokine Milieus

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In primary infection, $CD8^+$ T cells are important for clearance of infectious HSV from sensory ganglia. We present evidence of $CD4^+$ T-cell-mediated clearance of infectious HSV-1 from neural tissues. In immunocompetent mice, HSV-specific $CD4^+$ T cells were present in sensory ganglia and spinal cords coincident with HSV-1 clearance and remained detectable at least 8 months post-infection. Neural $CD4^+$ T cells isolated at the peak of neural infection secreted IFN-γ, TNF-α, IL-2, or IL-4 after stimulation with HSV antigen. HSV-1 titers in neural tissues were greatly reduced over time in $CD8^+$ T-cell-deficient and $CD8^+$ T-cell-depleted mice, suggesting $CD4^+$ T cells could mediate clearance from neural tissue. Clearance of infectious virus from neural tissues was not significantly different in $CD8^+$ T-cell-depleted, perforin-deficient or FasL-defective mice compared to wild-type mice. Virus titers in neural tissues of chimeric mice expressing both perforin and Fas or neither perforin nor Fas were significantly lower than in controls. Thus, perforin and Fas were not required for clearance of infectious virus from neural tissues. These results further define the HSV-specific $CD4^+$ T cell response. To determine the influence of differential TLR activation of DCs in development of appropriate $CD4^+$ T cell phenotype, magnitude, and memory, we established bone marrow-derived DCs that were 92.6% $CD11c^+CD11b^+$, and 94.0% $CD11c^+B220^-Ly\text{-}6c^-$ *in vitro*. Ligands for TLR3, -4, or -9 were applied to DCs, and cytokine and chemokine secretion was examined. Particular interest was paid to IL-12 and IFN-γ (important for T_H1 differentiation thought critical against HSV), and antiviral type I interferons. Proliferation and activation of the $CD4^+$ T cells co-cultured with TLR-ligand-stimulated DCs were assessed. $CD4^+$ T cell magnitude, effector function, and establishment of memory generated upon injection of TLR-ligand-stimulated peptide-pulsed DCs were examined. Stimulation of DCs through TLR3 enhanced $CD4^+$ T cell production of large amounts of T_H1-type cytokines and cytolytic molecules. Stimulation of DCs through TLR4 did drive this phenotype, and also enhanced memory $CD4^+$ T cell population formation within the genital tract. A vaccine able to elicit a vigorous, long-lasting $CD4^+$ T cell response may prove important in limiting disease and transmission of virus.

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CHAPTER 1: GENERAL INTRODUCTION

Herpes Simplex Virus (HSV) Types 1 and 2 are significant human pathogens. An estimated 80% of people are infected with at least one strain (52). HSV-2 is typically thought of as the virus responsible for causing genital lesions, while HSV-1 is associated with common cold sores (41). It is estimated that 20-40% of people in the United States suffer herpetic orolabial lesions due to HSV-1, though the estimated number of those infected ranges from 50 to 80% (41). Much like HSV-2, HSV-1 can also cause genital lesions, which is an increasing problem among adolescents (225). However, HSV-1 can also cause corneal scarring and loss of vision, and is the leading cause of infectious blindness in the United States (52, 127, 128). Most troubling is the transport of the virus to the central nervous system, which can occasionally result in encephalitis and devastating neural damage (52, 127, 128). Additionally, infection with HSV-2 also results in increased risk of acquisition of human immunodeficiency virus (HIV) (14, 20, 44, 110).

HSV-1 and -2 have a wide range of clinical manifestations in addition to genital lesions. Gingivostomatitis, or herpes labialis, most often due to HSV-1 infection, and results in the development of vesicles or open lesions on lips, tongue, gums, hard and soft palette during primary infection, and the commonly known “cold sores” during recurrent outbreaks (31, 275). Herpes labialis can cause discomfort during eating, as well as psychosocial issues. Another clinical manifestation attributed to HSV-1 infection is herpes gladiatorum. This infection results in the development of a rash on the hands,

face, neck, or thorax, and is transmitted through direct contact with infected skin of another athlete during contact sports like wrestling (275, 289). Infection is typically mild, resulting in fever and malaise, which clears within 10 to 14 days (5). Eczema herpeticum is a manifestation of HSV-1 due to contact of damaged skin due to eczema, burns, or other skin damage, and in severe cases can lead to dehydration and death (126, 289). Herpetic whitlow, also known as digital herpes simplex as it commonly affects fingers or toes, can be caused by direct contact with open HSV-1 or -2 lesions (43, 289). Herpetic whitlow is a concern in dental and medical offices, as healthcare workers can contract the infection from oral or genital fluids and later pass the infection on to other patients (289).

Virus

HSV-1 and -2 are members of the family Herpesviridae, subfamily Alphaherpesviridae. The genome of HSV is large, around 152 kb, and composed of double-stranded DNA encoding (an estimated) up to 200 genes (252). The dsDNA core is contained within a capsid with T = 16 icosahedral symmetry. This is surrounded by a tegument layer containing around 20 proteins that are thought to play roles in virus replication and structure (124). The tegument layer is surrounded by a lipid envelope, which contains multiple viral glycoproteins (252). Glycoprotein B (gB) and gC function in the binding of the virus to heparan sulfate on host cells, which in turn allows for the binding of viral gD to the host cell via herpesvirus entry mediator (HVEM), nectin-1, and nectin-2, thus initiating fusion of the viral envelope with the host cell (252).

Upon binding of the glycoproteins and fusion of the viral envelope and host cell, the virus capsid and portions of the tegument are transported to the host cell nucleus, where the immediate early (IE) or α proteins are synthesized (91). Members of the α proteins are then responsible for the initiation of the infectious cycle, promoting virus replication and lifting repression of the early (E, or β) and late (L, or γ) genes (91). The β proteins are important for the replication of the viral DNA, may play a critical role in viral replication within special tissues such as neural tissues, and function to suppress themselves while simultaneously enhancing synthesis of the γ proteins (91). The γ proteins are mostly structural, and include the glycoproteins, capsid, and tegument components (91). Nucleocapsid packaging ensues following the assembly of the empty capsid and DNA cleavage to the appropriate genome length (91). The full nucleocapsid obtains its lipid bilayer envelope by attaching to the nuclear membrane, and the virions are then released from the cell surface (91).

The virus invades the local nerve termini via retrograde axonal transport (14, 127, 128). In this way the virus is able to gain access to the neuronal cell bodies within the sensory ganglia, where the virus again undergoes acute replication which is shut down by host cell-mediated events of unknown mechanism, and establishes a lifelong, persistent infection in up to 10% of neurons (14, 52, 110, 128). Reactivation from latency, initiated by viral genes such as the infected cell protein 10 (ICP10), can occur during times of emotional or physical stress, and can cause recurrent disease (14, 128). During periods of reactivation, the virus is shed from the infected host, sometimes in the absence of clinical symptoms, and thus may have an increased chance of infecting additional susceptible hosts (115, 128).

HSV has evolved many immune evasion mechanisms which prevent its recognition by the host immune system during replication. For example, the viral glycoprotein C has been implicated in the resistance of attack by complement (14). Further, glycoproteins E and I prevent the binding of antibody (14). Glyoproteins J and D have been implicated in the prevention of apoptosis of infected cells (297). Also, the serine/threonine protein kinase US3 has been shown to inhibit caspase activation, and therefore effectively blocks the CTL response known to be important for the clearance of HSV (14).

During a reactivation event, the virus causes a down-regulation of class I MHC expression on keratinocytes via the inhibition of the transporter associated with antigen presentation (TAP) by ICP47, thereby interfering with the recognition of the infected cells by $CD8^+$ T cells (14, 110, 286). HSV-specific T cells cannot produce IFN-γ during the prodrome or reactivation stages, and are therefore not able to stimulate NK-mediated cytotoxicity (14). IFN-γ appears important for the activation of NK cells and the induction of cytolytic activity, as inhibition of IFN-γ-mediated activation of NK cells results in reactivation of HSV (14). Interestingly, these HSV-specific T cells regain their ability to produce IFN-γ after lesion healing (14). It is postulated that, during reactivation, the virus evades the immune system by shifting the immune response away from the antiviral T_H1-type response (14). Therefore, by inducing a more T_H2-type cytokine bias, the molecules responsible for the maturation and function of antigen-presenting dendritic cells are downregulated, thereby inhibiting the efficient priming of CTLs (14). Alternatively, there is also increasing support for the idea that reactivated virus may induce T regulatory cells, thereby shifting the immune response away from the

previously described T_H1-type anti-HSV response and thus suppressing the immune system (14).

T cell response to infection with HSV

Cell infiltrates in recurrent human lesions include monocytes, macrophages, dendritic cells, and $CD4^+$ T cells, followed by the infiltration of $CD8^+$ T cells that correlates with lesion resolution (52, 133, 301). Zhu et al demonstrate the importance of HSV-specific $CD8^+$ T cells in the resolution of HSV-2 from the genital mucosa (301). $CD8^+$ T cells infiltrate preferentially the dermis and epidermis surrounding the site of infection, where they remain for at least 2 months at the dermal-epidermal junction even after lesion resolution and decrease in inflammatory response (301). Further, HSV-2-specific $CD8^+$ T cells may contribute to controlling reactivation, as they are positioned between nerve endings in the skin and adjacent basal keratinocytes (301). Therefore, virus-specific $CD8^+$ T cells located between nerve endings in the skin and the adjacent basal keratinocytes may act as sentinels, alerting the immune system to the reactivation event in the subclinical phase, often preventing the extensive replication of virus, disruption of the epidermis, and lesion formation (301). Previous studies have further defined the mechanisms employed by specific $CD8^+$ T cells in the clearance of HSV, demonstrating the necessity of a lytic mechanism and the presence of IFN-γ (55).

Depletion of either the $CD4^+$ or $CD8^+$ T cell subset delays clearance of HSV, but indicates that either subset is capable of clearance (189, 250). Simmons and Tscharke

note that in animals in which $CD4^+$ T cells have been depleted, or in human patients with $CD4^+$ T cell deficiencies such as persons with acquired immunodeficiency syndrome (AIDS), HSV cannot be cleared from skin, and lesion resolution is impossible (246). This may indicate that $CD4^+$ T cells are important for resolution of HSV (246). However, the exact role of $CD4^+$ T cells in HSV resolution (i.e. providing 'help' through costimulatory or cytokine signals to other immune cells, or through direct effector mechanisms) remains to be determined.

While $CD8^+$ T cells have a demonstrated role in the clearance of HSV from the genital tract, and there is evidence for involvement of $CD4^+$ T cells, the cell-mediated events that are important for clearance of virus within the nervous system are not as well defined. Since neurons do not express MHC antigens, it is thought that they are therefore protected against lytic killing (246). However, data presented by Simmons and Tscharke suggest the loss of some neurons during acute HSV infections (246). Since infected neurons do not express MHCI but can upregulate the expression of MHCII, it might be reasoned that, in infected neural tissues, $CD8^+$ T cells maintain the integrity of the nervous system by resolving HSV via non-lytic mechanisms, while $CD4^+$ T cells, perhaps when virus reaches a critical threshold, employ lytic mechanisms to limit the spread of virus.

Vaccines

Even with the availability of antiviral medication for the treatment of genital HSV, infections remain common (20). The development of an effective HSV vaccine is important for reducing social and economic burden, controlling the rate of neonatal herpes infections, as well as for decreasing the increased risk of contracting HIV associated with HSV infection (14, 20, 110, 115). Vaccines against HSV have not been completely protective in all groups, due to the many obstacles created by the virus including: latent infection that evades host immunosurveillance, other viral methods of immune evasion, lack of understanding of the important immune mechanisms and how to induce them via vaccination, as well as determining the best method of vaccine delivery (19, 20, 254). While a vaccine against HSV will probably not prevent infection, immune resistance may play an important role in limiting the spread of virus to the sensory ganglia, resulting in lower latent virus loads which may ultimately alleviate a patient's pain and suffering caused by recurrent lesions, as well as diminishing viral shedding and passage of the virus to additional susceptible hosts.

In designing an effective vaccine against HSV, it becomes important to examine and consider the various responses of the immune system to natural infection. An effective HSV vaccine will likely need to elicit immune responses at both the site of infection (often the genital mucosa) as well as in the neural tissues. Further, it is important to note that an effective immune response at the site of infection is thought to limit the amount of virus that accesses and becomes latent in neurons, which therefore

translates to a decrease in the number of reactivation events and a decrease in viral shedding and passage to further susceptible hosts (14, 159, 239, 246). Other important factors to consider in the design of an effective vaccine include formulation, route of inoculation, site of inoculation, and protocol, as, undoubtedly, these factors will influence the magnitude, composition, duration, and compartmentalization of the induced immune response (14). A vaccine should also ideally be stable, cost-effective, and safe for use in immunocompromised populations (14).

The development of an effective HSV vaccine is hampered by a lack of knowledge regarding the immune correlates of protection, identification of immunogenic epitopes, and the design of a safe and effective immunization scheme (19). HSV-1 glycoprotein D (gD) is a good candidate antigen because it is highly conserved between HSV-1 and HSV-2, and is a main target for $CD4^+$ T cells (19). Further, a recombinant gD vaccine administered with alum and 3'-*O*-deacylated-monophosphoryl lipid A (MPL) adjuvants has been shown to provide significant protection in double-seronegative women (19, 20, 254). Importantly, antibody response to the gD-MPL vaccine was similar to or greater than that induced by a natural HSV infection, further indicating the effectiveness of this vaccine candidate (19, 20, 254). Importantly, when immunized with T_H2 peptide epitopes, mice were not able to survive lethal challenge with HSV-1, but when immunized with T_H1 peptide epitopes, animals developed $CD4^+$ T cell-dependent protective immunity (19). It is hypothesized that combining several of the highly immunogenic gD epitopes, which have been shown to induce a strong T_H1-type response characterized by the production of high levels of the cytokines IL-2 and IFN-γ, might

lead to the creation of a broader, more potent T cell response (19). gD epitopes have also been shown to induce strong antibody responses (19).

Upon restimulation, effector memory cells undergo rapid maturation, can secrete large amounts of cytokines, and are able to quickly enter inflamed tissues. Central memory cells, however, circulate through the secondary lymphoid tissues, take longer to mature into effector cells, and require more time to secrete large amounts of cytokines. Our preliminary data illustrate the important role of $CD4^+$ T cells in protection of the genital tract, sensory ganglia, and spinal cord. Thus, a vaccine with the ability to elicit a vigorous and long-lasting $CD4^+$ T cell response may be important. Such a response would therefore be beneficial to the patient, by reducing neural damage, reducing recurrent lesions at the original site of infection, as well as reducing the passage of virus to new hosts, including neonates. As the development of varied $CD4^+$ T cell responses and phenotypes are possible, the studies presented here aimed to determine 1) how $CD4^+$ T cells protect at genital and neural sites and 2) how to elicit $CD4^+$ T cells at these sites to respond appropriately to infection.

Outline of dissertation

In animal models, cell mediated immunity has proven important for controlling HSV infection. Both HSV-specific $CD4^+$ and $CD8^+$ T cells have been isolated from the lesions of human patients, and these cells are important for the clearance of virus from the genital epithelium (187). Our previous studies and preliminary data demonstrate the

presence of $CD4^+$ and $CD8^+$ T cells in infected sensory ganglia and spinal cords following primary infection with HSV-1, where they accumulate and persist (Fig. 2 and 3). Further, we have shown that HSV-specific $CD4^+$ T cells were present in secondary lymphoid and neural tissues at least through day 168 after primary infection with HSV-1 (Fig. 4). $CD4^+$ T cells are important for maximizing an immune response, including the activation of other important immune cells, including macrophages, B cells, NK cells, and $CD8^+$ T cells. By constructing experiments in which $CD8^+$ T cells were either depleted or genetically absent from animals, we were able to show that $CD4^+$ T cells were sufficient for clearance of HSV-1 from both genital and neural sites after primary infection (Fig. 6). Further, by adoptively transferring $CD4^+$ T cells into genetically B- and T cell-deficient $Rag1^{-/-}$ mice, we again demonstrated that $CD4^+$ T cells appear to be sufficient for clearance of HSV-1 from genital and neural sites (Fig. 7). Our results with $CD4^+$ T cells challenge current thinking, which emphasizes the importance of $CD8^+$ T cells in controlling neural HSV infection. How $CD4^+$ T cells are able to eliminate infectious virus from these tissues remains to be determined, but our results strongly suggest the involvement of nonlytic mechanisms of clearance.

The goal of the studies presented here was to delineate the natural $CD4^+$ T cell response to infection with HSV. This question was addressed through studies examining (a) the activation and persistence of virus-specific $CD4^+$ T cells within genital and neural tissues, (b) expression of lytic granzyme B by virus-specific $CD4^+$ T cells in genital and neural tissues, (c) virus clearance by $CD4^+$ T cells in genital and neural tissues in the absence of TNF, perforin, Fas/FasL, or both perforin and Fas/FasL mechanisms of clearance, and (d) the development of IFN-γ-independent mechanisms of clearance.

Further, we wanted to explore how to elicit an appropriate $CD4^+$ T cell response, thus enhancing the memory $CD4^+$ T cell population and immunity afforded by a less immunogenic vaccine. This was addressed through determining the type of $CD4^+$ T cell response generated *in vitro* to dendritic cells (DCs) activated through various Toll-like receptors (TLRs). These studies included determination of (a) the activation of DCs and (b) cytokine secretion by DCs, in response to different TLR ligands. These *in vitro* studies were extended to examine the effect of TLR-stimulated DCs on $CD4^+$ T cells as determined by $CD4^+$ T cell (a) activation, (b) proliferation, (c) expression of lytic molecules, (d) expression of chemokine receptors and integrins important for trafficking to the genital mucosal and neural sites of infection, and (e) cytokine secretion as a possible determinant of $CD4^+$ T cell lineage commitment. We then attempted to translate these findings to an *in vivo* system, where we examined (a) the numbers of T_H1-, T_H2-, and T_H17-producing antigen-specific $CD4^+$ T cells in secondary lymphoid and genital tissues and (b) cytolytic activity of antigen-specific $CD4^+$ T cells, after immunization with TLR-stimulated DCs.

CHAPTER 2: EFFECTOR CD4$^+$ T LYMPHOCYTE RESOLUTION OF ACUTE HSV INFECTION AT GENITAL AND NEURONAL SITES

Introduction

Herpes Simplex Virus (HSV) types 1 and 2 are significant human pathogens. An estimated 80% of people are infected with at least one strain (52). 16-18% of adults in the United States are seropositive for HSV-2, while estimates for some developing nations are as high as 97% (77, 284). It is estimated that 20-40% of people in the United States suffer herpetic orolabial lesions due to HSV-1, though the actual number of those infected may range from 50 to 80% (41). While seroprevalence of HSV-1 in the United States appears to be declining, the actual number of genital herpes cases attributed to HSV-1 is on the rise (226, 284). Several reports note an increase in the number of genital herpes cases caused by HSV-1, a phenomenon seen in developed nations including the United States (163, 202, 240, 267, 284). Although HSV-2 is typically thought of as the virus responsible for causing genital lesions, the current trend in the United States, especially among adolescents, is HSV-1 as the most common cause of newly diagnosed genital HSV infections (226). Like HSV-2, HSV-1 has been shown to infrequently result in encephalitis and devastating neural damage (52, 127, 128, 253). Severe disease can occur among immunocompromised persons and in newborns, and infection with HSV-2 also results in increased risk of acquisition of human immunodeficiency virus (HIV) (14, 20, 44, 110, 278).

Primary HSV infection is commonly initiated in the genital epithelium, where the virus undergoes acute replication in epithelial cells (128). The virus is then able to invade the local nerve termini, travel via retrograde axonal transport, and gain access to neuronal cell bodies within the sensory ganglia, where the virus establishes a lifelong, persistent infection (14, 52, 110, 127, 128). During times of emotional or physical stress, reactivation from latency can occur, in which the virus travels via anterograde axonal transport back to or near the original site of infection and can cause recurrent disease (14, 128). During periods of reactivation, the virus is shed from the infected host, sometimes in the absence of clinical symptoms, and thus may have an increased chance of infecting additional susceptible hosts (115, 128, 278). It is estimated that among HSV-2-positive individuals, only 20% experience typical lesions, while 60% demonstrate atypical presentations, and 20% are asymptomatic (11, 137). It is estimated that up to 70% of heterosexual transmission events occur during periods of asymptomatic shedding (61, 137, 181). There are 500,000 new cases of HSV-2 reported each year (61, 137, 181).

HSV infections have great psychosocial and economic impact. While the development of medications such as acyclovir have aided the healing of individual herpetic lesion outbreaks, and therefore eased psychosocial burden, the number of HSV infections and the economic cost of these HSV infections continues to increase (37, 76, 260). Szucs et al estimate the direct (clinic consultations, laboratory exams, and medications) and indirect costs (loss of productivity) due to HSV-2 infections in the United States in 1996 at around $1.2 billion (260). Further, the Centers for Disease Control and Prevention report a steady increase in the number of primary cases of HSV infection (37). Thus, the economic burden of HSV must also be on the rise, and

emphasizes the need for an effective vaccine against HSV. Such a vaccine, while unlikely to prevent infection, will limit neural damage, limit reactivation events, and importantly, help limit spread of HSV to additional hosts. However, the development of an effective HSV vaccine is hampered by a lack of understanding of the cell-mediated events associated with the immune response to natural HSV infection.

Cell-mediated immunity

Cell mediated immunity has proven important for controlling HSV infection. Both HSV-specific $CD4^+$ and $CD8^+$ T cells have been isolated from the lesions of human patients, and these cells have been shown to be important for the clearance of virus from the genital epithelium (187). Further, HSV-specific T cells reside in the skin near previous HSV lesion sites, as well as at the junctions between the epithelium and sensory neurons (301). Effector functions mediated by these HSV-specific $CD4^+$ and $CD8^+$ T cells are thought to be responsible for clearance of infectious virus, termination of viral shedding, and resolution of lesions at epithelial sites of recurrent infection (135, 216). $CD4^+$ and $CD8^+$ T cells secreting type I cytokines and possessing cytolytic capabilities are present at the genital site of HSV-2 infection in animal models (187, 192). Human HSV-specific T cells have also been noted to possess these characteristics upon infiltration of active genital lesions (134). As $CD4^+$ T cells are known to be important in supporting the functions of both $CD8^+$ T cells and B cells, an effective vaccine against HSV can be reasoned to require the use of immunodominant $CD4^+$ T helper cell epitopes

(19, 221). However, the exact role of $CD4^+$ T cells in HSV resolution (i.e. providing 'help' through costimulatory or cytokine signals to other immune cells, or through direct effector mechanisms) remains to be determined.

$CD4^+$ T cell help

Important questions in examining $CD4^+$ T cell help for $CD8^+$ T cell responses is when these effects are exerted, and for which $CD8^+$ T cell phase (activation or memory) these interactions are required (21). It has been shown that $CD4^+$ T cells are not required to generate a robust $CD8^+$ T cell response in acute infections, but $CD4^+$ T cell help is required for the maintenance of virus-specific responses in chronic infections (46, 221, 261, 281). $CD8^+$ memory T cells, when transferred to a $CD4^+$ T cell-deficient environment, gradually lost the ability to rapidly respond during rechallenge, produced less interferon (IFN)-γ, and proliferated less efficiently, demonstrating that $CD4^+$ T cells are required for the maintenance of virus-specific $CD8^+$ T cell memory cells (22, 125, 281). Therefore, in designing an effective vaccine against HSV, one must consider the generation of Ag-specific T cells in terms of optimal number, phenotype, and effector function (281). Further, it appears that a vaccine that stimulates only $CD8^+$ T cells will not be as effective as a vaccine that is able to stimulate multiple components of the immune system, including $CD4^+$ T cells (22).

T cell cytotoxicity

While $CD8^+$ T cells are generally thought to be cytotoxic, there is evidence that cytolytic mechanisms may also be important for $CD4^+$ T cell control of infection (7, 12). The presence of $CD4^+$ CTL appears to correlate with inflammatory conditions (and increase over time in chronic, untreated conditions), and these cells are thought to exercise perforin (Pfp)-mediated killing (7, 12, 59, 283, 287). For example, Appay et al. show that HIV-infected persons have increased numbers of $CD4^+Pfp^+$ T cells (compared to healthy individuals), that these cells are cytolytic, and that the number of $CD4^+Pfp^+$ T cells in HIV-infected patients correlates with the stage of disease (7). Aslan et al., in studying cells from patients infected with hepatitis C virus, conclude that the presence of $CD4^+Pfp^+$ T cells correlates with advanced inflammation (12). It has been suggested that $CD4^+$ CTLs may be an important factor in the control of HSV infection, as the immediate early protein ICP47 interferes with the loading of class I MHC molecules with peptides in the endoplasmic reticulum (14, 286). Further, HSV-specific $CD4^+$ CTLs have been isolated from HSV lesions, suggesting they play a role in the clearance of HSV from the genital tract (133). These cells were shown to possess high levels of granule membrane protein (GMP-17) and granzyme A, demonstrating that they contain the necessary components (i.e. lytic granules and cytotoxic effector molecules) for lytic killing (7). Altogether, the phenotype, cytokine profile, and function of these $CD4^+$ CTLs resemble that of differentiated $CD8^+$ T cells, and thus demonstrate the necessary components for lytic killing (7, 81).

There are two major pathways by which CTLs exert cytotoxicity (50). One pathway requires the specific recognition of targets via class I MHC-peptide complexes (cytotoxic granule exocytosis), while the other is MHC-nonrestricted (production of death receptor ligands and cytokines (IFN-γ, TNF-α, FasL) that initiates the caspase cascade) (50). CTLs are known to contain cytotoxic granules within their cytoplasm (82). Perforin, proteoglycans, and serine proteases (granzymes) contained within these granules are cytotoxic effector molecules involved in the lysis of target cells (82). Upon CTL recognition of and adhesion to the target cell, degranulation in the CTL is initiated in a calcium-dependent process (286). Polyperforin creates a pore in the target cell membrane through which granzymes are delivered (286). The target cell is induced to undergo cell death through either osmotic lysis or the delivery of cytotoxic effector molecules (granzymes) through the pore that initiate the apoptotic caspase cascade (169, 273, 282). Granzymes then induce DNA fragmentation, and initiate death of the infected cell (286). Granzyme B mediates apoptosis partly through the mitochondria by inducing the permeabilization of the mitochondrial membrane and release of pro-apoptotic proteins such as cytochrome c (which initiates the caspase cascade) (28). Perforin is important for defense against invading pathogens, and is thought to have a role in homeostasis as suggested by the fatal disregulation of the immune system associated with familial hemophagocytic lymphohistiocytosis (FHL), a homozygous deficiency in perforin (169).

In addition to granzymes which direct apoptosis and perforin which delivers the granzymes to the cytosol of target cells, there is a third component of the lytic granule expressed by natural killer (NK), $CD4^+$, and $CD8^+$ T cells in humans. Known as granulysin, this third effector protein has the ability to distinguish between eukaryotic

and prokaryotic cell membranes, thus exerting its lytic effects on pathogen while sparing host cells (17, 223). Granulysin has been shown to play a role in the defense against intracellular bacteria, fungi, and parasites, either alone or in combination with perforin (255). Granulysin is thought to exert its effects through damaging the mitochondrial membrane of the target cell, thus altering mitochondrial function, or through the induction of caspase-3 (120, 150). Expression of granulysin by $CD8^+$ T cells can be induced upon stimulation with IL-21 and/or IL-15 (96). Interestingly, infection with HIV-1 has been shown to suppress both $CD4^+$ and $CD8^+$ T cell expression of granulysin, which may be important for the HIV-1-induced suppression of T cell function and the development of opportunistic infections during this immunosuppression (96, 296). With respect to HSV, it has been shown that infection with the virus similarly upregulates the expression of granzyme B and granulysin (277).

A CTL can also initiate cell death via Fas/FasL. Upon activation through the TCR, CTLs express FasL (that is normally stored in the lytic granules) on their surface, while Fas is constitutively expressed in many tissues (27, 121). The surface expression of FasL is dependent on de novo synthesis of RNA and protein, and the transport of FasL through the Golgi apparatus (121). Newly synthesized FasL is stored in the lytic granules before delivery to the cell surface (27). FasL on the CTL interacts with Fas expressed on the target cell, target cell recognition results in the polarized delivery of the lytic granule toward the immunological synapse formed between the CTL and the infected cell, and cell death is thus initiated (27, 121). Granule exocytosis is thought to be the main cytotoxic pathway utilized by HSV-specific CTLs (287), and further, it is thought that

HSV might encode genes that prevent cytolysis via the Fas/FasL pathway as well as the function of granzyme B (110).

In addition to FasL and perforin, a CTL can initiate death in a target cell via the release of TNF-α, a pathway which may be especially important in considering the control of acute virus infection of neural tissues (107). Glutamate induces the release of TNF-α from glial cells, which in turn produces caspase-8 and initiates a caspase cascade that involves the recruitment of TNF-R1 (a death receptor) and Fas, thus leading to apoptosis of the neuron (136). An increase in the concentration of TNF-α may lead to the destruction of neural tissues in neurodegenerative disorders as well as in response to pathogens (136). Thus, in considering the elimination of infectious HSV from neural tissues, TNF-α-mediated apoptosis may prove important.

While perforin is considered the main pathway through which CTLs lyse infected target cells, Chen et al have demonstrated that Fas plays an important role in the absence of perforin (38). This demonstrates the importance of investigating the potential for other lytic mechanisms to function in the absence of one or more lytic pathway, suggests a compensatory role for the lytic pathways, and begs the question as to whether noncytolytic pathways may be preferentially utilized in situations where cell lysis may not be beneficial (i.e. the lysis of infected neurons that cannot be regenerated).

One noncytolytic mechanism that is employed by CTLs is the release of the cytokine IFN-γ (107). It has been shown that $CD8^+$ T cells can control HSV infection in neural tissues, as well as prevent reactivation, an effect that may be due to the production and action of IFN-γ (110). IFN-γ also recruits additional immune cells, such as macrophages, to the site of infection (107). IFN-γ has been demonstrated to have an

important function in protection against HSV. Studies utilizing IFN-γ-deficient mice or employing *in vivo* neutralization of IFN-γ demonstrated significantly delayed virus clearance from the genital tract (30, 87, 188, 250). Further, IFN-γ stimulation of parenchymal, rather than of hematopoietic, cells is important for rapid resolution of genital HSV-2 infection in mice (23, 102). In humans, previous studies suggest that upon HSV infection, IFN-γ produced by T cells can synergize with type I interferons to directly inhibit replication of the virus (185, 230). Further, T cell-produced IFN-γ may reverse the downregulation of MHC-I induced by ICP47 on HSV-infected cells (107, 265).

Though IFN-γ clearly plays an important role in protection against HSV, evidence for IFN-γ-independent mechanisms has also been presented. The presence of IFN-γ-independent mechanisms of clearance stem from previous studies demonstrating that, while delayed compared to intact animals, clearance of infectious HSV-2 from the murine genital tract is possible in the absence of either IFN-γ or its receptor (23). Further, in a cutaneous model of HSV-1 infection, studies suggested the presence of a T cell-mediated IFN-γ-independent response (292). The precise role of IFN-γ in developing an appropriate immune response against HSV remains to be fully characterized.

While the exact mechanism(s) of T cell-mediated clearance of infectious HSV remains to be determined, evidence suggest that clearance by HSV-specific T cells requires virus-specific lytic activity in addition to contributions of multiple cytokines such as IFN-γ, TNF-α, IFN-α/β, and IL-15 (9, 30, 55, 104, 188, 214, 229, 250, 271).

Impact

Candidate vaccines against HSV have not been completely protective in all groups, providing, at best, partial protection in double-seronegative women (19, 20, 254). This may be attributed to the many obstacles created by the virus including: latent infection that evades host immunosurveillance, other viral methods of immune evasion, lack of understanding of the most important immune mechanisms and how to induce them via vaccination, as well as determining the best method of vaccine delivery.

How $CD4^+$ T cells protect at genital and neural sites and the ability of vaccine-elicited $CD4^+$ T cells to respond appropriately to infection are not understood. Our preliminary data illustrate the important role of $CD4^+$ T cells in protection of the genital tract, sensory ganglia, and spinal cord (see Figure 4 and Figure 5) (113). Thus, a vaccine with the ability to elicit a vigorous and long-lasting $CD4^+$ T cell response may be important. Such a response would therefore be beneficial to the patient, by reducing neural damage, recurrent lesions at the original site of infection, as well as reducing the passage of virus to new hosts, including neonates.

In conclusion, the development of an effective vaccine against HSV is hampered by several factors, including a lack of understanding of the important cell types and mechanisms required for induction of an appropriate immune response. An effective vaccine against HSV will most likely need to elicit immune responses at both the site of infection and in the neural tissues, as well as eliciting responses from several cell types. In addition to supporting the roles of other immune cells, $CD4^+$ T cells appear sufficient

for clearance of HSV from genital and neural sites (107, 113). HSV-specific $CD4^+$ T cells, by supporting other important immune cell responses and/or through direct effector mechanisms, may prove important for clearance of acute HSV. Studies of the immune responses to natural infection with HSV will help elucidate key events that an effective vaccine will need to elicit.

The goal of the studies presented here was to further examine the natural $CD4^+$ T cell response to infection with HSV. We hypothesized that $CD4^+$ T cells act as effector cells, directly impeding acute replication of HSV-1 in genital and neural tissues, and further, that the effector mechanism of $CD4^+$ T cells does not involve the cytolytic mechanisms Fas, perforin, or TNF-α, but does involve IFN-γ. These questions were addressed through studies examining (a) the activation and persistence of virus-specific $CD4^+$ T cells within genital and neural tissues, (b) expression of lytic granzyme B by virus-specific $CD4^+$ T cells in genital and neural tissues, (c) virus clearance by $CD4^+$ T cells in genital and neural tissues in the absence of TNF, perforin, Fas/FasL, or both perforin and Fas/FasL mechanisms of clearance, and (d) the development of IFN-γ-independent mechanisms of clearance.

Materials and Methods

Virus

HSV-1 strain SC16 and HSV-2 strain 186 were obtained from Dr. Lawrence Stanberry (Columbia University, New York, NY). HSV-2 $333tk^-$ was originally obtained from Dr. Mark McDermott (McMaster University, Ontario, Canada) (178). Virus stocks were prepared by infection of Vero cell monolayers at a multiplicity of infection of 0.01, as

previously described (187, 191). The virus was released from the Vero cells by three freeze–thaw cycles (187, 191). Cell debris was removed by centrifugation, and virus stocks were stored at -80 °C (187, 191).

Mice

C57BL/6J (B6), B6.129S7-Ifng^{tm1Ts}/J (IFN-$\gamma^{-/-}$), CD8$^{-/-}$, perforin-deficient C57BL/6-Pfp$^{-/-tm1Sdz}$ (Pfp$^{-/-}$), T cell receptor transgenic C57BL/6-Tg(TcraTcrb)425Cbn/J (OT-II) containing CD4^{+} T cells specific for the chicken ovalbumin peptide $OVA_{323-339}$, B6Smn.C3H-Fasl^{gld} (*gld*), B6.MRL-Fas^{lpr} (*lpr*), B6.129S7-*Rag1*^{tm1Mom}/J (Rag1$^{-/-}$), and B6.129-*Tnfrsf1a*^{tm1Mak}/J (TNFR$^{-/-}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific-pathogen-free conditions at the University of Texas Medical Branch Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved animal facility. Experiments were conducted with Institutional Animal Care and Use Committee approval and oversight by staff veterinarians.

Virus inoculation and quantification

Mice were inoculated intravaginal (ivag) as described previously (186). Briefly, six days prior to inoculation, mice were treated with 2.0 mg medroxyprogesterone acetate (SICOR Pharmaceuticals, Inc., Irvine, CA) subcutaneously. Hormonal pretreatment was necessary to induce susceptibility of mice to genital HSV-1 inoculation (178, 187), which

may reflect thinning of the genital epithelium (122) or induction of the HSV entry receptor, nectin-1, on vaginal epithelial cells (156). Mice were anesthetized with sodium pentobarbital, and inoculated as previously described (39).

To quantify infectious HSV-1 within the vaginal tract, mice were swabbed with calcium-alginate swabs as described previously (39). Swabs were placed in 1 mL media (medium 199, 2% newborn calf serum, 2% penicillin/streptomycin, 2% amphotericin) and frozen at -80 °C until titration of Vero cell monolayers (113). Clearance of infectious virus from spinal cords and dorsal root ganglia, and spread of virus to hindbrains was determined by quantification of virus by standard plaque assay, as previously described (187). Briefly, groups of five HSV-1-infected B6 mice were euthanized, and the lumbosacral region of the dorsal root ganglia and the adjacent areas of the spinal cord, as well as hindbrains (including cerebellum, medulla oblongata, and brain stem), were harvested, weighed, and frozen at -80 °C in 1 mL media (medium 199, 2% newborn calf serum, 2% penicillin/streptomycin, 2% amphotericin). Tissues were later thawed, homogenized, clarified by centrifugation, and titrated on monolayers of Vero cells. Though polymerase chain reaction (PCR) has proven highly sensitive for detection of HSV, this method does not allow for the delineation of infectious versus latent virus (68). As the current study focused on infectious virus within the spinal cord and sensory ganglia, and therefore the previously established (though less sensitive) method of titration on Vero cell monolayers was employed.

Enrichment of $CD4^+$ and $CD8^+$ T cells from neural tissues

Mice were sacrificed on day 9 after ivag inoculation with 10^5 PFU HSV-1 SC16. Lymphocytes were isolated from pooled spinal cords or dorsal root ganglia, by a modification of the method previously described (171). Briefly, tissues were pushed through mesh screens and stirred for 30 minutes in calcium- and magnesium-free PBS (Invitrogen Corp., Grand Island, NY) at ambient temperature. Samples were then resuspended in a 30% Percoll (Sigma-Aldrich, St. Louis, MO) solution, layered over a 70% Percoll cushion and centrifuged at 500 × g for 20 minutes at ambient temperature. After centrifugation, the upper layer containing dispersed neural tissue was removed, and again spun over a 70% Percoll cushion. Lymphocytes from the Percoll interface of both preparations were then combined.

Flow cytometry

Lymphocytes were stained with fluorochrome-labeled antibodies against CD4, CD8, CD25, CD44, or CD69 (Pharmingen, San Diego, CA). Data were acquired on a Becton Dickson FACSCanto (BD Biosciences, San Diego, CA) at the University of Texas Medical Branch Flow Cytometry Core Facility, and analyzed with FlowJo software (Treestar, Inc., Ashland, OR). The number of activated $CD4^+$ T cells in uninfected or HSV-1 infected iliac lymph node, spinal cord, and dorsal root ganglia was determined using FlowJo software and is presented as number of $CD4^+$ T cells that were also $CD25^+$, $CD44^{hi}$, or $CD69^+$ per 10^6 total isolated cells.

Depletion of $CD8^+$ T cells *in vivo*

Mice received 1.0 mg anti-CD8 (2.43) Ab i.p. on the two days prior to infection (d^-2 and d^-1). Following ivag inoculation with 10^6 PFU HSV-1 SC16, mice also received 0.5 mg anti-CD8 Ab i.p. every other day throughout the study. Depletion was assessed by flow cytometry and was typically greater than 98%.

Neutralization of IFN-γ *in vivo*

Mice received 1.0 mg anti-CD8 (2.43) Ab i.p. on the two days prior to infection. Following ivag inoculation with 10^6 PFU HSV-1 SC16, mice received 0.5 mg anti-CD8 Ab i.p. every other day throughout the study. Additionally, mice were treated with 2.0 mg anti-IFN-γ (XMG) Ab or control (SFR.8) Ab on days 6 and 8 post-infection, and then with 1.0 mg daily throughout the remainder of the study. Treatment with anti-IFN-γ Ab (or control) was began on day 6 post-infection in an attempt to not alter critical IFN-γ-dependent mechanisms of clearance occurring within the genital tract, as the focus of this study was to determine the role of IFN-γ in clearance of infectious virus within the neural tissues. Animals were sacrificed on days 7 and 11 post-infection, and clearance of infectious virus from spinal cords and dorsal root ganglia was examined by quantification of virus by standard plaque assay.

Quantification of HSV-specific T lymphocytes

ELISPOT analysis was performed similarly to the procedure described (187, 191). B6 mice were infected vaginally with 10^5 PFU HSV-1 SC16. Lymphocytes were isolated from iliac lymph nodes, spinal cords, and dorsal root ganglia on days 6; 8; 11; 14; and 42, 69, or 168 days post-infection. Iliac lymph nodes were pooled from five animals, and a single-cell suspension was created by pushing the tissues through a mesh screen. Spinal cords and dorsal root ganglia were pooled from five animals, and processed as described above. Lymphocytes from all tissues were incubated in anti-IFN-γ, anti-TNF-α, anti-IL-2, anti-IL-4, or anti-IL-17 antibody-coated nitrocellulose plates (Millipore Corporation, Billerica, MA) with 5×10^5 mitomycin C (Sigma-Aldrich)-treated feeder cells per well, with and without UV-killed HSV-1 antigen (191). Plates were incubated for 40h, and developed as previously described, by the sequential addition of corresponding biotinylated antibody (Pharmingen), streptavidin peroxidase (Sigma-Aldrich), and 3-amino-9-ethyl-carbazole plus sodium acetate (58, 187). Spot-forming cells (SFC) were quantified with the aid of a dissecting microscope.

