The role of dendritic cells in the cornea in the adaptive immune response following Herpes Simplex Virus-1 ocular infection

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Herpes simplex virus (HSV)-1 infection of the cornea results in expansion of $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes (DLN), and causes a $CD4^+$ T cell – mediated immunopathological disease in the cornea called herpes stromal keratitis (HSK). During steady state, the cornea possesses a resident population of $CD11c^+$ $CD11b^-$ dendritic cells (cornea-resident DCs). $CD11c^+$ DCs infiltrate the cornea after HSV-1 infection (cornea-infiltrating DCs), but their contribution to T cell expansion and the progression of HSK, as well as the role of DCs present in the DLN (DLN-resident DCs), following corneal infection is unknown. We employed mice that express high-affinity diphtheria toxin (DT) receptors from the CD11c promoter to selectively deplete $CD11c^+$ dendritic cells present in the cornea and the DLN. We depleted cornea-resident and cornea-infiltrating DCs by timed local (subconjunctival) injection of DT into murine corneas. Corneal and DLN – derived DCs were depleted by systemic (intraperitoneal) DT treatment. The studies outlined in this thesis demonstrate the following: 1) DCs resident in the cornea and DLNs at the time of infection are not essential to $CD4^+$ and $CD8^+$ T cell expansion in the DLN, nor are they necessary for HSK development. 2) Cornea-infiltrating DCs are responsible for most of the $CD8^+$ T cell expansion measured at 3 and 7 days post infection (dpi), and contribute to the prevention of lethal encephalitis. 3) Both cornea-infiltrating DCs and DLN – derived DCs participate in $CD4^+$ T cell expansion at 3 dpi, with cornea-infiltrating DC stimulating $CD4^+$ T cell expansion in the DLN at 7 dpi. Lastly, 4) although DCs infiltrate the

cornea at the onset of disease, the development of HSK between 7 and 21 dpi did not require corneal DCs. In its place, associations of $CD4^+$ T cells with MHC II – expressing corneal epithelial cells and macrophages may promote HSK progression in corneas depleted of DCs.

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1.0 INTRODUCTION

1.1 HERPES SIMPLEX VIRUS TYPE-1

1.1.1 Epidemiology

Herpes simplex virus (HSV)-1 is an evolutionarily ancient and ubiquitous human pathogen that affects the majority of the world's population. It is estimated that at least 80% of the world's population has been exposed to HSV-1 (1, 2). Though primary acquisition of HSV-1 is progressively delayed in industrialized countries, it occurs at a young age in developing nations (3). In the United States alone, the seroprevalence of HSV-1 has been estimated at 60% of the total population over the past 25 years (4), while in developing countries such as Tanzania , 90% of the population is seropositive by twenty years of age (5). Following initial primary infection of the orofacial region that includes the cornea, HSV-1 infects the sensory nerve termini at the site of infection, and travels to the innervating trigeminal ganglia (TG). There the virus establishes a state of latency, where nonreplicative viral genomes are maintained within neuronal nuclei, and no infectious viral particles are produced (6). HSV-1 has the ability to reactivate from latency, where replication of latent genomes occurs and infectious virus progeny is produced and shed towards the original site of infection. A combination of host and virus-related factors, as

well as amount of viral load, influences the ability of HSV-1 to reactivate and to retain latent viral genome (7-10).

Loss of neuronal viral control is often asymptomatic, but the virus may undergo reactivation frequently and may cause viral or immune pathology at the site of primary infection. The spectrum of diseases caused by HSV-1 includes herpes labialis, genital lesions, herpes simplex encephalitis, gingivostomatitis, and herpes stromal keratitis (HSK). Infections with HSV-1 last for the lifetime of the host, and there is yet no known treatment. Current drug therapies such as acyclovir, valcyclovir, and famciclovir inhibit viral DNA synthesis and are only effective during productive infection but do not prevent latent virus reactivation and re-infection (1, 11). Prophylactic and therapeutic candidate vaccines have been tested on animal models of herpes infection and have been largely unsuccessful in clinical trials (12, 13). Current strategies at vaccine development have been aimed at inducing humoral and cellular-mediated immunity for better protection (14).

1.1.2 Viral structure

HSV-1 is a double stranded DNA virus with a 152-kilobase pair genome that encodes at least 84 viral proteins (15). The HSV-1 genome can be divided into six important regions: (i) the ‘a’ sequences located at the end of the linear genome and necessary for viral DNA circularization and DNA packaging; (ii) the long repeat (LR) region that encodes immediate early regulatory proteins and latency associated transcripts; (iii) the unique long (UL) region that contains genes for DNA replication enzymes and capsid proteins; (iv) the short repeats (RS) that encode a very important immediate early protein, infected cell protein 4 (ICP4); (v) the origins of replication found in the middle of the UL and of RS; (vi) and the unique short region that encodes

glycoproteins, kinases, and other proteins that are critical for viral host range and response to host defense.

The viral genome is encased in an icosadeltahedral structure of viral proteins called the capsid, that is surrounded by an amorphous layer of proteins required to effectively initiate viral transcription and is collectively designated as the tegument. A lipid bilayer envelope derived from the host cell membrane and embedded with glycoproteins surrounds the tegument and the capsid. These glycoproteins aid in release of the virus from infected cells and in binding and entering new susceptible targets (1).

The life cycle of herpes viruses can be divided into three segments: (i) lytic infection where infectious virions are produced and the host cell undergoes lysis; (ii) latent infection where there is limited viral gene transcription and no infectious virions are formed; and (iii) reactivation from latency where there is minimal viral gene transcription that may lead to DNA replication and production of infectious viral particles de novo.

1.1.3 Cell entry

HSV-1 enters the cell via fusion at the cell membrane (16, 17), endocytosis (18), or phagocytosis (19). The initial phase of infection involves the attachment of viral proteins glycoprotein B (gB) and glycoprotein C (gC) to heparan sulfate proteoglycan molecules on the host cell surface. Then glycoprotein D (gD) associates with certain cell surface receptors such as the herpes virus entry mediator (HVEM), nectin-1, or 3-O-sulfated heparan sulfate (20-23). The last step involves an intermediate of gB, gD, glycoprotein L (gL), and glycoprotein H (gH) to induce fusion of the viral envelope and the host cell plasma membrane (22). Upon entry into the host cell, HSV-1 shuts off cellular protein synthesis by degrading host mRNA molecules through the virion host

shutoff (vhs) proteins (24). Using the microtubule network of the host cell, the nucleocapsid is transported towards the nucleus where viral DNA is introduced into the nucleus through an entry pore. The linear viral genome circularizes and the virus utilizes the host RNA polymerase II to transcribe its viral genes (25, 26).

The transcription of HSV-1 genes is highly sequential and tightly regulated. Within 1-2 hours of infection, HSV-1 expresses the first gene products, which are termed the immediate early (IE), or α-genes. These do not need de novo viral protein synthesis. The IE genes are critical for subsequent early viral protein expression and include infected cell protein (ICP) 0, ICP4, ICP22, ICP27, ICP47, and Us1.5. ICP0 facilitates viral infection and expression of all genes without directly binding DNA (27, 28). ICP4 upregulates expression of the early (E) or β-genes and late (L) or γ-genes (29, 30). ICP47 inhibits the transport of viral antigens into the endoplasmic reticulum, thus avoiding antigen presentation (31, 32). Four to eight hours after infection, the β-genes get expressed and initiate viral DNA replication. These genes include ICP8 (a DNA-binding protein), ICP6 (a subunit of ribonucleoreductase), and viral thymidine kinase (TK) (1). Viral DNA replication is necessary to activate expression of the γ genes, which can be subdivided into two groups. γ1 genes, such as gB, can be transcribed before DNA replication but increase expression after DNA synthesis, while γ2 genes on the other hand, need DNA replication to occur for their transcription. As a whole γ gene products comprise viral structural proteins, glycoproteins, and tegument components required for final viral assembly (1). Capsids are assembled and viral DNA is packaged into the capsids. The virus acquires tegument components in the cytoplasm and utilizes the host cell transport machinery for cell egress as newly formed virions. The entire viral replication transpires in about 12 hours.

1.1.4 Latency

Depending on the kind of cell infected, HSV-1 can lead to cell death or establishment of latency. Latency is defined as viral genome retention in neuronal cells without virion production or host cell destruction. During latency, viral DNA circularizes into an extra-chromosomal episome that is closely associated with histones (33). It is unclear how latency is established and what factors contribute to maintain it. It may involve circularization of the viral genome induced by downregulation of the IE gene ICP0 (34). It has also been suggested that the regulation and maintenance of latency is dependent on a dynamic relationship between the neuron, virus, and the host immune system (35). In our infection model of the cornea, latency is established in the trigeminal ganglion that innervates the eye, nose, and mouth. There is minimal expression of viral genes and there is difficulty in detecting viral proteins (36).

While there are no viral proteins detected during HSV-1 latency, there is abundant expression of latency associated transcripts (LAT) in infected neurons (37-39). Because of this, at a molecular level, latency is defined as viral gene expression limited to LAT transcription. Studies have shown, however, that LATs are not absolutely necessary for the establishment of latency or viral reactivation (40-42). LATs are critical in enhancing neuronal survival by blocking neuronal apoptosis (43, 44), and may promote survival of latently infected neurons by inhibiting transforming growth factor (TGF)-β signaling (45). LATs are also important for controlling viral reactivation by encoding microRNAs that impair expression of the IE gene ICP0 (46). LATs are also involved in determining what subtypes of neurons will be amenable to latent infection (47).

It is not well-understood how HSV-1 exits from a latent state to enter into productive lytic infection or reactivation. Reactivation leads to production of infectious virus particles that

travel down the axon by anterograde transport back to the primary site of infection, which leads to recurrent disease. Specific conditions, such as immunocompromise, UV-B irradiation, hypothermia, invasive surgery, and psychological stress have been associated with reactivation from latency (48-54).

The paradigm has promoted a central role for the neuron and changes in its physiology in influencing reactivation, but studies from our lab and others have shown that the immune response is actively participating in maintaining HSV-1 latency (6, 55). Reactivation can be inhibited by the antiviral cytokines expressed in the latently infected ganglia (56, 57), such as interferon (IFN) γ and tumor necrosis factor (TNF) α (6, 58).

$CD8^+$ T cells closely associate with neurons in the latently infected ganglia and can prevent reactivation ex vivo (59-61) by secretion of cytokines IFNγ and TNFα that may lead to neuronal apoptosis (60, 62) or by secretion of noncytotoxic lytic granules that do not kill neurons (63). These $CD8^+$ T cells have been shown to be specific for HSV-1 antigens (64, 65).

1.2 CORNEA

Primary infection with HSV-1 involves mucosal surfaces, which includes ocular sites such as the cornea. The cornea is a transparent and avascular tissue that forms the outermost layer of the eye. It functions to protect the rest of the eye from foreign material and along with the lens, controls and focuses the entry of light into the eye. The corneal tissue is highly structured and is arranged in five basic layers (**Fig. 1**). The outermost region is the epithelium, which is a physical barrier that protects the eye and provides a smooth surface for absorption and redistribution of oxygen and other cell nutrients.

The epithelium is innervated by tiny nerve endings that make the cornea very sensitive to touch and pain. Below the epithelium is the Bowman's layer, which is composed of layered collagen fibers that help in retaining the structure of the layer beneath it, the stroma (66, 67). The stroma comprises about 90% of the thickness of the cornea, is devoid of blood vessels, and is made up primarily of water (78%) and collagen (16%), and less amounts of proteoglycans (68, 69). The collagen that makes up the stroma lends strength, elasticity, and form to the cornea, and its unique and highly organized arrangement and spacing are essential in minimizing light scattering thus permitting transparency. The collagen fibers of the stroma are arranged as orthogonal layers called lamellae, and in between the lamellae are keratocytes which function to synthesize collagen and proteoglycans. The keratocytes are also important in producing growth factors during development and tissue injury and repair (70).

Beneath the stroma is the Descemet's membrane, which is composed of collagen fibers different from those of the stroma. It is a thin but strong layer of tissue that protects against infection and injuries. Underneath the Descemet's membrane is the endothelium, which serves to produce the collagen fibers of the Descemet's membrane. It is the innermost layer of the cornea, and one of its other primary functions is to pump out excess liquid from the stroma, and transport cellular nutrients to the corneal cells (71, 72). This is critical because endothelial cells cannot be regenerated and when they are destroyed, the stroma swells with water, becomes hazy and eventually opaque, leading to corneal blindness. The cornea is avascular and does not depend on blood vessels for cell nutrients and tissue survival. A study has shown that the avascularity of the cornea is due to the constitutive expression of the soluble receptor for vascular endothelial growth factor (VEGFR)-1 in corneas, which prevents the formation of new blood vessels by binding VEGF (73).

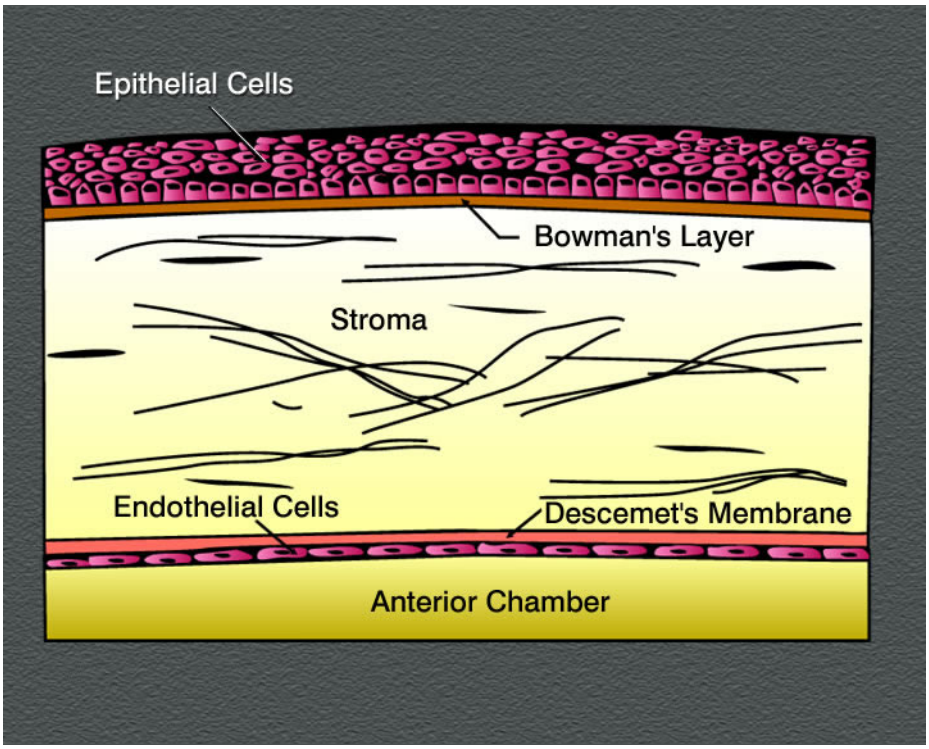

Figure 1. Schematic of the cornea

The diagram illustrates the basic layers of the cornea. The epithelium is the first layer on the anterior part of the cornea, followed by the Bowman's layer, the stroma, Descemet's membrane, and the endothelium.. Image was taken from: http://www.onset.unsw.edu.au/issue2/Contactlenses/Cornea.jpg.

1.2.1 Ocular immune privilege

Maintaining homeostasis of ocular tissues is critical to preserve vision. Inflammatory processes in the eye as a response to foreign insult can inflict injury to bystander cells of the retina or the corneal endothelium, and these may promote blindness because of an inability of these cells to regenerate. In some instances, the only recourse for remedy seems to be corneal transplantation. It is widely known that corneal allografts enjoy a remarkable advantage in that they are exempt

from some forms of immune-mediated inflammation, which makes them a highly successful type of organ transplant even in the absence of HLA-matching or immunosuppressive drugs (74-80). This phenomenon was recognized about sixty years ago and was termed as immune privilege (81, 82).

The immune privilege of the cornea is attributed to various anatomical, physiological, and immunological factors that limit immune-mediated inflammation in the eye. Part of the reason is because under non-pathological conditions, the cornea is avascular and is devoid of lymph vessels. Corneal epithelial and endothelial cells are equipped with various membrane bound molecules that may block immunological attack. Fas ligand (FasL or CD95L) is expressed on all layers of the cornea, iris, retinal pigmented epithelium, and ciliary body (83). FasL is a molecule that induces apoptosis of infiltrating neutrophils and lymphocytes that express its receptor Fas (CD95) (84), and this has been shown in corneal allografts as well (85, 86). Ligands that induce apoptosis that are expressed in the eye include tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (87, 88) and programmed death ligand – 1 (PD-L1) (89-91). Both of these molecules promote apoptosis of the cells expressing them upon engagement of their respective receptors.

The aqueous humor, which is the intraocular fluid of the anterior chamber, contains various neuropeptides, cytokines, and growth factors that contribute to immune privilege, including cell membrane bound (92, 93) or soluble complement regulatory proteins (CRP) (94), vasoactive intestinal peptide (VIP) (95), calcitonin gene-related peptide (CGRP) (96), thrombospondin (TSP) (97, 98), macrophage migration inhibitory factor (MIF) (99), alpha melanocyte stimulating hormone (a-MSH) (100, 101), TGFβ (102, 103), secreted lymphocyte

antigen-6/urokinase-type plasminogen activator (SLURP)-1 (104), and as previously mentioned, VEGFR1.

1.2.2 Immune cell populations of the cornea

Due to its immune-privileged nature, the cornea was thought to lack hematopoietic cells, however, studies from our lab and others in the past decade have shown that this is not the case. The cornea, like other mucosal surfaces, is a tissue that hosts a network of antigen – presenting cells (APCs) that include $CD11c^+$ dendritic cells (DCs) (105-109) and $CD11b^+$ $CD11c^-$ macrophages (110). This is found to be true in both mice and humans (111, 112). Almost all of these DCs constitutively express major histocompatibility class (MHC) II, and are located in the basal epithelium, with more DCs residing in the peripheral cornea and gradually decreasing in density towards the central cornea (107) (**Fig. 2**). According to Hattori, et al., like the skin, the DCs in the cornea include $langerin^+$ cells in the epithelium and in the stroma (113). These MHC II^+ dendritic cells form membrane nanotubes, which may serve as means to communicate between cells in the cornea (114). In our model of HSV-1 corneal infection, our lab has shown that these dendritic cells resident to the cornea are responsible for regulating the innate immune response that clears HSV-1 replication by facilitating natural killer (NK) cell migration to the central cornea where virus lesions are located (115). Our lab has also demonstrated that in humans, these corneal DCs rapidly mobilize to the site of lesions in an ex vivo infection model (116).

Beneath the layer of dendritic cells in the epithelium and towards the anterior side of the stroma, there are $CD11b^+$ $CD11c^-$ macrophages that express low levels of MHC II, while there are MHC II^- $CD11b^+$ macrophages located towards the posterior side of the stroma (107) (**Fig.**

2). This unique stratification of antigen presenting cells can also be appreciated in human corneas (116). Although the role of these dendritic cells in the innate immune response is recognized, it remains unknown how these resident DCs function in adaptive immune responses.

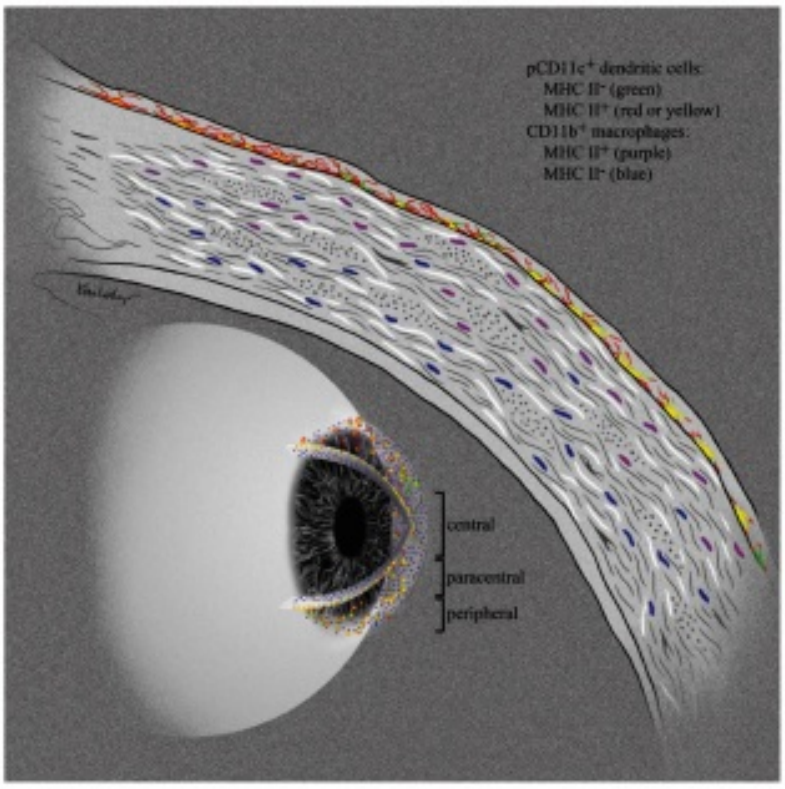

Figure 2. **The stratification of antigen presenting cells in the cornea**

The diagram depicts the unique stratification of antigen presenting cells in the normal cornea. Dendritic cells (green) expressing MHC II (red or yellow) are located in the epithelial basement membrane. Underneath, macrophages reside within the stroma, where MHC II-expressing macrophages (purple) are located in the anterior side, and MHC II-negative macrophages (blue) are in the posterior part. Image was obtained from Ophthalmol Eye Dis. 2009; 1:45-54 (107).

1.2.3 Innate immune response

With regards to microbial pathogens and from an immunological stand point, the eye is an exceptional organ that must maintain a perfect balance between the host immune response for pathogen clearance and preservation of the visual axis. Because of the inflammatory response to infection, there is accompanying tissue destruction that may lead to devastating and irreversible outcomes such as vision impairment. In order to understand this pathogenic process, it is imperative to identify and characterize the key molecules and immune cells that associate virus recognition to the innate immune response of the host, as well as the critical factors in the adaptive immune response generated against HSV-1 corneal infection, which contributes to both the host protection and ensuing immunopathology (HSK).

1.2.4 Innate immune recognition of HSV-1 in the cornea

In our model of HSV-1 ocular infection, the virus replicates in a lytic fashion in the corneal epithelium. Virus recognition by the host cell is essential to elicit a strong immune response and HSV-1 has several mechanisms by which it can evade host cell recognition (117, 118). Pathogen-associated molecular patterns (PAMPs) are highly conserved pathogen motifs that are recognized by host cells through what are collectively called pathogen recognition receptors (PRRs). HSV-1 PAMPs include glycoproteins, tegument proteins, GC-rich viral genomic DNA, as well as some AT-repeat regions, and virus-derived RNAs that accumulate during productive lytic infections. PRRs for HSV-1 include Toll-like receptors (TLRs) that alert the innate immune system to the presence of virus, and trigger the initial host response (119). Among them, TLR-2, -3, -7, -8, and -9 are known to be involved in recognition of HSV PAMPs (120-124). TLR-3,

recognized as an endosomal sensor, is expressed on the cell surface of corneal epithelial cells and fibroblasts (125-127), and its loss in mice and humans result in an enhanced susceptibility to herpes simplex encephalitis (128, 129). TLR-9 expression was observed in human corneal epithelial cells and fibroblasts after exposure to HSV antigens (130). TLRs signal through adaptor proteins that drive downstream type I interferon and inflammatory chemokine production in a highly regulated process that leads to recruitment of inflammatory cells that can control HSV-1 replication (131).

There exists a redundancy in biological systems in that there are other innate sensors that are similar to TLRs and respond to pathogenic RNA or DNA stimuli. These intracellular sensors also specifically induce type I IFN expression (132), and in the cornea, an interferon-inducible transcriptional modulator called IFN-inducible protein (IFI)-16, was identified in corneal epithelial cells and was found to induce IFNα - mediated CCL2 production. CCL2 production allowed the infiltration of inflammatory monocytes secreting inducible nitric oxide synthase (iNOS), which along with type I IFN, suppresses HSV-1 replication (133, 134). Recent investigations have revealed that HSV-1 elicits type I IFN through a cytosolic DNA sensor system that involves the adaptor molecule Stimulator of IFN genes (STING) (135). IFI-16 may possibly be an innate immune sensor that can associate HSV-1 recognition to type I IFN responses dependent on STING (133, 136).

First responders

Viral recognition and replication in corneal epithelial cells induces the release of pro-inflammatory cytokines and molecules by infected cells, bystander cells, and infiltrating cells. These include interleukin (IL)-1α, IL-1β, IL-8, IL-6, IFNγ, TNFα, macrophage inflammatory protein (MIP)-2, monocyte chemoattractant protein (MCP)-1, IL-12, and MIP-1α (137-146).

These pro-inflammatory cytokines can act as chemotactic factors that attract dendritic cells, monocytes, macrophages, neutrophils, NK cells, and γδ T cells to the site of infection, which in turn are critical in initial control of the virus (147-154). Natural killer cells are a component of the innate immune system that recognize the absence of or reduced expression of surface MHC I molecules often found in tumor cells or virus-infected cells. Upon detection of these cells that don't express self-antigen, NK cells get activated and secrete lytic granules containing granzymes that cause the infected cell to undergo apoptosis. They can also clear viral replication by secreting antiviral cytokines such as IFNγ and TNFα, and attracting other inflammatory cells by expressing chemokines such as CCL2, CCL3, CCL4, CCL5, CXCL1, and IL-8 (155). Natural killer cells are not present in the naïve cornea and infiltrate as early as 1 day after HSV-1 infection (unpublished data) via interactions between the chemokines receptor CXCR3 and its ligands CXCL9 and CXCL10 (151). In the absence of NK cells during HSV-1 infection, there is impaired control of viral replication in the cornea (115, 151).

Another major immune cell population that infiltrates the cornea early after infection is neutrophils. Neutrophils are white blood cells that are part of the innate immune system and are recruited to the site of infection to facilitate viral clearance by phagocytosis of infected cells and production of mediators such as nitric oxide. Initial studies showed that depletion of neutrophils by using an antibody to Gr-1 delayed virus clearance after HSV-1 infection (154). However, Gr-1 is expressed in other immune cells such as monocytes (156), DCs (157), and $CD8^+$ T cells (158). Upon further investigation however, specific depletion of neutrophils by the antibody to Ly6G did not promote HSV-1 clearance (159), indicating a more critical role for other cells expressing Gr-1 such as inflammatory monocytes (115).

After HSV-1 infection, macrophages are stimulated by TLR to secrete type I interferons (IFNs) (160). These type I IFNs and the subsequent signaling cascade are critical in controlling early HSV-1 replication (146, 161). Macrophages are activated by IFNγ from NK cells to control HSV-1 replication by releasing iNOS (162, 163). Even with the antiviral cytokines expressed and the early responding cells that infiltrate after HSV-1 infection of the cornea, the virus still gains access to the sensory nerve termini found in the corneal epithelium. This virus is transported in a retrograde axonal fashion to the TG within 24-48 hours after infection (164), and replicates up until 4 days post infection (165). NK cells, macrophages, and γδ T cells enter the TG after the virus starts replicating (165-167).

Virus spread from infected to neighboring neurons is limited by nitric oxide (NO) and TNF produced by macrophages and by granzymes A expressed by NK cells (167, 168). Infiltrating NK cells secrete IFNγ to activate macrophages to produce NO and TNF, and γδ T cells secrete type II IFNs to limit viral replication. This is not enough however, as the adaptive immune response is necessary to completely eliminate virus replication. Without a functional adaptive immune system, mice lose control of the virus and develop encephalitis (169).

1.2.5 Dendritic cells

Dendritic cells are a population of hematopoietic cells with critical roles in immunity, activating and initiating the immune system to respond to immune tolerance and danger signals elicited by pathogens. These cells are located throughout the body, and bridge the innate and adaptive immune responses by sampling antigens in these peripheral tissues, transport them to the draining lymph nodes (DLNs), where they can present them to potential naïve responding T cells. They are highly specialized cells that can capture, process, and present antigens to T cells.

These interactions between naïve T cells and DCs fine tune complex immune responses by controlling the primary activation and subsequent fate of the T cells, and therefore, also the outcome of the immune response (170).

Dendritic cell subsets

It is now very well established that DCs are very heterogeneous and consist of phenotypically, developmentally, and functionally distinct subpopulations (171-173). It is thought that this diversity is necessary to encompass the various kinds of pathogens encountered by the immune system, and this is reflected by the differences in expression of pathogen recognition receptors on different DC subsets (174-176).

These DC subsets have been defined traditionally by precursor, anatomical location, function, and phenotype. Broadly, they can be categorized into pre-dendritic cells (pre-DCs), conventional dendritic cells (cDCs), or inflammatory or monocyte – derived DCs.

Pre-DCs most often need additional microbial or inflammatory stimuli to further develop into fully functional DCs. One example of a pre-DC is the plasmacytoid dendritic cell (pDC). Plasmacytoid dendritic cells do not present antigen as efficiently as the other DC subtypes but they migrate between the blood, spleen, and lymph nodes, and secrete type I IFNs during viral infections (177-180). Monocytes, which are precursors of macrophages, can act as pre-DCs and differentiate into inflammatory or monocyte – derived DC (181).

Conventional DCs already exhibit dendritic cell form and function in the steady state, and can be divided into lymphoid – tissue DCs and migratory non – lymphoid tissue DCs, where the latter has the ability to migrate to the DLNs through the lymphatics. The cDCs in lymphoid tissues can be categorized based on their expression of cell surface molecules: $CD8\alpha^{+}$

CD11b^{low}DCs and CD4^{+} CD11b^{hi} DCs (182). The counterpart of the CD8α^{+} CD11b^{low}DC subset is identified in humans as the DC expressing blood –dendritic cell antigen (BDCA3) (183-185).

Migratory DCs, depending on the tissue they populate, are distinguished by their differential expression of the integrins CD11b and CD103 (186, 187). An exception is the migratory DCs in the lamina propria of the intestines, which express both (188, 189).

Langerhans cells, which are the model migratory DCs, are tissue-resident DCs that are found in epithelium of the skin, cornea, as well as the intestinal, respiratory, and reproductive tracts (113, 190-193). Initially thought to be the only cell that expresses the C-type lectin langerin in the skin, it was recently demonstrated that CD103^{+} dendritic cells also express langerin (190).

As suggested previously, inflammatory DCs are not present in the steady state, but appear after inflammation or infectious stimuli both in the periphery and in lymphoid organs, and can be derived from CCR2^{+} Ly6c^{hi} monocytes (194). One example is the TNF/iNOS producing (Tip) DCs (195). Another example is the DC that develops after infection of mice with Listeria monocytogenes (196). A detailed characterization of these DCs is described in Table 1.