Adoptive transfer

$CD4^+$ T cells from spleens of B6, perforin (Pfp)-deficient, or OT-II (MHC-II T cell receptor transgenic mice specific for the ovalbumin peptide $OVA_{323-339}$) mice (18) were purified using positive selection magnetic bead separation (Miltenyi Biotech, Auburn, CA). Purity of the $CD4^+$ T cells isolations was determined by flow cytometric analysis and routinely found to be > 96%. 4×10^6 $CD4^+$ T cells were injected intravenously in

irradiated (900 Rad) B6 or *lpr* mice, thus creating mice that possessed both functional perforin and Fas (Pfp^+Fas^+), neither perforin nor Fas (Pfp^-Fas^-), and mice that expressed both perforin and Fas but were unable to mount a specific response to infection with HSV-1 (OT-II). Mice were allowed to rest for 7d, during which time they received one 0.5 mg dose of anti-CD8 Ab to ensure depletion of $CD8^+$ T cells. Mice were then inoculated vaginally with 5×10^3 PFU HSV-1 SC16.

In complementary experiments, $CD4^+$ T cells from spleens of B6, OT-II, Pfp-deficient, or FasL-deficient mice were purified using positive selection magnetic bead separation (Miltenyi Biotech). Purity of the $CD4^+$ isolations was determined by flow cytometric analysis. 4×10^6 $CD4^+$ T cells were injected intravenously into genetically B- and T cell-deficient $Rag1^{-/-}$ mice. Mice were allowed to rest for 7d, during which time they received one 0.5 mg dose of anti-CD8 (2.43) Ab to ensure depletion of $CD8^+$ T cells. Mice were then inoculated vaginally with 5 x 10^3 PFU HSV-1 SC16.

Quantification of HSV-specific T lymphocytes (IFN-$\gamma^{-/-}$ experiments)

HSV-specific cytokine-secreting $CD4^+$ T cells were quantified in HSV-immune B6, HSV-immune IFN-$\gamma^{-/-}$ (twice inoculated with 2 x 10^5 PFU HSV-2 $333tk^-$) and naïve mice on days 2 and 5 after ivag challenge with 10^5 PFU HSV-2 strain 186 by a modification of the preciously described method (187). Lymphocytes were harvested from HSV-2 challenged mice, and $CD4^+$ T cells isolated using CD4 magnetic isolation (Miltenyi). $CD4^+$ T cells were incubated in anti-IFN-γ, anti-TNF-α, anti-IL-4, or anti-IL-17 antibody-coated nitrocellulose plates (Millipore) with 5×10^5 mitomycin C-treated feeder cells per

well, with UV-killed HSV-2 antigen (191). Plates were incubated for 40h, and developed as previously described, by the sequential addition of corresponding biotinylated antibody (Pharmingen), streptavidin peroxidase (Sigma-Aldrich), and 3-amino-9-ethyl-carbazole plus sodium acetate (58, 187). Spot-forming cells (SFC) were quantified using an ImmunoSpot reader with ImmunoCapture software (Version 6.0) and analyzed with ImmunoSpot software (Version 4.0) from Cellular Technology Ltd. (Cleveland, OH).

Adoptive transfer of $CD4^+$ T cells from HSV-immune B6 or HSV-immune IFN-$\gamma^{-/-}$ mice

HSV-immune B6 and HSV-immune IFN-$\gamma^{-/-}$ animals were challenged with 5 x 10^5 PFU HSV-2 strain 186. Lymphocytes were harvested four days later from the spleens and iliac lymph nodes. 2 x 10^8 cells per 175 cm^2 flask were cultured for three days in the presence of UV-killed HSV-2 antigen and 100 U/mL recombinant IL-2. OT-II splenocytes were cultured similarly, using 1.2 x 10^8 mitomycin C-treated OT-II peptide-pulsed syngeneic splenocytes, for use as a control. Activated $CD4^+$ T cells were then harvested from the cultures, purified by CD4 magnetic isolation (Miltenyi), and adoptively transferred into irradiated (650 cGy) recipient mice at 2.25 x 10^6 cells per mouse. Recipient mice were then challenged ivag with 10^4 PFU HSV-2 333tk^-.

Statistical analysis

HSV-specific T cell quantification and HSV-1 titers were analyzed by Mann-Whitney U-test, or one-way ANOVA with the Bonferoni correction for multiple groups, as appropriate. Analyses were performed using GraphPad Prism Version 4.0 software (GraphPad Prism Software, San Diego, CA). *p* values of less than 0.05 were considered statistically significant.

Results

HSV-1 clearance from genital tract, dorsal root ganglia, and spinal cords

In animal models, cell mediated immunity has proven important for controlling HSV infection. To examine the kinetics of clearance of HSV-1 from neural tissues, groups of B6 mice were inoculated ivag with HSV-1, and infectious virus in the dorsal root ganglia and spinal cords was quantified. Figure 1 demonstrates a typical pattern of clearance of infectious HSV-1 from neural tissues. In the dorsal root ganglia, the mean virus titer declined greatly on days 8 and 10 post-inoculation (Fig. 1). Further, the incidence of samples with infectious virus declined over the course of the experiments from 7/8 animals on day 6, to 4/8 animals on day 8, and 2/8 animals on day 10 post-inoculation, demonstrating a trend toward clearance of infectious HSV-1 from dorsal root ganglia. The clearance pattern in spinal cords was even more dramatic. Virus titers greatly decreased between days 6 and 8, and were below the limit of detection by day 10 post-

inoculation. Further, while 6/8 animals contained infectious virus in the spinal cord on day 6 post-inoculation, the incidence of samples containing infectious virus decreased to 1/8 animals by day 8 and 0/8 animals by day 10 (Fig. 1). The decrease in the amount of infectious virus present over the course of the experiment was significant in both dorsal root ganglia (p = 0.004 from day 6 to day 8, and p = 0.001 from day 6 to day 10) and spinal cord (p = 0.019 from day 6 to day 8, and p = 0.002 from day 6 to day 10).

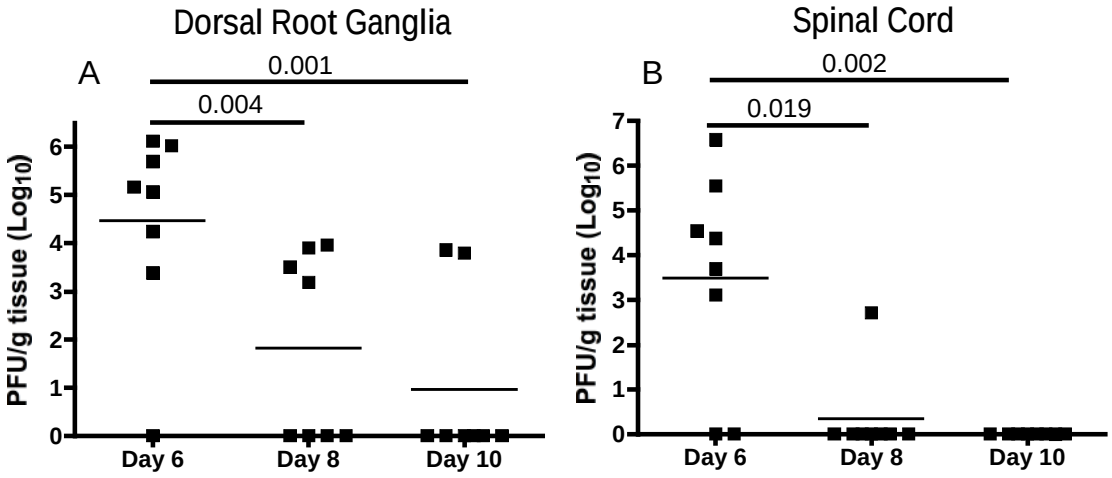

FIG. 1. HSV-1 clearance from dorsal root ganglia and spinal cord. B6 mice were inoculated ivag with HSV-1. Dorsal root ganglia (A) and spinal cords (B) were harvested on the indicated days for quantification of virus as described in Materials and Methods. The decrease in the amount of infectious virus present over the course of the experiment was significant in both dorsal root ganglia (p = 0.004 from day 6 to day 8, and p = 0.001 from day 6 to day 10) and spinal cord (p = 0.019 from day 6 to day 8, and p = 0.002 from day 6 to day 10).

$CD4^+$ and $CD8^+$ T cells are present in the HSV-1-infected spinal cord and dorsal root ganglia

Both HSV-specific $CD4^+$ and $CD8^+$ T cells have been shown to be important for the clearance of HSV-1 and HSV-2 from the genital epithelium (142, 187, 209), and an important role for $CD8^+$ T cells in the protection of nervous system tissues during a primary HSV infection has been suggested (246). To examine the role of T cells in protection of neurons in mice inoculated ivag with HSV-1, the presence of $CD4^+$ and $CD8^+$ T cell subpopulations in neural tissues during the course of HSV-1 infection was assessed. Few $CD4^+$ or $CD8^+$ T cells were detected in the dorsal root ganglia and spinal cords of uninfected mice (Fig. 2A, C). Increased numbers of both $CD4^+$ and $CD8^+$ T cells were detected in the spinal cords and dorsal root ganglia of HSV-1-infected B6 mice on day 8 post-infection (Fig. 2B, D), concurrent with the time of rapid virus clearance Fig. 1).

These findings were extended by examining the kinetics of cellular infiltration into draining lymph nodes and infected tissues. The number of $CD4^+$ T cells from iliac lymph nodes, spinal cords, and dorsal root ganglia expressing the activation markers CD25, CD44, or CD69 increased following ivag HSV-1 inoculation and, in all but one, peaked around day 6 or 8 post-infection (Fig. 3). Importantly, the peak of the activated $CD4^+$ T cell response correlated with the period of rapid clearance of infectious virus in immunocompetent mice, occurring around day 8 post-inoculation (Fig. 1).

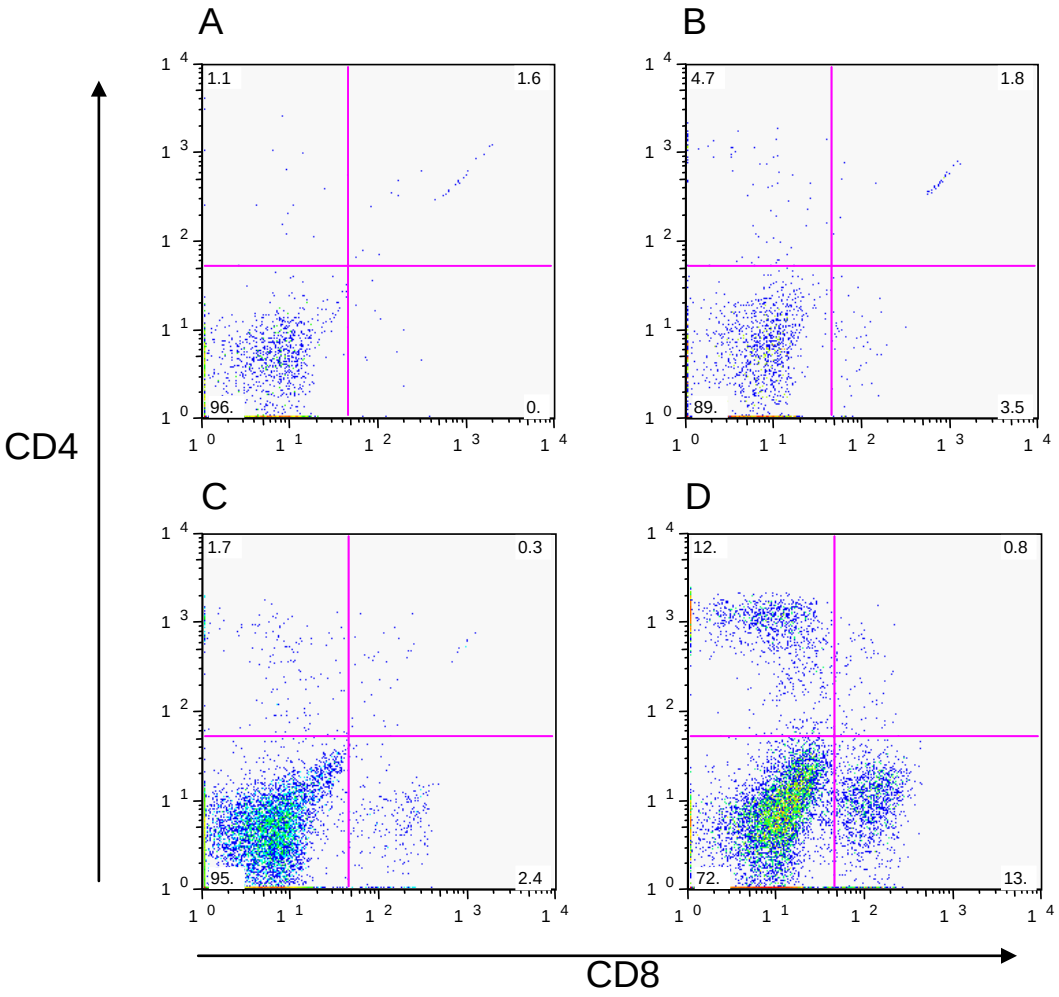

FIG. 2. Presence of $CD4^+$ T cells and $CD8^+$ T cells in HSV-1-infected spinal cords and dorsal root ganglia. B6 mice were infected with HSV-1, these mice and uninfected controls were sacrificed on day 8, and lymphocytes were isolated from pooled spinal cords or pooled dorsal root ganglia and stained with fluorochrome-labeled antibodies against CD4 and CD8. (A) Naïve dorsal root ganglia, (B) HSV-1-infected dorsal root ganglia, (C) naïve spinal cords, (D) HSV-1-infected spinal cords (D).

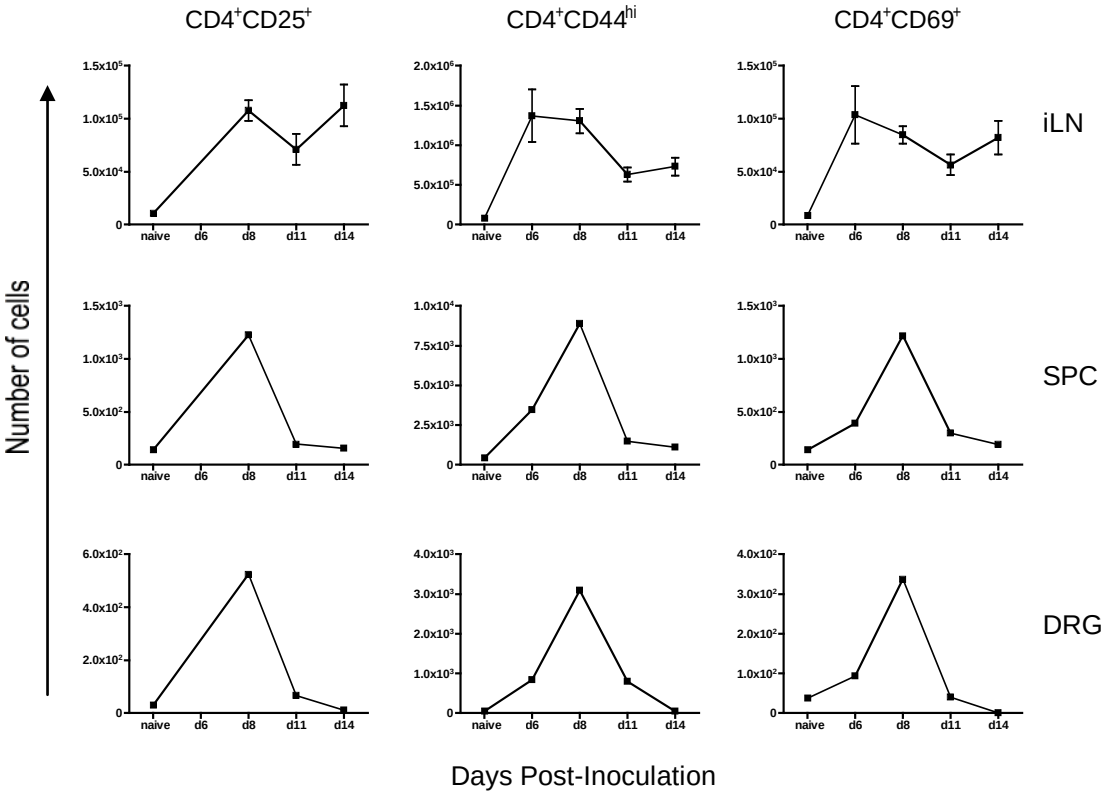

FIG. 3. $CD4^+$ T cells with activated phenotype are present in the spinal cord and dorsal root ganglia of HSV-1 inoculated mice. Lymphocytes were isolated from iliac lymph node, spinal cord, and dorsal root ganglia of HSV-1-infected mice on indicated days. Lymphocytes were stained with fluorochrome-labeled antibodies and analyzed by flow cytometry as described in Materials and Methods. The mean number of total $CD4^+$ T cells isolated from iliac lymph nodes ranged from 7.35×10^4 per 10^6 cells in naïve animals to 1.30×10^5 per 10^6 cells at the peak on day 8. The number of total $CD4^+$ T cells isolated from pooled spinal cords ranged from 7.63×10^2 per 10^6 cells in naïve animals to 1.02×10^4 per 10^6 cells at the peak on day 6. The number of total $CD4^+$ T cells isolated from pooled dorsal root ganglia ranged from 7.12×10^2 per 10^6 cells in naïve animals to 4.52×10^3 per 10^6 cells at the peak on day 8.

HSV-specific effector CD4+ T cells are present in neural tissues during resolution of acute HSV-1 infection

Because $CD4^+$ T cells expressing activation markers indicative of recent antigen exposure infiltrated the spinal cord and dorsal root ganglia of HSV-1-infected mice, the

antigen specificity of the $CD4^+$ T cells present at these sites was tested. B6 mice were inoculated ivag with HSV-1, sacrificed, and iliac lymph nodes, spinal cords, and dorsal root ganglia were harvested on various days post-inoculation. Lymphocytes were isolated, and cultured in the presence or absence of UV-killed HSV-1 antigen. The frequency of HSV-specific (IFN-γ-producing) $CD4^+$ T cells in iliac lymph nodes, spinal cords, and dorsal root ganglia of HSV-infected mice are provided in Figure 4. In all tissues tested, the number of HSV-specific $CD4^+$ T cells peaked around day 6 or 8 post-infection, followed by a gradual decline at each location examined. Importantly, these HSV-specific $CD4^+$ T cells persisted in the secondary lymphoid and neural tissues tested, and were detectable through at least 8 months post-infection.

These results were extended by further defining the cytokines secreted by HSV-specific $CD4^+$ T cells in iliac lymph nodes, spinal cord, and dorsal root ganglia. The number of local HSV-specific $CD4^+$ T cells that produced IFN-γ, TNF-α, IL-2, IL-4, or IL-17 was quantified by ELISPOT analysis on day 8 after ivag inoculation with HSV-1. This time point corresponds to the peak of HSV-specific $CD4^+$ T cell infiltration, as well as with rapid clearance of infectious virus (Fig. 1, 2). HSV-specific $CD4^+$ T cells producing IFN-γ, TNF-α, IL-2, or IL-4 were detected in these tissues on day 8 post-inoculation (Fig. 4D). HSV-specific $CD4^+$ T cells producing IL-17 were detected in the iliac lymph nodes, but not in the spinal cords or dorsal root ganglia of HSV-1-inoculated mice (Fig. 4D). The frequency of IL-4-producing HSV-specific $CD4^+$ T cells was highest in the spinal cords (Fig. 4D).

As effector $CD4^+$ T cells have been shown to express cytolytic activity (187, 201, 285) during viral infections, including HSV infections, neural $CD4^+$ T cells in the present

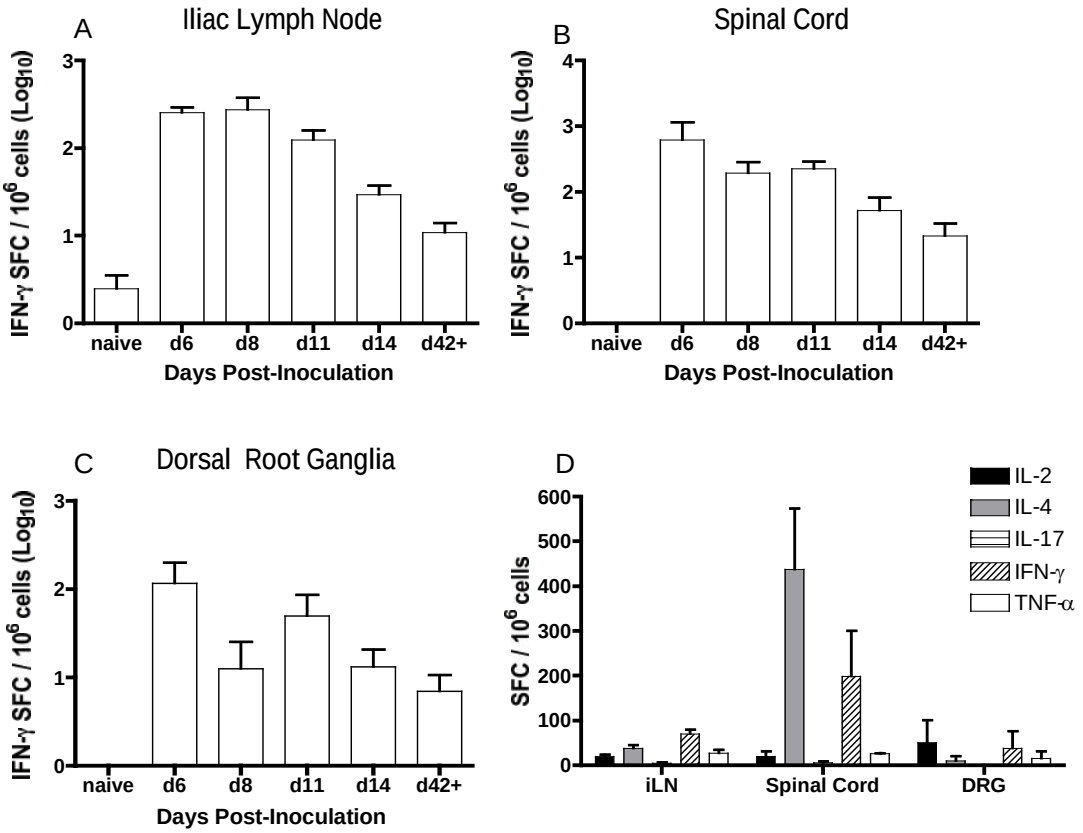

FIG. 4. HSV-specific $CD4^+$ T cells in iliac lymph nodes (A), spinal cords (B), and dorsal root ganglia (C) after infection with HSV-1. B6 mice were inoculated ivag with HSV-1, sacrificed on the indicated days, and isolated lymphocytes from these tissues were plated on anti-IFN-γ-Ab-coated ELISPOT plates. Extended time points labeled as 'd42+' represent three compiled experiments that were harvested at d42, d69, and d168 post-inoculation. Results are presented as the number of spot forming cells (SFC) per 10^6 lymphocytes. (D) Production of IFN-γ, TNF-α, IL-2, IL-4, and IL-17 by HSV-specific $CD4^+$ T cells in iliac lymph nodes, spinal cords, and dorsal root ganglia after infection with HSV-1. B6 mice were inoculated ivag with HSV-1 and sacrificed on day 8 post-infection. Pools of lymphocytes from spinal cords, dorsal root ganglia, or individual lymph nodes were quantified by ELISPOT. Mean cell counts of iliac lymph node lymphocytes from individual mice ranged from 1.1×10^6 for naïve mice, to 1.0×10^7 at the peak of the response on d6. Mean cell counts for spinal cord lymphocytes pooled from five mice ranged from 1.1×10^6 - 1.3×10^6. Mean cell counts for dorsal root ganglia lymphocytes pooled from five mice ranged from 4.15×10^5 - 6.07×10^5 cells.

study were examined for granzyme B on day 7 post inoculation with HSV-1. Figure 5 demonstrates that $CD4^+$ T cells isolated from the spinal cords and dorsal root ganglia of HSV-1-infected B6 mice expressed granzyme B. In experiments of identical design, detection of FasL on the surface of neural $CD4^+$ T cells was not consistent (data not shown).

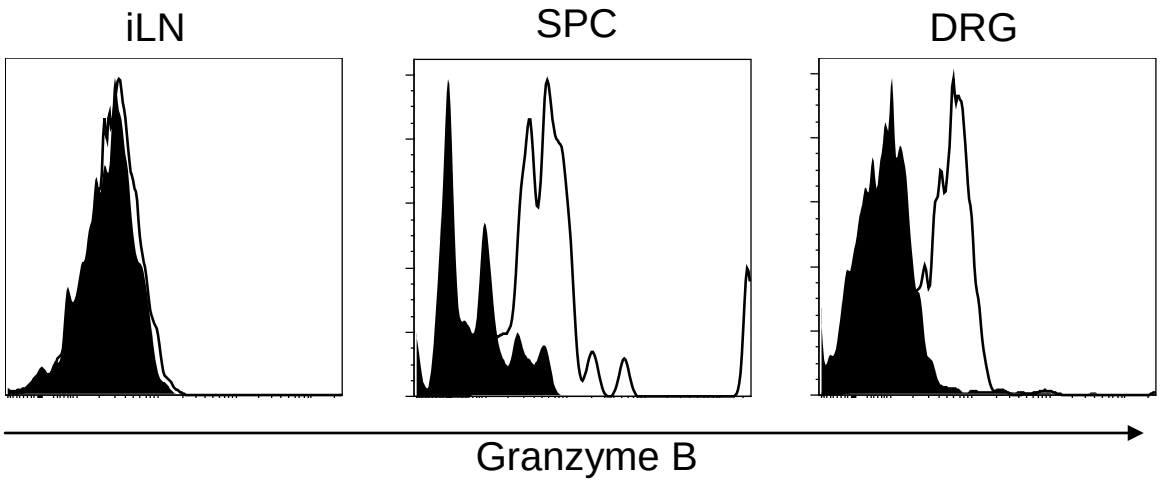

FIG. 5. Granzyme B expression by $CD4^+$ T cells. B6 mice were inoculated ivag with HSV-1. Mice were sacrificed on day 8 post-infection, and lymphocytes were isolated from spinal cords, dorsal root ganglia, or iliac lymph nodes. Pools of lymphocytes were stained with fluorochrome-labeled antibodies against CD4 and intracellular granzyme B. Solid histograms represent isotype controls, and open histograms indicate granzyme B staining in HSV-infected tissues.

Clearance of acute HSV-1 from genital and neural sites in the absence of $CD8^+$ T cells

HSV-specific $CD4^+$ T cells infiltrating the spinal cords and sensory ganglia of HSV-1-infected animals were hypothesized to be important for clearance of infectious virus from these sites. To test the ability of $CD4^+$ T cells to clear virus, mice from which $CD8^+$ T cells were either depleted or genetically absent ($CD8^{-/-}$) were inoculated ivag with HSV-

1. Wild-type B6 mice cleared infectious virus from the genital tract around day 6 post-infection (Fig. 6A). Both CD8-depleted and $CD8^{-/-}$ animals cleared virus from the genital tract by day 7 post-infection. This one day difference in clearance was not statistically significant and may reflect animal to animal variability rather than a true biological difference. The amount of infectious virus in the spinal cords and dorsal root ganglia of wild-type B6 mice decreased from day 7 to day 9 post-infection, and the incidence of spinal cords and dorsal root ganglia samples containing infectious HSV-1 also declined over this time from 8/8 on day 5 to 1/8 by day 9 post-infection (Fig. 6B, C). While both CD8-depleted and $CD8^{-/-}$ mice had higher virus titers in neural tissues on days 7 and 9 than did the wild-type B6 mice, these animals also showed a trend towards clearance of infectious virus from both the spinal cord and dorsal root ganglia (Fig. 6B, C). The CD8-depleted animals had a similar pattern of virus clearance from the neural tissues, with 6/8 having infectious virus in the spinal cord and 7/8 with infectious virus in the dorsal root ganglia on day 5. This was reduced to 1/8 (spinal cord) and 0/8 (dorsal root ganglia) by day 9 post-infection (Fig. 6). All (8/8) $CD8^{-/-}$ animals were found to have infectious HSV-1 in both the spinal cord and dorsal root ganglia on day 5, which was reduced to 1/8 (spinal cord) and 4/8 (dorsal root ganglia) by day 9 post-infection (Fig. 6). The difference in viral titer in the genital tract was found to be significantly different between B6 and CD8-depleted B6 mice on days 3 and 7 ($p < 0.001$). The difference viral titers on day 5 were found to be different between $CD8^{-/-}$ mice and B6 mice in spinal cord ($p = 0.0190$) and dorsal root ganglia ($p = 0.0251$).

We also tested an alternative approach in which $CD4^{+}$ T cells were adoptively transferred into $Rag1^{-/-}$ mice. $CD4^{+}$ T cell-recipient $Rag1^{-/-}$ mice cleared virus from the

genital tract by day 10 post-infection (Fig. 7). Conversely, Rag1$^{-/-}$ mice that did not receive T cells were unable to clear the virus from the genital tract (Fig. 7). Mice were sacrificed on days 8 and 9/10 after intravaginal challenge with HSV-1, and spinal cords and dorsal root ganglia were harvested for quantification of infectious virus. CD4^{+} T cell-recipient Rag1$^{-/-}$ mice had lower viral titers and showed a trend towards clearance in

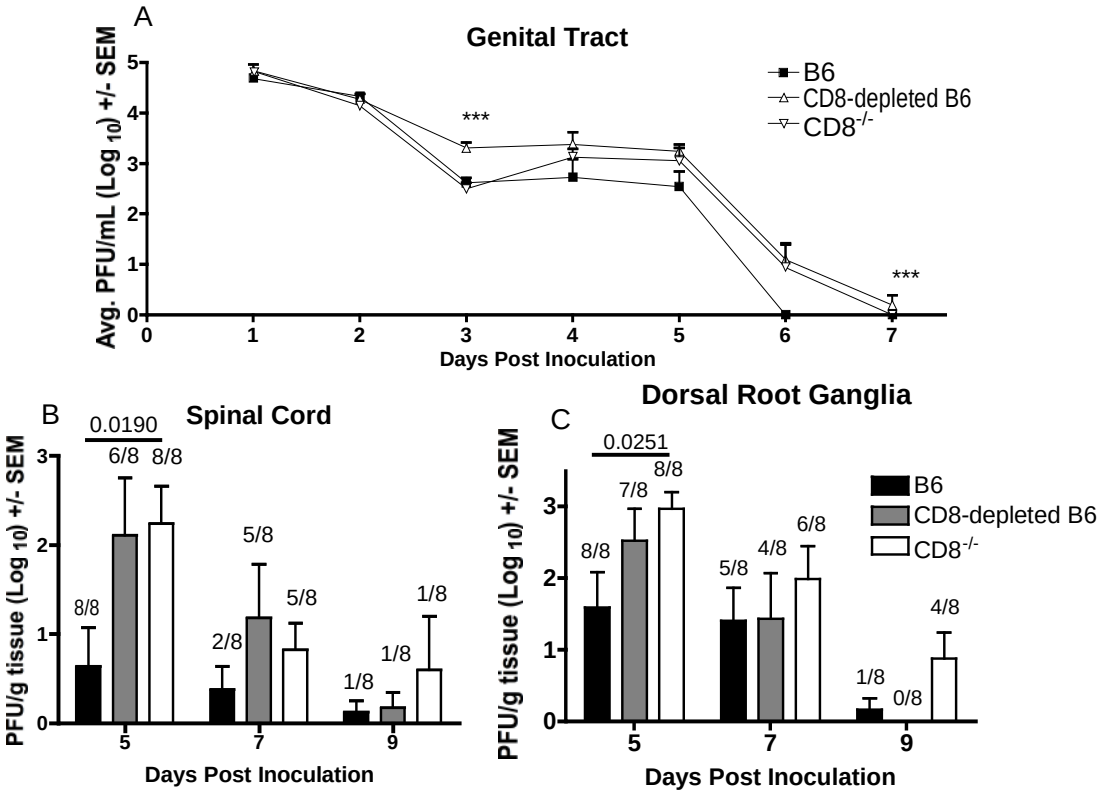

FIG. 6. CD4^{+} T cell-mediated clearance of HSV-1 from the genital tract, sensory ganglia, and spinal cord. B6 mice, CD8-depleted B6 mice, and CD8$^{-/-}$ mice were inoculated ivag with HSV-1. Mice were swabbed on the indicated days (A), and spinal cords (B) and dorsal root ganglia (C) were harvested on the indicated days, for quantification of virus. Numbers above bars in parts (B) and (C) equal the number of samples with infectious virus per total group samples. The difference in viral titer in the genital tract was found to be significantly different between B6 and CD8-depleted B6 mice on days 3 and 7 ($p < 0.001$). The difference viral titers on day 5 were found to be different between CD8$^{-/-}$ mice and B6 mice in spinal cord ($p = 0.0190$) and dorsal root ganglia ($p = 0.0251$).

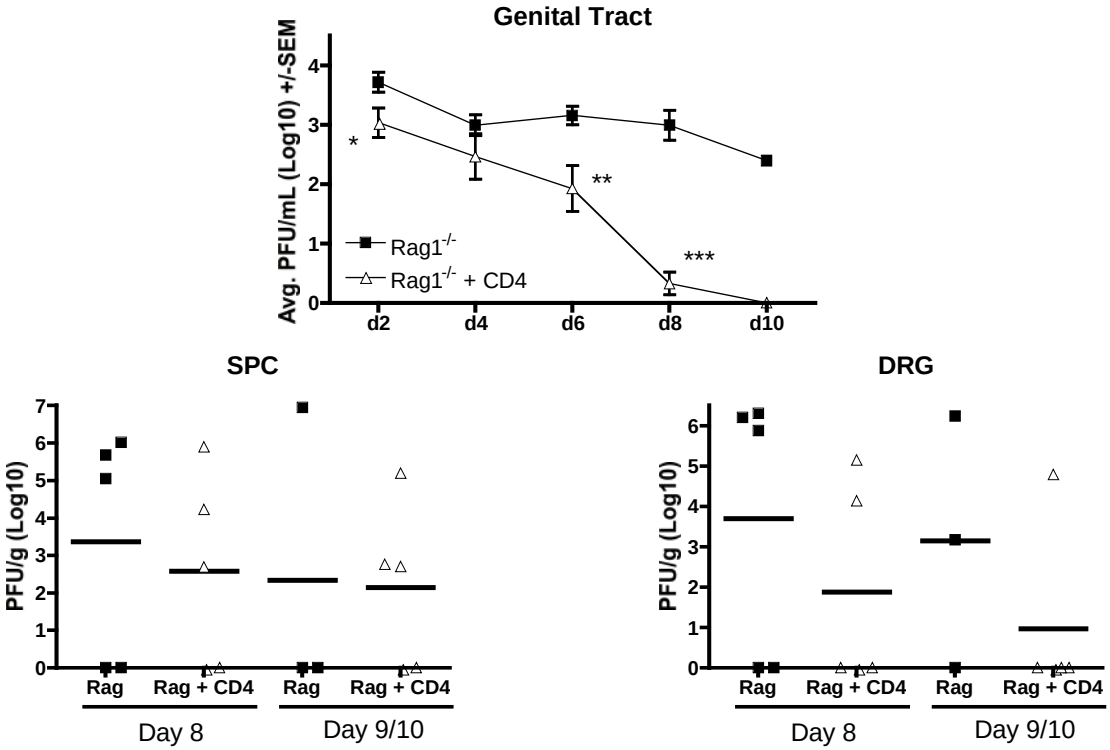

FIG. 7. $CD4^+$ T cell-mediated clearance of HSV-1 from the genital tract, sensory ganglia, and spinal cord. 4×10^6 $CD4^+$ T cells isolated from B6 mice were adoptively transferred into genetically B- and T cell-deficient $Rag1^{-/-}$ mice. $Rag1^{-/-}$ mice that did not receive T cells served as the control. Mice were challenged ivag with HSV-1 and swabbed daily. Spinal cords and dorsal root ganglia were harvested on given days, for quantification of virus. Viral titers in the genital tract were found to be significantly different on day 2 ($p = 0.0232$), day 6 ($p = 0.0089$), and day 8 ($p < 0.001$) post-infection.

both spinal cord and dorsal root ganglia, whereas $Rag1^{-/-}$ mice that did not receive T cells were not able to clear virus from these tissues (Fig. 7). 3/5 of the $CD4^+$ T cell-recipient $Rag1^{-/-}$ mice had infectious virus in the spinal cord and 2/5 in the dorsal root ganglia on day 8 post-infection. This remained the same (3/5) in the spinal cord but was reduced (1/5) in the dorsal root ganglia by day 10. 3/5 of the $Rag1^{-/-}$ mice that did not receive T cells were found to harbor infectious virus within the spinal cord and dorsal root ganglia

on day 8 post-infection. While this appears to be reduced to 1/3 (spinal cord) and 2/3 (dorsal root ganglia) by day 9/10, it is important to note that of the original five animals included in this group, two succumbed to infection prior to sampling, two were sacrificed early (day 9 rather than day 10) due to their severe disease, and only one animal survived for sampling on the intended day 10 time point. Viral titers in the genital tract were found to be significantly different on day 2 ($p = 0.0232$), day 6 ($p = 0.0089$), and day 8 ($p < 0.001$) post-infection.

$CD4^+$ T cell clearance of acute HSV from neural tissues does not require a lytic mechanism

Three approaches were used to test the involvement of cytolytic mechanisms in HSV-1 clearance, including the use of perforin-deficient or FasL-defective mice, adoptive transfer of perforin- or FasL-deficient $CD4^+$ T cells into $Rag1^{-/-}$ recipients, and the construction of short-term perforin/Fas radiation chimeras. The possible role for perforin in the $CD4^+$ T cell-mediated clearance of infectious HSV-1 was examined by comparison of mean virus titers in the genital tracts, dorsal root ganglia, spinal cords, and hindbrains of intact B6 mice, CD8-depleted B6, and CD8-depleted $Pfp^{-/-}$ mice. In the genital tract, infectious virus was not detected on day 6 in B6 mice (Fig. 8A). Significantly higher virus titers were detected on days 4 and 6 in CD8-depleted mice, and on day 6 in CD8-depleted $Pfp^{-/-}$ mice compared to B6 controls ($p < 0.001$). Virus clearance in CD8-depleted $Pfp^{-/-}$ and CD8-depleted B6 mice was very similar and infectious virus was not

detected in either group by day 9 after inoculation. Again, this slight difference in virus titers on day 8 between these two groups may reflect either a limited efficiency of plaque formation on Vero cell monolayers or an inherent variation in virus clearance among individual animals and is most likely not biologically significant. In the dorsal root ganglia, infectious virus was undetectable in B6 mice on day 9, while mean virus titers in both CD8-depleted B6 mice and CD8-depleted $Pfp^{-/-}$ mice dropped by nearly a log between days 7 and 9 (Fig. 8C). In the spinal cord, the B6 and CD8-depleted $Pfp^{-/-}$ mice were able to clear virus by day 9 post-infection, while all but a single CD8-depleted B6 animal (1 of 10) cleared virus by day 9 (Fig. 8B). The infection was apparently well controlled in dorsal root ganglia and spinal cords, as virus spread to the hindbrain was detected in only a single CD8-depleted B6 animal (Fig. 8D).

In a complementary approach, $CD4^+$ T cells isolated from wild-type B6, OT-II (OVA-specific), or perforin-deficient mice were adoptively transferred into groups of $Rag1^{-/-}$ mice (Fig. 9). $Rag1^{-/-}$ mice that received $CD4^+$ T cells from B6 or perforin-deficient mice cleared virus from the genital tract by day 9 after ivag challenge with HSV-1, whereas $Rag1^{-/-}$ mice that received $CD4^+$ T cells from OT-II donors were not able to resolve infectious virus from the genital tract (Fig. 9). Significantly lower virus titers were detected on days 6, 8, and 9 in $Pfp^{-/-}$ $CD4^+$ T cell-recipient mice, and on days 8 and 9 in B6 $CD4^+$ T cell-recipient mice compared to OT-II controls. Mice were sacrificed on days 7 and 9 after ivag challenge with HSV-1, and spinal cords and dorsal root ganglia were harvested for quantification of infectious virus. In the dorsal root ganglia, both $Pfp^{-/-}$ $CD4^+$ T cell-recipient mice and B6 $CD4^+$ T cell-recipient mice had lower mean virus titers than did the OT-II control mice. This difference in mean virus

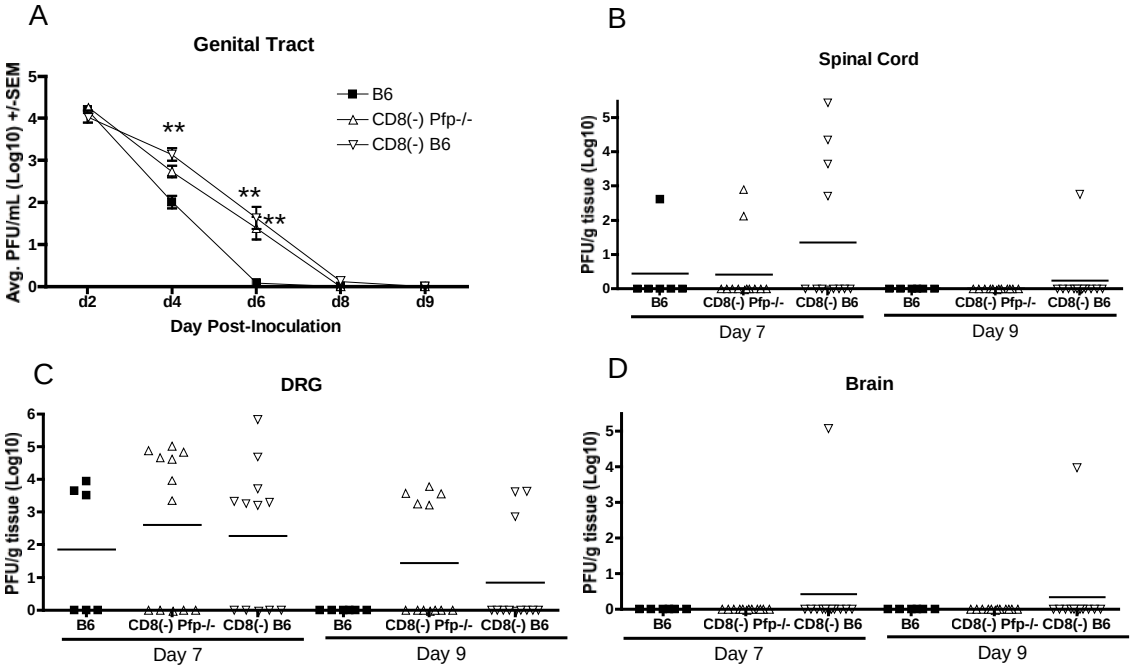

FIG. 8. $CD4^+$ T cell-mediated clearance of HSV-1 from the sensory ganglia and spinal cord does not require perforin. Groups of B6, CD8-depleted B6, or CD8-depleted $Pfp^{-/-}$ mice were inoculated ivag with HSV-1 and swabbed on the indicated days (A). Spinal cords (B), dorsal root ganglia (C), and hindbrain (D) were harvested on the indicated days, for quantification of virus. Values marked with two asterisks were found to be significantly different compared to B6 controls, with $p < 0.001$. 'CD8(-)' indicates depletion of $CD8^+$ T cells by injection of specific antibody.

titer was significant on day 7 between the B6 $CD4^+$ T cell-recipient mice and the OT-II control mice ($p = 0.0317$). In the spinal cord, again both $Pfp^{-/-}$ $CD4^+$ T cell-recipient mice and B6 $CD4^+$ T cell-recipient mice had lower mean virus titers than did the OT-II control mice. Interestingly, on day 9 post-infection 2/5 $Pfp^{-/-}$ $CD4^+$ T cell-recipient mice were found to contain detectable infectious virus, whereas 4/5 B6 $CD4^+$ T cell-recipient mice and 5/5 OT-II control mice harbored infectious virus. The difference in mean virus titer between $Pfp^{-/-}$ $CD4^+$ T cell-recipient mice and OT-II control mice was significant on day 9 ($p = 0.0079$) (Fig. 9).