Table 1. Dendritic cell subsets

DC subtype		**Location and migration**	**Markers in mice**	**Function**
Plasmacytoid DCs		Blood, spleen, lymph nodes	CD11c^{int}, CD11b^{low}, Gr-1^{+}, B220^{+}	Secretes type I interferons
Conventional DCs				
	Lymphoid tissue-resident DCs	Thymus, spleen, lymph nodes	CD11c, CD8$^{+/-}$, CD4$^{+/-}$,	Cross – presentation; capture of antigens from migratory DCs or from blood;

				antigen presentation
	Migratory tissue-resident DC	Peripheral tissues such as skin, lungs, intestine, cornea	CD11c, CD103$^{+/}$, CD11b$^{+/-}$	Sentinels in peripheral tissues; migrates to DLN for antigen presentation to T cells
Inflammatory dendritic cells or monocyte-derived DC		Anywhere, during inflammation, rarely during steady state	CD11c, CD11b, Ly6c, CD64, MAR-1	Antigen presentation; TNF and iNOS producing DCs; secretes IL-12, IL-23
Langerhans cells (an example of migratory tissue - resident DC)		Epithelial layers of different tissues	Langerin, CD11c^{lo}, CD11b^{int}, Birbeck granules, EpCAM^{hi},	Antigen presentation to T cells; deletion of self-reactive T cells; induction of Th2, Th17, Treg cells

1.2.6 HSV-1 infection of dendritic cells

Although the function of DCs in the cornea is not well characterized, the role of DCs in other peripheral tissues in response to infection with HSV-1 has been well studied. Dendritic cells are very effective at detecting pathogen and pathogen-associated molecular patterns (PAMP). DC recognition of HSV-1 is followed by secretion of vital antiviral cytokines such as IL-6 and IL-12 (197). It is intriguing that in response to some HSV-1 strains, cytokine release can be controlled by stimulation of TLR9 through viral DNA (197) or TLR2 through viral glycoproteins gH/gL or gB (198). Dendritic cells also elicit the release IFNα/β after HSV recognition, and this facilitates

inhibition of immediate-early HSV gene expression, prevention of virion release from infected cells, and impedance of the progression of infection from peripheral tissues to neurons (199). The importance of Type I IFNs in control of HSV-1 infections is illustrated in infected mice lacking the receptor for IFNα/β that are impaired in counteracting virus spread from primary infection to the central nervous system (133, 161, 200).

Plasmacytoid dendritic cells are regarded as a major producer of IFNα/β (201) through TLR9 stimulation (124). However, their importance in HSV-1 infections appears to be contingent on the route of inoculation. Effective control of vaginal submucosal (202) and systemic (203) HSV-1 infections appear to require pDCs. Plasmacytoid DCs however, do not seem to be necessary for clearing cutaneous HSV-1 infections (203). The fact that myeloid differentiation primary response gene 88 (MyD88), an adaptor molecule necessary for TLR2 and TLR9 signaling, does not influence viral replication after subcutaneous or corneal infection (133, 203), suggests that IFNα/β responses rely on various pattern recognition receptors. Additionally, after intracellular HSV-1 DNA recognition, type I IFN production by pDCs and conventional DCs require the adaptor molecule STING (135). These observations may imply that to activate cytosolic receptors, DCs have to be infected, while to stimulate various endosomal TLRs, DCs have to take up viral antigen.

As previously described, DCs have the ability for recognition of foreign antigens through PRRs (204). Once there is recognition, the DC undergoes maturation, upregulation of MHC II and costimulatory molecules, possible antigen uptake, egress from peripheral tissue, and migration to the DLNs for antigen presentation to T cells.

The case is different though if the DC gets infected. Various research groups have shown in vitro that upon HSV-1 infection of immature DCs there is a downregulation of expression of

costimulatory molecules such as CD40, and this is achieved by the formation of a complex between HSV-1 gene CD80, CD86, as well as MHC I molecules (205). MHC I-dependent antigen presentation to $CD8^+$ T cells is then partially blocked, product ICP47 and the cellular protein transporter associated with antigen processing (TAP). This inhibits the translocation of the MHC I-peptide complex to the cell surface in vivo (32, 206, 207). Overall, HSV-1-infected DCs, whether mature or immature, reduce their stimulatory capacity towards T cells (208). Infection with HSV-1 has been shown to reduce the expression of the invariant chain and HSV-1 glycoprotein B has been shown to interact with human leukocyte antigen (HLA)-DR and HLA-DM polypeptides, thus interrupting MHC II antigen processing and subsequently reducing $CD4^+$ T cell responses (209).

Initially believed to be due to viral cytopathic effects, studies have shown that HSV-1 can actively cause apoptosis of infected cells (210, 211). HSV-1 induces apoptosis in a two phase fashion where there is an early induction of anti – apoptotic mechanisms (212), and there is a late phase where pro – apoptotic mechanisms are activated (213). This inhibition of apoptosis in the early stages is to guarantee that the virus is able to complete viral replication before the host is able to establish defense mechanisms against the virus.

Migration of DCs from the infected site to the DLN is increased after HSV-1 infection (214-216). However, adoptive transfer studies with infected or uninfected DCs have shown that the infected DCs are impaired or prevented from migrating to the DLN (217). This is possibly due to its augmented attachment to fibronectin, which leads to increased adhesion to the extracellular matrix through lymphocyte function-associated factor (LFA) 1, as has been shown in vitro with HSV-infected human monocyte-derived DCs (218). In the skin, DC expression of molecules such as E-cadherin also tightly binds DCs to keratinocytes, and Langerhans cells from

HSV-infected skin downregulate E-cadherin for release to the DLN (219). HSV-1 is able to downregulate chemokines receptors CCR7 and CXCR4 on mature DC, which allow DC to migrate to the lymph nodes in response to chemokines CCL19 and CXCL12 respectively (220). Moreover, infected DCs are also not able to respond to CCL19 (221).

1.2.7 Dendritic cells and the adaptive immune response to HSV-1

The archetype mechanism for antigen presentation is that local tissue – resident DCs phagocytose microbial antigens and migrate to the DLNs to activate naïve T cells or to induce tolerance (222). This has been shown in an HSV-1 vaginal submucosal infection model (223), a skin lentivector immunization model (224), and a skin hypersensitivity model (225).

Still, it has also been shown that DCs that derive from infiltrating monocytes can stimulate effector $CD8^+$ T cells at peripheral sites of infection (226, 227). Consistent with these studies, it was also shown that the $CD11b^+$ DC subpopulation that infiltrate the vaginal submucosa after infection and not the skin-resident Langerhans cells, present antigen to $CD4^+$ T cells (228).

There is another prevailing view however, that DCs resident to the lymph node can present antigen to T cells (229), indicating one possibility that the migratory tissue – resident DCs ingested the peripheral antigen and ferried it to the DLNs for antigen presentation by lymphoid-resident DC. Inhibiting the migration of tissue-resident DC diminished the $CD8^+$ T cell responses in the DLN, indicating that antigen presentation on MHC I molecules relies on the egress of DC from the infected tissue (214). In these studies, blocking DC migration did not interfere with free virus passively draining to the lymph nodes, so presentation by $CD8^+$ DCs

was not a consequence of live virus infecting these DCs. These were shown in HSV-1 skin infection models (214, 230, 231).

Alternatively, the lymphoid – resident DC can acquire antigen by passive drainage through the lymphatic system, as antigens injected into the skin can access the DLNs within 30 minutes (232, 233). Indeed less than 24 hours after footpad challenge, HSV-1 antigens were detected in the lymph nodes (234), and lymphoid – resident DC was shown to mediate antigen presentation to $CD4^+$ and $CD8^+$ T cells after HSV-1 skin infection (223).

Another potential system is for both migratory tissue-resident DCs and lymphoid-resident DC to cooperate with each other. Identification of various DC subsets in the DLN after epidermal HSV-1 infection demonstrate that it is the lymph node resident $CD8^+$ DCs that present viral antigens to $CD8^+$ T cells (235). In a skin immunization model, DLN-resident DCs were necessary to initiate activation and trapping of cognate $CD4^+$ T cells, whereas migratory DCs were required for proliferation (236). In a skin vaccine model against fungi, it was shown that monocyte – derived DCs take up fungal antigen to the DLNs, while lymph node resident DCs, along with migrating skin-resident DCs, present antigen and prime $CD4^+$ T cells (237). This highlights that how the adaptive immune response is orchestrated by different DC subpopulations depends on the route of antigen entry, the nature of the pathogen, and the DC subset residing at the site of infection.

1.2.8 Cross-presentation of HSV-1-derived antigens by dendritic cells

For lymphoid-resident DCs to take up virally infected cellular material instead of being infected directly, these DCs have to take up exogenous antigen through the MHC class I presentation pathway through antigen cross-presentation (238-242). The requirement for cross-presentation of

HSV-derived antigens was shown in experiments utilizing mice that have a deficiency in gamma-IFI lysosomal thiol reductase (GILT), where HSV-specific cytotoxic T lymphocyte responses were impaired (243). GILT-less DCs are not able to present viral antigens to $CD8^+$ T cells even in the presence of other HSV-infected cells, unless they are directly infected with the virus (243). The ability of the $CD8^+$ DC subset in the lymph nodes to cross-present HSV antigen was established in vitro through an experiment incubating $CD8^+$ DCs with bystander cells infected with mutant HSV virus defective in virion formation (gH- or gB-deficient HSV), ensuring that only the bystander cells were productively infected, and not the $CD8^+$ DCs (244). These bystander cells were incapacitated in their antigen presentation, and upon incubation with $CD8^+$ T cells, proliferation could only be credited to the $CD8^+$ DCs.

After HSV infection, the capacity of $CD8^+$ DCs to stimulate $CD8^+$ T cells is dependent on their expression of TLR3 and TIR-domain-containing adapter-inducing IFNβ (TRIF) (245). Because TLR3 can be located in endosomes and HSV-1 infection produces a lot of viral RNA intermediates (246), it is likely that the TLR3 signaling pathway is involved in cross-presentation of antigen from dead or dying HSV-infected cells by $CD8^+$ DCs.

1.3 ADAPTIVE IMMUNE RESPONSE

Antigen presentation by DCs to naïve B cells, as well as to $CD4^+$ and $CD8^+$ T cells, initiates the adaptive immune response to HSV-1. Upon activation, B cells produce neutralizing antibodies that reduce viral spread (247). B cells can also function as APCs to induce HSV-specific $CD4^+$ T cell responses, and subsequently, B cell – deficient mice have impaired $CD4^+$ T cells and IFNγ responses (248).

1.3.1 $CD4^+$ T cells

$CD4^+$ T cells or T helper (Th) cells function, as their name suggests, in helping to activate and facilitate the survival of different immune cells. They aid B cells in antibody production and isotype switching. They also activate macrophages to develop their phagocytic and antimicrobial activities, as well as recruit neutrophils, eosinophils, and basophils to sites of infection and inflammation. Especially in the case of virus infections, they enable or "license" APCs to activate $CD8^+$ T cells through costimulatory interactions ($CD4^+$ T cell help), thus enhancing effector $CD8^+$ T cell responses and contributing to the maintenance of a functional $CD8^+$ T cell memory population. Through the cytokines and chemokines produced by $CD4^+$ T cells, they orchestrate a wide array of immune responses (249, 250).

Upon recognition of foreign antigen by naïve $CD4^+$ T cells in the context of MHC II on DCs, $CD4^+$ T cells undergo massive proliferation and differentiate into various $CD4^+$ T cell subtypes. Depending on the appropriate cytokine stimuli provided by the APCs, the activated transcription factors induced, and the sustaining cytokine environment at the time of presentation, these $CD4^+$ T cells expand and differentiate into various effector or regulatory phenotypes that can function to limit the pathogen, or serve as memory $CD4^+$ T cells that can combat pathogen after re-challenge. In certain cases, these $CD4^+$ T cells may be aberrantly activated and may contribute to immunopathology and autoimmunity.

The first discovered and consequently the best-characterized subsets are the Th1 and Th2 subtypes, which are known to secrete IFNγ and IL-4, respectively (249). The $CD4^+$ T cells generated in response to viral infections, including HSV-1, mainly have a Th1 phenotype and produce large amounts of IFNγ and IL-2 and express the transcription factor T-bet (251). This differentiation occurs in the presence of high levels of IL-12, type I IFNs, and IFNγ (252),

although Th1 cell responses may be generated in the absence of IL-12 and type I IFNs (253-255). Th1 helper $CD4^+$ T cells contribute to macrophage activation, inflammation, cell-mediated immunity and autoimmunity (256). On the other hand, Th2 cells secrete IL-4, IL-5, IL-13, and are generated in the presence of IL-4 (257). They function under the transcription factor GATA3 and induce allergic and helminth responses (258).

Another $CD4^+$ T cell subtype, Th17 cells, are characterized by IL-17 production and have been implicated in models of chronic inflammation, autoimmunity, and immunopathological conditions such as graft versus host disease (259), Type 1 diabetes (260), rheumatoid arthritis (261), and colitis (262). With regards to virus infections, Th17 effector responses have been characterized in models of HSV (263), mouse cytomegalovirus (MCMV) (264), influenza (265), and vaccinia virus (266). Th17 cells are generated in the presence of IL-6 and TGFβ and under the transcription factor retinoic acid receptor - related orphan γt (RORγt) (267, 268).

Another subset, the T regulatory cells (Tregs), suppress inflammatory responses by expressing molecules such as TGFβ, cytotoxic T lymphocyte activated (CTLA) – 4, IL-10, and galectin (269-271). Treg differentiation is induced by the transcription factor forkhead box P3 (FoxP3) (272, 273). Increased numbers of Tregs have been observed in human and animal models of virus infections and serve to both limit tissue damage and pathology especially in infections with West Nile Virus, respiratory syncytial virus (RSV), and influenza (274-276), and reduce overall magnitude of antiviral immune responses (277, 278).

The more recently characterized T cell subsets include follicular T helper (Tfh) cells, Th9 cells, and Th22 cells. T follicular helper cells are another specialized subset of $CD4^+$ T cells that provide B cell help through costimulatory CD40-CD40L interactions thus promoting B cell class switching. They are defined by the expression of transcription factor Bcl6, PD – 1, CXCR5,

inducible T cell costimulator (ICOS), and the cytokine IL-21 (279). Tfh cells are necessary for generating humoral responses against lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), influenza (280, 281), and human immunodeficiency virus (HIV) (282). Th22 cells, on the other hand, are defined by their expression of the cytokine IL-22. It is thought that these cells are involved in inflammatory and autoimmune diseases such as psoriasis, rheumatoid arthritis, Crohn's disease and atopic dermatitis (283). Lastly, Th9 cells are identified by their production of the cytokine IL-9 and require IFN-γ-regulatory factor 4 (IRF4) for their differentiation (284). These cells are implicated in the pathogenesis of autoimmunity, allergy, and asthma (285-287). There is not a lot known about the involvement of both Th9 and Th22 $CD4^+$ T cells in virus infections. There is substantial ambiguity however as to how many subsets there are, the relationships between the precursors of each subset and their outcomes, and the level of plasticity that each subset possesses.

The involvement of $CD4^+$ T cells in our model of HSV – 1 infection of the cornea will be elaborated on in a later section on the $CD4^+$ T cell-mediated immunopathology called herpes stromal keratitis, which is caused by HSV corneal infection.

1.3.2 $CD8^+$ T cells

$CD8^+$ T cells or cytotoxic T cells (CTLs) have the remarkable ability to specifically respond to pathogens by substantial expansion and differentiation into antigen-specific effector cells that can migrate to all tissues to clear the system of infections such as HSV-1. Indeed, it was work with LCMV-infected mice that initially showed the dual requirement for viral antigen plus self MHC I in presentation to $CD8^+$ T cells (288), as well as for the antigen-driven proliferation of these $CD8^+$ T cells (289, 290).

Based on T cell subset depletion studies and experiments with CD8-deficient mice, $CD8^+$ T cells contribute to resistance against intracellular infections with virus, bacterial, and protozoan pathogens. Without $CD8^+$ T cells in mice, HSV-1 clearance is impaired in the TG and leads to fatal encephalitis (291, 292).

During an infection, naïve $CD8^+$ T cells are primed by APCs in lymphoid organs such as the lymph nodes or spleen. $CD8^+$ T cells are located primarily in the T cell zones while DCs have an extensive network throughout the T and B cell zones and the subcapsular sinus of the draining lymph nodes (293). In parasite or virus infection models, it was shown that naive $CD8^+$ T cells encounter antigen-presenting DCs in the subcapsular sinus region or the interfollicular region of the DLNs (294, 295). Particulate antigen or pathogen is delivered via the lymphatics through passive migration or by migrating DC carriers. Even in the presence of other APCs such as $CD169^+$ macrophages lining the subcapsular sinus that get infected in the course of infection, naïve $CD8^+$ T cells preferentially interact with DCs (296).

Immediately after infection in the periphery, naïve $CD8^+$ T cells migrate quickly towards the peripheral regions of the T cell zone in response to either the gradient of cognate antigen (294) or to the dynamic changes in lymph node structure or chemokines levels (295, 297). Simultaneously, DCs relocate to the same area and acquire antigens from infected cells by direct infection or cross presentation. The proximity of the antigen – rich area to the subcapsular region maximizes the immune response in specialized areas of the lymph nodes.

At the peak of the primary response to the pathogen, there is a heterogeneous population of generated antigen-induced $CD8^+$ T cells that differ in phenotype and function. The majority of $CD8^+$ T cells are comprised of short lived effector cells (SLECs) that die after the infection is

cleared, while memory precursor effector cells (MPECs) that are less stimulated go on to survive and form a part of the memory $CD8^+$ T cell population (298, 299).

There is evidence that a single naïve $CD8^+$ T cell has the potential to differentiate into both effector and memory T cell subsets (300, 301). Contrastingly, it is also suggested that even before the first cell division, the fate of each $CD8^+$ T cell is already sealed as indicated by high degrees of variations in levels of yellow fluorescent protein (YFP) in responding $CD8^+$ T cells of IFNγ-YFP reporter mice (302). Later encounters with DCs and other factors such as inflammatory signals and costimulation may regulate the differentiation of effector $CD8^+$ T cells. Still, there are studies that imply that the heterogeneity in $CD8^+$ T cells is enforced at the time of the first cell division, as indicated by differences in expression of the transcription factor T-bet (303, 304).

Accordingly, once a naïve $CD8^+$ T cell divides, it can undergo as many as 19 cell divisions and this translates to a potential expansion of 500,000 fold (305). Maximal proliferation is achieved by the integration of multiple signals from the T cell receptor (TCR), costimulatory molecules, and inflammatory cytokines like IL-12 and type I IFNs (298, 299).

The first signal is recognized by the TCR as presented by the antigen-presenting cell in the context of MHC. The second set of signals comes from costimulation upregulated on APCs once it has received maturation signals (i.e. TLR activation). These costimulatory interactions include B7-CD28 or CD40-CD154 on DC – T cell respectively, and in their absence, T cells will experience antigen tolerance (306, 307). There are a lot of members of the TNF receptor (TNFR) family such as 4-1BB, OX40, glucocorticoid-induced TNF-related protein (GITR) and CD27, which function as important costimulatory molecules to $CD8^+$ T cells (308). The third set of signals originates from cytokines that activate T cells and promote differentiation into various

effector T cell subsets. IL-12 expressed by APCs supports the terminal differentiation of a naïve $CD8^+$ T cell capable of resisting intracellular pathogens like HSV-1 (309-312). IL-2 is another cytokine that promotes $CD8^+$ T cell differentiation by regulating T-bet and eomesodermin expression (304, 313) via a mammalian target of rapamycin (mTOR)-dependent signaling pathway (314).

Subsequently after antigen recognition, activated $CD8^+$ T cells upregulate chemokines receptors such as CXCR3 to allow infiltration into peripheral tissues (315). Another mechanism through which $CD8^+$ T cells are recruited into the site of infection is through $CD4^+$ T cell help. In an HSV-2 infection model, $CD4^+$ effector T cells infiltrate the vaginal tissue earlier than $CD8^+$ T cells, and express IFNγ to promote secretion of $CD8^+$ T cell – attracting chemokines CXCL9 and CXCL10 by the vaginal epithelium (316). This was also shown in the skin (317). Other peripheral tissues such as the lung and intestine do not exhibit the same $CD4^+$ T cell help requirement for effector $CD8^+$ T cell recruitment but there was no viral antigen detected in those models.

In our model of HSV-1 ocular infection, the sites where $CD8^+$ T cells migrate to are the cornea and trigeminal ganglia. T cell numbers peak 5 to 8 days after initial infection in C57Bl/6 mice, and at about 12 dpi in BALB/c mice. At the site of infection and upon recognition of an infected or foreign cell, $CD8^+$ T cells release lytic granules such as granzymes delivered to the target cell through pore-forming proteins called perforin, which allow entry of these proteins into the infected cell. Granzymes activate the cell's apoptosis machinery so it will undergo programmed cell death. $CD8^+$ T cells also express antiviral cytokines such as IFNγ and TNF.

T cell memory

After T cell expansion, T cells undergo contraction, where 90 – 95% of the effector $CD8^+$ T cells die. In the ensuing period, 5-10% of these T cells remain to become long-lived memory effector T cells. These memory $CD8^+$ T cells survive longer and rapidly respond upon re-challenge with the same antigen (318-321).

What signals do $CD8^+$ T cells receive to initiate and regulate the contraction phase? The inflammatory environment at the time of priming influences contraction. With limited inflammation, there is reduced effector T cell expansion and no contraction period (322). Among the factors that occur during priming which do not affect contraction include the duration of the initial TCR stimulus (323), and the kinetics of pathogen clearance (324, 325). One probable reason why contraction occurs is that there is a limited amount of cytokines that promote survival available for the expanded population of $CD8^+$ T cells. This deprivation may be responsible for cell death during contraction (326-330). Reintroducing these cytokines such as IL-2 (331) or IL-15 (329) during the contraction phase facilitates $CD8^+$ T cell survival and reduces the degree of contraction.

The second possibility could be that the fate of the cell is determined even before contraction transpires. It has been shown that there are two kinds of effector subsets that develops at the peak of expansion: a subset that is defined phenotypically as IL-7Rα^{high} and killer cell lectin-like receptor G1 (KLRG-1)^{low} and which preferentially survives the contraction phase (memory precursor cells); and a subset defined as IL-7Rα^{low} and KLRG-1^{high} which selectively undergoes cell death (terminally differentiated or short lived effector cells) (332). Terminally differentiated effector cells do not form functional memory cells and are eliminated in the contraction phase (321). After differentiating into memory cells, effector T cells maintain

expression of certain molecules and develop into either central memory or effector memory cells. Central memory cells are located within the lymphoid organs, are defined phenotypically by the expression of CD44, CD127, CD62L, and CCR7, and quickly respond to antigen re-challenge (333). Effector memory cells are found in peripheral tissues, are devoid of lymphoid organ homing receptors CD62L and CCR7, and maintain expression of CD44 and CD127. In comparison to central memory cells, effector memory cells are able to immediately secrete IFNγ and TNFα upon antigen stimulation (334).

HSV-1 and latency

As previously mentioned, the innate immune response in the form of macrophages and γδ T cells control viral replication in the trigeminal ganglia while the adaptive immune response is being developed. Once the virus establishes latency, the immune response is one of several factors that prevent viral reactivation. Following expansion of $CD8^+$ T cells in the DLNs, they migrate into the TG beginning 5-6 dpi, and peak in number by 8 dpi. These $CD8^+$ T cell numbers then decrease and maintain a stable population hereon to form the memory T cell pool even after latency is established. In both mice and humans, latently infected neurons are surrounded by activated $CD8^+$ T cells, indicating antigenic stimulation even in the absence of virion formation (60, 335, 336).

$CD8^+$ T cell expression of CD69 and granzyme B, and the inflammatory environment in the TG indicate dynamic involvement of the immune system during latency (56, 57, 64, 337). In the C57Bl/6 model of infection, approximately half of these $CD8^+$ T cells are specific for an immunodominant epitope on the viral protein glycoprotein B ($gB_{498-505}$) (64, 338). It has been recently discovered that the other half of these infiltrating $CD8^+$ T cells in the TG form a repertoire also specific for 18 other subdominant epitopes on 11 HSV proteins (339).

Differences in antigen presentation or processing, precursor frequency, and the route of infection may play a role in establishing this remarkable immunodominance.

Prior studies from our lab have demonstrated that HSV-specific $CD8^+$ T cells actively prevent the virus from reactivating in ex vivo latently infected neuronal cultures (59, 60). $CD8^+$ T cells protect from neuronal virus reactivation partly in a lytic fashion by secreting IFNγ (58, 340), and in a non-lytic mechanism by secreting lytic granules with granzyme B (63). IFNγ blocks reactivation by inhibiting the expression of immediate early genes ICP0, as well as blocking after the expression of late genes such as gH and gC (58, 340). Granzyme B is a lytic protein that enters the cell and induces apoptosis by cleaving caspases. In latently infected neurons, granzyme B cleaves an essential immediate early protein ICP4 thus halting virus replication and reactivation. More importantly, in an environment where it is imperative to eradicate the virus but protect the host, this mechanism does not kill the infected neurons that cannot regenerate.

1.4 IMMUNE RESPONSE AND IMMUNOPATHOLOGY

1.4.1 The pathogenesis and epidemiology of herpes stromal keratitis (HSK)

The research focus of our lab is the innate and adaptive immune response against HSV-1 infection of the cornea and the immunopathological consequence of this infection, herpes stromal keratitis. HSK is the leading cause of infectious blindness in developed countries. In the United States, ocular HSV-1 infections occur at about 37 years of age (341, 342), with about 12 out of 100,000 people getting newly infected annually (343). Although HSV-1 corneal infections

do not always lead to HSK, when HSK does develop, it presents as a progressive and recurring disease. The recurring nature of HSK is thought to be due to reactivation of latent HSV-1 in the TG, and shedding at the corneal surface (344). HSK may progress from either infectious epithelial keratitis, where epithelial dendritic shaped lesions are caused by replicating virus destroyed epithelial cells (345), or be the primary manifestation of HSK stromal involvement (346).

Clinically, HSK is characterized by opacity in the stroma, edema, and neovascularization believed to be triggered by persistent incidences of virus reactivation and shedding to the cornea (347-349). The robust immune response against viral proteins elicits the infiltration of immune cells, blood vessel ingrowth, and corneal stromal damage that contributes to opacity and edema. The inflammation can be suppressed by corticosteroids but subsequent scar tissue develops (350, 351). Clinical HSK is categorized as either necrotizing, characterized by necrosis, epithelial defect, active viral replication, and immune-mediated tissue damage in the stroma (345, 352); or non-necrotizing, defined by leukocytic infiltration and neovascularization, without viral replication (351).

Murine models of HSK have been developed to investigate disease pathogenesis. Like in human HSK, mouse HSK starts with dendritic epithelial lesions, and HSV also gains access to sensory neuron termini of mice during replication in the corneal epithelium. However, HSK in mice develops following primary infection and after viral clearance from the cornea, while HSK in humans is often accompanied by viral replication. Still, murine HSK closely resembles the immune or non-necrotizing keratitis in humans with dense leukocytic infiltrate and neovascularization.

In the mouse model, HSK starts at about 7 dpi, in the absence of viral replication. Stromal opacity worsens progressively and peaks in severity between 14 to 21 dpi. New blood vessels and lymph vessels creep into the avascular cornea and is induced by HSV-1 infection (348, 353, 354) via VEGF production of infected corneal epithelial cells and neutrophils (355). Blocking neovascularization by inhibition of VEGF or VEGF signaling is a possible therapeutic target to treat HSK (356-358).

The inflammatory infiltrate in the mouse cornea during HSK contain mostly neutrophils (~80 to 90%), DCs, $CD4^+$ T cells, and some $CD8^+$ T cells (148, 359-361). There is also a milieu of inflammatory and angiogenic factors which contribute to the stromal damage during HSK, including matrix metalloprotease 9 (MMP-9) (362), IL-17 (263, 363), IFNγ (364), and IL-2 (365).

1.4.2 The role of $CD4^+$ T cells in HSK

In earlier studies with HSK, it was shown that the disease did not develop in athymic nude mice that lack T cells (366) unless T cells were adoptively transferred into those mice, indicating that the immunopathology is T cell – dependent (367). Further investigation showed that $CD4^+$ T cells in particular were responsible for mediating HSK in mice (368-370), and that $CD4^+$ T cell – depleted mice or CD4 – deficient mice do not readily develop HSK (371, 372). This is logical since a majority of the T cells in HSK corneas are $CD4^+$ T cells rather than $CD8^+$ T cells (373). Research in our lab has shown nevertheless that in CD4 – deficient mice, $CD8^+$ T cells can mediate HSK, albeit transient and less severe, if the mice are infected at a higher dose (372).

Indeed, $CD4^+$ T cells that produce Th1 cytokines IL-2 and IFNγ induce neutrophil infiltration during HSK. IL-2 promotes chemotaxis and survival of neutrophils while IFNγ

upregulates platelet endothelial cell adhesion molecule (PECAM)-1 expression on blood vessel endothelial cells, thus encouraging neutrophil migration into the cornea (148, 365). Blocking either of these cytokines inhibits progression of the disease (364).

These $CD4^+$ T cells are also a source of IL-17 (Th17 $CD4^+$ T cells), and they appear to be required for the infiltration of $CD4^+$ T cells that secrete IFNγ (Th1 $CD4^+$ T cells). Also, inhibiting IL-17 early during the infection and late during HSK progression reduces HSK severity (263). These studies are consistent with the concept of $CD4^+$ T cells (Th1 and Th17) orchestrating HSK disease development. But what is stimulating $CD4^+$ T cell activity and how? Is viral antigen required for HSK to occur? The mechanism by which the disease starts is uncertain, but there have been three proposals to explain how $CD4^+$ T cells trigger HSK: 1) molecular mimicry, 2) bystander activation, and 3) HSV-1 specificity.

Cantor's group proposes HSK is a $CD4^+$ T cell - mediated autoimmune response to host antigens that have very similar epitopes to the HSV-1 viral protein UL6 (374-377). In their model of autoimmune HSK however, immunopathology was only demonstrated in infections with only one HSV-1 strain (KOS), and did not occur in another (RE). Moreover, another group showed that $CD4^+$ T cells specific for the UL6 protein was not able to induce HSK, and that there is no cross reactivity between the UL6 protein and corneal proteins (378). It was also shown that isolated $CD4^+$ T cell clones from HSK corneas in humans do not recognize UL6 or corneal antigens (379).