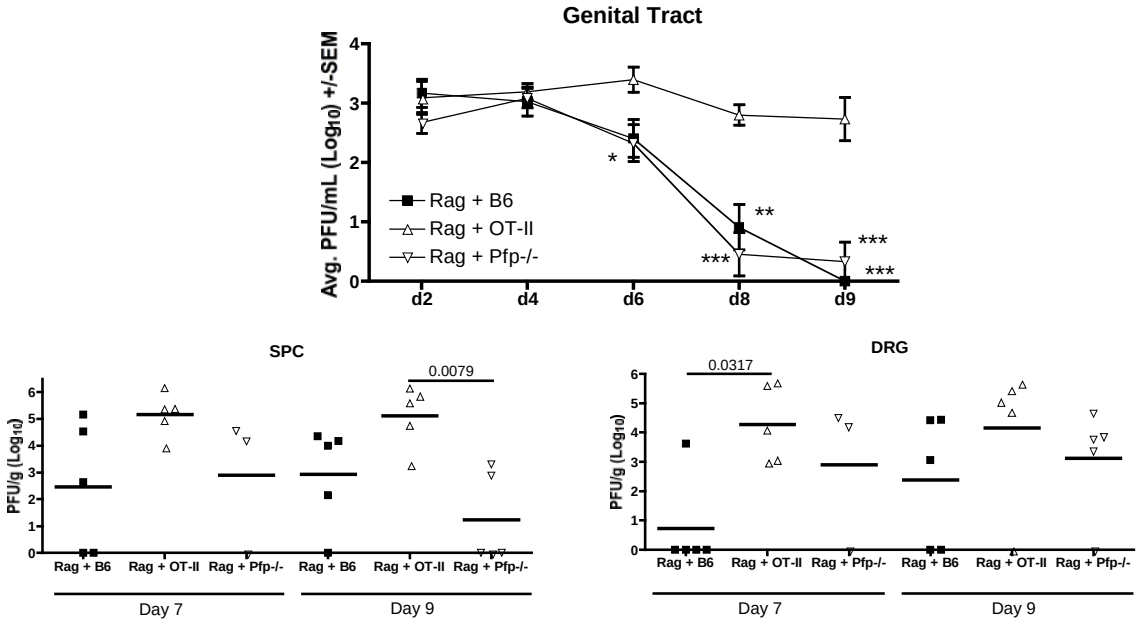

FIG. 9. Perforin-deficient $CD4^+$ T cells can mediate clearance of HSV-1 from neuronal tissue. 4×10^6 $CD4^+$ T cells isolated from B6, $Pfp^{-/-}$, or OT-II mice were adoptively transferred into genetically B- and T cell-deficient $Rag1^{-/-}$ mice. Mice were challenged ivag with HSV-1 and swabbed daily. Spinal cords and dorsal root ganglia were harvested on given days, for quantification of virus. Values marked with one asterisk ($p < 0.05$), two asterisks ($p < 0.01$), and three asterisks ($p < 0.001$) were found to be significantly different compared to OT-II controls.

The involvement of the FasL-mediated lytic pathway was examined by comparison of incidence of infection and virus resolution of CD8-depleted B6 and CD8-depleted FasL-defective (*gld*) mice. Both B6 and *gld* mice cleared virus from the genital tract, although titers were significantly higher on days 2, 4, and 6 in *gld* mice (Fig. 10A). Mean virus titers detected in the dorsal root ganglia dropped between days 7 and 9 in both B6 and *gld* mice (Fig. 10C). The mean HSV-1 titer was much higher in *gld* mice on day 7, but fell significantly on day 9 ($p < 0.05$), and infectious virus was detected in fewer mice on day 9 (9/10 on day 7 compared to 5/9 on day 9). Infectious virus was also

cleared from the spinal cords of both mouse strains, and infectious virus was detected in only a single CD8-depleted *gld* mouse, on day 9 (Fig. 10B). Again, no virus spread to the hindbrain was detected in either mouse strain on day 9 (Fig. 10D).

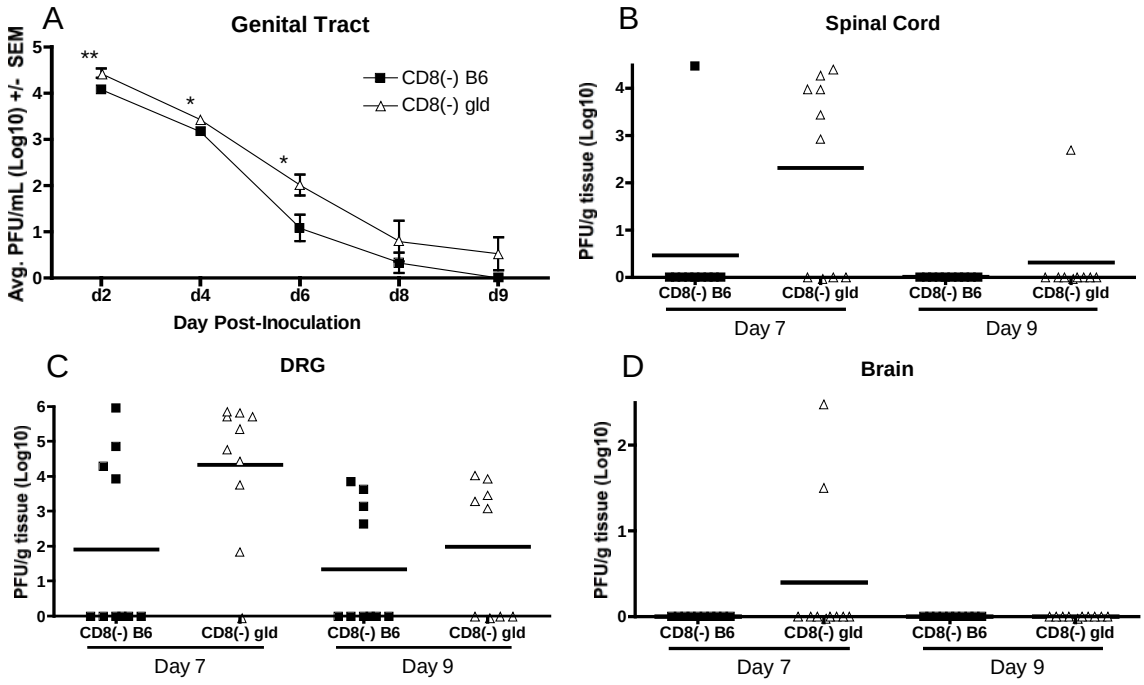

FIG. 10. $CD4^+$ T cell-mediated clearance of HSV-1 from the sensory ganglia and spinal cord does not require Fas/FasL interaction. Groups of CD8-depleted B6 or CD8-depleted *gld* mice were inoculated ivag with HSV-1 and swabbed on the indicated days (A). Spinal cords (B), dorsal root ganglia (C), and hindbrain (D) were harvested on indicated days, for quantification of virus. Values marked with one asterisk ($p < 0.05$), and the value marked with two asterisks ($p < 0.005$) were found to be significantly different compared to B6 controls. 'CD8(-)' indicates depletion of $CD8^+$ T cells by injection of specific antibody.

We next utilized an adoptive transfer model to demonstrate that HSV-specific $CD4^+$ T cells in infected neural tissues do not utilize the Fas/FasL lytic pathway in the resolution of infectious virus from these tissues. $CD4^+$ T cells isolated from wild-type B6, OT-II, or FasL-deficient mice (*gld*) were adoptively transferred into groups of $Rag1^{-/-}$ mice (Fig. 11). Both *gld* $CD4^+$ T cell-recipient and B6 $CD4^+$ T cell-recipient mice had reduced mean viral titer in the genital tract by more than one log between days 6 and 8 post-infection. Significantly lower virus titers were detected on days 8 and 9 in *gld* $CD4^+$ T cell-recipient mice, and on days 6, 8, and 9 in B6 $CD4^+$ T cell-recipient mice compared to OT-II controls. Mice were sacrificed on days 7 and 9 after ivag challenge with HSV-1, and spinal cords, dorsal root ganglia, and hindbrains were harvested for quantification of infectious virus. Though only the differences in mean virus titer in the spinal cord between B6- and OT-II-recipients were found to be significant ($p = 0.0089$ on day 7, $p = 0.0206$ on day 9), compared to $Rag1^{-/-}$ mice that received $CD4^+$ T cells from OT-II donors, $Rag1^{-/-}$ mice that received $CD4^+$ T cells from B6 or FasL-deficient mice had lower mean virus titers in spinal cord, sensory ganglia, and hindbrain, and showed a trend toward clearance from day 7 to day 9 post-infection (Fig. 11). In the dorsal root ganglia, 6/10 *gld* $CD4^+$ T cell-recipient mice, 6/10 B6 $CD4^+$ T cell-recipient mice, and 8/10 OT-II control mice were found to harbor infectious virus on day 7 post-infection. Incidence of infectious virus within the dorsal root ganglia was reduced by day 9 post-infection in the *gld* $CD4^+$ T cell-recipient mice (4/10) and B6 $CD4^+$ T cell-recipient mice (5/10), but increased in the OT-II control mice (7/8). In the spinal cord, 7/10 *gld* $CD4^+$ T cell-recipient mice, 6/10 B6 $CD4^+$ T cell-recipient mice, and 10/10 OT-II control mice were found to harbor infectious virus on day 7 post-infection. Incidence of infectious virus

within the spinal cord was reduced by day 9 post-infection in the B6 $CD4^+$ T cell-recipient mice (3/10), and OT-II control mice (7/8), but remained the same in *gld* $CD4^+$ T cell-recipient mice (no statistical significance) (7/8). Additionally, the mean virus titers in the spinal cords were found to be significantly different between the B6 $CD4^+$ T cell-recipient mice and the OT-II control mice on both day 7 ($p = 0.0089$) and day 9 ($p = 0.0206$). All groups appeared unable to prevent spread of infectious virus to the brain. However, *gld* $CD4^+$ T cell-recipient mice and B6 $CD4^+$ T cell-recipient mice had lower mean virus titers compared to OT-II controls on both days 7 and 9 post-infection, though these differences were not found to be statistically significant. All groups reduced their incidence of infection within the hindbrain between days 7 and 9 (B6 $CD4^+$ T cell-recipient mice, 3/10 on day 7, 1/10 on day 9; *gld* $CD4^+$ T cell-recipient mice, 5/10 on day 7 and 4/10 on day 9; OT-II controls, 6/10 on day 7, 4/8 on day 9) (Fig. 11). It is important to note, however, that two of the $Rag1^{-/-}$ mice that received $CD4^+$ T cells from OT-II donors succumbed to infection prior to day 9 tissue sampling.

Studies of perforin- and FasL-mediated mechanisms clearance of infectious HSV-1 were extended by constructing perforin/Fas irradiation chimeras. $CD4^+$ T cells isolated from wild-type B6 (perforin-positive), perforin-deficient ($Pfp^{-/-}$), or as a control, OT-II mice were adoptively transferred into groups of irradiated B6 (Fas-positive) or Fas-deficient (*lpr*) mice. This resulted in mice that had both functional perforin and Fas pathways (Pfp^+Fas^+), mice that had neither perforin nor Fas pathways (Pfp^-Fas^-), and mice that had both pathways but whose T cells were unable to recognize the virus (OT-II negative control). Mice were sacrificed on days 7 and 9 after ivag challenge with HSV-1, and spinal cords, dorsal root ganglia, and hindbrains were harvested for quantification of

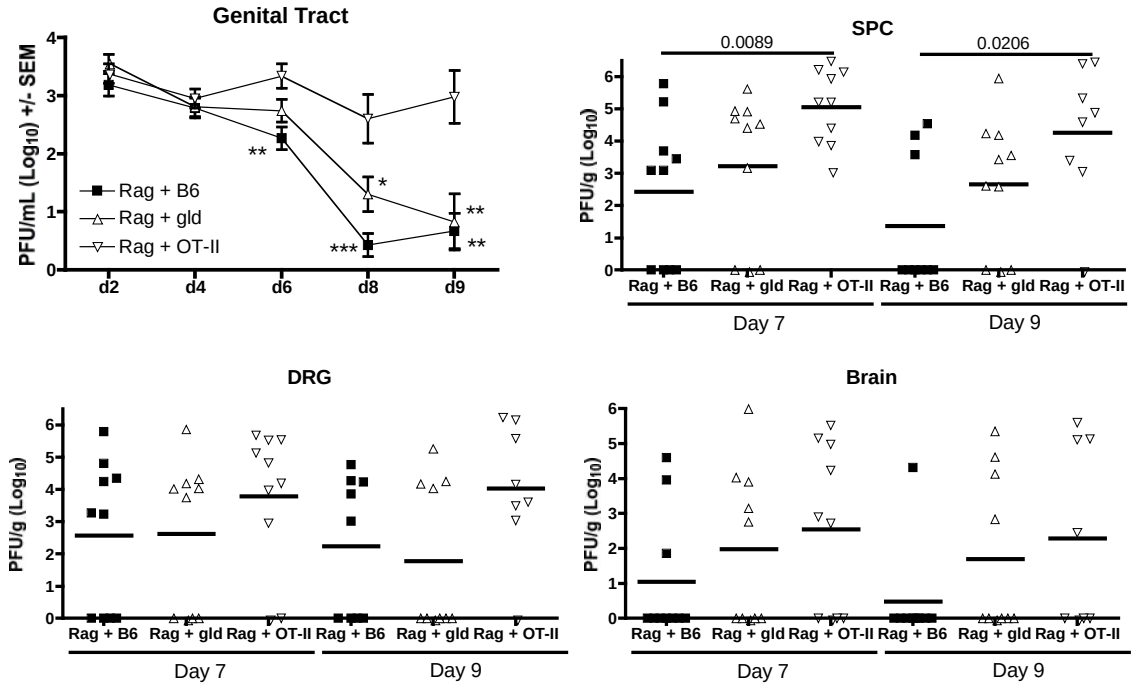

FIG. 11. $CD4^+$ T cells from FasL-defective mice can mediate clearance of HSV-1 from sensory ganglia and spinal cord. 4×10^6 $CD4^+$ T cells from B6, $FasL^{-/-}$, or OT-II mice were adoptively transferred into genetically B- and T cell-deficient $Rag1^{-/-}$ mice. Mice were challenged ivag with HSV-1 and swabbed daily. Spinal cords, dorsal root ganglia, and hindbrain were harvested on given days, for quantification of virus. Values marked with one asterisk ($p < 0.05$), the value marked with two asterisks ($p < 0.01$), and the value marked with three asterisks ($p < 0.001$) were found to be significantly different compared to OT-II controls.

infectious virus. Both of Pfp^-Fas^- and Pfp^+Fas^+ mice had significantly lower viral titers within the spinal cords on day 7 ($p = 0.0031$ and $p = 0.0101$, respectively) and on day 9 ($p = 0.0030$, $p = 0.0098$) compared to OT-II controls. Virus titers were similar in the sensory ganglia of Pfp^-Fas^-, Pfp^+Fas^+, and OT-II mice on day 7. However, compared to OT-II controls on day 9, virus titers were significantly lower in the Pfp^-Fas^- mice ($p = 0.0074$) and Pfp^+Fas^+ mice ($p = 0.0260$) (Fig. 12C). The incidence of samples containing infectious virus also fell on day 9 in these groups. While the virus infection was not

controlled and spread to the hindbrain in OT-II control mice, infectious virus was detected in the hindbrain of only a single $Pfp^{+}Fas^{+}$ mouse on day 9 (Fig. 12D). Both of $Pfp^{-}Fas^{-}$ and $Pfp^{+}Fas^{+}$ mice had significantly lower viral titers within the hindbrain on day 7 ($p = 0.0381$ and $p = 0.0423$, respectively) and on day 9 ($p = 0.00231$, $p = 0.034$) compared to OT-II controls.

Possible lytic killing via TNF in the $CD4^{+}$ T cell-mediated resolution of infectious HSV-1 in genital and neural tissues was examined utilizing $TNFR^{-/-}$ mice. CD8-depleted B6 mice cleared infectious HSV-1 from the genital tract by day 6 post-intravaginal inoculation, while CD8-depleted $TNFR^{-/-}$ mice cleared virus from the genital tract by day 8 (Fig. 13). Mice were sacrificed on days 7 and 9 after challenge, and spinal cords, dorsal root ganglia, and hindbrains were harvested for quantification of infectious virus. CD8-depleted $TNFR^{-/-}$ mice demonstrated higher mean virus titers than did the CD8-depleted B6 mice in spinal cord, dorsal root ganglia, and hindbrain on days 7 and 9, though none of these differences was found to be statistically significant. Both groups demonstrated a reduction in incidence of infection between days 7 and 9 within the neural tissues studied (CD8-depleted B6, spinal cord from 2/6 (day 7) to 0/6 (day 9), dorsal root ganglia from 1/6 to 0/6, and hindbrain from 2/6 to 0/6; CD8-depleted $TNFR^{-/-}$, spinal cord from 4/6 (day 7) to 1/6 (day 9), dorsal root ganglia from 2/6 to 1/6, and hindbrain from 3/6 to 2/6 (Fig. 13).

The examination of a possible role of non-lytic IFN-γ was attempted utilizing antibody neutralization. CD8-depleted B6 mice were treated with XMG (anti-IFN-γ) antibody or SFR.8 (control) antibody. Treatment with anti-IFN-γ Ab (or control) began on day 6 post-infection in an attempt to not alter critical IFN-γ-dependent mechanisms of

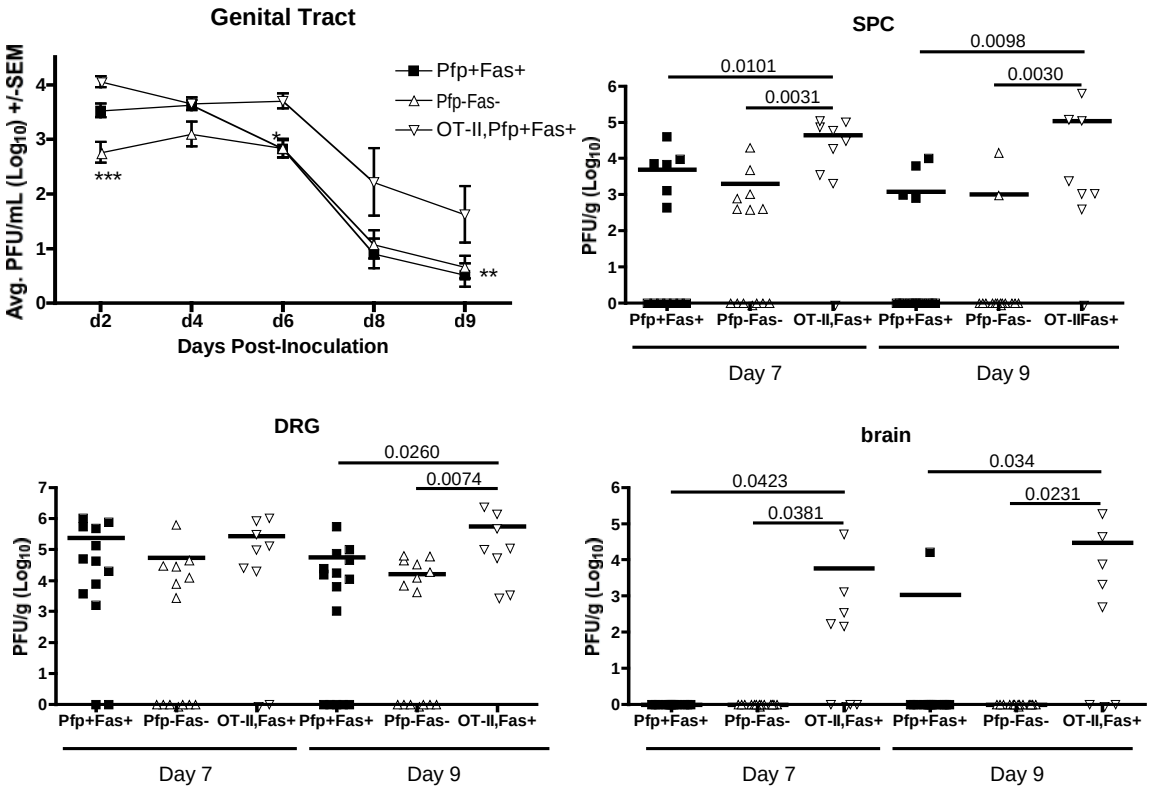

FIG. 12. $CD4^+$ T cells reduce infectious virus titers in spinal cords and dorsal root ganglia in the absence of perforin and Fas. Radiation chimeras were constructed as described in Materials and Methods. Mice were challenged ivag with HSV-1 and swabbed on the indicated days (A). Values marked with one asterisk ($p < 0.01$), the value marked with two asterisks ($p < 0.05$), and the value marked with three asterisks ($p < 0.001$) were found to be significantly different compared to OT-II controls. Spinal cords (B), dorsal root ganglia (C), and hindbrain (D) were harvested on indicated days, for quantification of virus. Both of Pfp^-Fas^- and Pfp^+Fas^+ mice had significantly lower viral titers within the spinal cords on day 7 ($p = 0.0031$ and $p = 0.0101$, respectively) and on day 9 ($p = 0.0030$, $p = 0.0098$) compared to OT-II controls. Compared to OT-II controls on day 9, virus titers were significantly lower in the Pfp^-Fas^- mice ($p = 0.0074$) and Pfp^+Fas^+ mice ($p = 0.0260$). Both of Pfp^-Fas^- and Pfp^+Fas^+ mice had significantly lower viral titers within the hindbrain on day 7 ($p = 0.0381$ and $p = 0.0423$, respectively) and on day 9 ($p = 0.00231$, $p = 0.034$) compared to OT-II controls.

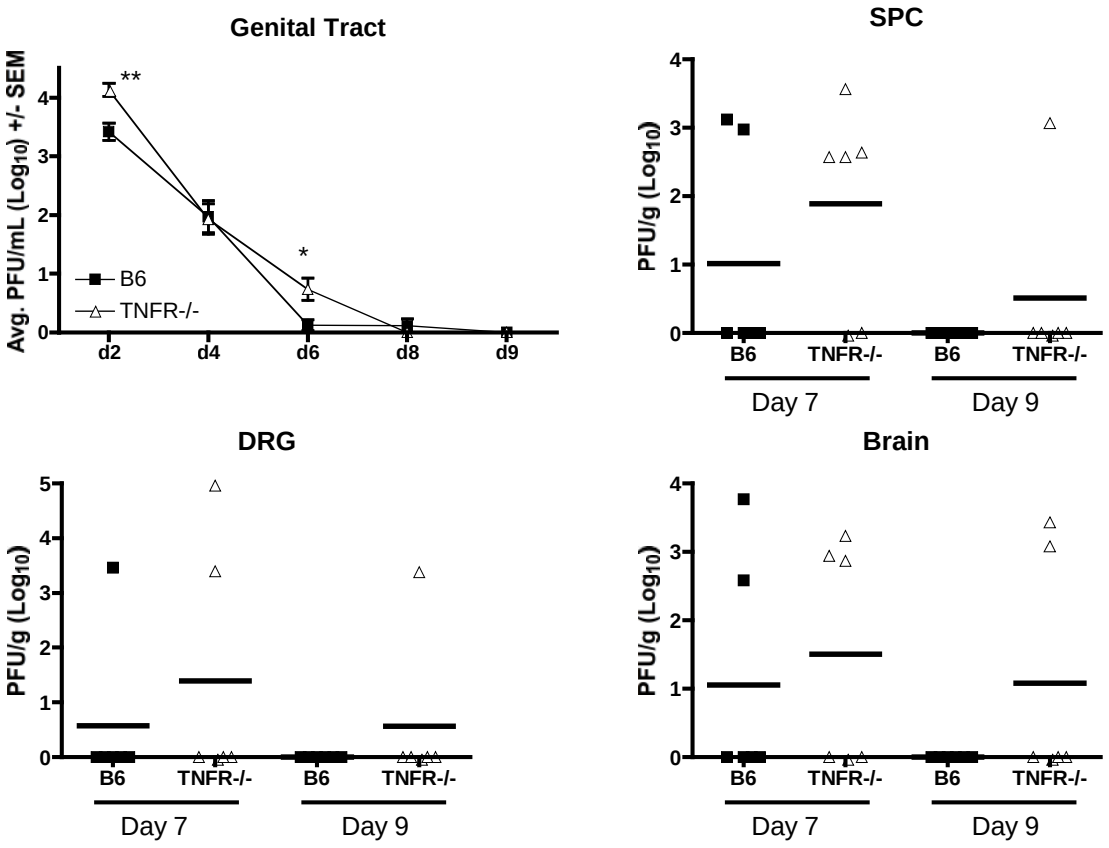

FIG. 13. $CD4^+$ T cell-mediated clearance of HSV-1 from the sensory ganglia and spinal cord does not require TNF. Groups of CD8-depleted B6 or CD8-depleted $TNFR^{-/-}$ mice were inoculated ivag with HSV-1 and swabbed on the indicated days (A). Spinal cords (B), dorsal root ganglia (C), and hindbrain (D) were harvested on indicated days, for quantification of virus. Values marked with one asterisk ($p < 0.05$), and the value marked with two asterisks ($p < 0.005$) were found to be significantly different compared to B6 controls. 'CD8(-)' indicates depletion of $CD8^+$ T cells by injection of specific antibody. Mean viral titers were found to be significantly different on day 2 ($p = 0.0014$) and day 6 ($p = 0.0249$) post-infection.

clearance occurring within the genital tract, as the focus of this study was to determine the role of IFN-γ in clearance of infectious virus within the neural tissues. Mice were sacrificed on days 7 and 11 after intravaginal inoculation with HSV-1, and spinal cords and dorsal root ganglia were harvested for quantification of infectious virus. On day 7

post-inoculation, 2/6 control mice and 4/6 anti-IFN-γ-treated mice were found to harbor infectious virus within the spinal cord (Fig. 14). Further, on day 7, 5/6 control mice and 4/6 anti-IFN-γ-treated mice were found to harbor infectious virus within the dorsal root ganglia (Fig. 14). Neither of these differences was found to be significant. By day 11 post-inoculation, infectious virus was not detectable in either group of mice within the spinal cord or dorsal root ganglia. It is important to note that this experiment should not immediately be interpreted to mean that IFN-γ is not involved in clearance of infectious HSV-1 from neural tissues. Rather, it is more likely that the IFN-γ was not completely neutralized, or that the XMG antibody was not able to gain access to the neural tissues in order to affect the IFN-γ acting within these tissues.

$CD4^+$ T cell-mediated immune responses in HSV-immune B6 and IFN-$\gamma^{-/-}$ mice to test for IFN-γ-independent mechanisms of protection

IFN-γ has been previously determined to play an important role in the clearance of HSV-2. T cell-produced IFN-γ has been noted within the HSV-2-infected genital tract of immune mice within 24 hours, where it can function in the direct clearance of virus or in the recruitment of additional immune cells, which are then activated to clear virus (188, 210). Both IFN-γ-producing $CD4^+$ and $CD8^+$ T cells are present in the draining lymph nodes after genital infection in mice (189, 210), as well as in herpetic lesions of humans (135). While the depletion of αβT cells destroys the ability of mice to clear HSV-2, depletion of either the $CD4^+$ or $CD8^+$ T cell subset only delays clearance,

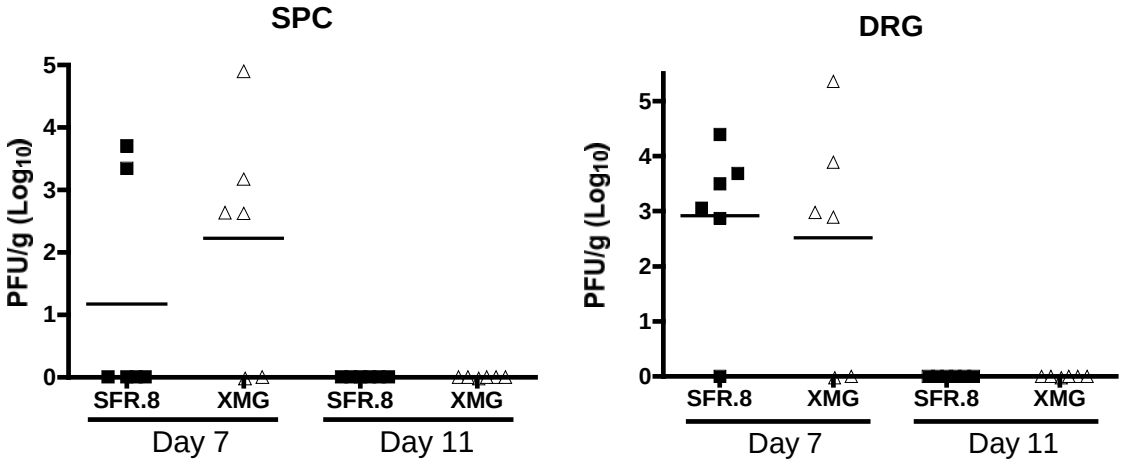

FIG. 14. Role of IFN-γ in the $CD4^+$ T cell-mediated clearance of HSV-1 from the sensory ganglia and spinal cord. Mice received 1.0 mg anti-CD8 (2.43) Ab i.p. on the two days prior to infection. Following ivag inoculation with 10^6 PFU HSV-1 SC16, mice received 0.5mg anti-CD8 Ab i.p. every other day throughout the study. Additionally, mice were treated with 2.0 mg anti-IFN-γ (XMG) Ab or control (SFR.8) Ab on days 6 and 8 post-infection, and then with 1.0 mg daily throughout the remainder of the study. Animals were sacrificed on days 7 and 11 post-infection, and clearance of infectious virus from spinal cords (A) and dorsal root ganglia (B) was examined by quantification of virus by standard plaque assay. There were no statistically significant differences found between the mean the mean virus titers of the two groups.

suggesting that either T cell subset is capable of virus clearance (189, 250). Importantly, specific antibody neutralization of IFN-γ results in the significantly delayed clearance of infectious HSV-2 from the genital tract (189, 190). Further, in the absence of IFN-γ or the IFN-γ receptor, previous work has demonstrated delayed virus clearance (23). Therefore, because clearance is delayed rather than completely prevented in the absence of IFN-γ, it appears that alternative, IFN-γ-independent mechanisms may also play a role in the clearance of virus.

The cytokine production of HSV-specific $CD4^+$ T cells in HSV-immune B6 and HSV-immune IFN-$\gamma^{-/-}$ mice after challenge with virulent HSV-2 strain 186 was examined by ELISPOT analysis. The dominant $CD4^+$ T cell response in the iliac lymph nodes of HSV-immune B6 was the production of IFN-γ. HSV-immune B6 $CD4^+$ T cells also produced IL-4, IL-17, and TNF-α, but at a lower magnitude (Fig. 15). HSV-immune IFN-$\gamma^{-/-}$ mice demonstrated a significantly higher number of IL-4-secreting $CD4^+$ T cells on all days compared to HSV-immune B6 mice ($p < 0.05$, day 2; $p < 0.001$, day 5 and day 7). The numbers of IL-17- and TNF-α-secreting $CD4^+$ T cells in the iliac lymph nodes of HSV-immune B6 and HSV-immune IFN-$\gamma^{-/-}$ mice were comparable on day 5 and day 7 after challenge. These cytokine responses were mirrored within the vaginal tracts of these animals (Fig. 15).

Inability of HSV-specific $CD4^+$ effector T cells from HSV-immune IFN-$\gamma^{-/-}$ mice to clear HSV-2 $333tk^-$ from the vaginal tract

It has been demonstrated previously that $CD4^+$ T cells are the predominant T cell subset responsible for the clearing of HSV from the genital epithelium in immune animals (88, 142, 189). In order to test the ability of HSV-specific $CD4^+$ T cells from HSV-immune IFN-$\gamma^{-/-}$ mice to clear HSV-2 from the genital epithelium, HSV-specific $CD4^+$ T cells from HSV-immune B6 and HSV-immune IFN-$\gamma^{-/-}$ mice were activated *in vitro*, and then adoptively transferred into irradiated recipients. Recipient mice were then challenged with attenuated HSV-2 $333tk^-$. Control mice, which received $CD4^+$ T cells from OT-II TCR transgenic mice, were not able to clear virus from the vaginal tract even through day

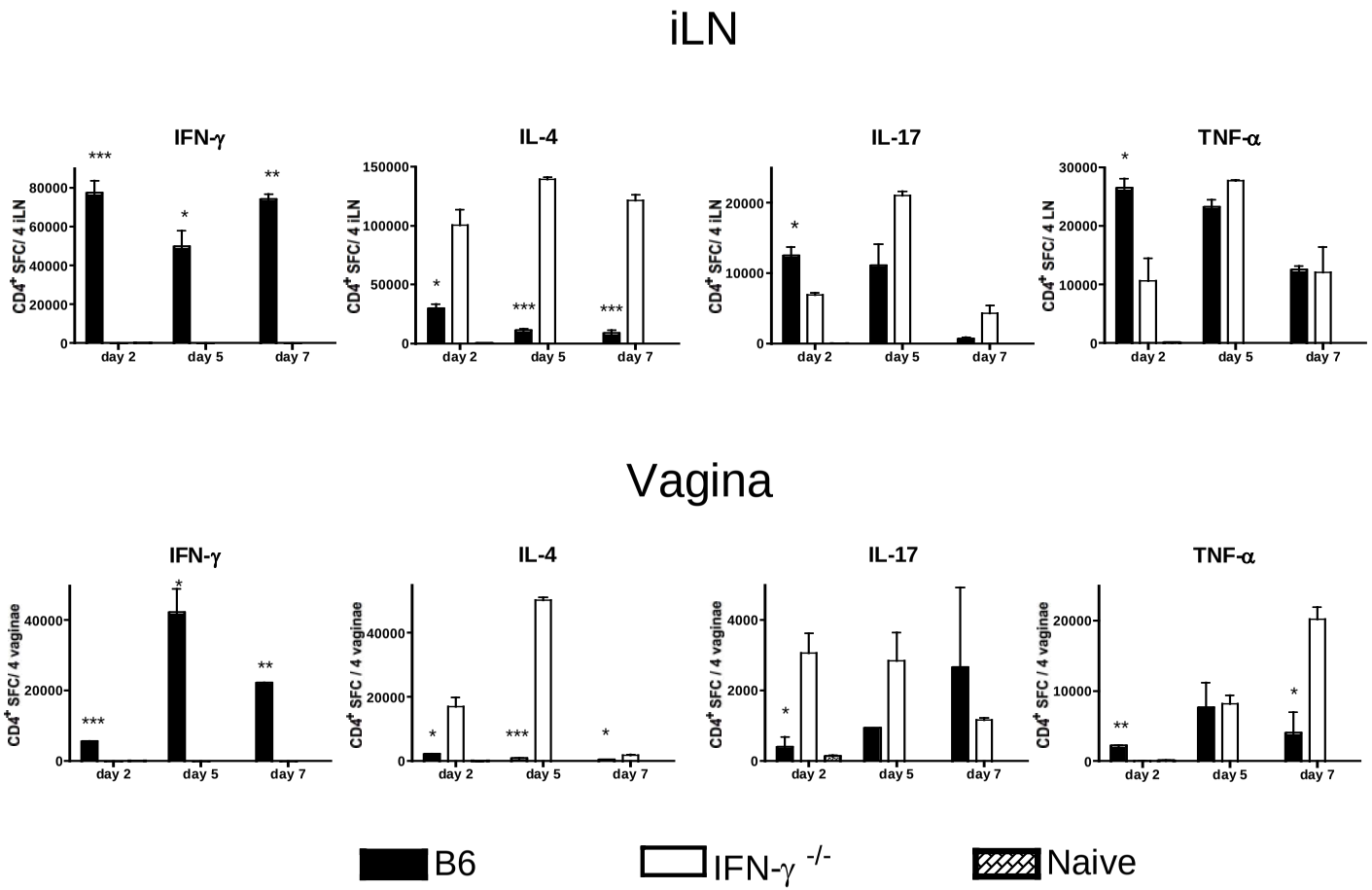

FIG 15. Cytokine secretion by HSV-specific $CD4^+$ T cells from HSV-immune B6 and IFN-γ-/- mice. $CD4^+$ T cells were isolated and pooled from iliac lymph nodes and vaginal tracts of HSV-immune B6, -immune IFN-$\gamma^{-/-}$ and naïve B6 mice on the indicated day after challenge with 10^4 PFU HSV-2 186. Results are expressed as the total number of cells obtained from the tissues from 4 mice and are representative of 3 experiments performed (*$p <$ 0.05; ** $p < 0.01$; *** $p < 0.001$ for comparisons between immune B6 and immune IFN-$\gamma^{-/-}$ mice; Student's *t* test).

12 after challenge (Fig. 16). In animals that received $CD4^+$ T cells from HSV-immune B6 mice, HSV-2 titers within the vaginal tract began to decrease around day 6 after challenge, with all mice clearing the virus by day 10 after challenge. Conversely, in animals that received $CD4^+$ T cells from HSV-immune IFN-$\gamma^{-/-}$ mice HSV-2 titers within the vaginal tract remained high throughout the course of the experiment, and were comparable to the OT-II $CD4^+$ T cell-recipient controls through day 12 after challenge. Further, 9 of the 10 HSV-immune IFN-$\gamma^{-/-}$ $CD4^+$ T cell-recipients were unable to clear virus from the vaginal tract within this time (Fig. 16).

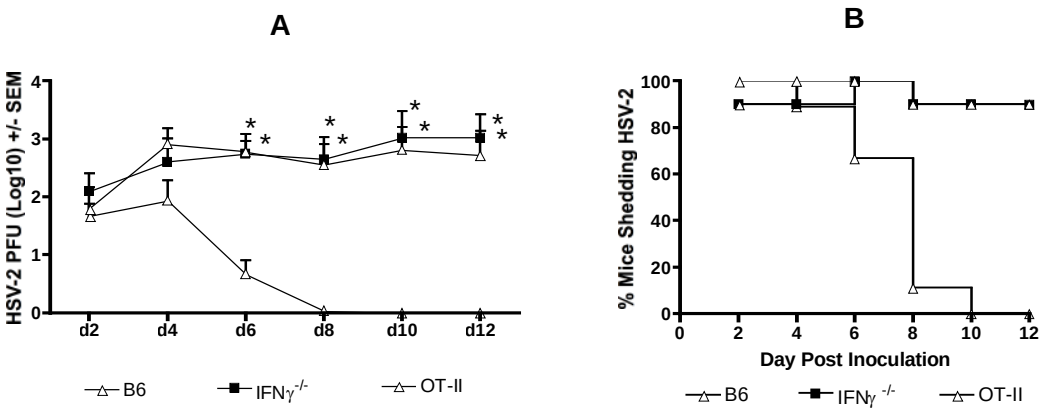

FIG 16. Adoptive transfer of $CD4^+$ T cells from HSV-immune B6, but not HSV-immune IFN-$\gamma^{-/-}$ mice, results in clearance of HSV-2 333tk- from the genital epithelium. Lymphocytes from HSV-immune B6, -immune IFN-$\gamma^{-/-}$, and naïve OT-II mice were activated in culture for 4 days with specific antigen. $CD4^+$ T cells were isolated and transferred to irradiated B6 recipients as described in Methods. Mice were immediately challenged with 5×10^3 PFU HSV-2 $333tk^-$ and virus was quantified on the indicated days. (A) Virus titers following HSV-2 challenge (*$p < 0.001$ compared to HSV-immune B6 mice; ANOVA). (B) Percent of mice shedding virus. Results are pooled from 2 experiments of identical design.