The second theory by Rouse's group proposes that antigens specific to HSV are not particularly required for HSK and that the inflammatory environment induced during HSK is enough to stimulate effector T cell activity and induce bystander $CD4^+$ T cells to mediate disease. Their studies were performed in HSV-infected mice with $CD4^+$ T cells specific for

ovalbumin (OVA). These mice developed severe disease after HSV-1 infection, and OVA-specific $CD4^+$ T cells were found in their corneas. It was also shown that depleting these $CD4^+$ T cells abrogated the disease (380). Yet because these mice were not capable of mounting an immune response against HSV-1, virus clearance was impaired and HSV infection was lethal to the mice. When acyclovir was employed to limit virus replication, inflammation was diminished in comparison (381). This indicates that infectious virus can stimulate bystander activation of $CD4^+$ T cells to induce inflammation, but not necessarily in mice that can control virus replication. Bystander $CD4^+$ T cells may contribute to the disease but the aforementioned studies also suggest that HSV-1 specific $CD4^+$ T cell effector function is required for initiating and prolonging HSK.

Our lab proposes that this is the case. $CD4^+$ T cells specific for HSV antigens have been derived from human (379, 382) and mice corneas (our lab's unpublished observation). Furthermore, when mice were tolerized to HSV-1 antigens at the time of infection, the mice were less vulnerable to disease (383). The likelihood is bystander activation of non HSV-specific $CD4^+$ T cells and stimulation of HSV-specific $CD4^+$ T cells both contribute to disease progression and maintenance. In the absence of infectious virus at the time of HSK onset, it is possible that HSV-1 antigens are shed into the corneal surface for HSV-specific $CD4^+$ T cells to respond to and this initiates disease. The cytokine milieu that is established may play a role in bystander activation of other $CD4^+$ T cells and this may prolong disease.

1.4.3 Antigen – presenting cells in HSK

Since $CD4^+$ T cells orchestrate HSK, and it is apparent that there is a requirement for HSV antigen specificity to establish the disease, what presents viral antigen to these $CD4^+$ T cells? Do

the APCs present in the cornea during HSK play a role in disease progression? Interestingly, there is a large influx of DCs that enter the cornea that coincides with when the disease starts. A previous study from our lab has suggested that APCs in the cornea during HSK might play an important role. In the experiment, presumed Langerhans cells (as defined by MHC II) were ablated from mice corneal epithelium by UV-B irradiation in one eye, and left untouched in the other, and then the mice were infected bilaterally in both eyes. This allowed a normal adaptive immune response to develop against HSV-1 because one of the mice eyes had intact APCs and this allowed the question of would HSK develop in the absence of APCs. Disease developed in the non-depleted eye, but not in the depleted one; which indicated a requirement for APCs to stimulate $CD4^+$ T cells during HSK (384). We now know that both DCs and macrophages present in the cornea express MHC II, and this study did not detail which population was ablated and for how long. In a study comparing HSV-1 strains with distinct differences in inducing HSK severity, it was shown that HSV-1 KOS infected corneas, the virus of which generates lower occurrences and milder levels of HSK severity in mice than the strain HSV-1 RE, has less DCs infiltrating into the central cornea (369).

Additionally, a critical factor for T cell activation is the engagement of costimulatory molecules. Further proof for the requirement for APCs is that blocking the costimulatory molecule B7.1 in the cornea deterred the development of HSK (385), and so did blocking 4-1BB and 4-1BB ligand interactions (386). Neither interactions between OX40 and OX40L nor CD40 and CD40L were essential however (387, 388). By and large, even if the importance of APCs in HSK has been implied, the question of which particular antigen presenting cells and how they contribute to HSK has not been elucidated completely.

1.5 THE ROLE OF COSTIMULATION IN ADAPTIVE IMMUNITY

The initiation of antigen-specific T cell responses relies on the interaction between the T cell receptor and peptide-MHC complexes. Apart from TCR signaling, signals mediated through cell surface costimulatory receptors and their respective ligands expressed on APCs are required for proliferation and survival of T cells. There is differential expression of costimulatory receptors on T cells, and ligand expression is tightly regulated, indicating specific roles for each costimulatory interaction between receptor and ligand.

Costimulatory molecules are generally categorized into two families: the immunoglobulin (Ig) superfamily of CD28 and ICOS (CD278) and their ligands (389); and the TNFR and TNF superfamily (CD40-CD40L, CD27-CD70, OX40/CD134-OX40L/CD252, and 4-1BB/CD137-4-1BBL/CD137L) (390). This introduction will focus on two of the best-characterized costimulatory interactions: between CD28 and CD80/CD86, and CD40 and CD154.

1.5.1 CD28/CTLA-4 and CD80/CD86

CD28 is expressed constitutively on naïve T cells (391), and promotes proliferation after binding with ligands CD80 or B7.1 and CD86 or B7.2. These interactions are hampered by interactions of the B7 molecules with the inhibitory receptor CTLA-4 (CD152). CTLA-4 is the higher affinity receptor for CD80 and CD86 (392-394), and its expression is immediately upregulated after T cell activation (395). On most APCs, CD86 is constitutively expressed at low levels and is rapidly upregulated, whereas CD80 expression is induced only after APC maturation and activation (396, 397). The expression of CD80 and CD86 is modulated by the activation state of

the APC. Innate receptors on APCs recognize stress, infection, and cellular damage, which lead to induction of transcription, translation, and recruitment of CD80 and CD86 to the cell surface. The expression of CD80 and CD86 is modulated by the activation state of the APC (397, 398).

Members of the CD28 family are characterized by a variable Ig like extracellular domain and a short cytoplasmic tail. Upon binding of CD80 or CD86 on APCs to CD28 on T cells, signaling downstream is mediated through the phosphatidylinositol 3-kinase-protein kinase B (Akt) pathway and growth factor-receptor-bound protein 2 (Grb2) (399, 400). Ligation of the CD28 molecule promotes recruitment of lipid rafts to the immunological synapse which lowers the activation threshold of the T cell and enhances the magnitude of the overall T cell response (399). CTLA-4 expression is then upregulated instantly and regulates T cell responses negatively through different mechanisms. It is able to sequester CD80 and CD86 away from CD28 by its high affinity to these molecules. It can recruit phosphatases such as Src homology region 2 domain-containing phosphatase 1 (SHP-1) and SHP-2, thereby, dephosphorylating downstream signals of the TCR. It transduces a signal into the APCs, consequently enhancing the catabolism of tryptophan, thereby creating an inhibitory environment for the T cell (401, 402). Once CTLA-4 is upregulated, the expression of CD28 is subsequently downregulated by endocytosis (403).

CD80 and CD86 have largely redundant functions. Synergized with signals from the TCR, they both provide critical co-stimulatory signals to increase and maintain a T cell response through interacting with CD28 (306). CD28 ligation by itself does not have an effect on T cell activation, but with TCR signaling it regulates the threshold for T cell activation and considerably decreases the amount of TCR engagements required for T cell activation (404). The outcome of this interaction is increased IL-2 production, upregulation of CD25 (the IL-2 receptor α chain), entry of the T cell into the cell cycle, and enhanced T cell survival through the

upregulation of the antiapoptotic molecule Bcl-XL (399, 400). CD28 signaling leads to augmentation and sustaining of T cell responses by promoting T cell survival to enable cytokines to effect expansion and differentiation of T cells (405-409). The responses of previously activated T cells are also optimized by CD28 through enhancing IL-2 production and T cell survival.

Costimulatory molecules orchestrate critical interactions to enhance antiviral T cell responses. Various characteristics of viral infections establish which costimulatory molecules are necessary for the immunity of the host. Additionally, viruses have evolved numerous mechanisms of immune evasion to hinder T cell costimulation, therefore studying and targeting T cell costimulation improves antiviral immunity. The absence of CD28 signaling negatively impacts $CD4^+$ and $CD8^+$ T cells (410). Several murine models of acute viral infection depend heavily on CD28 costimulation such as infections with influenza A virus (281, 411), VSV (412), and vaccinia virus (413). During influenza infections, signals downstream of CD28 ligation are necessary for IL-2 mediated T cell survival and expansion (414), IL-2 production by memory T cells (415), and secondary immune responses are impaired when CD28 and CD80/CD86 interactions are inhibited (416). Lack of CD28 interactions was shown to promote reduction of the immune response specific to subdominant epitopes to influenza (411). Furthermore, memory $CD8^+$ T cell responses and viral clearance depend on CD28 costimulation during secondary responses against influenza and HSV-1 (417).

For viruses that are able to establish latency such as murine gamma herpes virus-68 (HV-68) (413, 418, 419) and murine cytomegalovirus (MCMV) (420), $CD8^+$ T cell responses and control of virus replication require CD28-CD80/CD86 costimulation. $CD4^+$ and $CD8^+$ T cell

responses specific to HSV-1 were also ablated in infected mice where CD28 interactions were blocked (421).

This dependence on CD28 costimulation does not apply for all virus infections however. For instance, the magnitude of acute $CD8^+$ T cell responses specific for LCMV does not change when there is a lack of CD28 and CD80/CD86 interactions (407). Subsequent studies have shown that in models where there is strong TCR signaling, (like in acute LCMV infections), CD28 costimulation may not be as critical (422), and the requirement for costimulation is overcome (404, 423). There is rapid and extensive replication of LCMV and persistence of virus and viral antigen at high levels that may provide a strong and sustained TCR signal to override the need for CD28 costimulation. The lack of dependence for CD28 may possibly be due to differences in the duration of TCR signaling (404). Nevertheless, without CD28 costimulation, LCMV-specific memory precursors are found to be defective phenotypically and qualitatively (424, 425). Indeed, CD28 costimulation widely impacts the development of virus-specific T cell responses.

1.5.2 CD40-CD40L/CD154

Due to its critical role in immunity, one of the best-described costimulatory molecules is the receptor CD40. CD40 is a member of the TNFR family, and is expressed by B cells, professional APCs, and non-immune cells and tumors. It binds CD40L or CD154, which is expressed on activated T cells and in other non-hematopoietic cells under inflammatory conditions. CD40 signaling regulates a variety of molecular and cellular processes that include the initiation and progression of cellular and humoral immunity.

CD40 is a hydrophobic transmembrane protein with an extracellular domain that is homologous to other TNF family members. Its expression was initially described on B cells, and now has been characterized on DCs, monocytes, macrophages, platelets, and non-immune cells such as epithelial and endothelial cells, as well as myofibroblasts and fibroblasts (426-428). Although its expression is constitutive on some cell types, CD40 expression may be regulated by secreted factors such as IFNγ, IL-1, IL-3, IL-4, TNFα (429-436), granulocyte/macrophage colony-stimulating factor (GM-CSF) (437), HIV (438), Epstein-Barr virus (EBV) (439), phorbol esters (440), Mycobacterium tuberculosis bacilli (441), 12-O-tetradecanoy phorbol-13-acetate (TPA) (442), antibodies against IgM or CD20 (442), as well as ultraviolet light exposure (443).

CD40L/CD154 is a type II transmembrane protein, the expression of which has been observed on activated mature T cells and γδ T cells. However, an inflammatory environment can induce CD154 expression on monocytes, NK cells (444), B cells (445), macrophages, and DCs (446). CD154 expression has been induced on vascular endothelial and smooth muscle cells (447-449), basophils (450, 451), eosinophils (452), platelets (453, 454), and mast cells (450, 455).

The ligation of CD40 to CD154 facilitates the trimerization of CD40 and the direct or indirect recruitment of the adapter proteins TNFR-associated factors (TRAFs) to the cytoplasmic domain of CD40 (456). TRAF protein recruitment leads to the activation of the canonical and non-canonical nuclear factor (NF)-κB pathways, as well as the mitogen-activated protein kinase (MAPKs), phosphoinositide 3-kinase (PI3K), and phospholipase Cγ (PLCγ) pathways. Upon ligand binding of CD40L to CD40, TRAF family members TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6 are recruited to CD40 cytoplasmic domains. Depending on the cell type involved, CD40 mediates signaling through TRAF proteins and can either activate or inhibit various

signaling pathways. On the other hand, CD40 signaling has been shown to occur independent of the TRAF proteins and through the Janus family kinase 3 proteins (Jak3). Direct binding of Jak3 to the CD40 cytoplasmic domain induces STAT5 phosphorylation (457, 458).

Besides being a key regulator in T cell costimulatory signals, CD40 ligation upregulates expression of other numerous costimulatory ligands on antigen presenting cells, such as CD80, CD86, 4-1BBL, OX40L, and CD70 (459-464). Therefore CD40 interactions influence T cell costimulation indirectly by activating APCs. CD40/CD40L binding is critical for the formation of germinal centers and antibody class switching. It is also involved in generating $CD8^+$ T cell memory (465-467). Nonetheless, CD40 is indispensable in primary $CD8^+$ T cell responses against VSV infections, but not of LCMV (468). $CD4^+$ T cell responses such as cytokine production and expansion specific to LCMV however, depend on CD40 interactions. It has also been shown that CD40-CD154 costimulation is required for generating antiviral $CD4^+$ T cell responses but is dispensable for $CD8^+$ T cell responses (469). Similarly, blockade of CD40/CD40L interactions has a greater impact on $CD4^+$ T cells than $CD8^+$ T cells in the HSV-1 skin infection model (421). Therefore, the requirement for $CD4^+$ and $CD8^+$ T cell costimulation is varied, and depends on the nature of the virus and the ensuing viral infection. Additionally, one or more costimulatory molecules are critical for the development of T cell-dependent immunity.

2.0 STATEMENT OF THE PROBLEM

Dendritic cells are a heterogeneous lineage of innate immune cells that perform critical functions in host defense against attacking pathogens, which include HSV-1. They are strategically located at the interface of self and non-self, in lymphoid organs, and in mucosal tissues such as the skin and the cornea. Though its presence in an immune privileged site such as the cornea has long been confirmed, in an environment that lacks blood and lymphatic vessels, and immunosuppressive and anti-inflammatory factors are present, it is intriguing what role these antigen-presenting cells would play.

Interaction between dendritic cells and herpesviruses mainly takes place by contact with host mucosa. Although this association promotes lifelong protective immunity, in the immediate scenario, it can lead to colonization of the host instead. This suggests that dendritic cell function is not completely abolished by infection and that the mechanism by which protective immunity is achieved should be investigated. How the DCs in the cornea may respond to HSV infections to generate immunity is highly relevant for conceiving novel antiviral therapies and vaccines against these pathogens.

Consequently, studying the role of corneal dendritic cells in the context of herpes stromal keratitis is also important. There is no live virus detected in the cornea once disease is initiated, and $CD4^+$ T cells mediate the neutrophilic infiltrate that cause the tissue scarring and destruction

in the cornea. Simultaneous with the development of opacity, dendritic cells enter the cornea as well. Whether they contribute to disease persistence and establishment is not known.

3.0 SPECIFIC AIMS

1. Determine the relative contribution of cornea-resident dendritic cells, cornea-infiltrating dendritic cells, and lymph node-resident dendritic cells in expanding the $CD8^+$ T cells in the draining lymph nodes after infection.

Hypothesis: Cornea-derived (cornea-resident and cornea-infiltrating) dendritic cells are necessary in generating HSV-specific $CD8^+$ T cells in the DLNs after infection.

2. Determine the role of cornea dendritic cells in expanding the $CD4^+$ T cells in the draining lymph nodes after infection, as well as in the re-stimulation of $CD4^+$ T cells in the cornea during herpes stromal keratitis.