Discussion

Cell-mediated immunity is important for controlling HSV infection and previous studies have demonstrated the importance of $CD8^+$ cytolytic T lymphocytes (CTLs) in the clearance of HSV-2 from the genital epithelium of experimental animals (55, 209) and humans (135, 215). Kuklin et al demonstrated that $CD4^+$ T cells are critically important in the prevention of disease following vaginal infection of mice with HSV-1 (142). It has been suggested that $CD4^+$ T cells may be an important factor in the control of HSV infection in humans. IFN-γ, which may be supplied by $CD4^+$ T cells, has been shown to reverse the effects of the HSV immediate early protein ICP47, which interferes with the loading of MHC class I molecules with peptides (14, 265). Further, while $CD8^+$ T cells are classically thought to be the cell type responsible for cytolytic effects, there is evidence that cytolytic mechanisms may also be employed by $CD4^+$ T cells in control of various viral infections, including HIV and hepatitis C (7, 12). An important role for cytolytic $CD4^+$ T cells in infections with HSV has also been suggested (57, 135, 192). Further, HSV-specific $CD4^+$ T cells have been isolated from HSV lesions of humans (133, 135) and the genital epithelium of mice (187), suggesting they play a role in the clearance of HSV from the genital tract.

HSV is also a neural pathogen, and its replication in immune competent individuals following primary infection is normally controlled. Simmons and Tscharke presented evidence for $CD8^+$ T cell-mediated clearance of HSV-1 from thoracic dorsal root ganglia in the murine zosteriform model, as mice selectively depleted of $CD8^+$ T cells demonstrated increased neural destruction and virus spread (246). In the present

study, the increased presence of activated, HSV-specific $CD4^+$ T cells in the spinal cords and dorsal root ganglia of mice in primary HSV-1 infection is clearly demonstrated (Fig. 2, 3, 4). $CD4^+CD25^+$ T regulatory cells (T_{reg}) have previously been shown to be important participants in the T cell response to HSV (259). While the $CD4^+CD25^+$ T cells noted in the spinal cords and dorsal root ganglia of HSV-1 infected mice in the present study could in part include T_{reg}, the results of this study demonstrate $CD4^+$ effector T cell activity (Fig. 4). Further, $CD4^+$ T cells were sufficient for clearance of infectious HSV-1 from the genital tract, as well as possessing the ability to clear infectious virus from the spinal cords and dorsal root ganglia of these mice (Fig. 6). Our data align with one study of ocular HSV-1 infection, in which clearance of infectious HSV-1 form the trigeminal ganglia was apparently achieved by either the $CD4^+$ or the $CD8^+$ T cell population (72). Although either subset appears sufficient for clearance of infectious HSV, it is important to emphasize that both the $CD4^+$ and the $CD8^+$ T cell populations are likely to function in clearance in an intact animal. The results from the present study are consistent with this idea in that, although CD8-depleted B6 mice cleared virus from sensory ganglia, they did so more slowly than did B6 mice containing both $CD4^+$ and $CD8^+$ T cell subsets (Fig. 6). The variations in these experiments in comparison to their complementary experiments in which $CD4^+$ T cells were adoptively transferred to genetically B- and T-cell-deficient $Rag1^{-/-}$ mice (Fig. 7) may reflect the fragility of the $Rag1^{-/-}$ model. These animals may easily be overwhelmed by the infection, thus making the results not as apparent as in the complementary experiments. It is important to note that the ability to detect virus clearance in these studies was limited by the efficiency of plaque formation on Vero cell monolayers. Although some virus

may have been present in genital or neural samples beneath our level of detection, the use of this assay nonetheless allowed a quantification of infectious virus for comparison among groups while being mindful of the inherent limitations of the sensitivity of the assay.

In the genital model of HSV-2 infection, the requirement for either perforin- or Fas/FasL cytolytic mechanisms in $CD8^+$ T cell-mediated clearance from the genital epithelium has been previously demonstrated (55). Perforin is important for CTL-mediated defense against invading pathogens, and is thought to have a role in homeostasis as suggested by the fatal disregulation of the immune system associated with a homozygous deficiency in perforin (169). CTLs can also initiate cell death via Fas/FasL interaction. FasL on the surface of the CTL interacts with Fas expressed on the target cell, where target cell recognition results in the initiation of cell death pathways (83). In several other viral diseases affecting the central nervous system (CNS), including lymphocytic choriomeningitis, Theiler's, and West Nile viruses, the absence of perforin or Fas/FasL pathways has been correlated with loss of control of the virus and persistence of the virus in the CNS (118, 197, 228, 244). Granule exocytosis is thought to be the main cytotoxic pathway utilized by HSV-specific CTLs. Evidence for the importance of these mechanisms is bolstered by the fact that HSV encodes genes that prevent cytolysis via the Fas/FasL pathway as well as the function of granzyme B (110, 287).

In studies of latently HSV-1-infected human and murine trigeminal ganglia, it was noted that virus-specific $CD8^+$ T cells interacting with the neurons appeared to be capable of cytolysis, as they expressed granzyme B and perforin, though the amount of neural

damage in these tissues was reported as "limited" (258, 274). In the current study, $CD4^+$ T cells within the HSV-1-infected spinal cord and sensory ganglia expressed granzyme B, and thus appeared to possess the components necessary for a lytic T cell (Fig. 5). However, through three different experimental designs (specific antibody depletion, transfer of deficient $CD4^+$ T cells to genetically B-and T cell-deficient mice, and radiation chimeras), neither perforin nor Fas/FasL interactions was an absolute requirement for the $CD4^+$ T cell-mediated clearance of infectious HSV-1 from neural tissues (Fig. 8, 9, 10, 11, 12). These data do not exclude some role for perforin or Fas/FasL interactions, as complete clearance of infectious HSV-1 was not always seen in all animals tested.

In addition to Fas/FasL interactions and granule exocytosis, a CTL can initiate death in a target cell via the release of TNF-α. The release of TNF-α results in the production of caspase-8 and initiation of a caspase cascade that involves the recruitment of TNF-R1 (a death receptor) and Fas, thus leading to apoptosis of the target cell (136). Thus, TNF-α-mediated apoptosis may prove important for the elimination of infectious HSV from neural tissues. Lundberg et al previously reported an important role for p55 (TNF-R1) in limiting HSV-1 replication in the eye, ganglia, and brainstem (167). However, an increase in the concentration of TNF-α may result in pathology from destruction of neural tissues in neurodegenerative disorders as well as in response to pathogens (136). Interestingly, in the present study utilizing the genital model of HSV-1 infection in mice, a definitive role for TNF-α in controlling HSV-1 replication in the genital tract, spinal cord, or sensory ganglia using TNF-R1 deficient mice was not demonstrated (Fig. 13). This is consistent with the observation that relatively few TNF-

α-secreting $CD4^+$ T cells infiltrated the HSV-infected dorsal root ganglia and spinal cords in our experiments (Fig. 4).

One noncytolytic mechanism that may be employed by HSV-specific T cells to control virus replication in neural tissues is the release of the cytokine IFN-γ. It has been shown that $CD8^+$ T cells can control HSV infection in neural tissues, as well as prevent reactivation, an effect that may be due to the production and action of IFN-γ (34, 110). IFN-γ directly inhibits virus replication, can upregulate MHC-I and MHC-II expression, and could potentially be involved in recruitment of additional immune cells, such as macrophages, to the site of infection (265). Evidence for IFN-γ-mediated clearance of infectious HSV from neural tissues was previously reported in a study by Lewandowski et al, in which a 10-fold increase in intracerebral IFN-γ correlated with a 5000-fold increase in LD_{50}, as well as a rapid decrease in HSV titer in neural tissues (155). Thus, the $CD4^+$ T cells found in the HSV-1-infected neural tissues in our experiments may employ IFN-γ in clearance of infectious virus. This is an attractive idea, in that a nonlytic mechanism may be preferable in neural tissues that cannot be regenerated. In the trigeminal ganglia, the inflammatory cell-infiltrate has been shown to consist largely of $CD8^+$ T cells and a relatively small number of $CD4^+$ T cells, accompanied by both T_H1 and T_H2 cytokines, but in the absence of neural destruction (160, 264). Liu et al hypothesize that the IFN-γ and TNF-α produced in the infected trigeminal ganglia function to control virus replication, while the IL-4 and IL-10 found in these tissues limit the infiltration of polymorphonuclear leukocytes and thus destruction of neural tissues (160). This hypothesis is intriguing, and is consistent with the IFN-γ- and IL-4-producing $CD4^+$ T cells detected in neural tissues in the current experiments (Fig. 4D).

The large number of IL-4 producing cells detected in HSV-infected neural tissues is somewhat surprising as HSV induces a dominant T_H1-type response in the draining lymph nodes (187). The reason for this prominent IL-4 response is unclear at present although it should be pointed out that IL-4 secreting cells are commonly detected in secondary lymph nodes and genital tracts despite the presence of a dominant IFN-γ response (114, 187).

Another example of a polyvalent $CD4^+$ T cell-produced cytokine response is the studies by Querec et al. Using a live attenuated vaccine against yellow fever virus (known as YF-17D), Querec et al demonstrated an innate response that was characterized by the secretion of IL-12(p40), IL-6, and IFN-α, and resulting in an adaptive $CD4^+$ T cell response composed of both T_H1 and T_H2 subsets (220). Further, it is suggested that this polyvalent cytokine response, and the balance within this response, is critical for generation of an appropriate immune response and memory formation (220).

In the present study, the long-term presence of HSV-specific $CD4^+$ T cells in spinal cord and dorsal root ganglia of mice following HSV-1 inoculation was demonstrated (Fig. 4). It has been previously shown that HSV-specific $CD8^+$ T cells are retained in HSV-infected trigeminal ganglia (159). It has been proposed that the presence of T cells in these neural sites may limit the spread of the virus to additional uninfected neurons, reduce the number of reactivation events, secrete neurotropic factors, or simply be an effect of repeated reactivation events (99). The mechanism by which these adaptive immune cells are maintained in neural tissues remains to be defined. In murine trigeminal ganglia infected with HSV-1, it has been shown that rare neurons (approximately one neuron every ten days) within the latently infected ganglia do

produce productive cycle genes, and that these rare neurons appear to be responsible for a local immune response involving the infiltration of inflammatory cells (63). Thus, the maintenance of a T cell population within HSV-infected neural tissues, as was seen in the current study, may be in response to sporadic translation of viral message during "latent" infection.

IFN-γ has previously been shown to have an important function in the resolution of primary HSV-1 infections within the murine genital tract (30, 250). Further, IFN-γ has been shown to be important in the resistance to primary genital HSV-2 infection (86, 188). IFN-γ is also important for resistance in HSV-immune animals as well, despite the presence of HSV-specific antibody and cytolytic T cell responses (189, 192, 209). Within 24 hours of intravaginal re-challenge with HSV-2, IFN-γ is detectable in HSV-immune animals and is capable of altering HSV titers within the vaginal tract at this time (189, 209). IFN-γ is thought to influence upregulation of MHC class II expression of genital epithelial cells, to synergize with type I interferons, and alter the expression of cell adhesion molecules, thus enhancing the infiltration of additional immune cells to the genital site of HSV infection (185, 211, 230).

Inoculation of mice with attenuated HSV-2 333tk$^-$ has previously been used as a model for successful immunization against HSV (178, 189, 207). Here, the clearance of HSV-2 333tk$^-$ by IFN-$\gamma^{-/-}$ mice and subsequent development of an adaptive immune response allowed for further examination of IFN-γ-independent immune mechanisms. In similar studies, Yu et al. found that IFN-$\gamma^{-/-}$ mice could be completely immunized by inoculation with HSV-2 333tk$^-$, and further that T lymphocytes from these animals were completely protective in transfer recipients in a zosteriform challenge model with HSV-1

(292). In our hands, immunization in this manner was only able to induce partial resistance of IFN-$\gamma^{-/-}$ mice to challenge with virulent HSV-2, as HSV-immune $CD4^+$ T cells from these animals were not able to clear attenuated virus from the vaginal tracts of transfer recipients. The differences in finding between studies by Yu et al. and our lab may be attributed to the use of different strains of HSV or different strains of mice.

The cytokines present during activation of naïve $CD4^+$ T cells are important for their differentiation into T_H1 and T_H2 effector subsets (269). In the presence of IL-12, $CD4^+$ T cells are directed to adopt a T_H1 phenotype, thus producing IFN-γ and TNF-α (36). IFN-γ, which activates antimicrobial macrophages, also synergizes with DC-produced IL-12 to activate CTLs (16). In the presence of IL-4, $CD4^+$ T cells become T_H2 cells, secreting IL-4 (eosinophil activation) and IL-5 (B cell antibody production) (16). IL-4-secreting $CD4^+$ T cells (T_H2) have previously been detected in HSV-immune IFN-$\gamma^{-/-}$ mice (30, 292). In addition to these IL-4-secreting $CD4^+$ T cells, we were also able to detect IL-17-secreting $CD4^+$ T cells (T_H17) and IFN-γ-secreting $CD4^+$ T cells (T_H1) within the iliac lymph nodes and genital tracts of HSV-immune B6 and HSV-immune IFN-$\gamma^{-/-}$ mice. In reference to HSV specifically, a T_H1-type response has been shown to play a role in clearance of virus both *in vivo* and *in vitro* (104, 229). A T_H17-type response is more typically associated with defenses against extracellular bacteria, but may function in the recruitment of granulocytes to the site of infection, thus limiting spread of the virus (13, 132, 190, 272, 288). While a direct anti-HSV role for a T_H2-type response is not known, it is possible that these $CD4^+$ T cells enhance the immune response in other ways, such as enhancing B cells and the production of specific antibody, promoting activation and migration of additional innate and adaptive immune

cell types, or directly impeding virus spread. Despite the presence of specific $CD4^+$ T cells secreting potentially antiviral cytokines in the current studies, resistance against HSV-2 re-challenge was not established.

Candidate vaccines against HSV have not been completely protective in all groups, suggesting new strategies should be pursued. In designing an effective vaccine against HSV, it may be useful to examine and consider the various responses of the immune system to natural infection. The data included here illustrate the important role of $CD4^+$ T cells in clearance of infectious virus from the genital tract, dorsal root ganglia, and spinal cord during primary infection. In addition to playing an effector role in clearance of HSV from genital and neural sites, $CD4^+$ T cells may also support maintenance, activation, and expansion of other adaptive immune cells at peripheral sites as has been shown in other viral infection models. $CD4^+$ T cells in association with either $CD8^+$ T cells or B cells are required for rapid clearance of measles virus from the CNS (266). A role for $CD4^+$ T cells in supporting protective antibody within the CNS has been shown in studies of West Nile virus (248). In studies of infection with Sindbis virus and mouse hepatitis virus, antibody has been shown to be critical for the prevention of virus reactivation within the CNS (33, 222). In our HSV model, we noted greatly diminished HSV-specific B cell and $CD8^+$ T cell responses following inoculation of $CD4^+$ T cell-depleted mice (data not shown). $CD4^+$ T cells are also known to support activation of sensory ganglia-resident, memory $CD8^+$ T cells (276). Such $CD4^+$ T cell responses at neural sites of HSV infection might therefore be beneficial to the immunized individual by limiting HSV infection of the sensory ganglia. This in turn might result in limitation in the amount of virus establishing latency, reduction in recurrent lesions at the

original site of infection, thus impacting the transmission of virus to new hosts, including neonates. The results of the present study demonstrate that HSV-specific $CD4^+$ T cells are recruited to HSV-infected sensory ganglia, where they are maintained long after viral infection, along with HSV-specific $CD8^+$ T cells (160) and plasma cells (193). Our findings further demonstrate an important role for these $CD4^+$ T cells in protection of the sensory ganglia and spinal cord suggest that an effective HSV vaccine may need to elicit immune responses at both the site of epithelial infection, as well as in the neural tissues.

CHAPTER 3: MANIPULATION OF $CD4^+$ T CELL RESPONSES VIA TLR-INDUCED PROINFLAMMATORY CYTOKINE MILIEUS

Introduction

Despite the availability of antiviral medications such as acyclovir and valacyclovir for the treatment of genital HSV, infections remain common (20). The development of an effective HSV vaccine is important for reducing social and economic burden, controlling the rate of neonatal herpes infections, and lowering the elevated risk of contracting HIV associated with HSV infection (14, 20, 110, 115). The development of an effective HSV vaccine is hampered by incomplete knowledge of the immune correlates of protection, identification of immunogenic epitopes, and the design of a safe and effective immunization schedule (19).

An effective vaccine should exhibit both efficacy and safety, generating an appropriate, long-lived immune response with limited inflammation. The quest for a vaccine that is both effective and safe often falls short. For example, subunit vaccines, consisting of an isolated target viral protein alone, or vectored vaccines, consisting of viral protein inserted into another virus, have proven safe even in the immunocompromised, though the increased safety comes at the cost of reduced immunogenicity. Alternatively, immunization with whole bacterial products can induce a robust immune response, though severe systemic inflammation can also result. In the design of a vaccine that is effective yet also safe, one strategy is to utilize adjuvants as a

means to customize, enhance, or prolong the immune response to a less than optimal immunogen.

In comparison to responses elicited by antigen alone, the use of adjuvants supplied in conjunction with antigen for T cell activation has been shown to induce a significantly greater number of activated T cells to accumulate and proliferate within the draining lymph nodes (206). Interestingly, the injection of soluble antigen without the accompaniment of an adjuvant has been shown to transiently activate antigen-specific T cells, with the majority of those T cells then being deleted (206). Thus, the generation of an activated, functional T cell response appears to require not only antigen, but also additional signals such as pathogen components (recognized through pathogen-associated molecular patterns (PAMPs), as discussed below): components that can be mimicked by adjuvants (206).

Vaccination and the mucosal immune system

While the use of topical microbicides can be employed to prevent attachment of HSV and passage of the virus to additional hosts (294), the disadvantages of this strategy for disease control include a strict and demanding regimen requiring a high degree of patient compliance, a contraceptive effect, toxicity, and a possible proinflammatory response, which could lead to disruption of the mucosal surface and allow for greater susceptibility to other sexually transmitted pathogens such as HIV (177). This highlights the need for an effective vaccine against HSV to limit spread of HSV and reduce susceptibility to other sexually transmitted diseases.

The mucosal immune system is seen as compartmentalized, as there appears a physical barrier between systemic and mucosal immune systems, as well as boundaries established within the mucosa that restrict immune responses to or near the induction site (111). The female genital tract mucosa also presents an interesting challenge in the induction of a protective immune response, in that the lower female genital mucosa does not contain sites of immune initiation and dissemination (to distant sites) such as the Peyer's patches found in the intestinal epithelium, while the endocervix does contain lymphoid aggregates that can initiate an immune response (182). No lymphocyte homing markers or chemokine receptors specific for trafficking to the genital tract have been identified. The known lymphocyte homing molecule utilized within gut mucosal tissues is MAdCAM-1 expressed by endothelial cells (which interacts with α4β7 expressed on T cells), which has been identified to play a role in activating T cells within the Peyer's patches of the intestinal mucosa (256). MAdCAM-1 has not been identified within the human genital mucosa (112). The non-mucosal specific adhesion molecules VCAM-1, ICAM-1, and E-selectin have been noted within the genital mucosa, but a role in lymphocyte homing to the genital tract has not been demonstrated (112). The induction of an HSV-specific $CD4^+$ T cell response that can be directed to home to the genital mucosa may prove a critical feature for an effective vaccine against HSV.

A common feature among many of the failed HSV vaccines is that they are not applied to the (often) site of infection at the genital mucosa, and thus, while the vaccines are able to induce robust systemic immune responses, they may not be capable of producing strong immune responses within the genital tract (145). However, while past studies in which non-replicating (i.e. subunit protein) antigens were delivered

intravaginally were capable of generating specific immunity, these responses were not robust, leading to the conclusion that the genital tract is not able to respond robustly to intravaginal immunization with non-replicating antigens (84, 145, 208). Therefore, one strategy for the development of an effective vaccine against HSV involves the use of adjuvants in the manipulation of the ensuing immune response, guiding the type and strength of response. An effective vaccine against HSV will likely need to induce a virus-specific $CD4^+$ T cell response that exhibits appropriate effector function (including the development of a T_H1-type cytokine response and cytolytic ability), homing to the site of infection (in the case of genital HSV, homing to the genital mucosa, but perhaps also to neural sites of virus infection), as well as the development of a robust, long-lived memory population.

Dendritic cells

Dendritic cells (DCs) are important mediators of the immune response. Immature DCs are fully capable of phagocytosis, macropinocytosis, and adsorptive endocytosis, processes for sampling the environment for the presence of invaders (16). As antigen presenting cells (APCs), they capture and process antigens, and activate T cells through surface expression of large amounts of peptide-MHC complexes and costimulatory molecules (16). DCs are great amplifiers of the immune response, as one dendritic cell has the capability to activate between 100 and 3000 T cells (16). Interestingly, the concentration of MHC-peptide complexes has been found to be 10-100× higher on DCs than on other APCs such as B cells and monocytes (103). Upon capture of antigen, DCs

begin maturation and migrate to the lymph nodes (16). Within the lymphoid organs (spleen and lymph node), DCs complete maturation (129), attract B and T cells through release of chemokines (1), and enhance survival of circulating T cells (29). Due to their amplification and enhancement of the T cell response, DCs may prove critical for effective vaccination and an important consideration in vaccine design.

Dendritic cells are activated via interaction of PAMPs with toll-like receptors (TLRs), and enrich T cell responses through production of inflammatory cytokines and upregulation of MHC molecules (16). Enhanced immune responses to a range of pathogens including HIV-1 (270), hepatitis B (49), *Leishmania major* (224), influenza (101), and tumor antigens (138), have been observed during T cell priming upon application of various TLR ligands and are often attributed to the establishment of optimal cytokine milieus. The cytokines produced upon TLR activation are important for the clonal expansion of the T cells, as well as directing their differentiation into T_H1 and T_H2 effector subsets (269). Mature DCs produce IL-12 to persuade T cells to adopt a T_H1-type phenotype, thus producing IFN-γ (36). IFN-γ, which activates antimicrobial macrophages, also synergizes with DC-produced IL-12 to activate CTLs (16). In the presence of IL-4, DCs direct T cells to become T_H2-type cells, secreting IL-4 (eosinophil activation) and IL-5 (B cell antibody production) (16). Activated T cells further the immune response through their interactions with B cells (antibody production), macrophages (cytokine production), and target cells (direct lysis by the T cells) (16). Signals delivered through different TLRs can be interpreted by different subsets of APCs (218), allowing for the manipulation of the immune response through utilization of different TLRs.

With respect to infection with HSV-2, vigorous T cell immunity is elicited by vaginal DCs. $CD11c^+CD11b^+$ DCs are rapidly recruited to the submucosa beneath the infected epithelial cells (295). Though there are three types of DCs that have been identified within the vaginal epithelium, it is the submucosal DCs that have been determined to migrate to the lymph nodes and become the major activators of both a $CD8^+$ and a $CD4^+$ T cell response against HSV-2 (290, 295). The development of a T_H1-type immune response, thought to be crucial for defense against HSV, has been noted within the iliac lymph node, which drains the upper two-thirds of the vaginal tract (295). Further, upon infection with HSV-2, plasmacytoid DCs are recruited from the blood to the vaginal mucosa, where they produce the antiviral cytokine IFN-α, exhibit direct antiviral effects, and can influence $CD4^+$ T cells to produce IFN-γ and differentiate into T_H1 cells (165, 235). These DCs have been found to be critical for protection against lethal challenge with HSV-2, an effect that is mediated through the recognition of HSV-2 CpG oligodeoxynucleotides via TLR9 on the DCs (242). Previous studies have demonstrated the natural ability of HSV-2 to stimulate TLR2, -3, and -9 (73, 236). Though the vaginal epithelium is known express TLR1, -2, -3, -5, -6, and to some extent TLR4, the migratory DC recognition of natural HSV-2 CpG motifs through TLR9 is the initial innate response against the virus (165). Stimulation through different TLRs has been shown to lead to the development of distinct cytokine milieus, thus influencing and directing the ensuing $CD4^+$ T cell response.

$CD4^+$ T cell lineage development

Almost a quarter century ago evidence for separate effector $CD4^+$ T cell lineages, a heterogeneous $CD4^+$ T cell population, emerged. 1986 brought the establishment of the T_H1- T_H2 paradigm: the proposal that two different types of $CD4^+$ T cells were responsible for the observed delayed-type hypersensitivity (T_H1) and allergic reactions (T_H2) attributed to $CD4^+$ T cell responses (40). These studies further defined the two lineages by their cytokine production (IL-2 and IFN-γ for T_H1, and IL-4 for T_H2), and suggested that these cytokines promoted their own lineage development, while inhibiting development of the other (40). Since the birth of the T_H1-T_H2 paradigm, several other $CD4^+$ T cell subsets and/or lineages have been described, including $CD4^+$ T cells that are involved in inflammatory responses to invading pathogens, as well as $CD4^+$ T cells that naturally suppress the immune system. Some of the major points concerning the more well-researched $CD4^+$ T cell lineages will be further discussed below, as reviewed by Wan and Flavell, and Zhou et al (279, 298).

The dominant T_H1 cytokine is IFN-γ, though IL-2 and TNF-α are also produced (279, 298). The innate cell production of IL-12 and the production of IFN-γ by NK cells and T cells drive the differentiation of $CD4^+$ T cells toward the T_H1 phenotype (298). Development of T_H1 cells depends on T box transcription factor, or T-bet (298). T_H1 cells have been shown to be important for the immune response against intracellular pathogens including intracellular bacteria, such as *Salmonella typhimurium* (66), protozoa, such as *Leishmania major* (231), and viruses, such as vaccinia virus (293), as well as against tumors (184). T_H1 cells have also been suggested as the dominant $CD4^+$ T cell subtype responsible for clearance of HSV (152). Though T_H1 cells are effective at

clearing intracellular pathogens and protecting against tumors, their characteristic inflammatory cytokines have also been implicated in the development of autoimmune diseases, inflammatory diseases, and graft-versus-host reactions (47, 97, 279, 280).

T_H2 cells are defined by their production of IL-4, IL-5, IL-9, IL-10, and IL-13 (279, 298). The development of the T_H2 lineage is attributed to IL-4 within the microenvironment, with GATA-3 being the important transcription factor (279). T_H2 cells have been shown to be important in the defense against extracellular pathogens, such as helminthes (51). Like T_H1 cells, T_H2 cells themselves have been described as pathogenic in certain circumstances. The diseases associated with an overzealous T_H2 population include asthma and allergies (279, 291).

T_H17 $CD4^+$ T cells are characterized by their production of IL-17, IL-21, and IL-22 (279, 298). A T_H17-type $CD4^+$ T cell population develops in the presence of IL-6 and TGF-β together, IL-21, and IL-23, with the transcription factor ROR-γt being important for development (279, 298). T_H17 cells are important for the clearance of bacteria, such as *Klebsiella* in the lung, and fungi, such as *Candida albicans*, and are important for defense at mucosal surfaces (85, 98, 279, 298). Like T_H1 and T_H2 subtypes, T_H17 cells can also lead to pathogenesis themselves and are thought to be important in the development of autoimmune diseases such as autoimmune experimental arthritis (198).

Regulatory T cells (T_{reg}) are another $CD4^+$ T cell subset that has recently drawn much interest. Induced T_{reg} are characterized by the production of large amounts of IL-10 and TGF-β (279). They are also induced to differentiate in the presence of IL-10 and TGF-β, and the important transcription factor for this lineage is FoxP3 (279). T_{reg} are unlike T_H1, T_H2, and T_H17 $CD4^+$ T cells in that they are not antigen specific, and they are

immunosuppressive (279). T_{reg} are thought to be involved in maintaining homeostasis, for monitoring the immune response to infection (and thus preventing the development of a pathogenic T_H1 or T_H2 response, as mentioned above), and for returning the $CD4^+$ T cell populations to homeostatic levels following resolution of infection (279, 298). In reference to HSV, T_{reg} cells have been implicated in the attraction of additional immune cells to the site of infection (164).

In the activation of naïve $CD4^+$ T cells, a three signal approach has been proposed. The first signal involves the interaction of the TCR on the $CD4^+$ T cell with the peptide-bound MHC-II molecule on the antigen presenting cell, the strength of which has proven important in the determination of lineage commitment of the T cell (249). The second signal involved with T cell activation is the interaction of costimulatory molecules. Costimulation has also been shown to influence $CD4^+$ T cell lineage commitment (279). For example, costimulation mediated through CD28—B7 has been shown to result in an enhanced T_H1 response, while in humans, signaling through inducible T cell costimulatory (ICOS) and its ligand directs the development of a T_H2 response (279). It has been argued that the third signal in T cell activation, the cytokine milieu present during priming, is the dominant signal in determining $CD4^+$ T cell lineage commitment (279). The cytokine milieu present during immunization, as discussed above, influences the type of $CD4^+$ T cell response that develops. Therefore, manipulation of the cytokine milieu present during immunization may allow for the directed development of a $CD4^+$ T cell response. One way that the cytokine milieu can be altered is through the innate immune system, and activation of antigen presenting cells through the various Toll-like receptors (TLRs), which will be discussed further below.

The innate immune response and pattern recognition receptors

Pathogen-associated molecular patterns (PAMPs) are largely invariant among different groups of pathogens, are essential for survival of the pathogen, and are present on viral and bacterial invaders but not on host cells (179). Recognition of PAMPs by host pattern recognition receptors (PRRs) can initiate direct effector functions of innate immune cells, such as phagocytosis or production of nitric oxide synthase, or can induce expression of inflammatory cytokines and chemokines, thus triggering the adaptive immune system and prompting an infiltration of leukocytes to the site of infection (3, 143, 179). Further, recognition of PAMPs through PRRs represents a connection between the innate and adaptive immune systems, as this recognition of invading pathogens has been shown to upregulate the APC expression of costimulatory molecules in preparation for activation of the adaptive immune response (143, 179).

TLRs are the most extensively studied family of PRRs and can detect a wide range of pathogen-associated molecules including carbohydrates, nucleic acids, lipids, and proteins (3). TLRs are membrane-bound either at the cellular or endosomal membrane, and are composed of an extracellular (or luminal) domain containing leucine-rich repeats important for binding to PAMPs, and a cytoplasmic Toll/interleukin-1 (IL-1) receptor homology (TIR) domain important for intracellular signal transduction, induction of inflammatory cytokine and chemokine secretion, and leukocyte infiltration (64, 204).

TLRs have been identified in most cell types, including APCs, such as macrophages, B cells, and DCs, though TLR expression varies among cell types and anatomical location (106, 194). In addition to triggering cells of the innate immune

system, ligands of various TLRs can have direct effects on adaptive immune cells (232). The expression of various TLRs was noted in general to be of higher magnitude in $CD8^+$ T cells than in $CD4^+$ T cells, as well as being higher in innate cells versus adaptive immune cells, though TLR expression was dependent on both species (animal) and cell type (232). An appropriate and effective T cell response is dependent on the TLR that is engaged (232). It is important to note that pathogens contain multiple PAMPs, which are then recognized by various PRRs (TLRs) (194).

Expression of TLRs 1 through 9 has been detected in the murine vaginal tract (251), and the expression of TLRs 1 through 6 has been differentially noted within the different epithelial regions of the human female genital tract (62). It is of important note that, upon infection, the infiltration of various immune cells, which may express additional or alternate TLRs, may enhance the available TLR repertoire (73).

The presence of TLR2 (147) and TLR4 (146, 153) have been documented within the rodent central nervous system. Within the human central nervous system, the expression of TLRs was found to be broad within glial cells, the level of expression varied between individuals, and is perhaps subject to dynamic regulation (32). *In vitro*, human adult microglia have been shown to express TLR 1-8 (32). Human astrocytes and oligodendrocytes have been shown to express TLR 1-4, whereas murine astrocytes and oligodendrocytes appear not to express TLR4, thus demonstrating a potentially important difference between the human and murine immune systems (153). Interestingly, while dendritic cells are known to express TLR3, it has now also been shown that human microglia, astrocytes, and oligodendrocytes also express TLR3 (32, 199).

Human neurons are noted to express TLR3 even in the absence of glia and, when stimulated with polyinosinic-polycytidylic acid (PIC), a synthetic dsRNA mimic, mount an immune response that includes the production of IFN-β, chemokines, and inflammatory cytokines (217). Further, these neurons can mount an immune response to HSV-1 that also includes the production of inflammatory cytokines including IL-6, but does not include production of IFN-β (217). The expression of TLR3 in human neurons is constitutive and, like in DCs (174), is confined mostly to the cytoplasm, although neurons may also express some TLR3 at the membrane (217). Because human neurons express TLR3 and are reactive to PIC, dsDNA could be a major target for the sensing of viral invaders (such as HSV) within the human nervous system (217). This is important when considering infection with HSV, as important immune events occur within neural tissues as well as at the original site of infection (such as the vaginal mucosa). These data also underscore the importance of TLR3 and dsDNA recognition in the defense against HSV.

TLRs provide critical services in the induction of the adaptive immune response to invading pathogens. They are capable of mediating the maturation of dendritic cells, thus leading to enhancement of antigen presentation, polarization of $CD4^+$ T cell into T_H1 and T_H2 lineages, and alleviation of T_{reg} suppression (117). During a primary T cell response, the TLR and MyD88 pathways are essential for counteracting the suppressive activity of T_{reg} (212). Of special interest, signaling through TLR2 has been shown to result in the proliferation of the T_{reg} subset, in conjunction with the loss of FoxP3 expression and suppressive capabilities (158, 257). In the absence of a greatly enhanced T cell proliferative response, the application of either a ligand for TLR3 or TLR9 has

been shown to prolong survival of activated $CD4^+$ T cells (70). The enhanced survival of TLR-activated $CD4^+$ T cells has been attributed to the increased expansion and reduced contraction rate of the population (70). It is of note that certain viruses, such as vaccinia virus (60) and mouse mammary tumor virus (116), that feature a dsRNA intermediate in their life cycle, are capable of suppressing an effective $CD4^+$ T cell response (70). Employment of TLR ligands that are known to enhance the $CD4^+$ T cell response may be able to restore this important immune response (70).

Polyinosinic-polycytidylic acid as an adjuvant

A natural product of replication in many viral lifecycles, dsRNA (or its synthetic mimic, PIC (175, 183) has been identified as a ligand for TLR3 (4). TLR3 expression has been demonstrated on dendritic cells, macrophages, NK cells (16), and $CD4^+$ T lymphocytes (70). Activation of TLR3 through PIC (or viral dsRNA) results in the strong activation of the IFN-β promoter and a weaker activation of NF-κB (4). This is unique, in that the activation of other TLRs (2, 5, 7, and 9) results in the signaling through MyD88 thus activating NF-κB (2). PIC is also capable of utilizing a MyD88-independent pathway which leads to activation of the transcription factors NF-κB and interferon regulatory factor (IRF)-3 (94, 238), which are known potent inducers of the antiviral type I interferons (4, 56). Indeed, activation of TLR3 upon treatment with PIC results in the production of IFN-α and -β, T_H1-type cytokines and chemokines, MIP-1α, and RANTES, and induces NK cell production of IFN-γ (10, 64, 93, 94). IFN-α (mainly produced by plasmacytoid DCs) can influence T cells to produce IFN-γ and DCs to

produce IL-15 (235), thus enhancing the type I interferon effects on NK cells resulting in proliferation, cytotoxicity, and tissue trafficking (24), as well as the maturation of macrophages (237). Further, type I interferons affect DC maturation and promote their expression of high levels of MHC and costimulatory molecules (235), thus enhancing proliferation and survival of T cells (172). Therefore, PIC or another ligand that stimulates TLR3 may prove to be an effective adjuvant.

Treatment with PIC at the time of vaccination with peptide has been shown to enhance the antigen-specific $CD8^+$ T cell response by tempering the contraction phase and improving survival of the $CD8^+$ T cell population (233), though a slight delay of peptide delivery, even of just 4h post-priming with PIC can negate this moderation of immune response (234). This is likely due to the noted short duration (declining after only 1-4h) of enhanced inflammatory cytokine response due to treatment with PIC (234). Thus, PIC or another ligand that activates DCs though TLR3 has potential as an adjuvant in the successful protective against HSV because of the enhanced activation of DCs, production of type I interferons, and thus the activation of $CD4^+$ T cells.

CpG oligodeoxynucleotides as adjuvants

Another TLR ligand with potential as an adjuvant is CpG-oligodeoxynucleotides (CpG), a bacterial DNA mimic. CpG activates APCs via TLR9, resulting in the a) upregulation of MHC class II and costimulatory molecules on human B cells and plasmacytoid dendritic cells allowing for stronger APC—T cell interactions (161), b) secretion of T_H1-type cytokines, promoting the production of IFN-γ by NK cells and

allowing for T cell recruitment that is critical for memory formation after immunization (35, 130), c) proliferation of and antibody production by B cells (130), and d) expansion of B cells and naïve and memory T cells as a result of induction of anti-apoptotic effects within these populations (48, 130). Intravaginal delivery of CpG has been shown to induce rapid infiltration of inflammatory cells to the vaginal epithelium, proliferation and thickening of the genital epithelium (8), and IL-15 production, known to be protective against HSV-2 vaginal challenge (74).

Further, CpG treatment enhances survival of the activated $CD4^+$ T cell population in a MyD88 and PI-3 kinase-dependent manner, resulting in increased IL-2 production and proliferation (69). It is also of note that treatment with CpG DNA can alleviate the suppressive effects of T_{reg} (213). CpG DNA play a functional role in the $CD4^+$ T cell response, as they deliver a MyD88-dependent, direct costimulatory signal which allows the $CD4^+CD25^-$ effector population to evade T_{reg} suppression (149). Within a colitis model, suppression of T_{reg} effects has also been noted, and is thought to occur through the TLR2-mediated downregulation of FoxP3, which was not found in treatment with CpG (149, 158). It is thought that T_{reg} may exert their suppressive effects through the inhibition of IL-2 expression, and thus, the suppressive effects of T_{reg} may be alleviated by the CpG DNA-mediated increase in IL-2 production (149). Activation through CpG, like PIC, has been shown to enhance the survival of activated $CD4^+$ T cells through their activation of NF-κB (70).

It is thought that the resistance to HSV-2 as a result of CpG application occurs through induction of T_H1-type cytokines and chemokines and recruitment of dendritic cells (93, 242). Utilizing the immune response initiated through TLR9, Kwant et al were

able to induce significant and protective immune responses against HSV-2, as well as reduce recurrences and shedding, after intravaginal immunization with CpG and recombinant HSV-2 glycoprotein B (145). Due to the induction of a T_H1-type cytokine milieu and the upregulation of MHC-II molecules, CpG (or another ligand that activated DCs through TLR9) may prove a powerful tool for enhancing the $CD4^+$ T cell response generated by vaccination against HSV.

Higher MHC-II binding affinity was noted among T cells recruited during vaccination with dispersible adjuvants such as CpG or monophosphoryl lipid A (MPL), while T cells recruited in response to vaccination with depot agents such as alum, incomplete Freund's adjuvant (IFA), or complete Freund's adjuvant (CFA) possessed antigen-specific TCRs with lower MHC-II binding affinity, an effect not dependent on antigen dose (170). This finding further highlights the ideas that a) different adjuvants will induce varied T cell responses and b) if a strong T cell response is desired (such as a strong T_H1-type response desired in an effective HSV vaccine), choice of vaccine adjuvant may be critical in guiding the ensuing immune response (71, 170). It is noted that MHC-II expression by dendritic cells is also a reflection of the inflammatory context under which the dendritic cell was primed, and thus can also be a function of adjuvant composition (170, 180). Especially with protein vaccination, as is proposed for protection against HSV, an adjuvant with the ability to elicit a strong T_H1-type response may prove critical (78, 170).

Lipopolysaccharides as adjuvants

LPS is thought to exert its effectiveness through an induction of an inflammatory environment, including the production of TNF-α or IL-1 (206). Further, LPS has been shown to induce production of IL-12, which is thought to be important in supporting the IFN-γ production by T cells and thus development of a dominant T_H1-type response that could be important in the defense against HSV (206, 268). However, induction of an immune response through TLR4 has not been proven effective in an HSV-2 vaginal challenge (73). While both TLR3 and TLR4 have been shown to induce antiviral genes, it appears that signaling through TLR3 induces a more intense and prolonged gene induction compared to that of TLR4 (10). Interestingly, it has been shown that the genes activated by TLR9 signaling are a portion of the genes that are activated by TLR4 signaling (65). More specifically, signaling through TLR4 or TLR9 results in recruitment of the MyD88 adaptor protein and activation of the transcription factors NFκB and AP-1, resulting in the production of TNF-α (65). TIRAP, another adaptor protein, is involved with signaling through TLR4 but not TLR9, activation of RNA-dependent protein kinase (PKR), and resulting in the production of type I interferons (65).

One concern involved in the use of a TLR4 ligand as an adjuvant relates to the observation that LPS leads to the development of endotoxic shock (123). However, monophosphoryl lipid A (MPL), a derivative of LPS, is a TLR4 agonist that does not induce shock (173). It is of note that MPL is one component of the GlaxoSmithKline vaccine against HSV-2 (20) that is postulated to not drive an appropriate T cell response. While MPL may drive a T_H2-type response rather than the desired T_H1 response, another nontoxic TLR4 ligand may prove to enhance the desired response. Therefore, due to

their ability to drive the development of a dominant T_H1-type response, TLR4 ligands may prove capable of enhancing the immune response to a less immunogenic vaccine against HSV. While LPS itself is not a candidate for an adjuvant for use in humans due to toxicity, LPS used in the experiments here represents a model for a TLR4 ligand.

Factors in vaccine design

Important factors to consider in the design of an effective vaccine include formulation, route of inoculation, and schedule, as, undoubtedly, these factors will influence the magnitude, composition, duration, and compartmentalization of the induced immune response (14). A vaccine should also ideally be safe for the immunocompromised, stable, and cost-effective (14). Of additional concern, the marketing, feasibility of delivery, and moral perceptions of potential recipients pose great obstacles to the administration of an intravaginal vaccine. Therefore, a vaccine with the capability to induce the desired long-lasting, multifaceted immune response via a more conventional route (i.e. intramuscular) is of significant public health importance.

Though high levels of HSV-2 neutralizing antibodies have been achieved, previous candidates for HSV vaccines have not been completely protective in all groups, possibly due to the lack of a T cell response generated upon vaccination (42, 254). Previous studies have demonstrated the necessity of cell-mediated immunity (187-190) and the importance for a T_H1-type cytokine response (especially IFN-γ and IL-12) (86, 188) in limiting genital HSV-2 disease. It seems that an adjuvant-generated T_H1-type

cytokine milieu incorporated with a vaccine that elicits a robust T cell response will provide better protection against HSV-2 (219).

Adjuvants can be employed to alter the ensuing immune response, utilizing Toll-like receptors (TLRs) as a means to direct and heighten the immune response to a less immunogenic vaccine (170). Selecting an adjuvant able to induce specific qualities within an immune response may help to overcome the immature neonatal immune system and provide immune protection such is seen in adults (176, 245). TLR ligands can alter the innate environment (such as the cytokine milieu present during activation), as well as stimulate adaptive responses (such as enhancing the capacity of T cells for activation, proliferation, and IFN-γ production), thus allowing for great manipulation of the immune response through vaccination and providing important consideration for vaccine design (232).

Therefore, the use of TLR ligands as adjuvants may allow for the manipulation and enhancement of the ensuing immune response to a less immunogenic vaccine. We hypothesized that TLR ligand-induced proinflammatory cytokine milieus could be used to manipulate the ensuing $CD4^+$ T cell responses. More specifically, we wanted to explore the possibility of influencing the $CD4^+$ T cell response to mimic that seen in natural infection with HSV, thus enhancing the memory $CD4^+$ T cell population and immunity afforded by a less immunogenic vaccine. This was addressed through determining the type of $CD4^+$ T cell response generated *in vitro* to dendritic cells (DCs) activated through various Toll-like receptors (TLRs). It is important to emphasize that this is a *model* system, and not meant to advocate the use of DCs as a vaccine in this manner. This model system allowed us to carefully examine the indirect effects of TLR

ligands on $CD4^+$ T cell function and memory by conscientiously controlling for the affinity of the TCR for antigen, the DC subset, the antigen (OT-II peptide), as well as concentration and type of TLR ligand signal. These studies included determination of (a) the activation of DCs and (b) cytokine secretion by DCs, in response to different TLR ligands. These *in vitro* studies were extended to examine the effect of TLR-stimulated DCs on $CD4^+$ T cells as determined by $CD4^+$ T cell (a) activation, (b) proliferation, (c) expression of lytic molecules, (d) expression of chemokine receptors important for trafficking to the genital mucosal and neural sites of infection, and (e) cytokine secretion as a possible determinant of $CD4^+$ T cell lineage commitment. We then attempted to translate these findings to an *in vivo* system, where we examined (a) the numbers of T_H1-, T_H2-, and T_H17-producing antigen-specific $CD4^+$ T cells in secondary lymphoid and genital tissues and (b) cytolytic activity of antigen-specific $CD4^+$ T cells, after immunization with TLR-stimulated DCs.

Materials and Methods

Mice

C57BL/6J (B6), Balb/Cj, and C.Cg-Tg(DO11.10)10Dlo/J (DO.11; MHC-II T cell receptor transgenic mice specific for the ovalbumin peptide $OVA_{323-339}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific-pathogen-free conditions at the University of Texas Medical Branch Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved

animal facility. Experiments were conducted with Institutional Animal Care and Use Committee approval with oversight from staff veterinarians.

Bone marrow-derived dendritic cells

Femurs from one B6 or one Balb/c mouse were harvested. Tissue was removed from bone with the aid of a scalpel, and the ends of the femurs were carefully cut away. A small 27-guage needle and 5 mL syringe were used to flush bone marrow from each femur in approximately 4 mL Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich) containing 5% new born calf serum and 1% penicillin/streptomycin. Bone marrow was pushed through a mesh screen to create a single-cell suspension. Cells were washed once in HBSS, and then treated with 0.5 mL Red Cell Lysis Buffer (Sigma-Aldrich) for 5 minutes at ambient temperature. Cells were washed twice in HBSS and counted in trypan blue. Cells were resuspended at 2×10^6 cells/mL (for B6 mice) or at 3×10^6 cells/mL (for Balb/C mice) in DC media: RPMI containing 10% fetal calf serum, 1% sodium pyruvate, 1% penicillin/streptomycin, 10 U or 20 ng/mL recombinant mouse IL-4 (Pharmingen), and 400 U or 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D Systems, Minneapolis, MN). Previous studies have demonstrated that in the presence of IL-4, which inhibits monocyte development, the addition of GM-CSF supports the development of large aggregates of proliferating DCs (108). 10 mL of resuspended cells were plated to each 100 mm culture dish. Cells were incubated at 37°C, 5% CO_2, for 10 days. On day 3 of incubation, an additional 10 mL fresh DC media were added to each culture dish. On days 6 and 8 of incubation, 10 mL of

supernatant were removed from each culture dish, centrifuged, pelleted cells were resuspended in fresh DC media, and 10 mL fresh DC media were added back to the original culture dishes.

Phenotype of cultured bone marrow dendritic cells

On day 10 after culture in the presence of IL-4 and GM-CSF, cells were harvested and stained with fluorochrome-labeled antibodies against CD11b, CD11c, CD3, B220, and Ly6c, and examined by flow cytometry. Cultured cells were found to be 92.6% $CD11c^{+}CD11b^{+}$, and 94.0% $CD11c^{+}B220^{-}Ly\text{-}6c^{-}$. Similar results were obtained when culturing bone marrow harvested from Balb/c mice. This phenotype is consistent with other studies examining the submucosal DC population involved in the activation of T_H1-type responses against HSV-2 (295).

Magnetic purification of $CD11c^{+}$ dendritic cells

$CD11c^{+}$ dendritic cells were purified by positive selection using a Magnetic Antibody Cell Separation (MACS) $CD11c^{+}$ Isolation Kit (Miltenyi). $CD11c^{+}$ cells were washed and counted. Purity of the $CD11c^{+}$ cells population was analyzed by flow cytometry upon completion of the study. The magnetic separation was found to increase the yield of $CD11c^{+}CD11b^{+}$ DC from 92.6% pre-separation to 93.5% post-separation. Because this increase in purity was not significant, magnetic purification of DCs after culture was discontinued.

In vitro stimulation of dendritic cells with TLR ligands

On day 10 of dendritic cell culture from bone marrow, adherent dendritic cells were scraped from Petri dishes. Both adherent and non-adherent DCs were washed, counted, and resuspended at 2×10^6 cells/mL in RPMI containing 10% fetal calf serum, 1% sodium pyruvate, 1% penicillin/streptomycin. 50 µL (1×10^5) cells were then plated to each of 168 wells of 96-well U-bottom plates. Four dilutions of each TLR ligand were prepared in RPMI containing 10% fetal calf serum, 1% sodium pyruvate, 1% penicillin/streptomycin so that final ligand concentrations were as follows: PIC: 100, 50, 25, or 10 µg/mL (Sigma-Aldrich); CpG 1826: 20, 10, 5, or 1 µg/mL (InvivoGen, San Diego, CA); LPS: 5, 1, 0.5, or 0.1 µg/mL (Sigma-Aldrich). 50 µL of each ligand and concentration were added to appropriate wells. Experiments also included DCs that received an additional 50 µL media alone, or that were stimulated with UV-inactivated HSV-2 stain 186 antigen at 5 MOI. All samples were incubated at 37°C, 5% CO_2, over seven days. A portion of the samples for each condition (each ligand at each concentration) was harvested at 24 hours, 72 hours, and on day 7 post-stimulation. Samples were spun at 1300 rpm for 8 minutes. Supernatants were frozen at -20 °C in multiple aliquots per condition, in order to reduce added freeze-thaw cycles while conducting ELISA and Bio-Plex assays. Cells were resuspended in FACS media (RPMI containing 10% new born calf serum and sodium azide) for flow cytometry.

Secretion of type I interferons by DCs

Secretion of type I interferons by DCs cultured in the presence of TLR ligands was examined by plating culture supernatants on VeriKine™ Mouse Interferon Alpha and Mouse Interferon Beta Kits (PBL Interferon Source, Piscataway, NJ). Briefly, after sample incubation on the pre-coated plates, plates were developed by sequential addition of antibody, anti-secondary antibody conjugated to horse radish peroxidase, and finally the substrate tetramethylbenzidine. Absorbance at 450 nm was determined on a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA) and compared to standard values over the linear portion of the curve. Antibody concentrations were calculated using Softmax Software (Molecular Devices). The IFN-α kit has a range of 12.5 – 500 pg/mL, while the IFN-β kit has a range of 15.6 – 1000 pg/mL.

Secretion of cytokines and chemokines

DC-TLR ligand culture supernatants or DC-TLR + $CD4^+$ T cell co-culture supernatants were tested for the presence of cytokines and chemokines using the Bio-Plex Mouse Cytokine 23-plex Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Briefly, colored beads coated with different antigens were incubated with standards, controls, and sample supernatants. This was followed by the sequential addition of detection antibody and streptavidin-PE. Data were acquired on a BioPlex 200 and analyzed with Bio-Plex Manager Software version 5.0 (Bio-Rad).

Upregulation of costimulatory molecules by DCs

TLR-stimulated DCs were stained with fluorochrome-labeled antibodies against CD11c, CD80, CD40, or MHC-II (Pharmingen, San Diego, CA). Data were acquired on a Becton Dickson FACSCanto (BD Biosciences, San Diego, CA) at the University of Texas Medical Branch Flow Cytometry Core Facility, and analyzed with FlowJo software (Treestar, Inc., Ashland, OR). The number of $CD11c^+$ cells upregulating CD80, CD40, or MHC-II was determined by FlowJo and is presented as number of double-positive cells per 10^6 total cells.

Dendritic cell - $CD4^+$ T cell co-culture

On day 10 of DC culture (see above), cells were harvested from Petri dishes, washed, counted, and resuspended at 10^6 cells/mL in RPMI containing 10% fetal calf serum, 1% sodium pyruvate, 1% penicillin/streptomycin (no IL-4 or GM-CSF). DCs were plated in Petri dishes with TLR ligand to yield a final concentration 50 μg/mL PIC, 5 μg/mL CpG, 0.5 μg/mL LPS, or media alone. These concentrations of TLR ligands were determined in previous experiments to induce the greatest response, and are from here on referred to as 'the optimal concentrations' of the TLR ligands. Cultures were incubated overnight at 37 °C.

TLR-stimulated DCs were harvested, washed, and pulsed with 10 μM OT-II peptide, irrelevant peptide, or media alone for 2h at 37 °C. DCs were washed, counted, and resuspended at 2×10^5 DC/mL in RPMI containing 10% fetal calf serum, 1% sodium pyruvate, 1% penicillin/streptomycin.

$CD4^+$ T cells were purified from DO.11 mice by negative selection using a Magnetic Antibody Cell Separation (MACS) $CD4^+$ T cells Isolation Kit (Miltenyi). Purity of the $CD4^+$ T cells population was analyzed by flow cytometry upon completion of the study. $CD4^+$ T cells were resuspended at 2×10^6 cells/mL in RPMI containing 10% fetal calf serum, 1% sodium pyruvate, 1% penicillin/streptomycin.

2.5×10^6 $CD4^+$ T cells were plated with 1.25×10^5 DCs in 6-well plates. TLR ligands were added back to the appropriate cultures to yield the optimal final concentrations. Cultures were incubated over 4 days at 37 °C.

A schematic drawing demonstrating the DC—$CD4^+$ T cell co-culture can be found in Figure 17.

Cytokine secretion by $CD4^+$ T cells (*in vitro*)

On day 4 of DC-$CD4^+$ T cell co-culture, $CD4^+$ T cells were harvested and restimulated with PMA/Ionomycin for 2h at 37 °C. Monensin (Pharmingen) was then added to the cultures for an additional 4h at 37 °C. $CD4^+$ T cells were then washed, and stained with fluorochrome-labeled antibodies against CD4 and the DO.11 T cell receptor (KJ1-26) for 30 min at 4 °C. Samples were washed and resuspended in Cytofix/Cytoperm (Pharmingen) for 20 min at 4 °C. Samples were washed twice with PermWash (Pharmingen) and stained intracellularly for IFN-γ, TNF-α, IL-2, IL-4, Il-5, IL-17, or granzyme B.

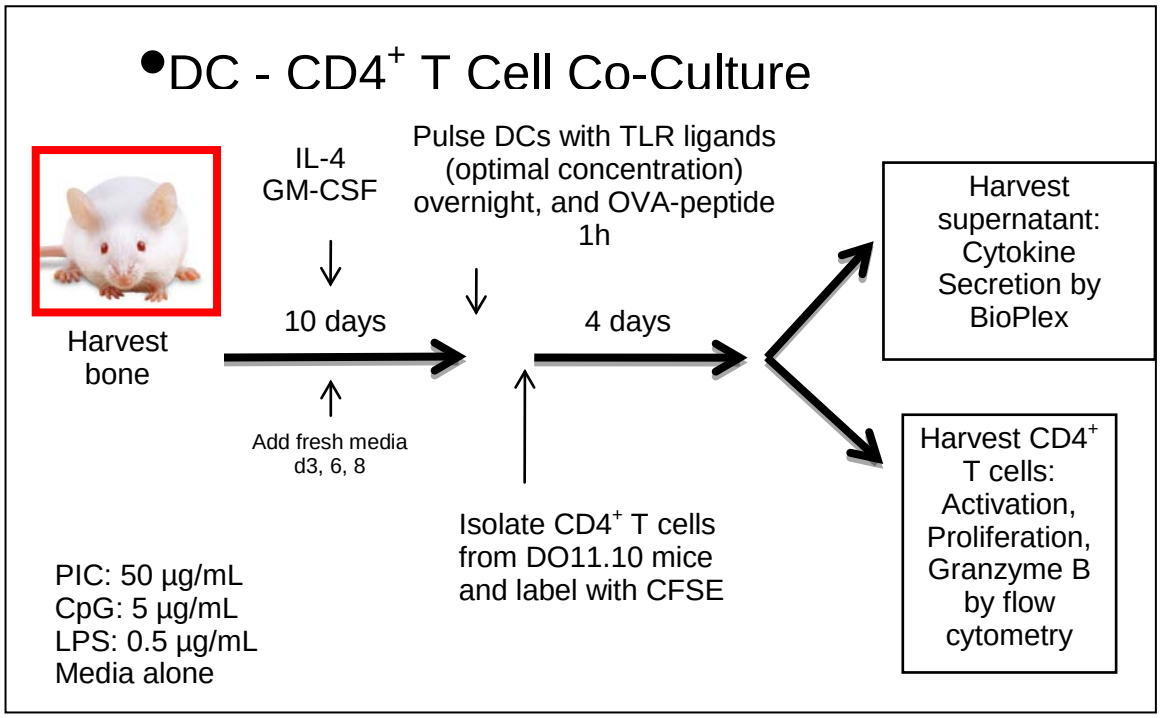

FIG. 17. Schematic representation of the method for DC—$CD4^+$ T cell co-culture. Bone marrow was harvested from the femurs of one B6 or Balb/c mouse and cultured 10 days in the presence of IL-4 and GM-CSF. On day 10 of DC culture, cells were harvested from Petri dishes, washed, and plated in Petri dishes with TLR ligand to yield a final concentration 50 µg/mL PIC, 5 µg/mL CpG, 0.5 µg/mL LPS, or media alone. Cultures were incubated overnight at 37 °C, then harvested, washed, and pulsed with 10 µM OT-II peptide, irrelevant peptide, or media alone for 2h at 37 °C. $CD4^+$ T cells were purified from DO.11 mice by negative selection using a Magnetic Antibody Cell Separation (MACS) $CD4^+$ T cells Isolation Kit (Miltenyi). 2.5×10^6 $CD4^+$ T cells were plated with 1.25×10^5 DCs in 6-well plates. TLR ligands were added back to the appropriate cultures to yield the optimal final concentrations (as mentioned above). Cultures were incubated over 4 days at 37 °C.

Activation of $CD4^+$ T cells cultured in the presence of TLR ligand-stimulated DCs

On day 4 of DC-$CD4^+$ T cell co-culture, $CD4^+$ T cells were stained with fluorochrome-labeled antibodies against CD4 and KJ1-26, and CD25, C44, CD62L, or CD69 and expression was examined by flow cytometry.

In vitro CCR staining

On day 4 of DC-$CD4^+$ T cell co-culture, $CD4^+$ T cells were harvested and labeled with fluorochrome-labeled antibodies against CD4 and KJ1-26, and CCR5, CCR4, CCR3, CXCR3, CD197, α4β7, CD18, or CD11a. Alternatively, $CD4^+$ T cells were incubated for 30 min at 37 °C in the presence of 5 μg/mL P selectin ligand or E selectin ligand (R&D). Cells were then washed and labeled with goat anti-mouse anti-$Fc(ab)^2$ PE (Southern Biotec) for 30 min at 37 °C.

In vitro proliferation of $CD4^+$ T cells

Prior to addition to the previously established DC-$CD4^+$ T cell co-culture, $CD4^+$ T cells isolated from DO.11 spleens were labeled with 20 μM CFSE. Cells were washed three times before addition to the DCs in culture. On day 4 of DC-$CD4^+$ T cell co-culture, $CD4^+$ T cells were harvested and resuspended in 1% formaldehyde for examination by flow cytometry.

Immunization of Balb/c mice with DC vaccine

Bone marrow-derived DCs were cultured as described above. Ten days after initial DC culture, DCs were harvested from Petri dishes, washed in HBSS, counted, and resuspended at 10^6 DC/mL in DC media (without IL-4 and GM-CSF). DCs were treated overnight with the previously determined optimal concentrations of TLR ligands: 50 μg/mL PIC, 0.5 μg/mL LPS, or media alone. Also at this time, recipient Balb/C mice

received 10^5 $CD4^+$ T cells i.v. These cells were magnetically isolated (negative selection, Miltenyi) from DO.11 mice (MHC-II T cell receptor transgenic mice specific for the ovalbumin peptide $OVA_{323-339}$). The next day, TLR-stimulated DCs were harvested from Petri dishes, pulsed with OVA peptide for one hour, washed in HBSS, and counted. 2×10^5 DCs were injected i.p. into the CD4-recipient mice. Seven days later, the CD4-recipient mice received a second immunization with DCs cultures as described above. This day of second immunization is referred to in these experiments as "Day 0."

A schematic drawing representing this immunization scheme can be found in Figure 18.

In vivo timecourse of the specific $CD4^+$ T cell response after immunization

Spleens, iliac lymph nodes, and inguinal lymph nodes were harvested from mice on days 0, 6, 9, and 11 after the second immunization with OVA-DCs or LPS-OVA-DCs (see immunization scheme for more detail). Lymphocytes were labeled with fluorochrome-labeled antibodies against CD3, CD4, CD8, and KJ1-26. The peak day of $CD3^+CD4^+KJ1\text{-}26^+$ cells in each of the tissues appeared to be d6. In a second experiment performed similarly, the peak day of $CD3^+CD4^+KJ1\text{-}26^+$ cells in each of the tissues appeared to be d9. Therefore both day 6 and day 9 timepoints were utilized in further experiments.

Additionally, spleens, iliac lymph nodes, and inguinal lymph nodes were harvested from mice on days 0, 3, 6, and 9 after immunization with LPS-OVA-DCs or

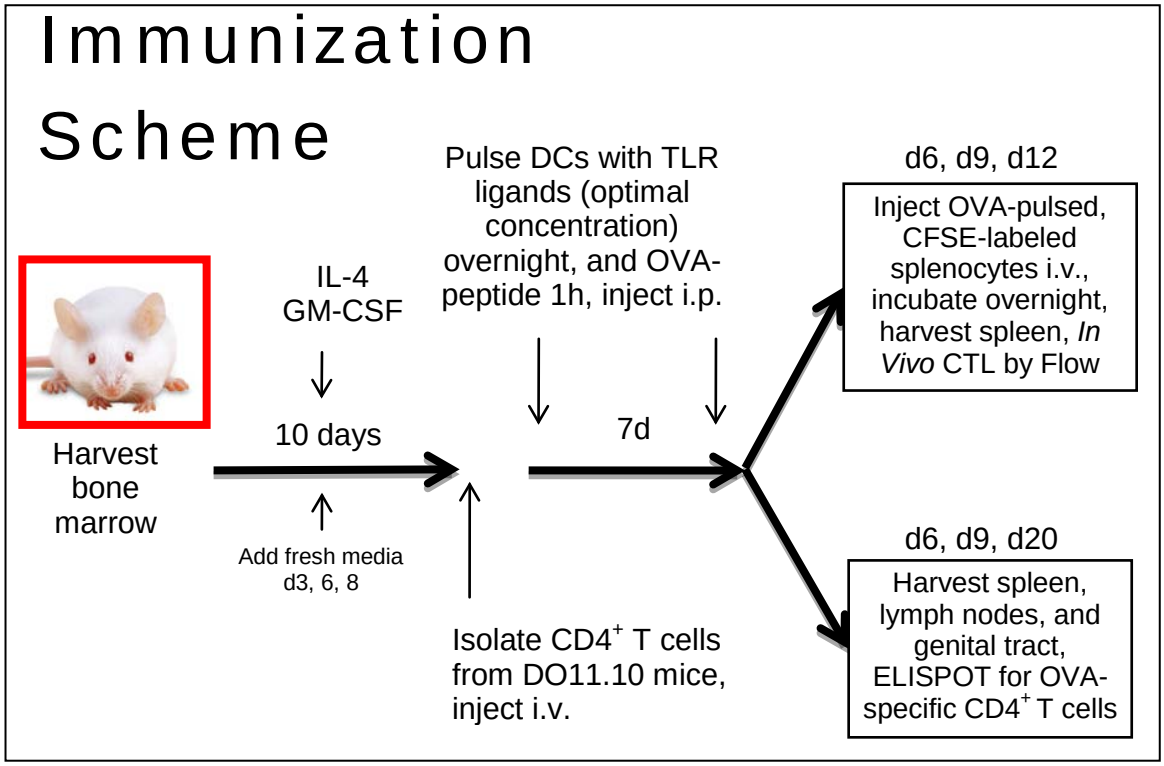

FIG. 18. Schematic representation of the DC immunization scheme. Bone marrow was harvested from the femurs of one B6 or Balb/c mouse and cultured 10 days in the presence of IL-4 and GM-CSF. Ten days after initial DC culture, DCs were harvested, washed, and treated overnight with the previously determined optimal concentrations of TLR ligands: 50 µg/mL PIC, 0.5 µg/mL LPS, or media alone. Also at this time, recipient Balb/C mice received 10^5 $CD4^+$ T cells (isolated form DO.11 mice) i.v. The next day, TLR-stimulated DCs were harvested from Petri dishes, pulsed with OT-II peptide for one hour. 2×10^5 DCs were injected i.p. into the CD4-recipient mice. Seven days later, the CD4-recipient mice received a second immunization with DCs cultures as described above. This day of second immunization is referred to in these experiments as "Day 0."

LPS-OVA-DCs plus 1.0 µg LPS and analyzed by flow cytometry for differences in the number of $CD3^+CD4^+KJ1\text{-}26^+$ cells induced by the two vaccine formulations.

Quantification of specific $CD4^+$ T cells after immunization with TLR ligand-stimulated DCs

ELISPOT analysis was performed similarly to the procedure described (187, 191). On days 6, 9, and 20 after the second immunization, spleen, iliac lymph nodes, and vaginal tracts were harvested from groups of 9 or 10 immunized Balb/c mice. Spleens and iliac lymph nodes were maintained as separate samples (except for naïve animals, in which 5 tissues were pooled), and single-cell suspensions were created by pushing tissues through mesh screens. Vaginal tracts were pooled within a group. Vaginal tracts were minced finely with scissors, and then incubated for 30 min at 37 °C in the presence of Blendzyme and DNase, before single-cell suspensions were prepared. Lymphocytes from all tissues were incubated in anti-IFN-γ, anti-IL-4, or anti-IL-17 antibody-coated nitrocellulose plates (Millipore Corporation, Billerica, MA) with 5×10^5 OT-II peptide-pulsed mitomycin C (Sigma-Aldrich)-treated feeder cells per well (191). Plates were incubated for 40h, and developed as previously described, by the sequential addition of corresponding biotinylated antibody (Pharmingen), streptavidin peroxidase (Sigma-Aldrich), and 3-amino-9-ethyl-carbazole plus sodium acetate (58, 187). Spot-forming cells (SFC) were quantified using an ImmunoSpot reader with ImmunoCapture software (Version 6.0) and analyzed with ImmunoSpot software (Version 4.0) from Cellular Technology Ltd. (Cleveland, OH).

In vivo cytotoxic T lymphocyte (CTL) assay

On day 6, 9, or 11 after the second DC immunization, mice received 10^7 cells labeled with 2 μM CFSE and 10^7 cells labeled with 20 μM CFSE and pulsed with 10 μM OT-II peptide i.v. Cells were incubated overnight (approximately 18 hours) *in vivo*. Spleens were harvested, and single-cell suspensions were created and spun over a Histopaque cushion. Lymphocytes were washed and resuspended in 1% formaldehyde for analysis by flow cytometry. Percent specific lysis was defined as (1 – ratio unimmunized control / ratio immunized) x 100, where the ratio is $\%CFSE_{low}/\%CFSE_{high}$ (55).

Statistical analysis

Differences among amounts of cytokines secreted by $CD4^+$ T cells or DCs in culture, or in numbers of OVA-specific $CD4^+$ T cells in various tissues, were examined by one-way ANOVA with the Bonferoni correction for multiple groups. Analyses were performed using GraphPad Prism Version 4.0 software (GraphPad Prism Software, San Diego, CA). *p* values of less than 0.05 were considered statistically significant.

Results

Herpes Simplex Virus type 2 (HSV-2) infects an estimated 20-25% of the adult population in the United States, with approximately 500,000 new cases reported annually. Importantly, around 80% of infected individuals do not experience the symptoms and

lesions typically associated with genital herpes (61, 137, 181). It is estimated that 70% of heterosexual transmission occurs during periods of asymptomatic shedding, highlighting the need for an effective vaccine to reduce economic and psychosocial burden (61, 137, 181). It has been suggested that previous vaccine candidates against HSV-2 have not been completely protective in all groups due to the vaccine's inability to stimulate an appropriate T cell response (42, 254). Dendritic cells (DCs) activated through Toll-like receptors (TLRs) have been shown to upregulate costimulatory molecules necessary for stimulating a robust T cell response (16). Further, the use of adjuvants, utilizing TLRs, supplied in conjunction with antigens for T cell activation has been shown to induce significantly greater numbers of activated and proliferating T cells within the draining lymph node (206). Therefore, the use of TLR ligands as adjuvants may allow for the manipulation and enhancement of the ensuing immune response to a less immunogenic vaccine. Here, we examined the potential of bone marrow-derived DCs activated through different TLRs to establish a strong T_H1-type response: a response that has been previously shown to be important for limiting genital HSV-2 disease.

Activation of TLR3 upon treatment with PIC (or through its natural counterpart viral dsDNA) results in the production of antiviral IFN-α and –β and T_H1-type cytokines (10, 64, 93, 94), and promotes DC expression of high levels of MHC and costimulatory molecules (235), thus enhancing proliferation and survival of T cells (172), and inducing T cell production of IFN-γ. Activation through TLR9 (CpG) results in the activation of B and T lymphocytes, monocytes, macrophages, and DCs, which combined induces a cytokine milieu characterized by a dominant production of IFN-γ and IL-12 that guides differentiation toward a T_H1-type response (15, 80, 130, 227). DCs recruited from the

blood to the vaginal mucosa have been found to be critical for protection against lethal challenge with HSV-2, an effect that is mediated through the recognition of HSV-2 CpG oligodeoxynucleotides via TLR9 on the DCs (242). PIC (TLR3) and CpG (TLR9) have been shown to enhance the survival of activated $CD4^+$ T cells, but this is not the case for LPS (TLR4) (70). Though LPS has been shown to induce production of IL-12, which is thought to be important in supporting the IFN-γ production by T cells and thus development of a dominant T_H1-type response that could be important in the defense against HSV (206, 268), induction of an immune response through TLR4 has not been proven effective in an HSV-2 vaginal challenge (73).

Due to the induction of a T_H1-type cytokine milieu and the upregulation of MHC-II molecules on DCs, activation through TLR3 and/or TLR9 may prove powerful tools for enhancing the $CD4^+$ T cell response generated by vaccination against HSV. Therefore, PIC and CpG were examined in the studies presented here. LPS treatment has also been shown to enhance the development of the T_H1-type response thought critical against HSV, though induction of an activated $CD4^+$ T cell response through TLR4 activation has not been successful. Thus, LPS was chosen for the experiments presented here, as a ligand thought to produce the correct type of innate response, but which had not been previously successful in developing the desired adaptive immune response.

To summarize: DCs activated through TLRs have previously been shown to augment the activation and proliferation of T cells within the draining lymph nodes (206). Activation through TLR3 (via PIC) or TLR9 (via CpG) allows for DC enhancement of the T_H1-type T cell response (10, 15, 64, 80, 93, 94, 130, 227). While activation through TLR4 (via LPS) has also been shown to induce the production of a cytokine milieu that

would be expected to drive the differentiation of $CD4^+$ T cells toward the desired T_H1-type response, an effective anti-HSV $CD4^+$ T cell response was not generated (73). Therefore, we hypothesized that DCs treated with PIC or CpG, but not LPS, would result in the development of a T_H1 phenotype and enhanced lytic activity of the $CD4^+$ T cells. In the studies described here, bone-marrow derived DCs were treated with TLR ligands. The activation and expression of costimulatory molecules were examined by flow cytometry. The cytokines and chemokines secreted by the DCs in response to TLR treatment were examined by Bio-Plex bead array. The ability of TLR-activated DCs to drive the $CD4^+$ T cell response toward the desired T_H1 phenotype was then tested. TLR-activated DCs were then combined with $CD4^+$ T cells *in vitro,* and $CD4^+$ T cells were examined for enhanced activation and proliferation, expression of markers important for trafficking of the T cells to genital and neural tissues (important sites of immune responses in HSV infections), differentiation of the T_H1 lineage, and enhanced lytic capability. These studies were then extended to examine the ability of TLR ligand-influenced DCs, when used in immunization *in vivo*, to enhance the development of the previously defined appropriate $CD4^+$ T cell response.

Phenotype of cultured bone marrow-derived DCs

The use of IL-4 and GM-CSF has previously been shown to inhibit monocyte development while concurrently enhancing the development of large aggregates of proliferating DCs (108). Flow cytometry was utilized to examine the phenotype of bone marrow-derived cells after 10 days in culture in the presence of IL-4 and GM-CSF. The

phenotype of the cultured cells was found to be 92.6% $CD11c^{+}CD11b^{+}$, and 94.0% $CD11c^{+}B220^{-}Ly\text{-}6c^{-}$ (Fig. 19). This initial culture was started from the bone marrow of B6 mice, though similar results were obtained when culturing bone marrow harvested from Balb/c mice.

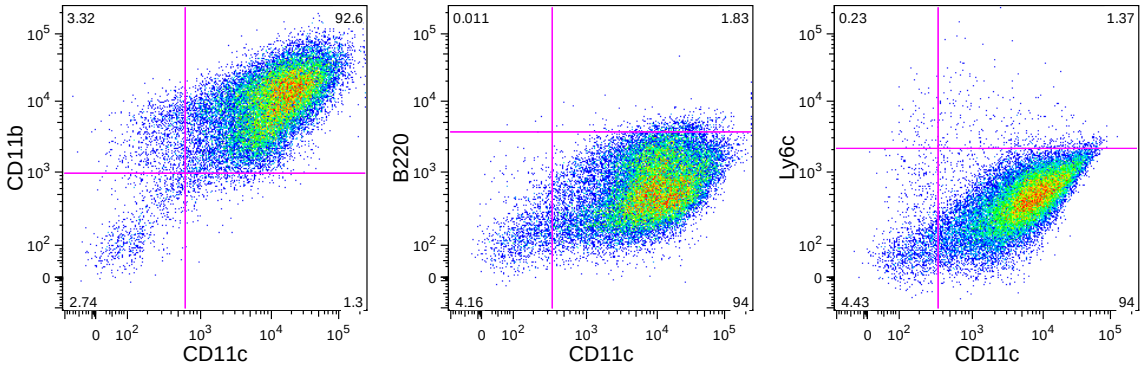

FIG. 19. Phenotype of cultured bone marrow dendritic cells. Bone marrow was harvested from the femurs of a B6 mouse. Cells were cultured at a concentration of 2 x 10^6 cells/mL in media containing 20 ng/mL IL-4 and 20 ng/mL GM-CSF. On day 10 after culture, cells were harvested and stained with fluorochrome-labeled antibodies against CD11b, CD11c, CD3, B220, and Ly6c. Cultured cells were found to be 92.6% $CD11c^{+}CD11b^{+}$, and 94.0% $CD11c^{+}B220^{-}Ly\text{-}6c^{-}$. Similar results were obtained when culturing bone marrow harvested from Balb/c mice.

Bone marrow DCs are mature and activated upon culture with TLR ligands

Dendritic cells activated via TLRs have previously been shown to enhance the immune response and further the priming of T cells to a wide range of pathogens through their upregulation of costimulatory molecules. In order to examine the differences among TLR ligands in influencing the mature, activated phenotype, and therefore the T cell response-enhancing ability of DCs, flow cytometry was utilized to examine the upregulation of costimulatory molecules on the surface of the cultures DCs. After 10

days in culture in the presence of IL-4 and GM-CSF, DCs were further cultured in the presence of four different concentrations each of PIC, CpG, or LPS, or media alone. The activation of the DCs was examined by flow cytometry at 24h, 72h, and 7d after stimulation with TLR ligands. In general, the mean fluorescence intensity (MFI) of the expression of both CD80 and MHC-II peaked at 24h after stimulation, and decreased through day7 (Fig. 20). This was true for all ligands, as well as media. The MFI was lowest for the upregulation of CD40 at 24h after stimulation, and increasing through day 7 (Fig. 20). This pattern also occurred for all ligands, including media. The activated phenotype noted within the DC samples stimulated with media only suggests the application of IL-4 and GM-CSF in culture provides an activation stimulus which was not due to the TLR ligand enhancement. The mean fluorescence intensities for each of the costimulatory molecule expressed by DCs in response to the TLR ligands can also be found in TABLE 1.

DCs secrete a wide range of cytokines and chemokines upon *in vitro* stimulation with TLR ligands

In addition to the enhancement of the immune response directed by DCs through their upregulation of costimulatory molecules, DCs are also important in establishing the type of cytokine milieu present during T cell priming, and thus directing the immune response. Differences in the DC-produced cytokine milieu induced by the application of the TLR ligands were examined by Bio-Plex bead array. After 10 days in culture in the presence

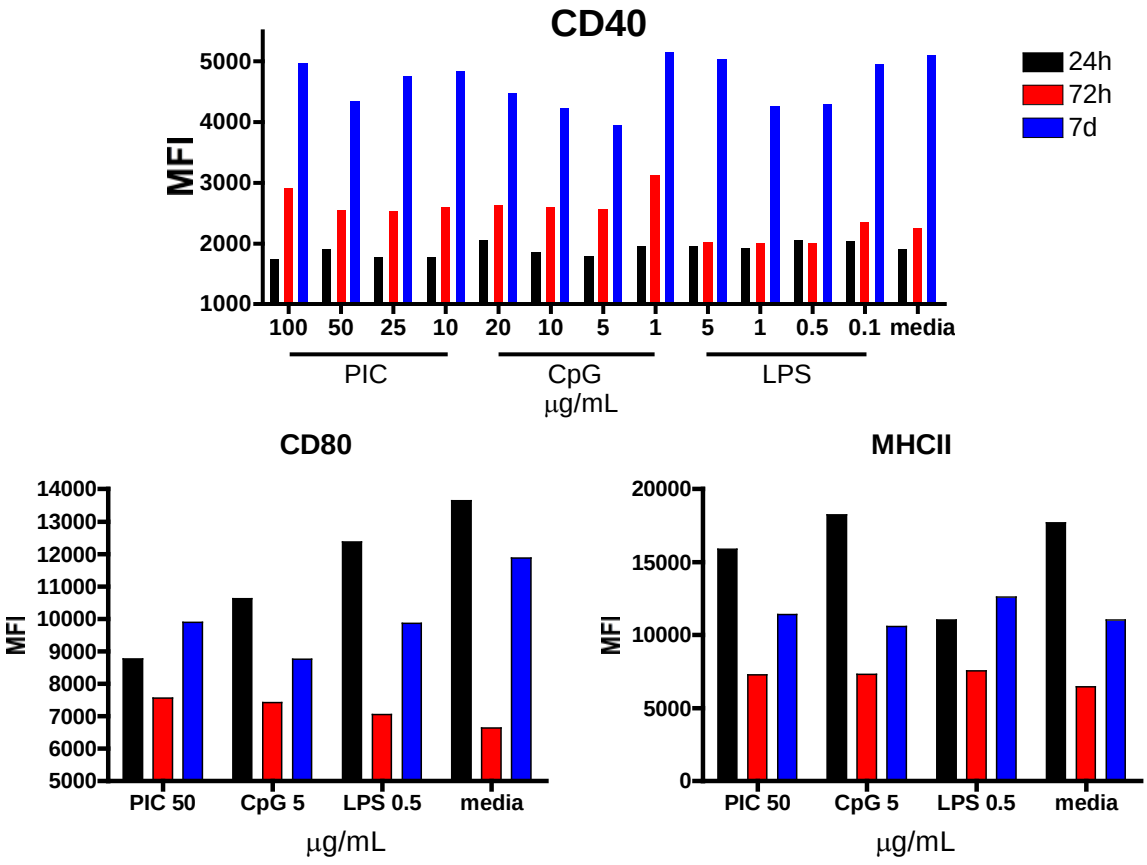

FIG. 20. Upregulation of costimulatory molecules by DCs. Bone marrow was harvested from the femurs of a B6 mouse. Cells were cultured at a concentration of 2 x 10^6 cells/mL in media containing 20 ng/mL IL-4 and 20 ng/mL GM-CSF. On day 10 after culture, cells were harvested and 10^5 DCs were cultured in the presence of PIC, CpG, LPS, or media alone. TLR-stimulated DCs were harvested at 24h, 72h, and 7d and stained with fluorochrome-labeled antibodies against CD11c and CD80, CD40, or MHC-II. The number of $CD11c^+$ cells upregulating CD80, CD40, or MHC-II was determined by FlowJo and is presented as number of double-positive cells per 10^6 total cells.

TABLE 1. Upregulation of costimulatory molecules by DCs.

	PIC			CpG			LPS		
	24h	72h	7d	24h	72h	7d	24h	72h	7d
CD40	1900	2540	4339	1784	2566	3948	2043	1999	4291
	1.00	1.13	0.85	0.94	1.14	0.77	1.08	0.89	0.84
CD80	8768	7579	9902	10629	7426	8766	12387	7061	10187
	0.64	1.00	0.83	0.78	0.98	0.74	0.91	0.93	0.86
MHC-II	15884	7277	12621	18233	7303	10607	19078	7546	11412
	0.90	1.13	1.14	1.03	1.13	0.96	1.08	1.17	1.03

Bone marrow was harvested from the femurs of a B6 mouse. Cells were cultured at a concentration of 2 x 10^6 cells/mL in media containing 20 ng/mL IL-4 and 20 ng/mL GM-CSF. On day 10 after culture, cells were harvested and 10^5 DCs were cultured in the presence of PIC, CpG, LPS, or media alone. TLR-stimulated DCs were harvested at 24h, 72h, and 7d and stained with fluorochrome-labeled antibodies against CD11c and CD80, CD40, or MHC-II. The number of $CD11c^+$ cells upregulating CD80, CD40, or MHC-II was determined by FlowJo and is presented as number of double-positive cells per 10^6 total cells. The numbers in the non-shaded rows in the table represent the mean fluorescence intensities (MFI) of each costimulatory molecule, while the shaded rows represent the 'fold change above media controls' defined as (MFI of costimulatory molecule expressed by DCs in the TLR-stimulated culture / MFI of costimulatory molecule expressed by DCs in the media-stimulated culture, for the same timepoint).

of IL-4 and GM-CSF, DCs were further cultured in the presence of four different concentrations each of PIC, CpG, or LPS, or media alone, in order to establish a dose-response curve for each ligand. The range tested for each ligand was based on concentrations used throughout the literature. It should be noted that the range tested for each TLR ligand allowed for the determination of an optimal ligand concentration that was consistent with what was previously utilized within the literature. Therefore, the following concentration for each ligand was utilized in further experiments: PIC = 50

μg/mL, CpG = 5 μg/mL, LPS = 0.5 μg/mL. These are also the concentrations utilized to generate the data presented in Figure 21 and Tables 2 – 4.