Hypothesis: Cornea-derived (cornea-derived and cornea-infiltrating) dendritic cells are necessary in generating HSV-specific $CD4^+$ T cells in the DLNs after infection. Dendritic cells present in the cornea during HSK are important in the progression of HSK.

4.0 MATERIALS AND METHODS

4.1 REAGENTS

The following fluorochrome-labeled antibodies were used: PerCP-conjugated anti-CD45 (clone 30-F11), phycoerythrin (PE)-Cy7-conjugated anti-CD4 (clone RM4-5), allophycocyanin-Cy7 (APC-Cy7)-conjugated anti-CD8 alpha (clone 53-6.7), fluorescein isothiocyanate (FITC)-conjugated anti-CD80 (clone 16-10A1), and PE-conjugated anti-I-A/I-E (clone M5/114.15.2) were purchased from BD Pharmingen (San Diego, CA).

eFluor 450-conjugated anti-CD3 (clone 17A-2), eFluor780-conjugated anti-CD3 (clone 17A-2), V450-conjugated anti-CD11b (clone M1/70), allophycocyanin-conjugated anti-CD11c (N418), PE-conjugated anti-CD70 (clone FR70), allophycocyanin-conjugated anti-CD80 (clone 16-10A1), allophycocyanin-conjugated anti-CD90.1 (also known as Thy1.1, clone HIS51), and PE-Cy7-conjugated anti-I-A/I-E (clone M5/114.15.2) were purchased from eBiosciences (San Diego, CA).

Pacific Blue-conjugated anti-CD40 (clone 3/23), PE-Cy7-conjugated anti-CD86 (clone GL-1), Alexa Fluor 780-conjugated anti-I-A/I-E (clone M5/114.15.2), and APC-Cy7-conjugated anti-Ly6c (cloneHK1.4) were purchased from Biolegend (San Diego, CA). The appropriate isotype control antibodies were purchased from their respective vendors.

4.2 MICE

4.2.1 Mice strains

Female wild-type (WT) C57BL/6J, BALB/cJ and CD11c-DTR [C.FVB-Tg(Itgax-DTR/EGFP)57Lan/J and B6.FVB-Tg(Itgax-DTR/GFP)57Lan/J] mice, 6–8 week old, were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice transgenic for the HSV glycoprotein B specific T-cell receptor (gB-T) were a gift from Francis Carbone (470). The gB-T mice were crossed onto Thy1.1 background by mating gB-T mice to Thy1.1^{+} mice and back crossing them for four generations to yield homozygous Thy1.1^{+} gB-T^{+} mice. All experimental animal procedures were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee and adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

4.2.2 Virus purification and HSV-1 infection of the cornea

HSV-1 strain RE was grown in Vero cells and intact virions were isolated on Optiprep gradients according to the manufacturer's instructions (Accurate Chemical and Scientific, Westbury, NY). For ocular HSV-1 infection, mice were anesthetized by intraperitoneal (i.p.) injection of 2.0 mg ketamine hydrochloride and 0.04 mg xylazine (Butler Schein, Pittsburgh, PA) in 0.2 ml HBSS (Mediatech, Manassas, VA). The abraded central corneas of anesthetized mice were infected by topical application of 3 μl RPMI 1640 (Lonza, Walkersville, MD) containing 1×10^5 plaque forming units (PFUs) of virus. All animal experiments were conducted in accordance with

guidelines established by the University of Pittsburgh Institutional Animal Care and Use Committee.

4.2.3 Generation of bone marrow chimeras

Bone marrow chimeras were created by transferring bone marrow from CD11c-DTR mice into lethally irradiated WT BALB/cJ or WT C57Bl/6 mice to avoid lethality associated with multiple DT treatments of CD11c-DTR mice (471). Briefly, BALB/cJ or C57Bl/6 hosts underwent two treatments of 500 rads in an animal γ-irradiator, and 2.0×10^6 bone marrow cells from CD11c-DTR respective donors were transferred intravenously. The resulting mice (referred to herein as CD11c-DTR chimeras) were housed under immunocompromised mouse conditions and treated regularly with 2 mg/ml neomycin from Sigma-Aldrich (St. Louis, MO) in their drinking water. The CD11c-DTR chimeras were fully reconstituted and ready for experimental use after 8 weeks.

4.2.4 In vivo diphtheria toxin treatments

Diphtheria toxin (DT) purchased from Sigma–Aldrich was prepared in a sterile solution of PBS at a concentration of 1 mg/ml. Transient DC depletion was effected in CD11c-DTR chimeras by a single i.p. injection of 175 ng DT; continuous DC depletion entailed additional i.p. treatments of 100 ng DT per mouse every 3 days. Local DC depletion in the cornea was effected by a single injection to the conjunctiva (subconjunctival or s.c.) of 30 ng DT; continuous DC depletion was done with additional subconjunctival treatments of 30 ng DT per mouse eye every 3 days.

4.2.5 In vivo antibody treatments

Systemic CD154 blockade was performed by an i.p. treatment with 250 ug hamster anti-MR-1 (BioXCell, West Lebanon, NH) at -1, 2, and 5 dpi. Systemic CD86 blockade was accomplished by an i.p. treatment with 200 ug rat anti-GL-1 (BioXCell, West Lebanon, NH) at -2, 1, and 4 dpi; local CD86 blockade was performed by an s.c. treatment with 20 ug of the antibody to the infected eye.

4.2.6 Scoring of herpes stromal keratitis

Mice were monitored for HSK on alternate days between 7 and 21 days post-infection (dpi) by slit lamp examination. A standard scale ranging from 1 – 4 based on corneal opacity was used: 1+ mild corneal haze, 2+ moderate opacity, 3+ complete opacity, 4+ corneal perforation. Disease incidence was defined as HSK score greater or equal to 2 by 15 dpi.

4.3 PHENOTYPIC ANALYSIS OF CELLS

4.3.1 Tissue preparation for flow cytometry

At indicated times, draining lymph nodes and corneas were harvested from euthanized mice. DLNs were minced to yield single cell suspensions and incubated with 500 µl DMEM (Lonza) containing 10% FCS (Atlanta Biologicals, Atlanta, GA) and 420 U/ml collagenase type I (Sigma-Aldrich) for 30 min at 37°C. For trigeminal ganglia excision, mice were anesthetized and

injected with 0.3 ml heparin (1000 U/ml) and then euthanized by exsanguination. Excised TG were digested in 100 µl per TG of DMEM (BioWhittaker) containing 10% FCS and 400 U/ml collagenase type I (Sigma-Aldrich) for 1 hour at 37°C, and dispersed into single cell suspensions by trituration through a p-200 pipette tip. Staining was performed with designated antibodies for 30 minutes at 4°C unless otherwise indicated. Data were collected on a FACSAria cytometer and analyzed by FACSDiva software (BD Biosciences).

4.3.2 Isolation of dendritic cells

The draining lymph nodes were removed from mice infected at the time periods indicated in the figures, and were minced and digested in 420 U/mL collagenase type 1 (Sigma-Aldrich) at 37°C for 30 min and treated with 120 IU/mL DNAse (Sigma) for 15 min at room temperature. Cell suspensions were prepared by processing through a 70-µm cell strainer (BD) and washed with PBS. The cells were then treated with 0.5 M EDTA and were incubated at room temperature for 5 min. Single cells were incubated with 100 uL antibody mix provided in the Dynabeads Mouse DC Enrichment Kit (Invitrogen) to deplete T cells, $mIgM^+$ B cells, NK cells, erythrocytes, and most granulocytes, under continuous shaking for 20 min at 4°C. Resuspended Depletion MyOne SA Dynabeads (Invitrogen) were incubated with the cells for 15 min at 4°C. Bead-absorbed cells were removed using a Dynal magnet (Invitrogen). Non-depleted cells were stained with the antibodies mentioned earlier after incubating for 10 min at 4°C in the presence of anti-CD16/32 (clone 2.4G2; BD) antibody to block Fc receptors. Stained cells were sorted and analyzed by FACSAria. Post isolation analyses indicate 70-85% purity of all DC populations.

4.3.3 Tracking of migratory dendritic cells through FITC painting

Mice were anesthetized and corneas were painted with 3 µl of a 1% FITC solution in acetone/dibutyl phthalate (1:1 ratio; Sigma-Aldrich). The cervical lymph nodes were isolated, and single cell suspensions were prepared as described in DC isolation and sorting.

Isolated lymph node cells were first enriched for DCs by depleting NK cells, T cells, B cells, and erythrocytes, and were stained with eFluor 450-conjugated anti-CD3 (clone 17A-2; eBioscience), V450-conjugated anti-CD11b (clone M1/70; eBioscience), allophycocyanin-conjugated anti-CD11c (clone N418; eBioscience), Pacific Blue-conjugated anti-CD40 (clone 3/23; Biolegend), FITC-conjugated anti-CD80 (clone 16-10A1; BD Pharmingen), and PE-Cy7-conjugated anti-CD86 (clone GL-1; Biolegend).

4.3.4 Tracking of surrogate antigen to the draining lymph nodes

In experiments using surrogate antigen, 20 ug of Alexa Fluor 594 conjugated to ovalbumin (OVA – AF594) (Life Technologies, Carlsbad, CA) in 3 uL PBS was applied topically onto mice corneas after infection.

4.3.5 Phenotypic analysis

For all phenotypic analyses, cells were stained for CD45 to permit gating exclusively on bone marrow-derived cells. Dendritic cells were defined as $CD11c^+$ $CD3^-$ cells. For analysis of $CD8^+$ and $CD4^+$ T cell populations and their phenotype, cells were stained with anti-CD3 to gate on T cells, and then with anti-CD8α and anti-CD4. For analysis of HSV-1 specific $CD8^+$ T cells (gB-

CD8), cells were additionally co-stained with anti-CD8α and with tetramers to glycoprotein B $(gB)_{498\text{-}505}$, ribonucleoreductase 1 $(RR1)_{822\text{-}829}$, $RR1_{982\text{-}989}$, and infected cell protein 8 $(ICP8)_{171\text{-}178}$.

4.3.6 Measurement of T cell proliferation

Infected mice received an i.p. injection of 1 mg bromodeoxyuridine (BrdU) per mouse to measure T cell proliferation. DLN and TG were excised 4 hours after BrdU injection, and dispersed cells were stained with anti-CD45, anti-CD8α, and anti-CD4 for 30 mins at 4°C, and $gB_{498\text{-}505}$ tetramer, $RR1_{822\text{-}829}$ tetramer, and $ICP8_{171\text{-}178}$ and $ICP8_{876\text{-}883}$ tetramers for 1 hour at room temperature. $CD4^+$ and $CD8^+$ T cells that divided over the 4 hour period were quantified by flow cytometry using a BrdU proliferation assay kit (catalog no. 559619; BD Pharmingen) according to manufacturers' instructions.

4.3.7 Measurement of T cell apoptosis

A Caspatag assay kit (Chemicon International, Cat # APT420) was used according to manufacturer's instructions to quantify cells $CD8^+$ T cells undergoing apoptosis. Single cell suspensions of DLN cells from infected mice were stained with antibodies to CD45, CD8, and CD4. Cells were then washed with FACS buffer, incubated with Caspatag FLICA reagent (1µl of the 30X FLICA reagent per TG and 10 uL of the 30X FLICA reagent per lymph node) for 1 hour at 37°C, and were washed twice with Caspatag Wash buffer before flow cytometry analysis.

4.4 WHOLE MOUNT FLUORESCENCE MICROSCOPY

Whole corneas were excised, flattened by making radial incisions, washed in PBS 4% FBS, and incubated with anti-CD16/CD32 for 1 hour at 4°C. They were then treated with 1% paraformaldehyde for 2 hours, washed two more times in PBS 4% FBS, incubated with the appropriate antibodies overnight at 4°C, washed in PBS 4% FBS for 15 min at 4°C three more times, and mounted. DLNs were excised, cut in half, carefully washed in PBS 4% FBS, incubated with anti-CD16/CD32 for 1 hour at 4°C, incubated with the appropriate antibodies overnight, and then were washed in PBS 4% FBS for 20 min five times. Lymph nodes were then treated with 2% paraformaldehyde for 2 hours at 4°C, and then washed in PBS 4% FBS for 20 min three times before mounting. Images were acquired on an Olympus Fluoview 1000× confocal microscope with a 1.4 NA 60× oil objective or a 0.85 NA 20× oil objective. Images were acquired by sequential scanning to avoid fluorescence crossover, and Z stacks were acquired at Nyquist sampling frequency through the tissue. All image reconstructions were made using Metamorph.

4.5 STATISTICAL ANALYSIS

All statistical analyses were computed with GraphPad Prism software, using one way ANOVA with Bonferonni's posttest and unpaired Student's *t* tests. The p values < 0.05 were considered statistically significant.

5.0 THE ROLE OF DENDRITIC CELLS IN THE EXPANSION OF $CD8^+$ T CELLS IN THE DRAINING LYMPH NODES AFTER CORNEAL INFECTION

Not unlike other mucosal surfaces, the immune-privileged cornea has its own resident dendritic cells located in the corneal epithelium of mice and humans. The dendritic cell paradigm suggests that these DCs can take up antigen and transport them to the draining lymph nodes for presentation to T cells. Various murine models have indicated that this is not always the case. There exists distinct DC subpopulations depending on phenotype, development, and function that may play specialized roles in antigen presentation and cytokine secretion, as well as induction of T cell tolerance and immunity. A particular DC subset is implicated depending on the antigen route, the nature of the pathogen, and the resident DC subset at the site of pathogen uptake. The roles of DCs in the cornea (cornea-resident DC), the ones infiltrating after HSV-1 infection (cornea-infiltrating DC), and the DC resident to the DLN (DLN-resident DC) are not well understood.

5.1 SUBCONJUNCTIVAL INJECTION OF DIPHTHERIA TOXIN DOES NOT REMOVE LYMPHOID RESIDENT CD11C^{HI} CELLS

CD11c-DTR chimeras (CD11c-DTR bone marrow into WT mice recipients) on both BALB/c and C57Bl/6 backgrounds were generated as described in Materials and Methods to circumvent issues of continuous diphtheria toxin (DT) treatment in CD11c-DTR mice (471). DCs expressing enhanced GFP under the CD11c promoter completely reconstituted the cornea and draining lymph nodes of CD11c-DTR chimeras as early as 6 weeks (115). All CD11c-DTR chimeras were used 8 weeks after bone marrow reconstitution.

DT treatment selectively ablates CD11c-expressing cells in the cornea. Resident macrophages in the cornea do not express CD11c, and therefore are not affected by DT treatment. Natural killer cells and plasmacytoid dendritic cells reportedly express some levels of CD11c (472-474). However, no pDCs are detected in the cornea after HSV-1 infection, and while natural killer cells do infiltrate, they are not depleted by DT treatment (115).

To address the importance of cornea-resident DCs, ensuring that local diphtheria toxin treatment into the conjunctiva of mice eyes (subconjunctival treatment) only depleted corneal dendritic cells, and that DT leakage doesn't occur in the DLN where it can deplete DLN – resident DCs were critical. Treatment with DT i.p. (systemic DC depletion) 2 days before infection effectively depleted CD11c^{+} DCs from the corneas (**Fig. 3A, 3B**) and lymph nodes (**Fig. 3C**) of reconstituted CD11c-DTR chimeras for 3 days. Subconjunctival (s.c.) treatment of DT (corneal DC depletion) 2 days before infection efficiently depleted CD11c^{+} DCs in the cornea (**Fig. 3A, 3B**) for 3 days, but did not ablate the CD11c^{+} DCs in the DLNs (**Fig. 3C**). Subconjunctival treatment of corneas with PBS (no DC depletion) does not affect DCs in the corneas (**Fig. 3A, 3B**) and lymph nodes (**Fig. 3C**) of CD11c-DTR chimeras.