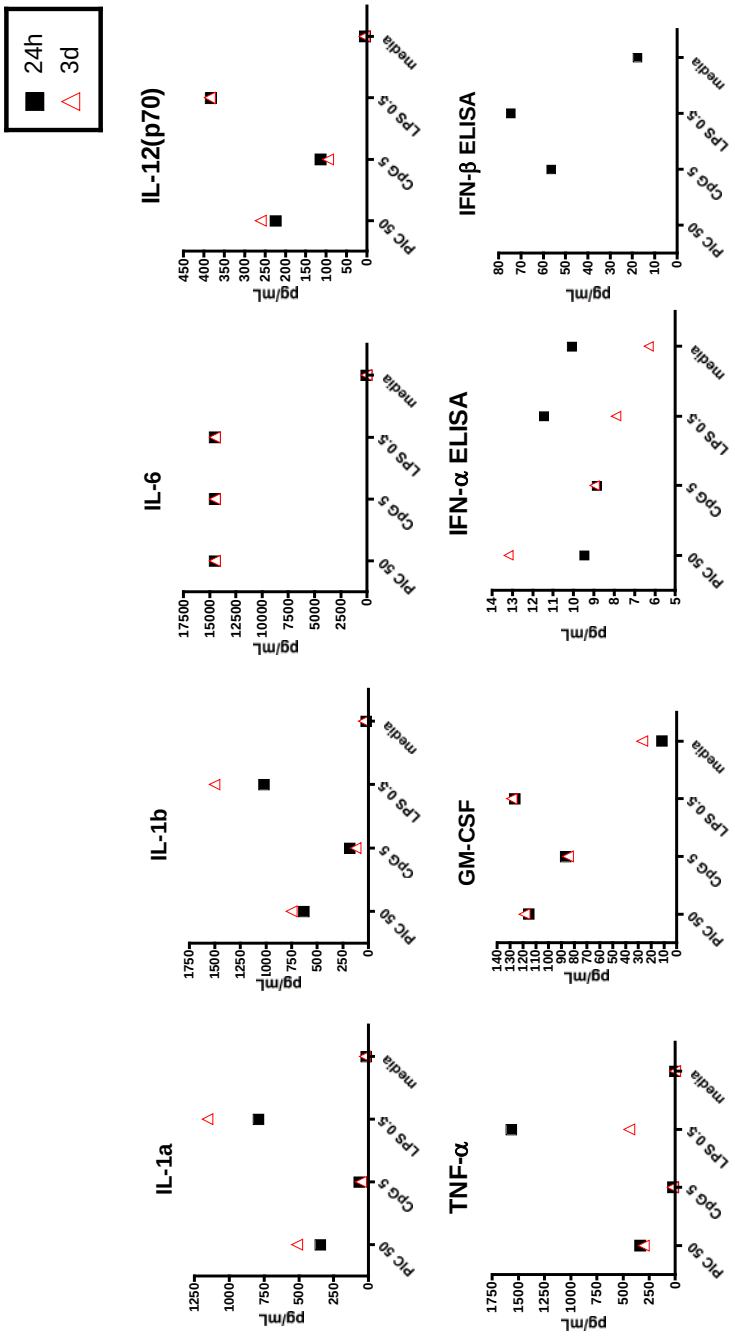

FIG. 21. Secretion of cytokines and chemokines by TLR ligand-stimulated DCs. Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing 20 ng/mL IL-4 and 20 ng/mL GM-CSF. On day 10 after culture, cells were harvested and 10^5 DCs were cultured in the presence of PIC (50 μg/mL), CpG (5 μg/mL), LPS (0.5 μg/mL), or media alone. DC-TLR ligand culture supernatants were harvested after 24 and 72h and tested for the presence of cytokines and chemokines using the Bio-Plex Mouse Cytokine 23-plex Assay (Bio-Rad). Briefly, colored beads coated with different antigens were incubated with standards, controls, and sample supernatants. This was followed by the sequential addition of detection antibody and streptavidin-PE. Data were acquired on a Bio-Plex 200 and analyzed with Bio-Plex Manager Software version 5.0 (Bio-Rad).

TABLE 2. Proinflammatory cytokines secreted by DCs in response to stimulation with TLR ligands.

	PIC			CpG			LPS		
Pro-Inflammatory	24h	72h	7d	24h	72h	7d	24h	72h	7d
IL-1α	346.31	512.9	434.81	64	53.96	49.7	791.38	1156.52	1311.11
	20.91	20.39	21.21	3.86	2.15	2.42	47.79	45.98	63.96
IL-1β	629.88	749.92	527.25	182.48	125.27	131.58	1019	1502.18	1174.43
	29.97	17.44	13.55	8.68	2.91	3.38	48.48	34.93	30.19
IL-6	14531.93	14531.93	14531.93	14531.93	14531.93	14531.93	14531.93	14531.93	14531.93
	471.97	333.53	339.61	471.97	333.53	339.61	471.97	333.53	339.61
IL-12(p40)	20865.46	21741.66	21741.66	21741.66	21741.66	21741.66	21741.66	21741.66	21741.66
	18.83	9.41	8.35	19.62	9.41	8.35	19.62	9.41	8.35
IL-12(p70)	223.5	260.98	187	114.28	95.69	59.89	383.4	385.84	354.95
	51.38	45.63	11.94	26.27	16.73	3.82	88.14	67.45	22.67
IL-17	35.29	31.68	22.85	21.57	18.78	17.77	38.12	41.73	29.16
	18.48	9.99	5.8	11.29	5.92	4.51	19.96	13.16	7.4
IFN-γ	57.63	52.1	37.9	41.94	35.56	29.93	61.46	72.24	54.32
	19.08	9.63	5.48	13.89	6.57	4.33	20.35	13.35	7.86
TNF-α	336.5	302.17	76.93	22	27.39	22.67	1564.12	439.52	114.36
	119.33	81.01	13.81	7.8	7.34	4.07	554.65	117.83	20.53
GM-CSF	115.28	118.76	93.16	86.58	84.86	79.82	126.28	128.71	115.72
	9.94	4.42	3.71	7.46	3.16	3.17	10.89	4.79	4.06
IFN-α	12.5	13.19	12.5	12.5	12.5	12.5	12.5	12.5	12.5
	1	1.06	1	1	1	1	1	1	1

Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing IL-4 and GM-CSF. On day 10 after culture, cells were harvested and 10^5 DCs were cultured in the presence of PIC (50 μg/mL), CpG (5 μg/mL), LPS (0.5 μg/mL), or media alone. DC-TLR ligand culture supernatants were harvested after 24h, 72h, and 7d and tested for the presence of cytokines and chemokines using the Bio-Plex Mouse Cytokine 23-plex Assay (Bio-Rad). Briefly, colored beads coated with different antigens were incubated with standards, controls, and sample supernatants. This was followed by the sequential addition of detection antibody and streptavidin-PE. Data were acquired on a Bio-Plex 200 and analyzed with Bio-Plex Manager Software version 5.0 (Bio-Rad). The numbers in the non-shaded rows in the table represent the concentrations in pg/mL of each proinflammatory cytokine, while the shaded rows represent the 'fold change above media controls' defined as (concentration of cytokine secreted by DCs in the TLR-stimulated culture / concentration of the same cytokine secreted by DCs in the media-stimulated culture, for the same timepoint).

TABLE 3. Anti-inflammatory cytokines secreted by DCs in response to stimulation with TLR ligands.

	PIC			CpG			LPS		
Anti-Inflammatory	24h	72h	7d	24h	72h	7d	24h	72h	7d
IL-4	20.7	15.01	9.01	11.09	5.96	5.96	24.63	17.9	12.74
	2.25	2.52	1.51	1.23	1	1	2.73	3	2.14
IL-5	27.89	29.56	24.94	19.6	21.03	18.48	35.68	31.69	33.23
	2.53	2.68	2.26	1.78	1.91	1.68	3.23	2.87	3.01
IL-10	30.91	34.4	25.09	29.13	110.75	113.16	65.29	67.66	35.05
	23.6	5.22	6.35	22.24	16.81	28.65	49.84	10.27	8.87
IL-13	273.59	222.51	171.49	147.1	171.49	120.52	333.58	295.8	242.49
	5.5	4.47	3.45	2.96	3.45	2.42	6.71	5.95	4.87
G-CSF	2665.32	3446.7	2680.69	1808.87	1705.02	639.81	3835.5	3537.32	3742.41
	353.49	385.11	286.7	239.9	190.51	68.43	508.69	395.23	400.26

Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing IL-4 and GM-CSF. On day 10 after culture, cells were harvested and 10^5 DCs were cultured in the presence of PIC (50 μg/mL), CpG (5 μg/mL), LPS (0.5 μg/mL), or media alone. DC-TLR ligand culture supernatants were harvested after 24h, 72h, and 7d and tested for the presence of cytokines and chemokines using the Bio-Plex Mouse Cytokine 23-plex Assay (Bio-Rad). Briefly, colored beads coated with different antigens were incubated with standards, controls, and sample supernatants. This was followed by the sequential addition of detection antibody and streptavidin-PE. Data were acquired on a Bio-Plex 200 and analyzed with Bio-Plex Manager Software version 5.0 (Bio-Rad). The numbers in the non-shaded rows in the table represent the concentrations in pg/mL of each anti-inflammatory cytokine, while the shaded rows represent the 'fold change above media controls' defined as (concentration of cytokine secreted by DCs in the TLR-stimulated culture / concentration of the same cytokine secreted by DCs in the media-stimulated culture, for the same timepoint).

TABLE 4. Chemokines secreted by DCs in response to stimulation with TLR ligands.

	PIC			CpG			LPS		
Chemokines	24h	72h	7d	24h	72h	7d	24h	72h	7d
MIP-1α	9541.41	815.77	252.41	1399.03	254.73	179.01	9541.41	9541.41	500.4
	144.54	4.51	2.28	21.19	1.41	1.62	144.54	52.77	4.52
MIP-1β	23446.22	1215.6	55.07	8820.84	522.16	151.57	23446.22	4336.58	82.97
	207.86	44.63	2.37	78.2	19.17	6.51	207.86	159.2	3.57
MCP-1	11880.4	20859.77	12761.69	3392.3	1585.93	1383.18	28204.07	49909.42	49904.42
	51.36	69.09	7.89	14.67	5.25	0.86	121.93	165.31	29.5
Eotaxin	1240.84	1145.06	866.81	847.71	828.53	618.07	1294.11	1422.67	1114.82
	6.14	5.66	4.29	4.19	4.1	3.06	6.4	7.03	5.51
KC	7689.08	11481.9	11481.9	6741.98	11481.9	10838.05	11481.9	11481.9	11481.9
	71.05	53.88	19.24	62.3	53.88	19.24	106.1	53.88	19.24
RANTES	3699.3	3699.3	3699.3	3699.3	1447.63	520.69	3699.3	3699.3	3699.3
	12.98	8.79	14.35	12.98	3.44	2.02	12.98	8.79	14.35

Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing IL-4 and GM-CSF. On day 10 after culture, cells were harvested and 10^5 DCs were cultured in the presence of PIC (50 μg/mL), CpG (5 μg/mL), LPS (0.5 μg/mL), or media alone. DC-TLR ligand culture supernatants were harvested after 24h, 72h, and 7d and tested for the presence of cytokines and chemokines using the Bio-Plex Mouse Cytokine 23-plex Assay (Bio-Rad). Briefly, colored beads coated with different antigens were incubated with standards, controls, and sample supernatants. This was followed by the sequential addition of detection antibody and streptavidin-PE. Data were acquired on a Bio-Plex 200 and analyzed with Bio-Plex Manager Software version 5.0 (Bio-Rad). The numbers in the non-shaded rows in the table represent the concentrations in pg/mL of each chemokine, while the shaded rows represent the 'fold change above media controls' defined as (concentration of cytokine secreted by DCs in the TLR-stimulated culture / concentration of the same cytokine secreted by DCs in the media-stimulated culture, for the same timepoint).

The cytokine and chemokine secretion by the DCs was examined by Bio-Plex at 24h, 72h, and 7d after stimulation with TLR ligands. In general, compared to DCs cultured in media alone, DCs cultured with PIC, CpG, or LPS greater amounts of a myriad of cytokines (Fig. 21). More specifically, the cytokines detected in the supernatants of cultures stimulated with the different TLR ligands did not seem to fall within the traditional definitions of the cytokine milieus required to drive a T_H1, T_H2, or T_H17 lineage. Rather, supernatants were found to contain cytokines that drive each of the T_H1 (IL-12, type I interferons), T_H2 (IL-4), and T_H17 (IL-6) (Fig. 21). Conversely, cytokines that are known inhibitors of each lineage, such as IL-4 for T_H1 or IFN-γ for T_H2, were also present (Fig. 21). These data are also represented as concentration (pg/mL) and as 'fold change above media' (concentration of cytokine present in TLR ligand-stimulated culture / concentration of cytokine present in media-only-stimulated culture, for each timepoint) in Tables 2 – 4.

More specifically, in considering the cytokines that may be important for the development of an appropriate anti-HSV $CD4^+$ T cell response, it was noted that LPS treatment induced the greatest amount of IL-12(p70) to be secreted by the DCs in these experiments (88.14 times the media control at 24h), followed by PIC (51.38 times), and with CpG inducing the least amount of IL-12(p70) to be secreted (26.27 times, Table 2). It has been previously observed that DCs induced to secrete IL-12(p70) can drive the differentiation of $CD4^+$ T cells toward the T_H1 lineage (168). IL-12 is also known to enhance production of IFN-γ, support the development of the T_H1 lineage, and together with IFN-γ to activate CTLs (16, 95, 298). Though the amounts of IFN-γ secreted by the DCs influenced by the three different TLR ligands tested in our experiments did not

appear vastly different, again, LPS treatment induced the greatest amount of IFN-γ to be secreted (20.35 times the media control at 24h), followed closely by PIC (19.08 times), and with CpG inducing the least amount of IFN-γ to be secreted (13.89 times, Table 2). Both IL-4 and IL-10 have been implicated in the development of a T_H2-type response (148, 279). The amounts of IL-4 secreted in response to the TLR ligands tested in our studies were not greatly enhanced over the amount of IL-4 secreted in the media controls (1.23 times the media control at 24h for CpG, 2.25 for PIC, or 2.73 for LPS, Table 3). The amounts of IL-10 secreted in these cultures were more markedly different (22.24 times the media control at 24h for CpG, 23.6 for PIC, or 49.84 for LPS, Table 2). IL-10, however, can also drive the development of T_{reg} (279). It should be noted that in these experiments, IL-6, which has been demonstrated to play a role in the development of a T_H17-type response, was secreted by DCs stimulated with PIC, CpG, or LPS in amounts that reached the maximum detected by the Bio-Plex assay at all timepoints examined (299). Also, the secretion of type I interferons, which can affect DC maturation, expression of MHC and costimulatory molecules (235), and enhance proliferation and survival of T cells (172) was not detected within our assays.

Chemokines are small host cell-secreted peptides that can be induced by infection, and play roles in the inflammatory response to infection and influence of the developing T cell response. In the studies presented here, the amount of macrophage inflammatory protein-1α (MIP-1α) secreted by $CD4^+$ T cells cultured in the presence of PIC- or LPS-stimulated DCs was greatly enhanced over media controls (144.54 times the media control for both PIC and LPS at 24h, Table 3). The amounts of regulated upon activation, normal T cell expressed and secreted (RANTES) secreted by $CD4^+$ T cells in

these cultures were found to reach the maximum detectable amount in cultures containing DCs stimulated with PIC, CpG, or LPS at 24h (12.98 times the media control Table 3). This amount remained the same throughout the course of the experiment for PIC- or LPS-influenced cultures, while the amounts in CpG-influenced cultures declined over time (3.44 times the media control at 72h, 2.02 at 7d, Table 2). MIP-1α and RANTES have previously been shown to influence the development of a T_H1-type $CD4^+$ T cell response (241, 247). Further, the upregulation of RANTES expression in genital and neural tissues has been noted upon HSV-2 infection (263).

To summarize: from these data it would appear that while PIC and LPS induced DC-produced T_H1-directing type cytokine milieus, LPS also induced a cytokine milieu that contained typically T_H2-type- or T_{reg}-type-enhancing cytokines. The enhanced secretion of IL-12(p70) and IFN-γ by LPS- or PIC-stimulated DCs may enhance an anti-HSV $CD4^+$ T cell response, though it is anticipated that the response directed by LPS influence may be less effective against HSV, as LPS induces T_H2- and T_{reg}-enhancing cytokines in addition to T_H1-type cytokines (in comparison to PIC).

TLR ligand stimulation is required for enhanced $CD4^+$ T cell activation

Activated T cells are important for furthering the immune response to infection. This is accomplished by $CD4^+$ T cells through the production of cytokines such as IFN-γ, interaction of the $CD4^+$ T cells with B cells (leading to production of antibodies), macrophages (production of cytokines), and direct lysis of target cells. Surface expression of activation markers on the $CD4^+$ T cells was examined by flow cytometry in

order to determine differences among $CD4^+$ T cells influenced by the different TLR ligand-stimulated DCs. Specific $CD4^+$ T cells cultured with TLR ligand-stimulated peptide-pulsed DCs upregulated their expression of the activation markers CD25, CD44, and CD69 by day 4 after co-culture, in comparison to those that were pulsed with irrelevant peptide or media alone (Fig. 22). These $CD4^+$ T cells also downregulated their expression of CD62L at this timepoint. More specifically, compared to those cultures pulsed with irrelevant peptide in which only 3.95% of the $CD4^+$ T cells upregulated CD25, or the media stimulated cultures in which 37.6% of the T cell upregulated CD25, those cultures stimulated with TLR ligands had greatly increased expression of the activation marker (PIC = 60.1%, CpG = 66.4%, and LPS = 62.9%) (Fig. 22). This pattern was similar for the expression of CD44 (irrelevant peptide = 11%, media = 41.6%, PIC = 58.8%, CpG = 63.3%, and LPS = 60.2%) and CD69 (irrelevant peptide = 11.8%, media = 36.5%, PIC = 55.1, CpG = 60.1%, and LPS =54.7%) (Fig. 22). The percentage of $CD4^+$ T cells downregulating CD62L was: for the irrelevant peptide-stimulated cultures 18.7%, media-stimulated 61.4%, PIC-stimulated 84.1%, CpG-stimulated 91%, and LPS 85.1% (Fig. 22). These data highlight the enhanced activation of specific $CD4^+$ T cells due to the influence of TLR ligand-stimulated DCs, above the activation seen due to the presence of activated DCs alone (media-stimulated DCs).

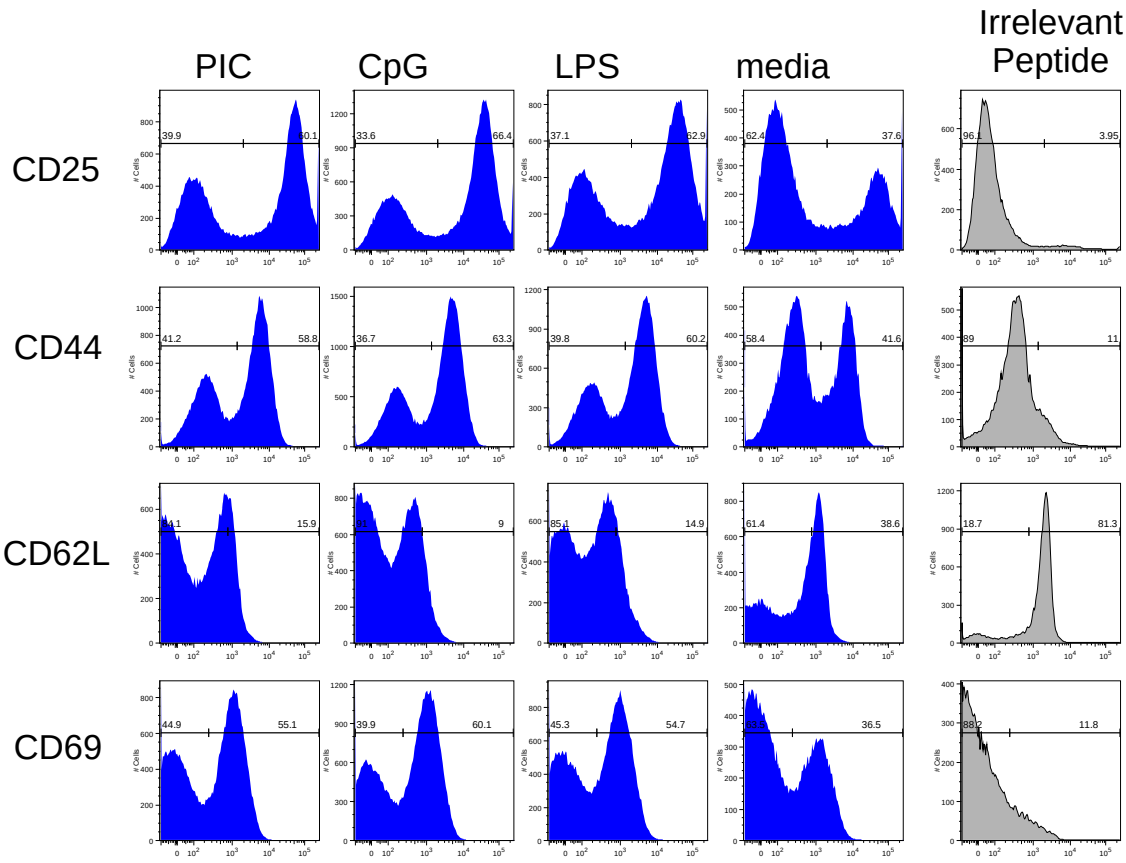

FIG. 22. Activation of $CD4^+$ T cells cultured in the presence of TLR ligand-stimulated DCs. Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing 20 ng/mL IL-4 and 20 ng/mL GM-CSF. On day 10 after culture, cells were harvested and DCs were cultured in the presence of PIC, CpG, LPS, or media alone overnight. DCs were harvested, pulsed with OT-II peptide, irrelevant peptide, or media alone for 2h. 2.5×10^6 $CD4^+$ T cells were then cultured with 1.25×10^5 TLR ligand-stimulated peptide-pulsed DCs in the presence of the appropriate TLR ligand. On day 4 of DC-$CD4^+$ T cell co-culture, $CD4^+$ T cells were labeled with fluorochrome-labeled antibodies against CD4 and KJ1-26, and CD25, C44, CD62L, or CD69.

TLR ligand stimulation resulted in greatly enhanced ability of DCs to drive naïve $CD4^+$ T cell proliferation *in vitro*

In order to examine the influence of TLR ligands on the proliferation of specific $CD4^+$ T cells, CFSE dilution was examined by flow cytometry. Naïve $CD4^+$ T cells cultured in

the presence of TLR ligand-stimulated, peptide-pulsed DCs proliferated to a greater extent in comparison to those that were stimulated without TLR ligand (i.e. media-stimulated DC only) over a four-day period. More specifically, on day 3 of $CD4^+$ T cell—DC co-culture, a difference in $CD4^+$ T cell proliferation among the various TLR ligand-stimulated cultures began to emerge. More specifically, 28.4% of the media-DC-stimulated $CD4^+$ T cells proliferated, whereas 53.8% of the PIC-DC-stimulated $CD4^+$ T cells proliferated, 48.0% of the CpG-DC-stimulates $CD4^+$ T cells proliferated, and 31.9% of the LPS-DC-stimulated $CD4^+$ T cells proliferated. On day 4 of $CD4^+$ T cell-DC co-culture, media-stimulated cultured demonstrated 35.2% proliferation as determined by CFSE dilution, whereas PIC-stimulated cultures demonstrated 57.4% proliferation, CpG-stimulated cultures 52.9%, and LPS-stimulated cultures 37.8% (Fig. 23). These data demonstrate an enhanced proliferation of specific $CD4^+$ T cells due to the influence of PIC- or CpG-stimulated DCs.

TLR ligand stimulation is required for enhanced expression of cytolytic granzyme B

Lytic $CD4^+$ T cells have previously been detected within the genital tract upon infection with HSV-2, where they may play a critical role in the rapid resolution of HSV (7, 12, 187). Further, glycoprotein J of HSV is able to inhibit granzyme B function, suggesting an important function for granzyme B in the defense against HSV (110). Flow cytometry was utilized to determine if lytic granule content of $CD4^+$ T cells was influenced by TLR activation of DCs. Specific $CD4^+$ T cells cultured with TLR ligand-stimulated, peptide-

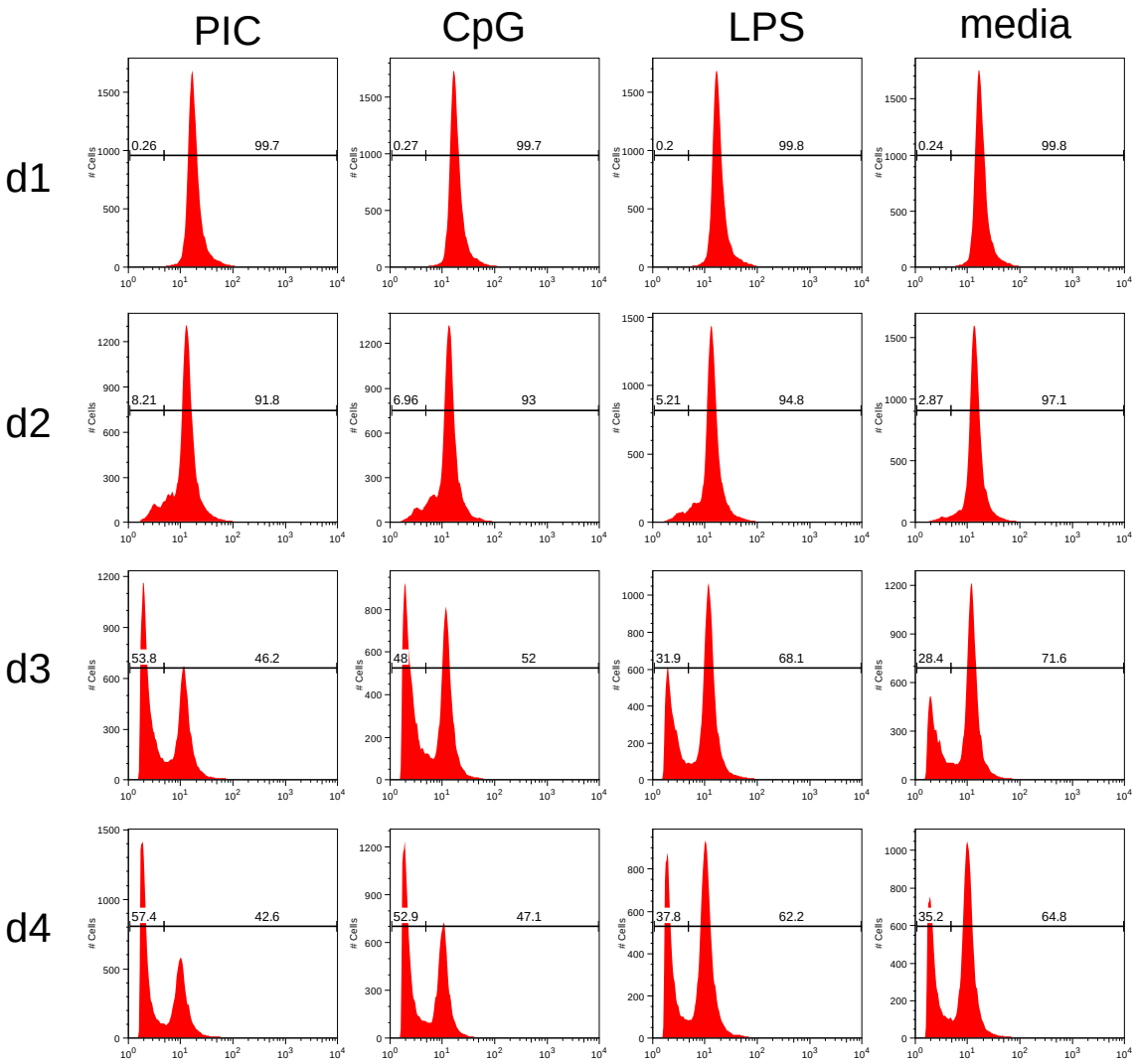

FIG 23. *In vitro* proliferation of $CD4^+$ T cells cultured with TLR ligand-stimulated DCs. Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing 20 ng/mL IL-4 and 20 ng/mL GM-CSF. On day 10 after culture, cells were harvested and DCs were cultured in the presence of PIC, CpG, LPS, or media alone overnight. DCs were harvested, pulsed with OT-II peptide, irrelevant peptide, or media alone for 2h. $CD4^+$ T cells isolated from DO.11 mice were labeled with 20 μM CFSE. 2.5×10^6 $CD4^+$ T cells were then cultured with 1.25×10^5 TLR ligand-stimulated peptide-pulsed DCs in the presence of the appropriate TLR ligand. On day 4 of DC-$CD4^+$ T cell co-culture, $CD4^+$ T cells were harvested for examination by flow cytometry.

pulsed DCs demonstrated a greater granzyme B expression in comparison to those that were stimulated with media alone or those pulsed with irrelevant peptide. More specifically, the expression of granzyme B in specific $CD4^+$ T cells in response to DCs pulsed with irrelevant peptide was 0.64%, media-stimulated DCs 8.91%, CpG-stimulated DCs 19.3%, and LPS-stimulated DCs 17.1% (Fig. 24). Interestingly, $CD4^+$ T cells from cultures containing PIC-stimulated DCs were found to be 63.7% positive for granzyme B expression (Fig. 24). This increased expression of granzyme B due to PIC may indicate the enhanced lytic capability of the $CD4^+$ T cells, and is one reason PIC was chosen for further experiments: because lytic $CD4^+$ T cells have previously been shown to be essential for rapid resolution of HSV from the genital epithelium, the enhanced lytic function of $CD4^+$ T cell due to the influence of PIC may prove beneficial in developing an effective vaccine against HSV.

TLR ligand stimulation is required for enhancement of chemokine receptor and integrin expression important for trafficking to genital and neural tissues

Chemokine receptor-ligand pairs play critical roles in the generation of effector T cell populations within the lymphoid tissues, and in trafficking of virus-specific immune cells to the primary site of infection (as well as to the central nervous system, another important site in HSV infections) (262). We utilized flow cytometry to examine the $CD4^+$ T cell expression of chemokine receptors known to be important for trafficking of the pathogen-specific T cell to skin (CCR7) and neural tissues (CCR3, CCR4, CCR5, and CXCR3). Upon examination of CCR4 upregulation, it was discovered that PIC (mean

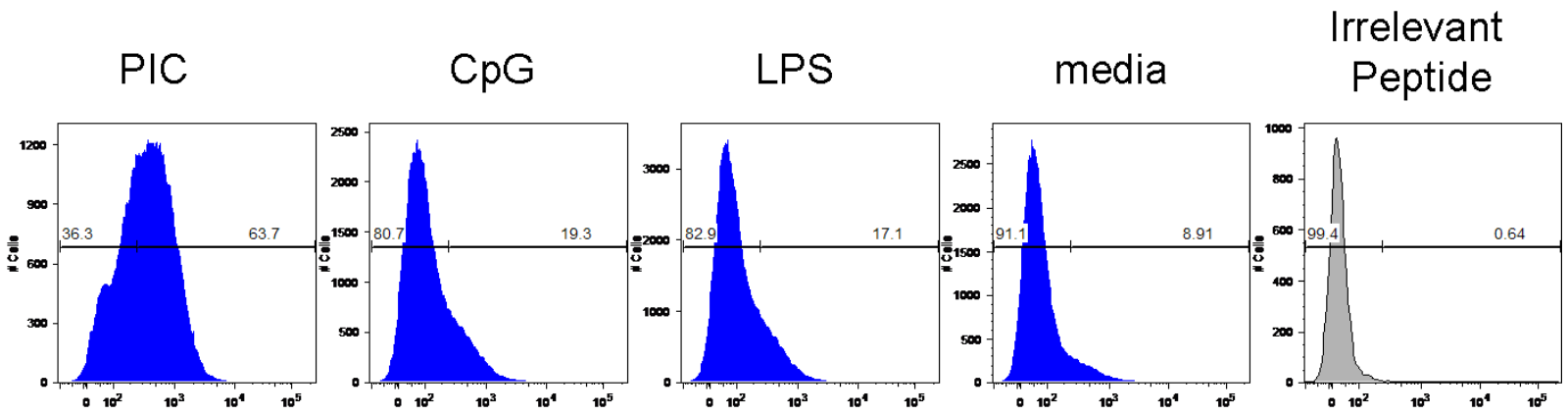

FIG. 24. Cytolytic capability of $CD4^+$ T cells cultured with TLR ligand-stimulated DCs. Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing 20 ng/mL IL-4 and 20 ng/mL GM-CSF. On day 10 after culture, cells were harvested and DCs were cultured in the presence of PIC, CpG, LPS, or media alone overnight. DCs were harvested, pulsed with OT-II peptide, irrelevant peptide, or media alone for 2h. 2.5×10^6 $CD4^+$ T cells isolated from spleens of DO.11 mice were then cultured with 1.25×10^5 TLR ligand-stimulated peptide-pulsed DCs in the presence of the appropriate TLR ligand. On day 4 of DC-$CD4^+$ T cell co-culture, $CD4^+$ T cells were harvested and labeled with fluorochrome-labeled antibodies against CD3, CD4, KJ1-26, and granzyme B and examined by flow cytometry.

fluorescence intensity = 1443), CpG (1034), or LPS (1290) induced an increased expression over media only (797, Table 4). PIC- or CpG-stimulated cultures demonstrated an upregulation of CCR5 expression by $CD4^+$ T cells (PIC mean fluorescence intensity = 70.9, CpG = 339), in comparison to the LPS- (11) or media-DC (12.4)-stimulated cultures. CpG- or LPS-stimulated cultures demonstrated an upregulation of CXCR3 expression by $CD4^+$ T cells (CpG mean fluorescence intensity = 435, LPS = 361), in comparison to the PIC- (248) or media-DC (272)-stimulated cultures. PIC-, CpG, or LPS-stimulated cultures demonstrated a slight upregulation of CCR7 expression by $CD4^+$ T cells (PIC mean fluorescence intensity = 287, CpG = 246, LPS = 240), in comparison to media-DC (220)-stimulated cultures. Specific $CD4^+$ T cells cultured with TLR ligand-stimulated peptide-pulsed DCs demonstrated a greater expression of chemokine receptors associated with neural trafficking in comparison to those that were stimulated with media alone.

The $CD4^+$ T cell expression of integrins known to be important for trafficking of the pathogen-specific T cell to vascular endothelial cells (CD18 and CD11a, P selectin ligand (P selectin glycoprotein ligand-1), and E selectin ligand) and gut ($\alpha4\beta7$) was also examined. The greatest expression of CD18 and CD11a by $CD4^+$ T cells was noted in cultures stimulated with PIC-DCs (CD18 MFI = 10487, CD11a MFI = 15145), followed by CpG-DCs (9148, 13120), then LPS-DCs (7827, 12014), and finally media-DCs (8391, 11688, Table 5). PIC- and CpG-stimulated cultures were found to contain $CD4^+$ T cells that upregulated their expression of $\alpha4\beta7$ (PIC MFI = 3088, CpG = 2800) in comparison to cultures stimulated with LPS (1700) or media-DCs (1937). The expression of P selectin ligand by $CD4^+$ T cells was most marked in cultures stimulated with PIC-DCs

TABLE 5. Upregulation of $CD4^+$ T cell chemokine receptors and integrins cultured with TLR ligand-stimulated DCs.

	PIC-OT	CpG-OT	LPS-OT	media-OT
CCR5	70.9	339	11	12.4
	5.72	27.34	0.89	1.00
CCR4	1443	1034	1290	797
	1.81	1.30	1.62	1.00
CCR3	133	193	95.6	69.2
	1.92	2.79	1.38	1.00
CXCR3	247	435	361	272
	0.91	1.60	1.33	1.00
CCR7	287	246	240	220
	1.30	1.12	1.09	1.00
α4β7	3088	2800	1700	1937
	1.59	1.45	0.88	1.00
CD18	10487	9148	7827	8391
	1.25	1.09	0.93	1.00
CD11a	15145	13120	12014	11688
	1.30	1.12	1.03	1.00
P selectin ligand	6740	4691	4865	3417
	1.97	1.37	1.42	1.00
E selectin ligand	752	606	440	698
	1.08	0.87	0.63	1.00

Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing IL-4 and GM-CSF. On day 10 after culture, cells were harvested and DCs were cultured in the presence of PIC, CpG, LPS, or media alone overnight. DCs were harvested, pulsed with OT-II peptide, irrelevant peptide, or media alone for 2h. 2.5×10^6 $CD4^+$ T cells isolated from spleens of DO.11 mice were then cultured with 1.25×10^5 TLR ligand-stimulated peptide-pulsed DCs in the presence of the appropriate TLR ligand. On day 4 of DC-$CD4^+$ T cell co-culture, $CD4^+$ T cells were labeled with fluorochrome-labeled antibodies against CD4 and KJ1-26, and CCR5, CCR4, CCR3, CXCR3, CD197, α4β7, CD18, or CD11a. Alternatively, $CD4^+$ T cells were incubated in the presence of 5 μg/mL P selectin ligand or E selectin ligand. Cells were then washed and labeled with goat anti-mouse anti-$Fc(ab)^2$ PE. The numbers in the non-shaded rows in the table represent the mean fluorescence intensities (MFI) of each chemokine receptor or integrin, while the shaded rows represent the 'fold change above media controls' defined as (MFI of chemokine receptor or integrin expressed by DCs in the TLR-stimulated culture / MFI of chemokine receptor or integrin expressed by DCs in the media-stimulated culture, for the same timepoint).

(MFI = 6740), followed by those cultures stimulated with LPS (4865) and CpG (4691), in comparison to cultures stimulated with media-DCs alone (3417). Interestingly, this pattern was mimicked by the number of $CD4^+$ T cells with 'high' P selectin ligand expression (i.e. the number of $CD4^+$ T cells demonstrating a 'high' level of P selectin ligand expression seemed to influence the MFI, Fig. 25). The expression of E selectin ligand was varied (PIC MFI = 752, CpG = 606, LPS = 440, media = 698). Specific $CD4^+$ T cells cultured with TLR ligand-stimulated peptide-pulsed DCs demonstrated enhanced expression of integrins associated with trafficking to the vascular endothelium and gut, in comparison to those that were stimulated with media alone.

In another way of looking at the data, PIC-stimulated DCs co-cultured with the $CD4^+$ T cells induced the highest expression of CCR5, α4β7, CD18, and CD11a, followed by CpG-DCs, LPS-DCs, and finally media (Fig. 25). The highest expression of CCR4 and P selectin ligand in $CD4^+$ T cells was also induced by PIC-DCs, but was then followed by LPS-DCs, then CpG-DCs, then media-DCs. LPS-DCs induced the highest expression of CXCR3 in the $CD4^+$ T cells, followed by PIC-DCs, then media-DCs, and finally CpG-DCs. PIC-DC stimulation of the $CD4^+$ T cells resulted in the highest expression of CCR3, followed by LPS-DCs, media-DCs, and finally CpG-DCs. The expression of E selectin ligand or CCR7 was upregulated similarly by any of the TLR ligands, and was above media-DC-only-stimulated cultures (Fig. 25). Thus PIC-stimulated DCs enhanced the specific $CD4^+$ T cell expression of chemokine receptors known to be important for trafficking to the genital mucosa and to neural tissues, and demonstrates the potential of PIC in driving a potentially anti-HSV $CD4^+$ T cell response. Therefore, *in vivo* studies were continued with PIC as a model for a TLR3 ligand.

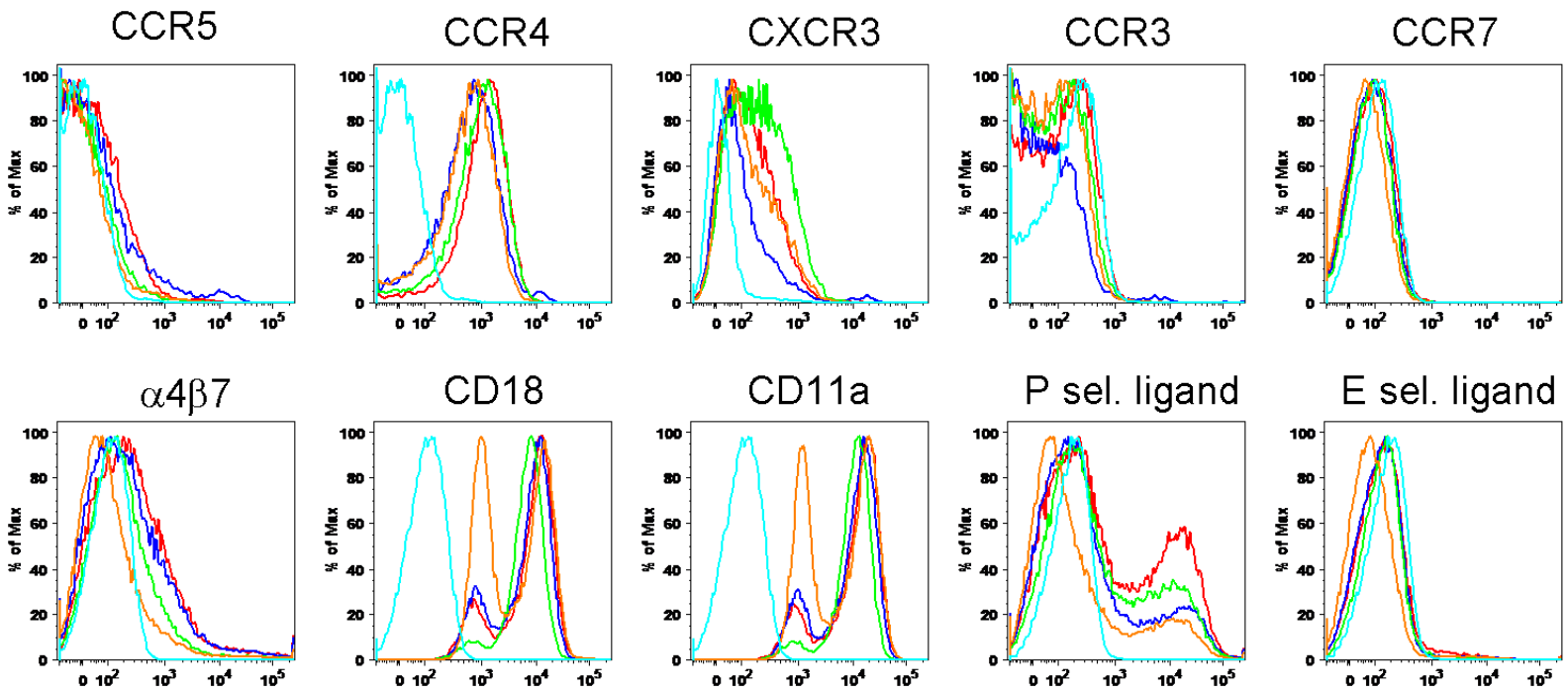

FIG. 25. Upregulation of $CD4^+$ T cell chemokine receptors and integrins cultured with TLR ligand-stimulated DCs. Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing 20 ng/mL IL-4 and 20 ng/mL GM-CSF. On day 10 after culture, cells were harvested and DCs were cultured in the presence of PIC, CpG, LPS, or media alone overnight. DCs were harvested, pulsed with OT-II peptide, irrelevant peptide, or media alone for 2h. 2.5×10^6 $CD4^+$ T cells isolated from spleens of DO.11 mice were then cultured with 1.25×10^5 TLR ligand-stimulated peptide-pulsed DCs in the presence of the appropriate TLR ligand. On day 4 of DC-$CD4^+$ T cell co-culture, $CD4^+$ T cells were labeled with fluorochrome-labeled antibodies against CD4 and KJ1-26, and CCR5, CCR4, CCR3, CXCR3, CD197, α4β7, CD18, or CD11a. Alternatively, $CD4^+$ T cells were incubated for 30 min at 37 °C in the presence of 5 μg/mL P selectin ligand or E selectin ligand (R&D). Cells were then washed and labeled with goat anti-mouse anti-$Fc(ab)^2$ PE (Southern Biotec) for 30 min at 37 °C. In the figure, the red line represents the PIC-stimulated cultures, blue equals CpG, green equals LPS, and orange represents the media-stimulated DC-$CD4^+$ T cell co-cultures, with the light blue representing the isotype control.