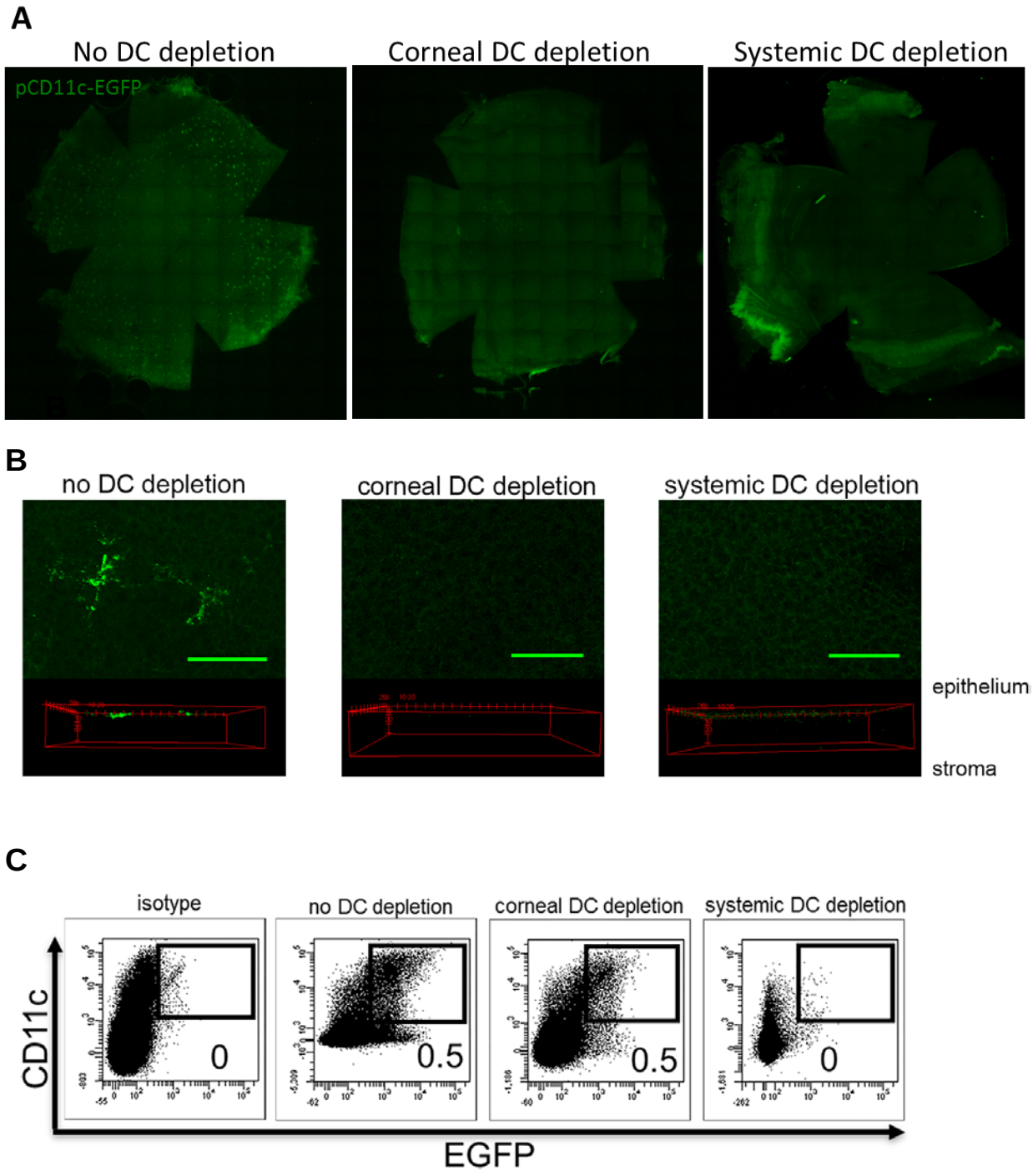

Figure 3. Selective depletion of resident corneal DCs.

BALB/c CD11c-diptheria toxin receptor (DTR) bone marrow chimeras were given subconjucntival injections of 30 ng diptheria toxin (corneal DC depletion), intraperitoneal injections of 150 ng DT (systemic DC depletion), or PBS

(no DC depletion). Two days after treatment, corneas were excised, fixed, and mounted for confocal microscopy imaging. A) Representative images taken of the whole corneas of mice that acquired no DC depletion, corneal DC depletion, or systemic DC depletion demonstrate effective depletion of dendritic cells with both DT treatments. B) Representative z-stack and three dimensional projections from the paracentral region of the cornea show both DT treatments effectively deplete DCs in the cornea. C) Lymph nodes were excised 2 days after treatment, treated with collagenase to yield a single cell suspension, stained with antibodies to CD11c, and analyzed for EGFP and CD11c expression. Representative flow cytometry plots show depletion of $CD11c^+$ $EGFP^+$ cells after systemic DT treatment, but not after corneal DT treatment. Numbers on flow cytometry plots are percentages of $CD11c^+$ $EGFP^+$ cells gated on $CD45^+$ cells. Data are representative of three experiments, with 3 to 5 mice per group.

5.2 DENDRITIC CELLS BEGIN TO RECONSTITUTE THE CORNEAL TISSUE 3 DAYS AFTER DIPHTHERIA TOXIN TREATMENT

Dendritic cell depletion only lasts for three days. After local or systemic DT treatment at -2 dpi, $CD11c^+$ $EGFP^+$ DCs repopulate the cornea (**Fig. 4A**) and DLN (**Fig. 4B**) of chimeras at 1 dpi.

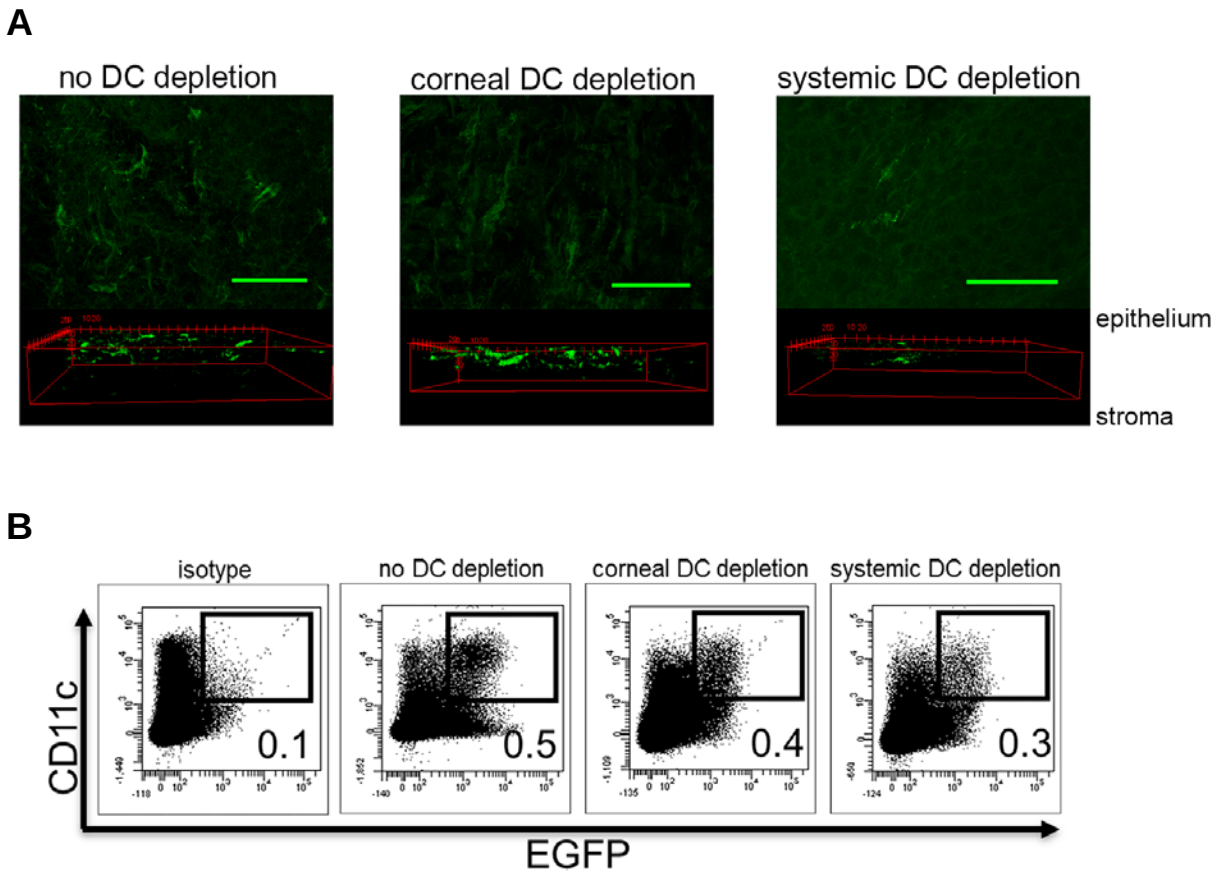

Figure 4. Reconstitution of dendritic cells into tissue of CD11c-DTR mice 3 days after diptheria toxin treatment.

BALB/c CD11c-DTR bone marrow chimeras were given subconjunctival injections of 30 ng DT (corneal DC depletion), intraperitoneal injecions of 150 ng DT (systemic DC depletion), or PBS (no DC depletion). Mice were infected with HSV-1 2 days after treatment. At 1 dpi (3 days after treatment), corneas were excised, fixed, and mounted for confocal microscopy imaging. A) Representative z-stack and three-dimensional projections from the paracentral region of the cornea show infiltrating cells expressing EGFP from the CD11c promoter at 1 dpi. B) Lymph nodes were excised at 1 dpi, treated with collagenase to yield a single cell suspension, stained with antibodies to CD11c, and analyzed for EGFP and CD11c expression. Representative flow cytometry plots show recovery of DC at 1 dpi. Numbers on flow cytometry plots are percetanges of $CD11c^+$ $EGFP^+$ cells gated on $CD45^+$ cells. Data are representative of three experiments, with 3 to 5 mice per group.

5.3 CORNEA-RESIDENT DC ARE NOT ESSENTIAL FOR $CD8^+$ T CELL EXPANSION IN THE DLN

To determine the importance of cornea-resident DCs in $CD8^+$ T cell expansion in the DLN, CD11c-DTR chimeras were treated with DT once 2 days before infection either locally through a s.c. injection or systemically through an i.p. injection to deplete DCs until 1 dpi. At 3 dpi, no significant difference was shown among the absolute numbers of $BrdU^+$ $CD8^+$ T cells in systemic DC depleted, corneal DC depleted, and non-DC depleted chimeras (**Fig. 5A**). No significant difference was observed among the absolute numbers of $BrdU^+$ $CD8^+$ T cells in systemic DC depleted, corneal DC depleted, and non-DC depleted chimeras when measured with a BrdU pulse from 0 to 3 dpi (**Fig. 5B**) at 3 dpi or after a 4 hour BrdU pulse at 7 dpi (**Fig. 5C**), thus suggesting that DC resident to the cornea are not necessary to expand $CD8^+$ T cells in the DLN.

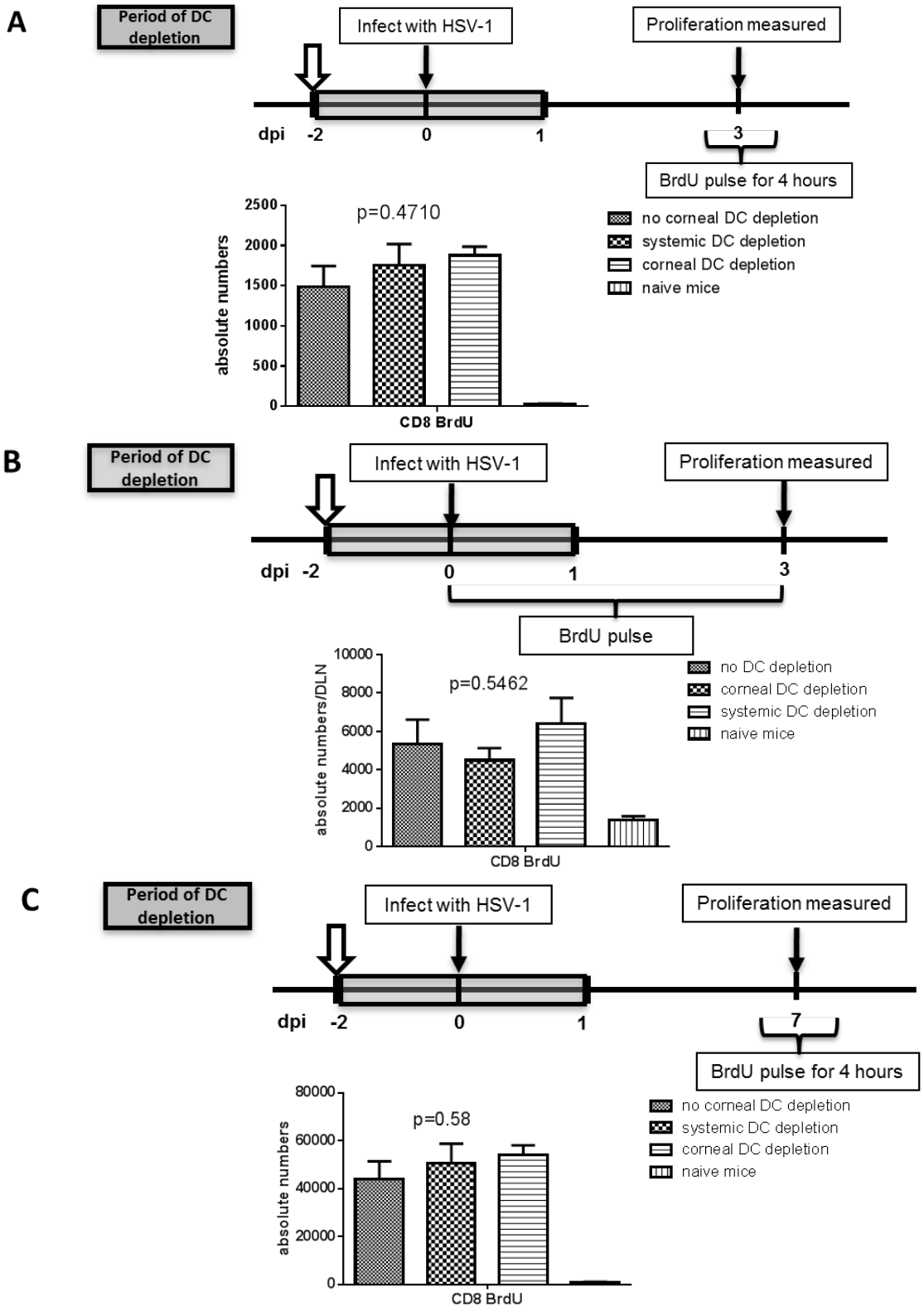

Figure 5. Cornea-resident DCs are not important for CD8$^+$ T cell expansion in the DLN.