$CD4^+$ T cells co-cultured with TLR-stimulated DCs produce a myriad of cytokines and chemokines

The cytokine milieu present during T cell activation drives the $CD4^+$ T cell toward one of several lineages possessing different immunogenic properties, and thus different immune responses (269). In the presence of IL-12, $CD4^+$ T cells are persuaded to adopt a T_H1 phenotype, thus producing IFN-γ and TNF-α (36). IFN-γ, which activates antimicrobial macrophages, also synergizes with DC-produced IL-12 to activate CTLs (16). In the presence of IL-4, $CD4^+$ T cells become T_H2 cells, secreting IL-4 (eosinophil activation) and IL-5 (B cell antibody production) (16). A T_H1-type response has been shown to play a role in clearance of HSV both *in vivo* and *in vitro* (104, 229). A direct role for a T_H2-type response in the defense against HSV is not known, though these $CD4^+$ T cells might enhance the immune response in other ways, such as enhancing B cells and the production of specific antibody, promoting activation and migration of additional innate and adaptive immune cell types, or directly impeding virus spread. A T_H17-type response is typically associated with defenses against extracellular bacteria, but may limit the spread of virus by recruiting granulocytes to the site of infection (13, 132, 190, 272, 288). While a T_H1-type immune response is thought to be critical for the resolution of HSV infection, the DCs in our system produced a myriad of cytokines that might be anticipated to drive several $CD4^+$ T cell lineages. Therefore, we examined the cytokine production of antigen specific $CD4^+$ T cells in our system, in response to stimulation with the different TLR ligand-stimulated DCs, in order to better define the $CD4^+$ T cell lineages that the TLR ligand treatments induced.

The cytokine production of the $CD4^+$ T cells in response to DCs stimulated in the presence of TLR ligands was examined to determine their lineage commitment, and thus their function. Culture supernatants in which specific $CD4^+$ T cells were cultured with TLR ligand-stimulated peptide-pulsed DCs (in comparison to those pulsed with irrelevant peptide or without any peptide) were found to contain a myriad of cytokines and chemokines; a specific TLR ligand was not found to influence $CD4^+$ T cells to continue down a single $CD4^+$ T cell lineage. For example, all cultures were found to contain IL-4 and IL-5 (T_H2), IFN-γ and TNF-α (T_H1), and IL-17 (T_H17) (Fig. 26). More specifically, in comparison to cultures stimulated with LPS, the amounts of IL-4, IL-10, IFN-γ, and IL-17 secreted in culture by PIC- or CpG-stimulated $CD4^+$ T cells were not significantly different, while PIC-stimulated cultured contained significantly less IL-5 ($p = 0.0360$) and TNF-α ($p = 0.0418$), and CpG-stimulated $CD4^+$ T cells secreted significantly more TNF-α ($p = 0.0198$) (Fig. 26). In reference to an appropriate $CD4^+$ T cell response against HSV, from the data described here, a TLR3 ligand appears a good candidate for an adjuvant due to its enhancement of $CD4^+$ T cell-produced IFN-γ (T_H1) combined with the lack of IL-17 induction (T_H17) in comparison to CpG (TLR9) or LPS (TLR4).

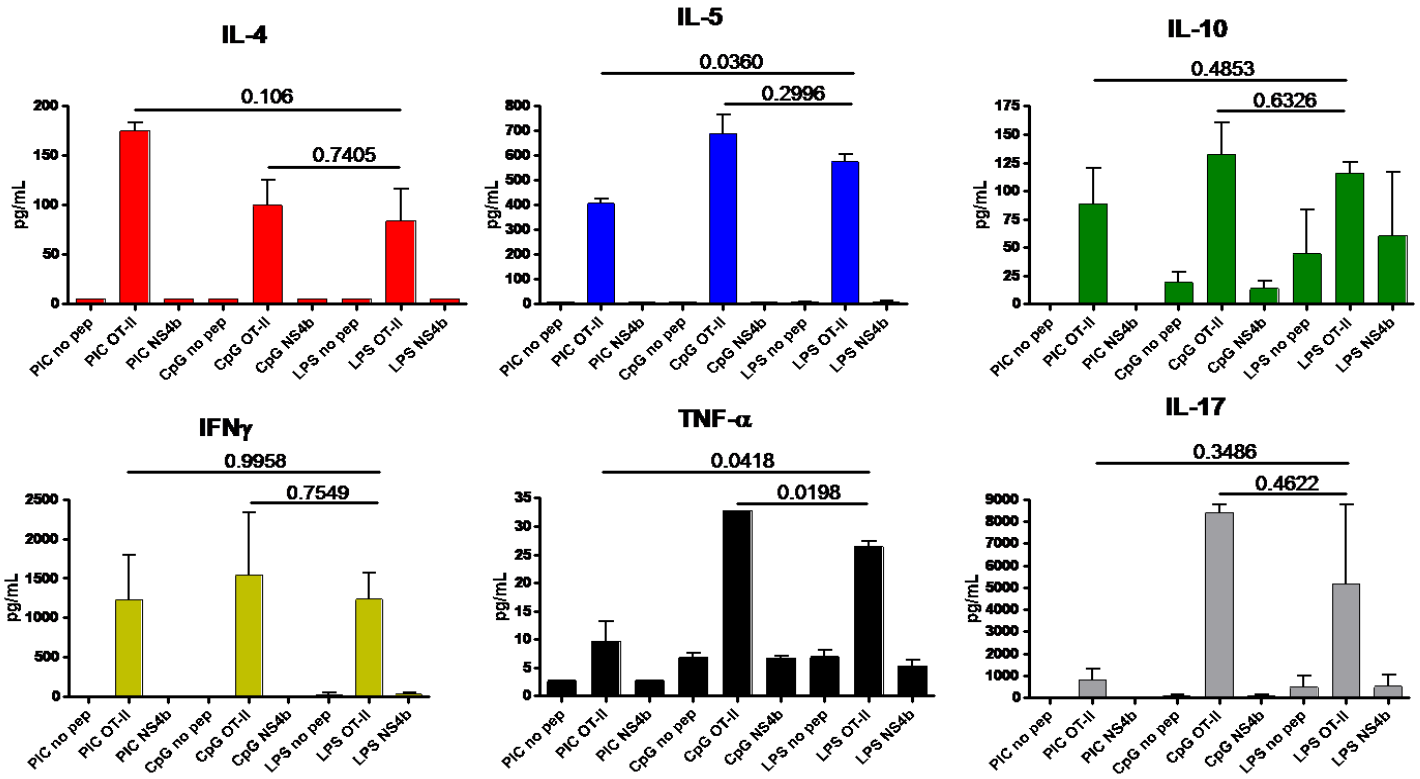

FIG. 26. Secretion of cytokines and chemokines by TLR ligand-influenced $CD4^+$ T cells. Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing 20 ng/mL IL-4 and 20 ng/mL GM-CSF. On day 10 after culture, cells were harvested and DCs were cultured in the presence of PIC, CpG, LPS, or media alone overnight. DCs were harvested, pulsed with OT-II peptide, irrelevant peptide, or media alone for 2h. 2.5×10^6 $CD4^+$ T cells were then cultured with 1.25×10^5 TLR ligand-stimulated peptide-pulsed DCs in the presence of the appropriate TLR ligand. DC-TLR + $CD4^+$ T cell co-culture supernatants were harvested after 4d and tested for the presence of cytokines and chemokines using the Bio-Plex Mouse Cytokine 23-plex Assay (Bio-Rad). Briefly, colored beads coated with different antigens were incubated with standards, controls, and sample supernatants. This was followed by the sequential addition of detection antibody and streptavidin-PE. Data were acquired on a Bio-Plex 200 and analyzed with Bio-Plex Manager Software version 5.0 (Bio-Rad).

In another way of looking at the data, in addition to the concentrations of each cytokine or chemokine, the 'fold change' (defined as the concentration in OT-II-pulsed culture / the concentration in irrelevant-peptide pulsed culture, for each TLR ligand) for each are presented in Tables 6 (pro-inflammatory cytokines), 7 (anti-inflammatory cytokines), and 8 (chemokines). Upon examination of secretion of the T_H1-type cytokine IFN-γ, the fold change was found to be greatest in PIC-DC-stimulated cultures (fold change = 974.22 times the irrelevant peptide-pulsed control), followed by CpG-stimulated cultures (163.86), and finally LPS-stimulated cultures (39.56, Table 6). The differences in secretion of another T_H1-type cytokine, TNF-α, among the various TLR ligand-stimulated cultures was not as marked (PIC fold change = 3.56, CpG = 4.82, LPS = 4.94). Interestingly, IL-17 secretion was also found to be greatest in PIC-stimulated cultures (fold change = 424.37), followed by CpG-stimulated cultures (107.37), and then LPS-stimulated cultures (10.00). A similar case was uncovered when examining the secretion of the T_H2-type cytokine IL-4 (PIC fold change = 29.51, CpG = 16.79, LPS = 14.23, Table 7). This pattern changed somewhat when examining another T_H2-type cytokine, IL-5 (PIC fold change = 37.07, CpG = 62.58, LPS = 48.30). MIP-1α and RANTES have previously been shown to influence the development of a T_H1-type $CD4^+$ T cell response (241, 247). When examining both the secretion of MIP-1α and RANTES in these experiments, PIC-stimulated cultures were found to have the greatest fold change (MIP-1α = 52.42, RANTES = 7.40), though CpG-stimulated cultures (MIP-1α = 1.11, RANTES = 1.22) and LPS-stimulated cultures (MIP-1α = 0.27, RANTES = 1.06) were not found to secrete enhanced amounts of the chemokines over their irrelevant peptide-pulsed controls (Table 8). Taken together, stimulation through TLR3 induced enhanced

secretion of T_H1, T_H2, and T_H17 cytokines, as well as chemokines known to support the development of the T_H1 lineage.

TABLE 6. Proinflammatory cytokines secreted by $CD4^+$ T cells in culture with TLR ligand-stimulated DCs.

	PIC		CpG		LPS	
Pro-Inflammatory	pg/mL	Fold Change	pg/mL	Fold Change	pg/mL	Fold Change
IL-1α	16.89	11.81	325.08	10.73	137.38	1.96
IL-1β	78.87	8.69	320.77	3.11	151.57	1.02
IL-6	283.88	289.67	4834.77	9.07	2034.17	3.12
IL-12(p40)	1117.32	12.38	8040.47	4.32	5426.18	2.67
IL-12(p70)	63.37	14.57	149.22	2.16	125.28	2.40
IL-17	810.55	424.37	8421.93	107.37	5186.00	10.00
IFN-γ	1237.26	974.22	1546.04	163.86	1241.11	39.56
TNF-α	10.05	3.56	32.83	4.82	26.49	4.94
GM-CSF	1525.37	31.65	3202.13	50.46	2505.27	12.25
IFN-α	8.16	1.26	13.73	0.84	12.33	1.35

Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing IL-4 and GM-CSF. On day 10 after culture, cells were harvested, washed, and cultured in the presence of PIC (50 μg/mL), CpG (5 μg/mL), or LPS (0.5 μg/mL) overnight. DCs were harvested, pulsed with OT-II peptide or irrelevant peptide for 2h. 2.5×10^6 $CD4^+$ T cells isolated from spleens of DO.11 mice were then cultured with 1.25×10^5 TLR ligand-stimulated peptide-pulsed DCs in the presence of the appropriate TLR ligand. On day 4 of DC-$CD4^+$ T cell co-culture, culture supernatants were harvested and tested for the presence of cytokines and chemokines using the Bio-Plex Mouse Cytokine 23-plex Assay (Bio-Rad). Briefly, colored beads coated with different antigens were incubated with standards, controls, and sample supernatants. This was followed by the sequential addition of detection antibody and streptavidin-PE. Data were acquired on a Bio-Plex 200 and analyzed with Bio-Plex Manager Software version 5.0 (Bio-Rad). The numbers in the non-shaded columns in the table represent the concentrations in pg/mL of each proinflammatory cytokine, while the shaded columns represent the 'fold change above irrelevant peptide-pulsed controls' defined as (concentration of cytokine secreted by $CD4^+$ T cells in the OT-II peptide-stimulated culture / concentration of the same cytokine secreted by $CD4^+$ T cells in the irrelevant peptide-stimulated culture, for the same TLR ligand).

TABLE 7. Anti-inflammatory cytokines secreted by $CD4^+$ T cells in culture with TLR ligand-stimulated DCs.

	PIC		CpG		LPS	
Anti-Inflammatory	pg/mL	Fold Change	pg/mL	Fold Change	pg/mL	Fold Change
IL-4	175.88	29.51	100.08	16.79	84.83	14.23
IL-5	408.87	37.07	690.21	62.58	577.47	48.30
IL-10	89.06	67.98	132.72	9.23	116.37	1.91
IL-13	2020.25	40.61	4177.77	71.31	3834.27	54.20
G-CSF	9.25	3.04	212.76	1.67	117.25	0.80

Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing IL-4 and GM-CSF. On day 10 after culture, cells were harvested, washed, and cultured in the presence of PIC (50 μg/mL), CpG (5 μg/mL), or LPS (0.5 μg/mL) overnight. DCs were harvested, pulsed with OT-II peptide or irrelevant peptide for 2h. 2.5×10^6 $CD4^+$ T cells isolated from spleens of DO.11 mice were then cultured with 1.25×10^5 TLR ligand-stimulated peptide-pulsed DCs in the presence of the appropriate TLR ligand. On day 4 of DC-$CD4^+$ T cell co-culture, culture supernatants were harvested and tested for the presence of cytokines and chemokines using the Bio-Plex Mouse Cytokine 23-plex Assay (Bio-Rad). Briefly, colored beads coated with different antigens were incubated with standards, controls, and sample supernatants. This was followed by the sequential addition of detection antibody and streptavidin-PE. Data were acquired on a Bio-Plex 200 and analyzed with Bio-Plex Manager Software version 5.0 (Bio-Rad). The numbers in the non-shaded columns in the table represent the concentrations in pg/mL of each anti-inflammatory cytokine, while the shaded columns represent the 'fold change above irrelevant peptide-pulsed controls' defined as (concentration of cytokine secreted by $CD4^+$ T cells in the OT-II peptide-stimulated culture / concentration of the same cytokine secreted by $CD4^+$ T cells in the irrelevant peptide-stimulated culture, for the same TLR ligand).

TABLE 8. Chemokines secreted by $CD4^+$ T cells in culture with TLR ligand-stimulated DCs.

	PIC		CpG		LPS	
Chemokines	pg/mL	Fold Change	pg/mL	Fold Change	pg/mL	Fold Change
MIP-1α	574.51	52.42	699.87	1.11	475.28	0.27
MIP-1β	1255.05	12.58	727.74	0.65	950.67	0.49
MCP-1	1035.28	1.07	1493.60	0.30	2457.73	0.58
Eotaxin	551.15	2.73	1139.61	3.45	860.24	3.23
KC	156.65	5.55	1558.40	1.22	671.15	0.99
RANTES	1625.56	7.40	2940.35	1.22	2921.09	1.06

Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing IL-4 and GM-CSF. On day 10 after culture, cells were harvested, washed, and cultured in the presence of PIC (50 μg/mL), CpG (5 μg/mL), or LPS (0.5 μg/mL) overnight. DCs were harvested, pulsed with OT-II peptide or irrelevant peptide for 2h. 2.5×10^6 $CD4^+$ T cells isolated from spleens of DO.11 mice were then cultured with 1.25×10^5 TLR ligand-stimulated peptide-pulsed DCs in the presence of the appropriate TLR ligand. On day 4 of DC-$CD4^+$ T cell co-culture, culture supernatants were harvested and tested for the presence of cytokines and chemokines using the Bio-Plex Mouse Cytokine 23-plex Assay (Bio-Rad). Briefly, colored beads coated with different antigens were incubated with standards, controls, and sample supernatants. This was followed by the sequential addition of detection antibody and streptavidin-PE. Data were acquired on a Bio-Plex 200 and analyzed with Bio-Plex Manager Software version 5.0 (Bio-Rad). The numbers in the non-shaded columns in the table represent the concentrations in pg/mL of each chemokine, while the shaded columns represent the 'fold change above irrelevant peptide-pulsed controls' defined as (concentration of chemokine secreted by $CD4^+$ T cells in the OT-II peptide-stimulated culture / concentration of the same chemokine secreted by $CD4^+$ T cells in the irrelevant peptide-stimulated culture, for the same TLR ligand).

In a complementary approach, $CD4^+$ T cells were harvested from day 4 DC-$CD4^+$ T cell co-cultures, restimulated, and then examined by intracellular flow cytometry for the production of IFN-γ, TNF, IL-2, IL-4, IL-5, and IL-17. Comparing $CD4^+$ T cells cultured with media-only-, PIC-, CpG-, and LPS-stimulated DCs, PIC-stimulated cultures influenced a greater production of IFN-γ, and CpG-stimulated cultures induced a greater expression of IL-2 and a much greater production of TNF (Fig. 27). The production of IL-4, IL-5, or IL-17 was not markedly different among the groups (Fig. 27).

In another way to examine the data, the mean fluorescence intensities (MFI) for this intracellular flow cytometry experiment can be found in Table 9. The production of the T_H1-type cytokine IFN-γ was greatest in PIC-stimulated cultures (MFI = 1114), followed by CpG-stimulated cultures (792), media-stimulated cultures (562), and finally LPS-stimulated cultures (513, Table 9). The production of TNF, another T_H1-type cytokine, was found to be greatest in CpG-stimulated cultures (MFI = 1707), followed by LPS-stimulated cultures (412), PIC-stimulated cultures (386), and media-stimulated cultures (359). CpG-stimulated cultures were also found to induce the greatest production of the T_H2-type cytokine IL-4 (MFI = 1609), while the other cultures resulted in similar intensities (PIC = 734, LPS = 751, and media = 833). The production of the T_H17-type cytokine IL-17 was not highly variant among the different TLR ligand stimulations (PIC MFI = 560, CpG = 604, LPS = 628, and media = 663). From this view of the data, it appears that $CD4^+$ T cell stimulation in the presence of TLR3-activated DCs results in the greatest production of T_H1-type cytokines while simultaneously producing lesser amounts of T_H2- or T_H17-type cytokines. In comparison, $CD4^+$ T cell stimulation in the presence of TLR9-activated DCs results in the production of both T_H1-

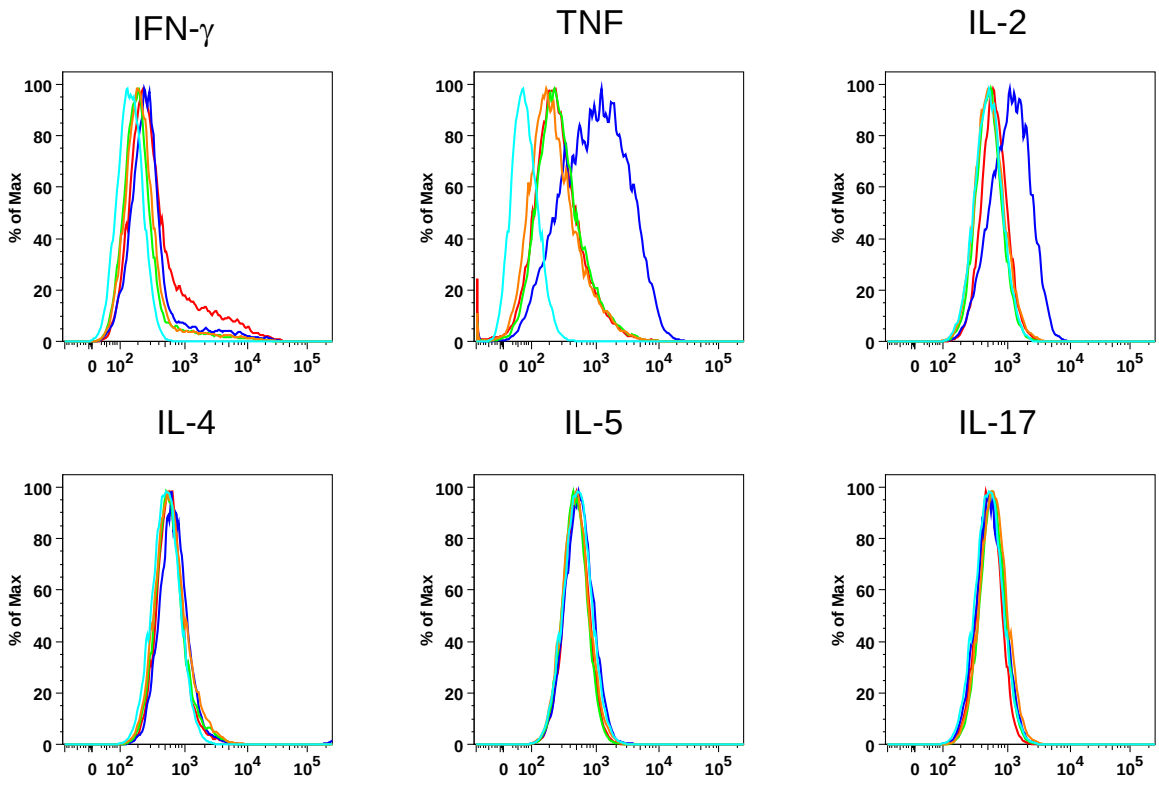

FIG. 27. Cytokine production of $CD4^+$ T cells cultured with TLR ligand-stimulated DCs. Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing 20 ng/mL IL-4 and 20 ng/mL GM-CSF. On day 10 after culture, cells were harvested and DCs were cultured in the presence of PIC, CpG, LPS, or media alone overnight. DCs were harvested, pulsed with OT-II peptide, irrelevant peptide, or media alone for 2h. 2.5×10^6 $CD4^+$ T cells isolated from spleens of DO.11 mice were then cultured with 1.25×10^5 TLR ligand-stimulated peptide-pulsed DCs in the presence of the appropriate TLR ligand. On day 4 of DC-$CD4^+$ T cell co-culture, $CD4^+$ T cells were harvested and labeled with fluorochrome-labeled antibodies against CD3, CD4, KJ1-26, and IFN-γ, TNF-α, IL-2, IL-4, IL-5, or IL-17 and examined by flow cytometry. In the figure, the red line represents the PIC-stimulated cultures, blue equals CpG, green equals LPS, and orange represents the media-stimulated DC-$CD4^+$ T cell co-cultures, with the light blue representing the isotype control.and T_H2-type cytokines, while $CD4^+$ T cell stimulation in the presence of TLR4-activated DCs results in intensities similar to, but slightly less than, those seen in cultures containing media-only-stimulated DCs.

TABLE 9. Cytokine production of $CD4^+$ T cells cultured with TLR ligand-stimulated DCs.

	PIC-OT	CpG-OT	LPS-OT	media-OT
IFN-γ	1114	792	513	562
	1.98	1.41	0.91	1.00
TNF	386	1707	412	359
	1.08	4.75	1.15	1.00
IL-2	664	1334	557	595
	1.12	2.24	0.94	1.00
IL-4	734	1609	751	833
	0.88	1.93	0.90	1.00
IL-5	532	600	506	531
	1.00	1.13	0.95	1.00
IL-17	560	604	628	663
	0.85	0.91	0.95	1.00

Bone marrow was harvested from the femurs of a Balb/c mouse. Cells were cultured in media containing IL-4 and GM-CSF. On day 10 after culture, cells were harvested and DCs were cultured in the presence of PIC, CpG, LPS, or media alone overnight. DCs were harvested, pulsed with OT-II peptide, irrelevant peptide, or media alone for 2h. 2.5×10^6 $CD4^+$ T cells isolated from spleens of DO.11 mice were then cultured with 1.25×10^5 TLR ligand-stimulated peptide-pulsed DCs in the presence of the appropriate TLR ligand. On day 4 of DC-$CD4^+$ T cell co-culture, $CD4^+$ T cells were harvested and labeled with fluorochrome-labeled antibodies against CD3, CD4, KJ1-26, and IFN-γ, TNF-α, IL-2, IL-4, IL-5, or IL-17 and examined by flow cytometry. Numbers in the non-shaded regions represent the mean fluorescence intensity for each cytokine, while numbers in the shaded regions represent the 'fold change above controls' defined as (MFI of cytokine produced by DCs in the TLR-stimulated culture / MFI of cytokine produced by DCs in the irrelevant peptide-stimulated culture, for the same timepoint).

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To summarize: $CD4^+$ T cells cultured in the presence of TLR ligand-stimulated DCs are activated, as demonstrated by their upregulation of the activation markers CD25, CD44, and CD69, as well as the loss of expression of CD62L (Fig. 22). Beginning on

day 3 and continuing on day 4 after DC-$CD4^+$ T cell co-culture, the antigen-specific $CD4^+$ T cells proliferated most in PIC- or CpG-stimulated cultures, followed by LPS-stimulated cultures, though all TLR ligand-stimulated cultures contained $CD4^+$ T cells that proliferated to a greater extent than did those containing media-stimulated DCs (Fig. 23). This demonstrates the enhancement of the $CD4^+$ T cell response. Though CpG- or LPS-stimulated cultures contained $CD4^+$ T cells that demonstrated an upregulation of the cytolytic molecule granzyme B compared to media-DC stimulated cultures, PIC-stimulated cultures demonstrated a marked upregulation of cytolytic molecule expression (Fig. 24). This is intriguing, in that an effective defense against HSV is known to require a lytic component. PIC-stimulated cultures also appeared most successful in the upregulation of chemokine receptors by the $CD4^+$ T cells that are known to enhance trafficking to genital and neural tissues (Fig. 25), which may prove important for defenses against HSV, as HSV affects not only the genital mucosa, but also establishes lifelong infection within the ganglia. Further, TLR-stimulated DCs enhanced the antigen-specific $CD4^+$ T cell secretion of a number of cytokines relevant to a T_H1-type immune response important for protection against HSV, as well as cytokines more relevant to a T_H2- and T_H17-type response. Therefore, antigen-specific $CD4^+$ T cells in the presence of TLR-stimulated DCs exhibit enhanced activation, proliferation, expression of cytolytic molecules, and expression of markers important for trafficking to genital and neural tissues in comparison to media-DC-stimulated controls *in vitro*.

Because the $CD4^+$ T cells cultured with PIC-stimulated DCs demonstrated the enhanced expression of chemokine receptors important for genital mucosal and neural tissue trafficking, as well as the upregulation of cytolytic granzyme B, *in vivo* studies

were continued with PIC, with LPS serving as a control. Do these enhancements of $CD4^+$ T cell function translate to *in vivo* studies? We hypothesized that immunization with PIC-stimulated DCs would result in a) a T_H1-dominant $CD4^+$ T cell response, b) an enhanced primary $CD4^+$ T cell response followed by the development of an effective, antigen-specific memory population, and c) enhanced cytolytic activity, in comparison to LPS- or media-stimulated DCs.

Immunization with TLR-stimulated DCs generates a specific $CD4^+$ T cell response in secondary lymphoid and genital tissues

Flow cytometry was used to determine the peak OVA-specific $CD4^+$ T cell response in secondary lymphoid tissue after immunization. The peak of the specific $CD4^+$ T cell response occurs between days 6 and 9 after the second i.p. immunization with TLR ligand-stimulated DCs. Spleens, iliac lymph nodes, and inguinal lymph nodes were harvested from mice on days 0, 6, 9, and 11 after the second immunization with OVA-DCs or LPS-OVA-DCs (see immunization scheme for more detail, Fig. 18). Lymphocytes were isolated and labeled with fluorochrome-labeled antibodies against CD3, CD4, CD8, and KJ1-26. The peak of the OVA-specific $CD4^+$ T cell response occurred around day 6 in all tissues examined (Fig. 28). In the repeat of this experiment, the peak day of $CD3^+CD4^+KJ1\text{-}26^+$ cells in each of the tissues of OVA-DC and LPS-OVA-DC occurred on d9. Therefore both day 6 and day 9 were examined in further *in vivo* experiments.

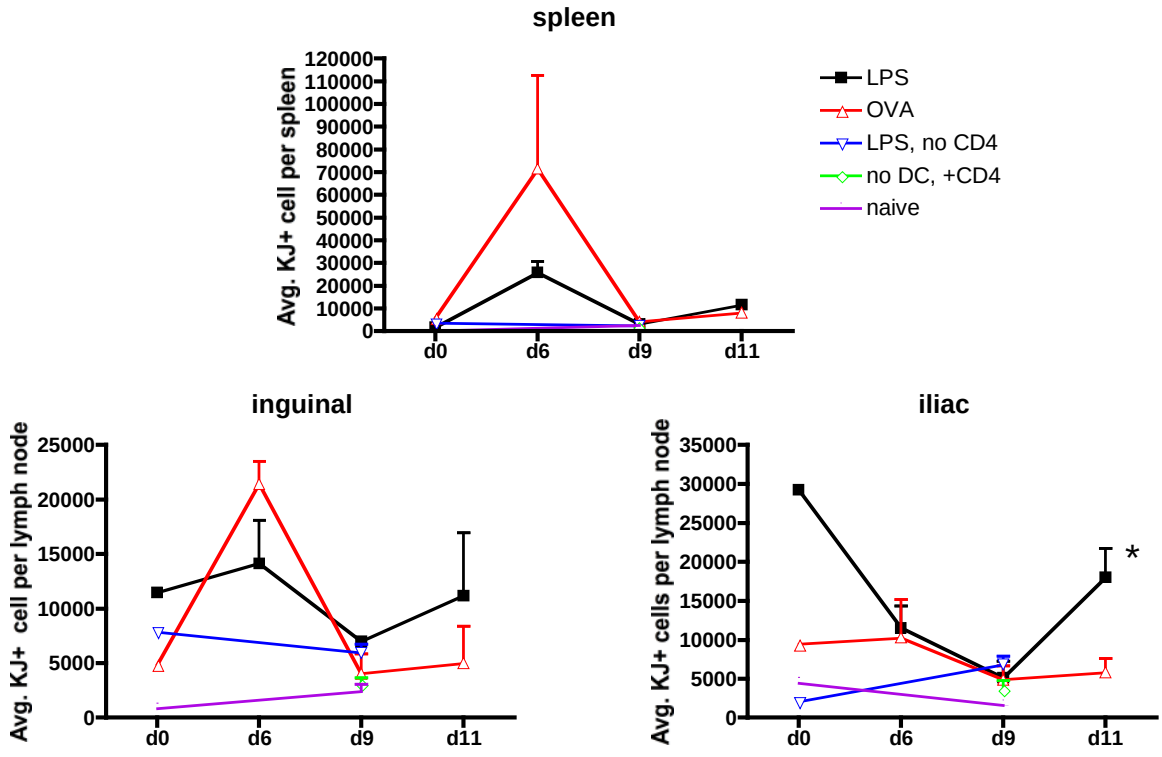

FIG. 28. The peak of the specific $CD4^+$ T cell response occurs around day 6 or 9 post-immunization. Spleens, iliac lymph nodes, and inguinal lymph nodes were harvested from mice on days 0, 6, 9, and 11 after the second immunization with OVA-DCs or LPS-OVA-DCs. Lymphocytes were labeled with fluorochrome-labeled antibodies against CD3, CD4, CD8, and KJ1-26. The peak day of $CD3^+CD4^+KJ1\text{-}26^+$ cells in each of the tissues appeared to be d6. In a second experiment performed similarly, the peak day of $CD3^+CD4^+KJ1\text{-}26^+$ cells in each of the tissues appeared to be d9. Therefore both day 6 and day 9 timepoints were utilized in further experiments. * = 0.0410.

Immunization with TLR ligand-stimulated DCs generates a specific $CD4^+$ T cell response not significantly different from immunization with TLR ligand-stimulated DCs plus injected TLR ligand

It has previously been proposed that direct injection of a TLR ligand with a DC vaccine (as opposed to simply immunizing with DCs stimulated with TLR ligands prior to

immunization) would allow for greater enhancement of the ensuing immune response (206). In order to optimize our immunization scheme, we tested the magnitude of the antigen-specific $CD4^+$ T cell response following immunization with (a) peptide-pulsed, TLR ligand-activated DCs or (b) peptide-pulsed, TLR ligand-activated DCs plus the TLR- ligand itself. To determine whether injecting TLR ligand with a DC vaccine would enhance the antigen-specific $CD4^+$ T cell response, the number of specific $CD4^+$ T cells in spleen, inguinal lymph node, and iliac lymph node were compared on days 0, 3, 6, and 9 after immunization with LPS-OVA-DCs or LPS-OVA-DCs plus 1.0 μg LPS. In none of the tissues at any of the timepoints was the difference between the two immunization formulations found to be significantly different (Fig. 29). Therefore, in order to reduce the amount of TLR ligand utilized in further experiments, as well as to avoid possible toxicity in response to the injected ligand, TLR ligand-stimulated DCs (without injected ligand) were utilized in the remaining experiments.

Immunization with TLR ligand-stimulated DCs generates a specific $CD4^+$ T cell response, as well as a memory population

The results of the initial flow cytometry timecourse experiments were complemented by quantifying the OVA-specific $CD4^+$ T cell cytokine response utilizing ELISPOT analysis. This method allows for not only the elucidation of the timing of the peak specific $CD4^+$ T cell response, but perhaps also to lend insight into the T_H lineage induced by the different TLR ligands. The cytokines present during activation of naïve

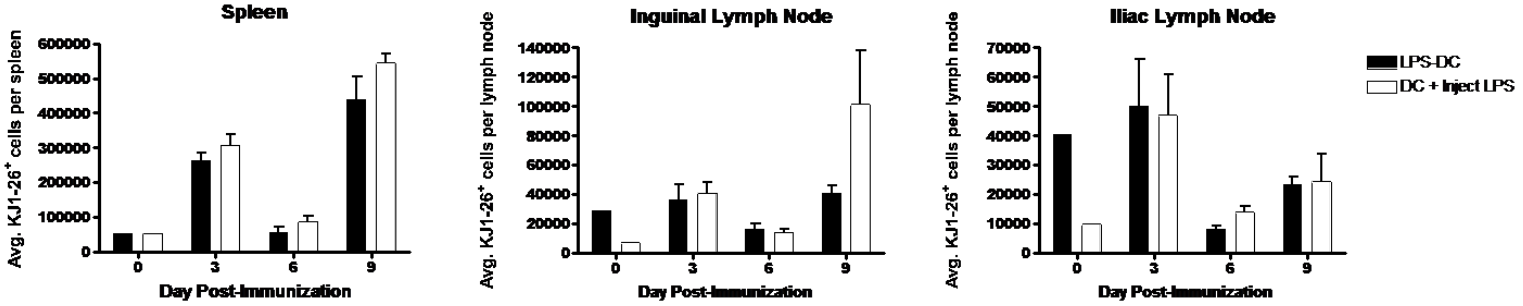

FIG. 29. Immunization with TLR ligand-stimulated DCs generates a specific $CD4^+$ T cell response not significantly different from immunization with TLR ligand-stimulated DCs plus injected TLR ligand. The numbers of specific $CD4^+$ T cells in spleen, inguinal lymph node, and iliac lymph node were compared on days 0, 3, 6, and 9 after immunization with LPS-OVA-DCs or LPS-OVA-DCs plus 1.0 μg LPS.

$CD4^+$ T cells are important for their differentiation into effector subsets (269). In reference to HSV specifically, a T_H1-type response has been shown to play a role in clearance of virus both *in vivo* and *in vitro* (104, 229). A T_H17-type response, though not typically associated with defenses against viruses, may function in the recruitment of granulocytes to the site of infection, thus limiting spread of the virus (13, 132, 190, 272, 288). While a direct anti-HSV role for a T_H2-type response is not known, these $CD4^+$ T cells might enhance B cells and the production of specific antibody, promote activation and migration of additional innate and adaptive immune cell types, or directly impede virus spread.

The peak of the specific $CD4^+$ T cell response in spleen, iliac lymph node, and vaginal tract was found to occur at day 6 for both LPS- and PIC-stimulated DC immunized mice. This was true for all cytokines (IL-4, IFN-γ, and IL-17) tested. Thus, all tissues examined contained specific $CD4^+$ T cells of the T_H1, T_H2, and T_H17 lineages generated in response to either the LPS- or PIC-stimulated DC immunization (Fig. 27). The greatest number of specific $CD4^+$ T cells was found to produce IL-4 (T_H2), followed by IL-17 (T_H17), and then IFN-γ (T_H1). This was true except in the spleen, in which the greatest number of specific $CD4^+$ T cells produced, IL-4, IFN-γ, and then IL-17. The two TLR ligand-stimulated DC immunization groups generated within the vaginal tract significantly different numbers of IL-4-producing specific $CD4^+$ T cells on day 6 ($p < 0.001$), and of IFN-γ-producing ($p < 0.01$) and IL-17-producing ($p < 0.05$) specific $CD4^+$ T cells on day 9 (Fig. 30).

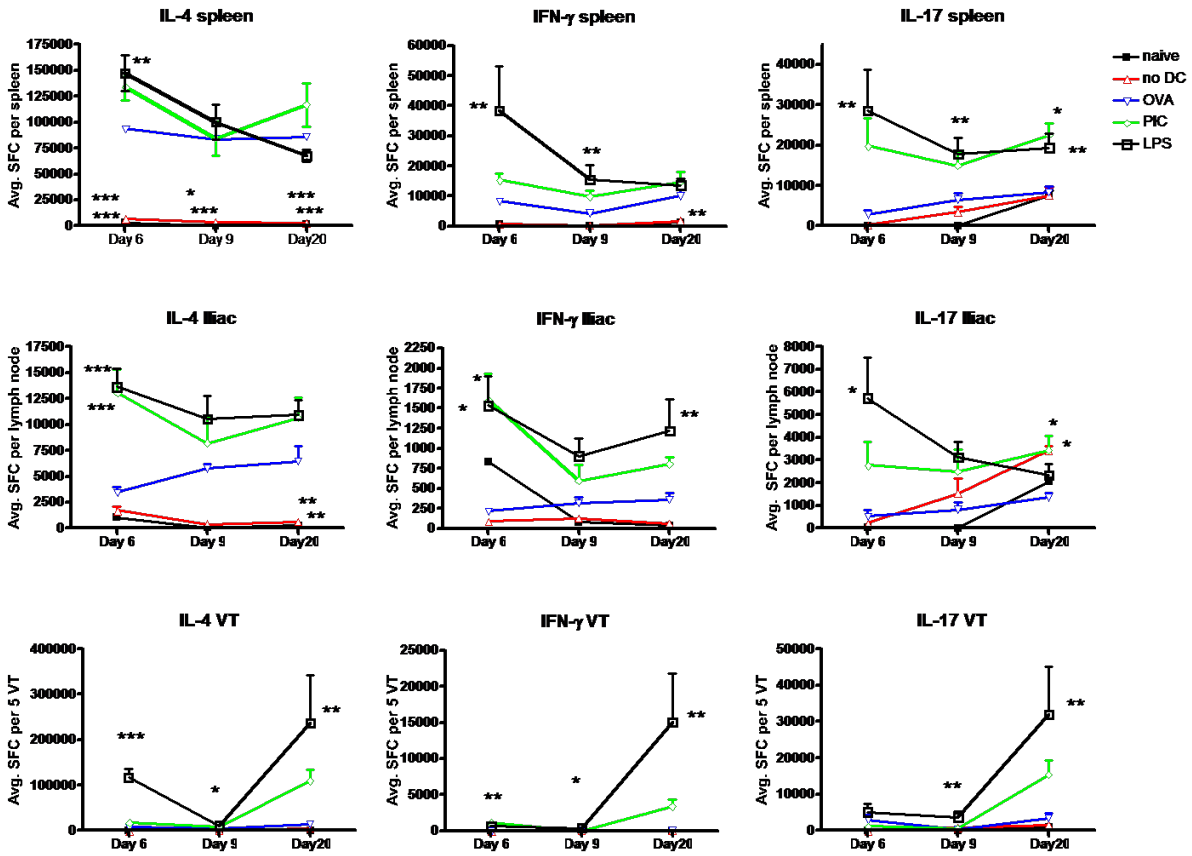

FIG. 30. Quantification of specific $CD4^+$ T cells after immunization with TLR ligand-stimulated DCs. On days 6, 9, and 20 after the second immunization, spleen, iliac lymph nodes, and vaginal tracts were harvested from groups of 9 or 10 immunized Balb/c mice. Spleens and iliac lymph nodes were maintained as separate samples (except for naïve animals, in which 5 tissues were pooled), and vaginal tracts were pooled within a group. Lymphocytes from all tissues were incubated in anti-IFN-γ, anti-IL-4, or anti-IL-17 antibody-coated nitrocellulose plates OT-II peptide-pulsed mitomycin C-treated feeder cells per well. Plates were incubated for 40h, and developed as previously described. Spot-forming cells (SFC) were quantified using an ImmunoSpot reader with ImmunoCapture software (Version 6.0) and analyzed with ImmunoSpot software (Version 4.0) from Cellular Technology Ltd. (Cleveland, OH). Data are presented as average SFC per tissue. *, $p < 0.01$; **, $p < 0.05$, ***, $p < 0.001$, comparing all groups to 'OVA".

This method was also utilized for examination of the establishment of a memory $CD4^+$ T cell population. Interestingly, both LPS- and PIC-stimulated DC immunized mice were found to contain specific $CD4^+$ T cells producing IL-4, IFN-γ, and IL-17 in spleen, iliac lymph node, and vaginal tract on day 20 following the second immunization (Fig. 30). As in previous timepoints, the greatest number of specific $CD4^+$ T cells was found to produce IL-4 (T_H2), followed by IL-17 (T_H17), and then IFN-γ (T_H1). The two TLR ligand-stimulated DC immunization groups harbored within the spleen significantly different numbers of IL-4-producing specific $CD4^+$ T cells on day 20 ($p < 0.05$) (Fig. 30).

Cytolytic activity generated in response to TLR ligand-stimulated DC immunization was not detectable

HSV-specific CTLs are postulated to mainly exercise granule exocytosis, as it is thought that HSV might encode genes that prevent cytolysis via the Fas/FasL pathway (110, 287). $CD4^+$ CTLs may prove important for control of HSV infection, as the immediate early protein ICP47 interferes with the loading of class I MHC molecules with peptides in the endoplasmic reticulum (14, 286). Indeed, HSV-specific $CD4^+$ CTLs have been isolated from HSV lesions in mice (192) and humans (133), suggesting they play a role in the clearance of HSV from the genital tract. Due to the observation of an increased expression of granzyme B in the TLR ligand-stimulated DC – $CD4^+$ T cell co-culture, the search for evidence of enhanced *in vivo* cytolytic activity altered by immunization with these TLR-stimulated DCs was undertaken. On day 6, 9, or 11 after the second DC immunization, mice received 10^7 cells labeled with 2 μM CFSE and 10^7 cells labeled

with 20 μM CFSE and pulsed with 10 μM OT-II peptide i.v. Spleens were harvested after approximately 18 hours. Unfortunately, there was no evidence of specific killing that could be detected with our assay in any of the treatment groups, compared to unimmunized controls, on any of the days tested (Fig. 31).

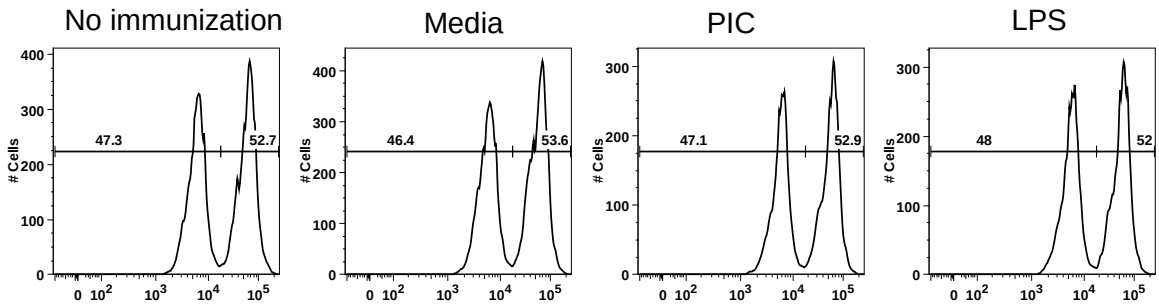

FIG. 31. *In vivo* cytotoxic T lymphocyte (CTL) assay. On day 6, 9, or 11 after the second immunization, mice received 10^7 cells labeled with 2 μM CFSE and 10^7 cells labeled with 20 μM CFSE and pulsed with 10 μM OT-II peptide i.v. Cells were incubated overnight (approximately 18 hours) *in vivo*. Spleens were harvested and spun over a Histopaque cushion. Lymphocytes were washed and resuspended in 1% formaldehyde for analysis by flow cytometry.

Discussion

Our previous studies have implicated $CD4^+$ T cells as important players in the immune response against HSV (113, 114). $CD4^+$ T cells were shown to be important for the protection of genital and neural sites, through production of cytokines that enhance the involvement of additional immune cells, as well as through clearance of virus at these sites. Likewise, it is anticipated that an effective vaccine against HSV will need to elicit these types of responses, establishing a vigorous and long-lived $CD4^+$ T cell response.

It has been proposed that $CD4^+$ T cells, after undergoing multiple rounds of stimulation within a defined cytokine milieu, undergo epigenetic changes at loci encoding the cytokines (6, 196, 299). The $CD4^+$ T cells are thus terminally differentiated and committed to one specific $CD4^+$ T cell lineage. Further, it has been suggested that T_H1 and T_H2 $CD4^+$ T cell lineages are mutually exclusive, as are T_H17 and T_{reg} lineages (279). For example, this notion is supported by evidence that T_H1 cytokines inhibit development of the T_H2 lineage, and vice versa (279). Additionally, there is evidence that the presence of either IFN-γ (T_H1) or IL-4 (T_H2) inhibits production of IL-17, and thus the development of the T_H17 lineage (89).

However, there is increasing evidence demonstrating the highly flexible nature of $CD4^+$ T cell populations. For example, Harrington et al demonstrated the ability of T_H1 memory cells *in vivo* to stop their own production of IFN-γ, thus appearing to prepare for a shift to a different response characteristic of an alternate lineage (90). In a model of lymphocytic choriomeningitis virus (LCMV) infection, when IL-4-producing LCMV-specific T_H2 $CD4^+$ T cells which had been generated *in vivo* were transferred to infected mice, the T_H2 cells began to produce IFN-γ (162). It has also been noted that T_{reg} $CD4^+$ T cells are capable of producing IFN-γ, IL-4, and IL-17 upon downregulation of FoxP3 expression, suggesting they may adopt characteristics of T_H1, T_H2, or T_H17 $CD4^+$ T cells (67). This idea of a highly plastic $CD4^+$ T cell population is intriguing when considering that T cells migrating throughout the body may require the ability to alter their defense tactics quickly against a myriad of encountered pathogens (279). Perhaps simply altering the inflammatory cytokine production profile of memory $CD4^+$ T cells is more efficient in defense against a newly encountered pathogen than is educating new T cell

populations. However seemingly committed $CD4^+$ T cells are able to alter their response, whether it depended on environmental cues or perhaps incomplete determination to a particular lineage, lineage plasticity will surely prove important for disease pathogenesis, as well as in the design and success of new therapeutics (205).

Considering the three-signal approach to T cell activation (TCR engagement, costimulation, and cytokine environment), in which the cytokine milieu present during activation has been postulated to have the most impact on the type of $CD4^+$ T cell effector and memory populations that result, we were interested in the types of cytokines that would be produced upon stimulation with different TLR ligands. It was hoped that each TLR ligand would lead to the development of a very defined cytokine milieu (T_H1, T_H2, T_H17, etc.), which would lead to the development of optimal anti-HSV effector function, lytic capability, homing to affected tissues, and the establishment of a long-term effective memory population. Thus, TLR ligands could be useful as adjuvants in vaccines, directing the immune response toward the most desirable for the given pathogen (109).

In our hands, in response to co-culture with DCs stimulated with PIC, CpG, or LPS, $CD4^+$ T cells produced a myriad of cytokines. These cytokine milieus did not fall within the previously defined distinct profiles for any one $CD4^+$ T cell lineage, but rather contained cytokines for T_H1, T_H2, and T_H17 lineages (Fig. 26 and 27, Table 6 - 9). One explanation for the observed presence of multiple lineages of $CD4^+$ T cells in our studies is that the $CD4^+$ T cells are not in fact completely committed. Other groups have noted the presence of $CD4^+$ T cells within their own systems that upregulate both T_{reg} and T_H17

transcription factors, suggesting that these $CD4^+$ T cells can alter their immune response in accordance with the pathogen encountered (300).

Another explanation is perhaps the $CD4^+$ T cells in our experiments were not receiving all signals required to drive the full commitment of the T cell toward a specific lineage, though the DCs presenting antigen in our experiments did appear to be activated, and upregulated costimulatory molecules such as CD80, as well as MHC class II at the time they were co-cultured with $CD4^+$ T cells. For example, McCarron and Reen demonstrate that TLR2 and TLR3 ligands not only induced the production of significant IL-2, but also that the secretion of said IL-2 was present at least 24h longer than stimulation without TLR ligands, demonstrating that the use of TLR ligands can influence not only the cytokine milieu present, but also the duration of the response, thus driving a potentially different environment for T cell priming (176). PIC (TLR3) and CpG DNA (TLR9) have been shown to enhance the survival of activated $CD4^+$ T cells, but this is not the case for peptidoglycan (TLR2) or LPS (TLR4) (70). Further, it is noted that activated $CD4^+$ T cells differ from their naïve counterparts in that they do not express TLR2 or TLR4, but have increased expression of TLR3 and TLR9 (70).

With respect to infection with HSV specifically, a characteristic T_H1-type response has been shown to play a role in clearance of virus both *in vivo* and *in vitro* (86, 104, 114, 188, 229). A T_H17-type response, though typically associated with defense against extracellular bacteria, may limit virus spread by recruiting granulocytes to the site of infection (13, 132, 190, 272, 288), although we were not able to demonstrate a role for T_H17 cells in HSV clearance (114). And though a direct role for a T_H2 $CD4^+$ T cells in the defense against HSV has not been demonstrated, it is possible that these cells may

augment the immune response by enhancing B cells and their production of specific antibody, promoting activation and migration of additional innate and adaptive immune cell types, or perhaps directly impeding virus spread. It is important to note, however, that antigen-specific $CD4^+$ T cells, when stimulated with TLR-influenced DCs, displayed greater production of cytokines. Of particular interest for studies in HSV, antigen-specific $CD4^+$ T cells, when stimulated with PIC-influenced DCs, displayed greater production of T_H1-type cytokines (IFN-γ) while not achieving a great enhancement of T_H17-type cytokines (IL-17), suggesting activation of DCs through TLR3 may prove beneficial for appropriately enhancing an immune response when combined with a less immunogenic vaccine against HSV (Fig. 26, Table 6).

Dendritic cells and plasmacytoid dendritic cells (pDC) are known to express TLR9 in mice (26). TLR9 detection has been implicated in the DC detection of genomic viral dsDNA in infections with HSV-1 (140, 166) and HSV-2 (166), and DCs have been shown to produce large amounts of IFN-α in response to HSV-1 (75), HSV-2 (166), and certain CpG motifs (141). HSV, with its relatively large genome, contains many CpG motifs (119). Further, recognition of HSV-2 and secretion of IFN-α by pDC was shown to rely on TLR9, as these processes could be blocked by applying an inhibitory CpG (166). Sato et al demonstrated that TLR2 and TLR9 recognition of HSV are not alternative pathways, but rather recognition of virus occurs first through TLR2 surface detection of virions, followed by internal (intracellular) TLR9 recognition of HSV dsDNA (236).

In another example of the initiation of the immune response through multiple TLRs, Querec et al demonstrate the activation of TLR2, 7, 8, and 9 in response to YF-

17D, a live attenuated vaccine against yellow fever (220). Querec et al demonstrated an innate response that was characterized by the secretion of IL-12(p40), IL-6, and IFN-α, which resulted in an adaptive $CD4^+$ T cell response composed of both T_H1 and T_H2 subsets (220). Further, it is suggested that this polyvalent cytokine response, and the balance within this response, is critical for generation of an appropriate immune response and memory formation (220). Therefore, the above mentioned study by Sato et al that demonstrated activation of TLR2 and TLR9 by HSV may indicate that infection with HSV might result in the differentiation of $CD4^+$ T cells to both T_H1 (as a result of induction of IL-12(p70) and IFN-α after activation of TLR9) and T_H2 or T_{reg} (as a result of IL-10 production upon TLR2 activation) (53, 105, 139, 200, 236). Taken together, these data might suggest that an appropriate immune response against HSV may not rely on a strictly T_H1 response, but rather requires a complex interplay of multiple $CD4^+$ T cell lineages (220). This underscores the importance of understanding the recognition of HSV at the innate level (i.e. which TLRs are important for the recognition of which viral components), so that this information can be utilized in the design of an effective vaccine (220).

TLR ligands have been utilized by other groups previously as therapeutic agent enhancers applied directly to the mucosal surfaces that require protection, or as adjuvants for peptide vaccines. A daily application of 100 μg PIC directly to the vaginal mucosa was found to result in increased protection from infection with HSV-2 in comparison to treatments involving only single applications (94). Delivery of PIC just prior to or just after challenge with HSV-2 was found to be the most effective at preventing infection (94). PIC treatment was noted to result in the upregulation of RANTES, thus

demonstrating the possibility for induction of a T_H1-type response thought to be important against HSV (94). Additionally, IFN-β, which is known to activate APCs, as well as directly inhibit HSV-2 gene expression, is also thought to be important for the PIC-mediated protection of the vaginal tract (94, 144). Importantly, the application of PIC (including studies which employed multiple treatments) was not shown to result in the long-term, high-level production of inflammatory cytokines that would demonstrate toxicity, as has been previously noted after repeated application of CpG (92, 94). Further, it is suggested that stimulation of TLR3 would significantly alter disease course in the event that infection was not prevented, possibly through reduction in recurrences and shedding (94).

Ashkar et al demonstrated that delivery of PIC resulted in the complete protection from a lethal challenge with HSV-2, when 100 μg was applied directly to the vaginal mucosa 24h prior to challenge with the virus (10). PIC delivered systemically (i.p.) was protective in 40% of animals, while PIC applied directly to the vaginal mucosa was found to be completely protective (10). Mice treated with PIC applied directly to the vaginal mucosa were 20% protected when treated 72h prior to infection with HSV-2, where 60% of animals were protected with the application of PIC occurring at 48h prior to infection, and 100% of animals survived lethal challenge when PIC was applied to the vaginal mucosa at 24h prior to challenge (10). Unlike results presented by Herbst-Kralovitz et al, the effectiveness of PIC treatment waned when applied immediately prior to challenge (10).

It is important to note that these studies by Ashkar et al and Herbst-Kralovitz et al examined alterations in the immediate, innate immune response to encounter of HSV,

whereas our studies were designed to examine alteration in the adaptive $CD4^+$ T cell response due to the influence of the TLR ligand. The studies by Ashkar et al and Herbst-Kralovitz et al sought to provide defense against HSV infection at the vaginal mucosa, while our studies were aimed at determining whether the use of TLR ligands could enhance the $CD4^+$ T cell response, and thus enhance the establishment and function of a memory response. The studies by Ashkar et al and Herbst-Kralovitz et al, however, do suggest that TLR3 stimulation enhances the immediate, innate immune response against HSV. This increased resistance to HSV infection may be due to the increased infiltration of the infection site by innate immune cells. An infiltration of innate cells including monocytes, macrophages, and DCs could enhance the T_H1-enhancing cytokine milieu described by Herbst-Kralovitz et al, and providing activation and enhancement of the $CD4^+$ T cell response (94). Indeed, in our hands $CD4^+$ T cells cultured in the presence of PIC-stimulated DCs demonstrated a T_H1-type phenotype based on their enhanced production of IFN-γ in the absence of an enhanced IL-17 production (Fig.26).

Haining et al found that DCs were significantly apparent at the injection site of CpG, and that these DC were activated based on the presence of both local and systemic IFN-α (79). Further, this activation of DC by CpG was sufficient for the migration of both $CD4^+$ and $CD8^+$ T cells (79). Thus, DCs are recruited upon injection of CpG, and the DCs then attract T cells into the tissues via secretion of chemokines, although the DC attraction of other cell types that could also release T cell chemoattractants has not been ruled out (79). However, though the immune system appeared to have been stimulated by the TLR adjuvant, the T cell response was found to be suboptimal (79). Injection of CpG results in the rapid collection of activated DC and lymphocytes at the local injection

site, the cells migrate to the draining lymph node, where the activated state of the pDCs is maintained (79, 195). A possible change in the composition of distant lymph nodes (which may be important for designing an HSV vaccine) has not been studied (79). CpGs or another TLR9 ligand could be used to "enforce lymphocyte migration" to a particular area of therapeutic interest (79).

In the studies presented here, the application of a TLR9 ligand (CpG in these studies) was explored as a possible adjuvant based on the previous finding that activation through TLR9 elicited a T_H1-enhancing DC response against HSV (166). Stimulation of $CD4^+$ T cells via CpG-stimulated DCs resulted in the enhanced proliferation of antigen-specific $CD4^+$ T cells (Fig. 23), the enhanced expression of activation markers (Fig. 22), an increase in the expression of the cytolytic molecule granzyme B (Fig. 24), and the secretion of a myriad of cytokines and chemokines (Fig. 26 and 27, Tables 6 – 9). It is noted, however, that these enhancements were not as marked as in cultures stimulated with PIC, which also appeared to stimulate the most T_H1-like cytokine milieu.

While treatment with PIC, CpG, and LPS are all capable of inducing an antiviral response to HSV-2, PIC was noted to produce the most potent response, with PIC and CpG alone being able to induce immune-mediated protection (10). With the application of only 25 μg of CpG to the vaginal mucosa, a large inflammatory cell infiltrate was noted in the vaginal cavity, while application of 100 μg of PIC resulted in little or no infiltration (10). Thus, it is thought that CpG may be the less acceptable adjuvant, as the large infiltration of immune cells may aid certain pathogens by providing additional cells for infection (10). However, intravenous injection of PIC in humans and subhuman primates has been demonstrated to lead to intolerable side effects such as impaired liver

function, fever, and leukopenia, though these effects are not seen in mice at equivalent PIC doses (45, 154). Based on the *in vitro* studies presented here, $CD4^+$ T cells cultured in the presence of PIC-stimulated DCs displayed the most desirable phenotype for defense against HSV (i.e. antigen-specific $CD4^+$ T cells were activated, secreted cytokines consistent with the T_H1-type response, upregulated chemokine receptors involved in trafficking to the genital mucosa as well as to neural tissues, and increased expression of cytolytic molecules). Therefore, one might speculate that a TLR3 ligand would be an effective adjuvant for an HSV vaccine. While PIC has been shown to induce toxicity in humans, there are TLR3 ligands which have not had this effect that are being tested as adjuvants for use in humans. For example, PIKA, a synthetic, stabilized dsRNA, has proven non-toxic in mice, and has been well tolerated causing few side effects when used in a phase I clinical trial (151). Therefore, while PIC itself may not be an acceptable adjuvant for use in humans, it serves as a model of a TLR3 ligand in our experiments, and may demonstrate the ability of a TLR3 ligand to induce the type of immune response that may enhance a less immunogenic HSV vaccine.

Induction of an immune response through TLR4 has not been proven effective in an HSV-2 vaginal challenge (73). LPS has been shown to induce production of IL-12, which is thought to be important in supporting the IFN-γ production by T cells and thus development of a dominant T_H1 type response that could be important in the defense against HSV (206, 268). In the present studies, LPS-stimulation of DCs appeared to enhance the type of $CD4^+$ T cell response thought to be important in the defense against HSV. For example, $CD4^+$ T cells cultured in the presence of LPS-stimulated DCs demonstrated enhanced expression of activation markers, enhanced proliferation,

upregulated chemokine receptors involved in trafficking to the genital mucosa and to neural tissues, and increased expression of cytolytic molecules. However, the proliferation and cytolytic molecule expression induced by LPS was not as pronounced as was the effect upon PIC stimulation. The $CD4^+$ T cell lineages established in response to treatment with LPS appeared to correspond to the previously defined T_H1, T_H2, and T_H17 lineages. Thus, LPS did not enhance the T_H1 lineages as well as PIC treatment appeared to in our cultures. Like TLR3 (PIC), signaling through TLR4 (LPS) can activate both IFN-β and NF-KB promoters (56). While both TLR3 and TLR4 have been shown to induce antiviral genes, it appears that signaling through TLR3 induces a more intense and prolonged gene induction compared to that of TLR4 (10). This may also help explain our data, in which stimulation of DCs through TLR3 appears to induce a more desirable $CD4^+$ T cell response, when considering what would enhance an appropriate immune response.

Stimulation of DCs through TLRs can alter the ensuing adaptive immune response, with stimulation of different TLRs leading to the development of different responses. This suggests that ligands capable of stimulating TLRs may make effective adjuvants, and may enhance and guide the development of the desired immune response. The use of adjuvants, plasmid DNA (via CpG motifs), cytokines, or other immunologically active molecules are being investigated for their potential in improving the immunogenicity of DNA vaccines, which are stable and relatively inexpensive to produce, but lack the immunogenicity of whole killed and recombinant protein vaccines (203). DNA vaccines appear to be most immunogenic when applied in a prime-boost regime, where priming of the immune system with DNA is followed by a boost with a

live or recombinant protein vaccine (203). For example, when IFN-γ is administered to the same injection site, it acts as an effective adjuvant (203). It has also been shown that injection of a Fas-encoding plasmid induces localized apoptosis, and importantly, the infiltration of dendritic cells and lymphocytes (203). When delivered as a fusion with antigen, FasL becomes a potent adjuvant, most likely by selectively inducing apoptosis of the cell containing the desired vaccine antigen (203).

HSV-1 glycoprotein D (gD) is a good candidate antigen because it is highly conserved between HSV-1 and HSV-2, and is a main target for $CD4^+$ T cells (19). Further, a recombinant gD vaccine administered with alum and 3'-*O*-deacylated-monophosphoryl lipid A (MPL) adjuvants has been shown to provide significant protection in double-seronegative women (19, 20, 254). Importantly, antibody response to the gD-MPL vaccine was similar to or greater than that induced by a natural HSV infection, further indicating the effectiveness of this vaccine candidate (19, 20, 254). The GSK vaccine consists of 20 μg gD, 50 μg MPL, and 500 μg alum (20). It is hypothesized that combining several of the highly immunogenic gD epitopes, which have been shown to induce a strong T_H1-type response characterized by the production of high levels of the cytokines IL-2 and IFN-γ, might lead to the creation of a broader, more potent T cell response (19). Importantly, when immunized with T_H2 peptide epitopes, mice were not able to survive lethal challenge with HSV-1, but when immunized with T_H1 peptide epitopes, animals developed $CD4^+$ T cell-dependent protective immunity (19). gD epitopes have also been shown to induce strong antibody responses (19).

When considering a vaccine that does not generate the desired T cell response, it is suggested that pre-sensitizing the immune system with a TLR ligand followed by the

release of antigen (for example, through the use of incomplete Freund's adjuvant) could lead to the development of a more robust T cell response (157). Haining et al suggest that antigen release coincident with peak TLR ligand effect would lead to the optimal immune system priming (79). In the studies presented here, the dominant $CD4^+$ T cell response upon immunization with antigen-loaded, PIC- or LPS-stimulated DCs was secretion of IL-4, suggesting a T_H2-type lineage development in spleen, iliac lymph nodes, and inguinal lymph nodes (Fig. 30). This was surprising, in that our earlier *in vitro* studies implied that PIC was most likely (compared to CpG or LPS) to induce the desired T_H1-type response. It is interesting to note, however, that most HSV-specific $CD4^+$ T cells within the spinal cord 8 days post-infection produced IL-4 (Fig. 4). This may indicate a benefit of a T_H2-type $CD4^+$ T cell response within the nervous system, or may simply be incidental.

The differences in our *in vitro* and *in vivo* studies imply the presence of additional factors capable of manipulating the developing T cell response within the murine model. These might include hormones, diet, age, co-infections, or simply the presence of additional immune cells that provide other stimuli that were not present in our cultures. For example, progesterone may result in increased susceptibility to HSV-2 and reduced antiviral immune responses through inhibiting the IFN-α-mediated activation of APCs and T cells (100). This may prove important for vaccine candidates that rely on TLR-mediated upregulation of type I interferons for induction of protection (100). In an example of how diet can alter the immune response, Sheridan and Beck describe an altered cytokine and chemokine production profile against HSV in vitamin E-deficient mice (243). Vitamin E-deficient mice demonstrated reduced trafficking of antigen-

specific T cells to neural sites of HSV infection, as well as increased numbers of T_{reg} in the periphery (243). Alterations in the immune response such as these, with the increased T_{reg} population dampening the desired T_H1-type response, as well as the suppression of the antigen-specific T cell response within the CNS, may prove detrimental to the establishment of an effective immune response to immunization against HSV.

Another consideration in the development of a TLR-adjuvanted vaccine is the genetic variation within TLRs in humans has been demonstrated to play a role in infection (25). The genetic variation within TLRs in humans may therefore be expected to alter susceptibility, magnitude of the innate immune response and subsequent adaptive immune response, and course of infection with HSV (64), and may thus prove important for vaccine design.

The model system utilized in the experiments presented here allowed us to carefully examine the effects of TLR ligands on $CD4^+$ T cell function and memory by conscientiously controlling the TCR affinity for antigen, DC subset, antigen (OT-II peptide), as well as concentration and type of TLR ligand signal. Based on our results, a ligand that stimulates TLR3 may on first glance appear to be the best option for use as an adjuvant to stimulate the type of response previously demonstrated to be important against HSV. For example, in our studies, PIC activation of DCs through TLR3 led to the development of a dominantly T_H1-type response while simultaneously leading to development of a limited T_H17-type response. Further, this activation of DCs through TLR3 also led to enhanced activation, proliferation, and lytic component content in the $CD4^+$ T cells. It is important to point out that, while DC TLR3 activation led to the most dramatic enhancement of this desired $CD4^+$ T cell phenotype, activation of DCs through

TLR4 or TLR9 also enhanced this development to some extent. An important consideration for the development of an effective vaccine is the memory T cell response that is induced and maintained after immunization. When adding this into the consideration of which TLR ligand may prove most effective when employed as an adjuvant in a vaccine, a TLR4 ligand may prove most beneficial, as the desired $CD4^+$ T cell phenotype was enhanced to some extent over media-stimulated DC controls, but also because of the enhanced memory $CD4^+$ T cell population that was noted 20 days after immunization within the genital tract, an important immune site when considering genital HSV infection. Further, while the LPS used in our studies to stimulate DC TLR4 would not be an acceptable adjuvant for use in humans due to its induction of endotoxic shock, MPL, a derivative of LPS, is a TLR4 agonist that does not induce shock (123, 173). Therefore, due to their ability to drive the development of a dominant T_H1-type response, as well as to induce a long-lasting antigen-specific memory population in the genital tract, TLR4 ligands may prove capable of enhancing the immune response to a less immunogenic vaccine against HSV.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

Cell infiltrates in recurrent human lesions include monocytes, macrophages, dendritic cells, and $CD4^+$ T cells, followed by the infiltration of $CD8^+$ T cells that correlates with lesion resolution (52, 133, 301). HSV-specific $CD8^+$ T cells play an important role in the resolution of HSV-2 from the genital mucosa (301). $CD8^+$ T cells infiltrate and remain at the dermal-epidermal junction for at least 2 months after lesion resolution (301). Due to their location at nerve ending in the skin, virus-specific $CD8^+$ T cells may function in immunosurveillance, alerting the immune system to the reactivation event and preventing the extensive replication of virus, disruption of the epidermis, and lesion formation (301). HSV-specific $CD8^+$ T cells have also been noted within the sensory ganglia of HSV-infected humans and mice, where they may function in limiting reactivation (131, 264). Previous studies have further defined the mechanisms employed by specific $CD8^+$ T cells in the clearance of HSV, demonstrating the necessity of a lytic mechanism and the presence of cytokines including IFN-γ, TNF-α, IFN-α/β, and IL-15 (9, 30, 55, 104, 188, 214, 229, 250, 271).

Depletion of either the $CD4^+$ or $CD8^+$ T cell subset delays clearance of HSV, indicating that either subset is capable of clearance (189, 250). In animals in which $CD4^+$ T cells have been depleted, or human patients with $CD4^+$ T cell deficiencies, HSV cannot be cleared from skin, nor can lesions be resolved (246). This indicates that $CD4^+$ T cells are important for resolution of HSV (246). However, the exact role of $CD4^+$ T cells in

HSV resolution (i.e. providing 'help' through costimulatory or cytokine signals to other immune cells, or through direct effector mechanisms) remains to be determined.

While $CD8^+$ T cells have a demonstrated role in the clearance of HSV from the genital tract, and there is evidence for involvement of $CD4^+$ T cells, the cell-mediated events that are important for clearance of virus within the nervous system remain to be explored. Infected neurons, which do not express MHCI but can upregulate the expression of MHCII, might indicate a necessity for $CD4^+$ T cell lytic mechanisms in limiting the spread of virus within the CNS. More information concerning the $CD4^+$ T cell response and mechanisms of clearance in both genital and neural tissues in a natural infection with HSV should help determine the type of response that an effective vaccine will need to elicit. The studies presented here were designed to further delineate the $CD4^+$ T cell responses to natural infection of genital and neural tissues with HSV. Further, the possibility of utilizing TLR activation of dendritic cells in order to direct an appropriate $CD4^+$ T cell response was examined, in hopes of utilizing this model to study how to influence the development of and appropriate $CD4^+$ T cell response within secondary lymphoid and genital tissues.

In the studies presented here, the increased presence of activated, HSV-specific $CD4^+$ T cells in the spinal cords and dorsal root ganglia of mice in primary HSV-1 infection was clearly demonstrated (Fig. 2, 3, 4). Further, $CD4^+$ T cells were sufficient for clearance of infectious HSV-1 from the genital tract, as well as possessing the ability clear infectious virus from the spinal cords and dorsal root ganglia of these mice (Fig. 6). It is important to emphasize, however, that both the $CD4^+$ and the $CD8^+$ T cell populations are likely to function in clearance in an intact animal. The results from the

present study are consistent with this idea in that, although CD8-depleted B6 mice cleared virus from sensory ganglia, they did so more slowly than did B6 mice containing both $CD4^+$ and $CD8^+$ T cell subsets (Fig. 6).

Granule exocytosis is thought to be the main cytotoxic pathway utilized by HSV-specific CTLs. Evidence for the importance of these mechanisms is bolstered by the fact that HSV encodes genes that prevent cytolysis via the Fas/FasL pathway as well as the function of granzyme B (110, 287). In studies of latently HSV-1-infected human and murine trigeminal ganglia, virus-specific $CD8^+$ T cells interacting with the neurons expressed granzyme B and perforin, though neural damage was limited (258, 274). In the current study, $CD4^+$ T cells within the HSV-1-infected spinal cord and sensory ganglia expressed granzyme B, and thus appeared to possess the components for lytic killing (Fig. 5). However, in studies utilizing either specific antibody depletion or the use of radiation chimeras, neither perforin nor Fas/FasL interactions was an absolute requirement for the $CD4^+$ T cell-mediated clearance of infectious HSV-1 from neural tissues (Fig. 8, 9, 10, 11, 12). These data do not, however, exclude some role for perforin or Fas/FasL interactions, as complete clearance of infectious HSV-1 was not always seen in all animals tested.

The cytokines present during activation of naïve $CD4^+$ T cells are important for their differentiation into effector subsets (269). While a T_H1-type response has previously gained recognition as the dominant $CD4^+$ T cell subset responsible for defense against HSV, we were able to detect IL-4-secreting $CD4^+$ T cells (T_H2), IL-17-secreting $CD4^+$ T cells (T_H17), and IFN-γ-secreting $CD4^+$ T cells (T_H1) within the iliac lymph nodes and genital tracts of HSV-immune B6 and HSV-immune IFN-$\gamma^{-/-}$ mice, as well as

in iliac lymph nodes and neural tissues of naïve B6 mice. In reference to HSV specifically, a T_H1-type response has been shown to play a role in clearance of virus both *in vivo* and *in vitro* (104, 229). A T_H17-type response is more typically associated with defenses against extracellular bacteria, but may function in the recruitment of granulocytes to the site of infection, thus limiting spread of the virus (13, 132, 190, 272, 288). A role for T_H17 cells in clearance of HSV-2 was not noted within the studies presented here, though they function in another fashion. While a direct anti-HSV role for a T_H2-type response is not known, it is possible that these $CD4^+$ T cells enhance the immune response in other ways, such as enhancing B cells and the production of specific antibody, promoting activation and migration of additional innate and adaptive immune cell types, or directly impeding virus spread.

The development of a polyvalent cytokine response, and thus the presence of multiple $CD4^+$ T cell lineages, seems to be in opposition to the idea that each $CD4^+$ T cell lineage is important for the defense against a certain type of pathogen. This idea may be inaccurate, as well as impractical from the standpoint of the immune response. The development of an innate response that was characterized by the secretion of IL-12(p40), IL-6, and IFN-α, and resulting in an adaptive $CD4^+$ T cell response composed of both T_H1 and T_H2 subsets in response to a live attenuated vaccine against yellow fever virus (YF-17D) has been reported (220). This suggests that a polyvalent cytokine response, and the balance of the $CD4^+$ T cell subsets within this response, may be critical for generation of an appropriate immune response and memory formation (220). Therefore, the development of IL-4- and IL-17-producing $CD4^+$ T cells seen in our experiments should not be ignored because they do not fit with the idea of a T_H1-mediated anti-HSV

response. Rather, these subsets deserve further investigation into their function in the immune defense against HSV, as they may represent important direct or indirect $CD4^+$ T cell functions that may become important in the design of an effective vaccine. For example, the different subsets may each have their own specific anti-HSV functions, may enhance the infiltration and activation of additional immune cells, or may simply serve to monitor each other, regulating the ensuing immune response, preventing toxicity due to overzealous cytokine secretion, and finally in resolution of the T cell response upon virus clearance. It is also important to note that the optimal $CD4^+$ T cell response may not be the same in all tissues. The role of these $CD4^+$ T cells in maintenance of the effector memory population within the genital tract and in neural tissues, as well as how these $CD4^+$ T cells aid in the recall of the antibody response should be examined.

Using the information gained from these first experiments in which the $CD4^+$ T cell response against HSV infection in genital and neural tissues was further defined, the possibility of directing the $CD4^+$ T cell response toward these specifications upon immunization was examined. More specifically, based on the earlier experiments, the possibility of development of a long-lived $CD4^+$ T cell response, characterized by T_H1-dominant response and cytolytic capabilities, through immunization with TLR-stimulated DCs was investigated. TLR ligands can alter the innate environment (such as the cytokine milieu present during activation), as well as stimulate adaptive responses (such as enhancing the capacity of T cells for activation, proliferation, and IFN-γ production), thus allowing for great manipulation of the immune response through immunization and providing important consideration for vaccine design (232).

In our hands, in response to co-culture with DCs stimulated with PIC, CpG, or LPS, $CD4^+$ T cells produced a myriad of cytokines. These cytokine milieus did not fall within the previously defined distinct profiles for any one $CD4^+$ T cell lineage, but rather contained cytokines for T_H1, T_H2, and T_H17 lineages (Fig. 26 and 27, Table 6 - 9). Stimulation of $CD4^+$ T cells via CpG-stimulated DCs resulted in the enhanced proliferation of antigen-specific $CD4^+$ T cells (Fig. 23), the enhanced expression of activation markers (Fig. 22), an increase in the expression of the cytolytic molecule granzyme B (Fig. 24), and the secretion of a myriad of cytokines and chemokines (Fig. 26 and 27, Tables 6 – 9). It is noted, however, that these enhancements were not as marked as in cultures stimulated with PIC, which also appeared to stimulate a more T_H1-like phenotype, based on their enhanced production of IFN-γ in the absence of an enhanced IL-17 production (Fig. 26). Based on the *in vitro* studies presented here, $CD4^+$ T cells cultured in the presence of DCs stimulated through TLR3 displayed the most desirable phenotype for defense against HSV (i.e. antigen-specific $CD4^+$ T cells were activated, secreted cytokines consistent with the T_H1-type response, upregulated chemokine receptors involved in trafficking to the genital mucosa as well as to neural tissues, and increased expression of cytolytic molecules). However, while not as marked, this desired response was also achieved in the presence of DCs stimulated through TLR4. Importantly, TLR4 ligand-stimulated DCs also enhanced the development of an antigen-specific memory $CD4^+$ T cell population within the genital tract. Therefore, a vaccine against HSV with the ability to stimulate DCs through TLR4, thus driving the development of a T_H1-type response, upregulation of cytolytic molecules, and development of an antigen-specific memory $CD4^+$ T cell response within the genital

tract, may prove essential in the defense against HSV. While LPS utilized in the studies presented here is not appropriate for use as an adjuvant in humans, it served as a model for a TLR4 ligand. MPL, a derivative of LPS, is a TLR4 agonist that does not induce shock and has previously been used in clinical trails (173)

Recognition of HSV does not occur via TLR2 or TLR9 exclusively, but occurs first through TLR2 surface detection of virions, followed by internal (intracellular) TLR9 recognition of HSV dsDNA (236). In another example of the initiation of the immune response through multiple TLRs, Querec et al demonstrate the activation of TLR2, 7, 8, and 9 in response to YF-17D, a live attenuated vaccine against yellow fever (220). This resulted in an innate response characterized by the secretion of IL-12(p40), IL-6, and IFN-α, which leads to a $CD4^+$ T cell response composed of both T_H1 and T_H2 subsets (220). Therefore, activation of TLR2 and TLR9 by HSV may indicate that infection with HSV might result in the differentiation of $CD4^+$ T cells to both T_H1 (as a result of induction of IL-12(p70) and IFN-α after activation of TLR9) and T_H2 or T_{reg} (as a result of IL-10 production upon TLR2 activation) (53, 105, 139, 200, 236). These data highlight the idea that an appropriate immune response against HSV may not rely on a strictly T_H1 response, but rather requires a complex interplay of multiple $CD4^+$ T cell lineages (220). This underscores the importance of understanding the recognition of HSV at the innate level (i.e. which TLRs are important for the recognition of which viral components), so that this information can be utilized in the design of an effective vaccine (220). Further, the recognition of HSV occurs through TLR2, TLR3, and TLR9 (73, 236), suggesting that the $CD4^+$ T cell response induced by immunization may also prove to be most beneficial when the vaccine is composed of adjuvants that stimulate multiple

TLRs. The optimal $CD4^+$ T cell response may also be influenced by the route of immunization, or the tissue under investigation, which are also important considerations in the development of an effective vaccine against HSV.

Future experiments that would be of interest include the use of recombinant glycoprotein D (produced in mammalian cells rather than bacteria in order to eliminate the presence of residual LPS in the preparation which may alter DC activation) in combination with different TLR ligands in the immunization of mice. The different TLR ligands could then be compared in their effects on 1) antibody (quantity and IgG subclass induced) and 2) memory (the development of central and memory T cells, as well as the magnitude of the memory T cell response in genital and neural tissues). Further, the effects of the different TLR ligands on 1) antibody-mediated prevention or limitation of infection, 2) virus clearance, and 3) protection of ganglia against the establishment of latency could then be examined upon challenge.

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Vita

Alison Joy Johnson was born on January 12, 1980 to Robert and JoAnn Johnson in Fargo, ND. Alison's interest in research began in her first year at the University of North Dakota (UND), when her proposal to examine crystal growth in microgravity was accepted by the NASA Reduced Gravity Student Flight Experiences Program. She was then awarded Howard Hughes Undergraduate Biological Sciences Apprenticeships, and chose to study the synthesis of structure of inorganic ring structures with Dr. Lothar Stahl at UND. Upon completion of her undergraduate studies, Alison accepted a National Institutes of Health (NIH) Post-Baccalaureate Intramural Research Training Award. At NIH, Alison studied changes in $CD4^+$ T cells associated with HIV infection, under the direction of Dr. Mark Connors. Alison then moved to Galveston in 2004 to attend the University of Texas Medical Branch UTMB. While completed her graduate work examining $CD4^+$ T cells in the immune response to Herpes Simplex Viruses with Dr. Gregg Milligan, Alison was honored to be supported by the Vale-Asche Foundation Pre-Doctoral Fellowship and the James W. McLaughlin Pre-Doctoral Fellowship.

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Abstracts

Manipulation of $CD4^+$ T cell Responses via TLR Ligand-induced Proinflammatory Cytokine Milieus. Presented at The Changing Landscape of Vaccine Development: Vaccines for Biothreats and Emerging & Neglected Diseases, 2009.

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Syntheses and Structural Characterization of the 1,3,2,4-Diazaphosphasiletidines $\{Me_2Si(^tBuN)_2P[N(H)Ph]=S\}$ and $\{Me_2Si(^tBuN)_2P[N(H)Ph]=N\textit{p}\text{-Tol}\}$. Presented at the 8th Annual North Dakota Science, Engineering and Mathematics Poster Session, 2000.