BALB/c CD11c-DTR chimeras were given subconjunctival injections of 30 ng DT (corneal DC depletion), i.p. injections of 150 ng DT (systemic DC depletion), or PBS (no DC depletion). Chimeras received a single treatment of DT 2 days before HSV-1 infection (as indicated by unfilled arrows) to deplete cornea-resident DCs locally or to deplete both cornea-resident and DLN-resident DCs systemically, and DCs that migrate into the tissue at 1 dpi. Chimeras received i.p. injections of 1 mg bromodeoxyuridine (BrdU) at indicated times. DLN were excised at 3 or 7 dpi and processed to yield single cell suspensions. DLN cells were stained with antibodies to CD3, CD8, and BrdU to measure $CD8^+$ T cell proliferation by flow cytometry. Bar graphs show mean ± SEM of absolute number of proliferating ($BrdU^+$) $CD8^+$ T cells per DLN. Depletion of cornea-resident DCs does not influence $CD8^+$ T cell proliferation when measured at 3 dpi using A) a 4 hour BrdU pulse or B) 3 day BrdU pulse, or C) when measured at 7 dpi using a 4 hour BrdU pulse. The *p* values for group differences were analyzed using a one-way ANOVA with Bonferroni posttests (all comparisons between groups are not significant). Data are representative of three experiments, with five mice per group.

5.4 CORNEA-INFILTRATING DC ARE IMPORTANT FOR EARLY EXPANSION OF $CD8^+$ T CELLS IN THE DLN AT 3 DPI

In CD11c-DTR chimeras, DCs reconstitute tissue 3 days after DT treatment (115). Thus, to deplete DCs that are recruited into the cornea after infection (cornea-infiltrating DC), DC depletion was extended by treating the chimeras with DT at 2 days before infection and 1 day after infection to allow ablation of DC until 4 dpi. At 3 dpi, there was a complete abrogation in the absolute numbers of $BrdU^+$ $CD8^+$ T cells after corneal DC depletion ($p<0.0001$, $p=0.0004$), and the numbers were comparable to that of naïve chimeras (**Fig. 6A**). No significant difference was observed between the absolute numbers of $BrdU^+$ $CD8^+$ T cells of corneal DC depleted and systemic DC depleted chimeras, indicating that the effect of depletion of corneal DC is enough to abrogate $BrdU^+$ $CD8^+$ T cell numbers (**Fig. 6A**). However, when DC were depleted through 3

dpi and DLN were harvested only at 7 dpi, the absolute numbers of $BrdU^+$ $CD8^+$ T cells recovered to the levels of non-DC depleted mice (**Fig. 6B**).

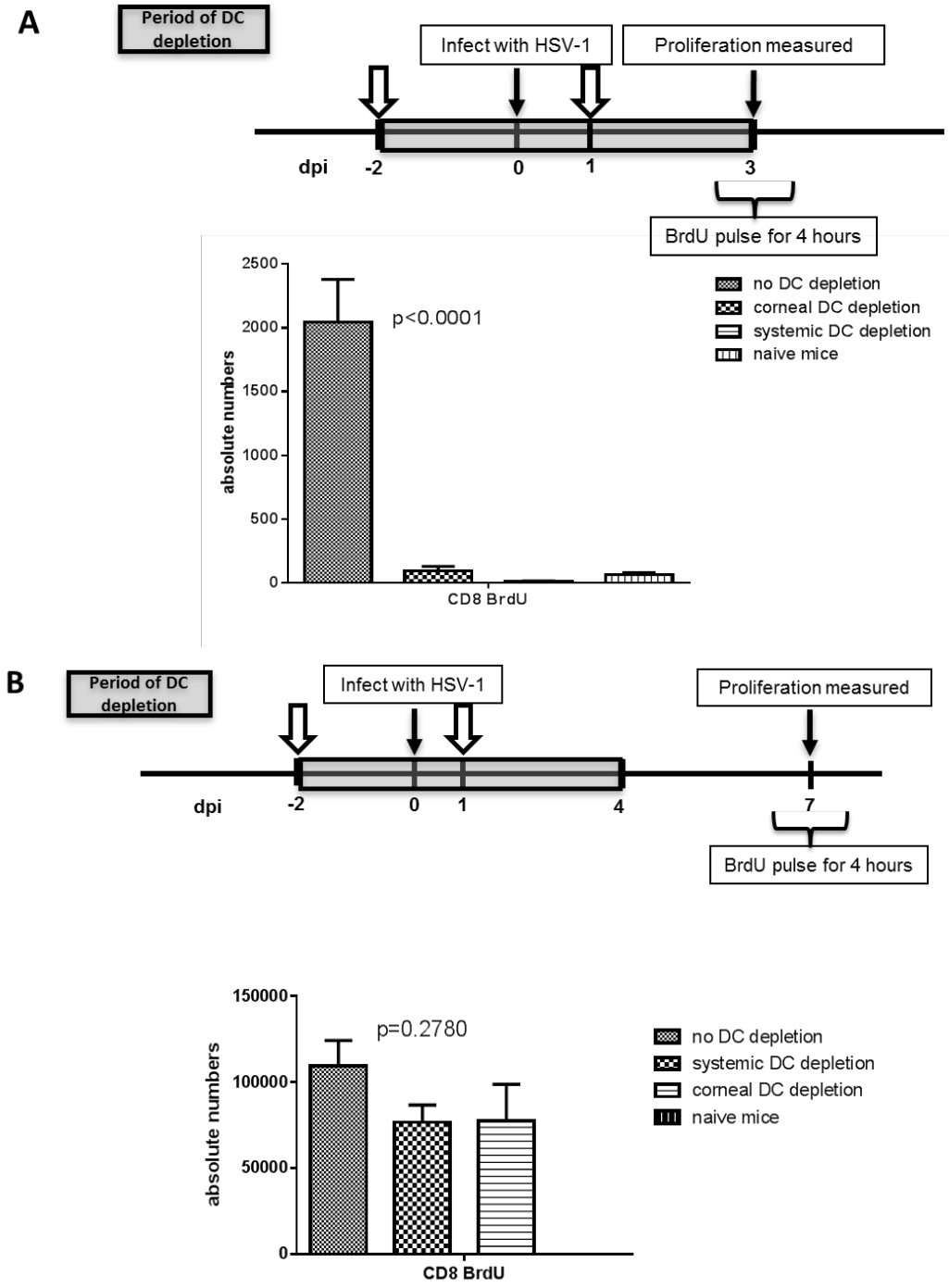

Figure 6. Early $CD8^+$ T cell expansion is solely dependent on infiltrating corneal DCs.

BALB/c CD11c-DTR bone marrow chimeras were given local (subconjunctival) or systemic (intraperitoneal) treatments of DT at -2 and +1 dpi (as indicated by unfilled arrows) to deplete cornea-derived DCs (corneal DC depletion) or both cornea-derived DCs and DLN-resident DCs (systemic depletion) up to 4 dpi, or PBS (no DC depletion). Chimeras received i.p. injections of 1 mg BrdU 4 hours before excision of DLN. Single cell suspensions of DLN were stained with antibodies to CD3, CD8, and BrdU to measure $CD8^+$ T cell proliferation by flow cytometry. Bar graphs show mean ± SEM of absolute number of proliferating ($BrdU^+$) $CD8^+$ T cells per DLN. A) $CD8^+$ T cell proliferation was significantly reduced by corneal DC depletion and systemic DC depletion. B) Corneal or systemic DC depletion up to 4 dpi did not influence $CD8^+$ T cell expansion when measured at 7 dpi. The *p* values for group differences were analyzed by a one-way ANOVA with Bonferroni posttests [A) no DC depletion vs corneal DC depletion and no DC depletion vs systemic DC depletion are $p < 0.05$, and corneal DC depletion vs systemic DC depletion is not significant, B) no depletion vs systemic DC depletion, no depletion vs corneal DC depletion, and systemic DC depletion vs corneal DC depletion are not significant]. Data are representative of three experiments, with five mice per group.

5.5 CORNEA-INFILTRATING DC ARE CRITICAL FOR EXPANSION OF IMMUNODOMINANT HSV-1-SPECIFIC $CD8^+$ T CELLS IN THE DLN AT 7 DPI

$CD8^+$ T cell proliferation recovered by 7 dpi, suggesting either stimulation by cornea-infiltrating DC reconstituting the cornea and then migrating to the lymph nodes after 3 dpi, or that DLN-resident DC are now able to stimulate T cells over time. Thus, to test the first possibility, we prolonged DC ablation to 7 dpi. Chimeras were treated with DT at 2 days before infection, 1 dpi, and 4 dpi before excision at 7 dpi. Corneal and systemic DC depletion up to 7 dpi abrogated the absolute numbers of $BrdU^+$ $CD8^+$ T cells when compared to numbers of non-DC depleted mice. There was no significant difference between absolute numbers of $BrdU^+$ $CD8^+$ T cells of corneal

DC depleted and systemic DC depleted chimeras (**Fig. 7A**) indicating that ablation of corneal DC is enough to abrogate generation of $CD8^+$ T cells in the DLN.

The immunodominant epitope in $CD8^+$ T cells against HSV-1 infection of C57Bl/6 mice is in glycoprotein B (gB). To determine if corneal DC depletion affects expansion of HSV-specific $CD8^+$ T cells, we employed CD11c-DTR chimeras on a C57Bl/6 background (generated as described in Materials and Methods). A significant reduction in proliferating gB-specific $CD8^+$ T cells is observed at 7 dpi after corneal or systemic DC depletion, as detected through tetramer staining, indicating that cornea-infiltrating dendritic cells are important in generating $CD8^+$ T cells specific to HSV after corneal infection (**Fig. 7B**).

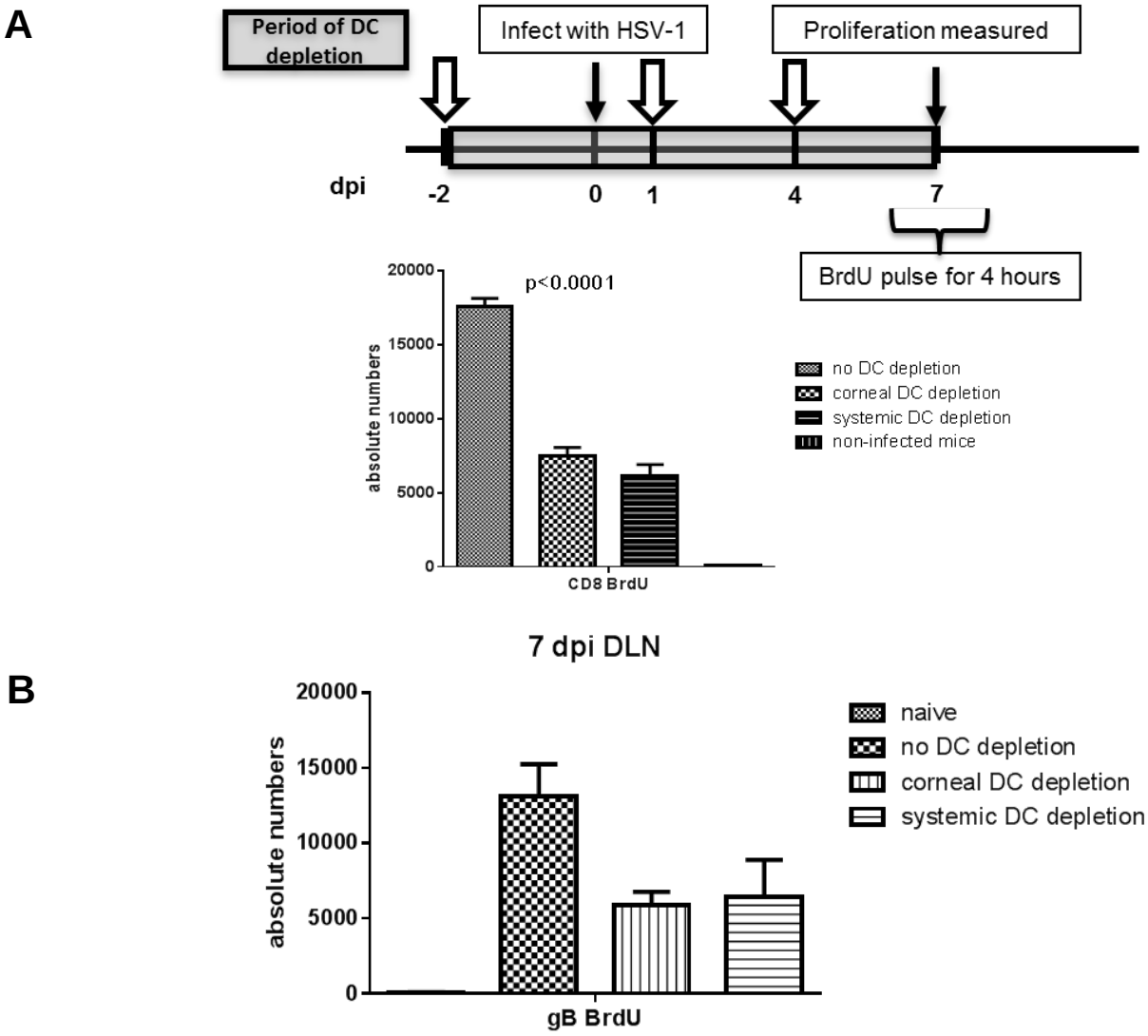

Figure 7. Late CD8$^+$ T cell expansion is dependent on infiltrating corneal DCs.

BALB/c or C57Bl/6 CD11c-DTR bone marrow chimeras were given local (subconjunctival) or systemic (intraperitoneal) treatments of DT at -2, +1, and +4 dpi (as indicated by unfilled arrows) to deplete cornea-derived DCs (corneal DC depletion) or both cornea-derived DC and DLN-resident DC (systemic DC depletion) up to 7 dpi, or PBS (no DC depletion). Chimeras received i.p. injections of 1 mg BrdU 4 hours before excision of DLN. A) Single cell suspensions of DLN from BALB/c chimeras were stained with antibodies to CD3, CD8, and BrdU to measure CD8$^+$ T cell proliferation by flow cytometry. B) Single cell suspensions of DLN from C57Bl/6 chimeras were stained with MHC class I tetramers containing the HSV-1 glycoprotein B $(gB)_{498\text{-}505}$ epitope. Bar graphs show mean ± SEM absolute numbers of proliferating (BrdU$^+$) CD8$^+$ T cells per DLN. The *p* values for group differences were analyzed by a one-way ANOVA with Bonferroni posttests [no DC depletion vs corneal DC depletion and no

DC depletion vs systemic DC depletion are $p < 0.05$, corneal DC depletion vs systemic DC depletion is not significant]. Data are representative of three experiments, with five mice per group.

5.6 CORNEA-INFILTRATING DC ARE CRITICAL FOR EXPANSION OF SUBDOMINANT HSV-1-SPECIFIC CD8$^+$ T CELLS IN THE DLN AT 7 DPI

To determine if local corneal DC depletion also affected the proliferation of CD8$^+$ T cells specific to subdominant epitopes as well, MHC I tetramers that contain the HSV-1 proteins ribonucleotide reductase 1 $(RR1)_{882\text{-}829}$, $RR1_{982\text{-}989}$, or infected cell protein 8 $(ICP8)_{171\text{-}178}$ epitopes were used to stain DLN of CD11c C57Bl/6 chimeras. At 7 dpi, both corneal and systemic DC depletion significantly reduces the absolute numbers of proliferating CD8$^+$ T cells specific for the $RR1_{982\text{-}989}$ epitope or a pool of CD8$^+$ T cells specific for both $RR1_{882\text{-}829}$ and $ICP8_{171\text{-}178}$ (**Fig. 8**)..

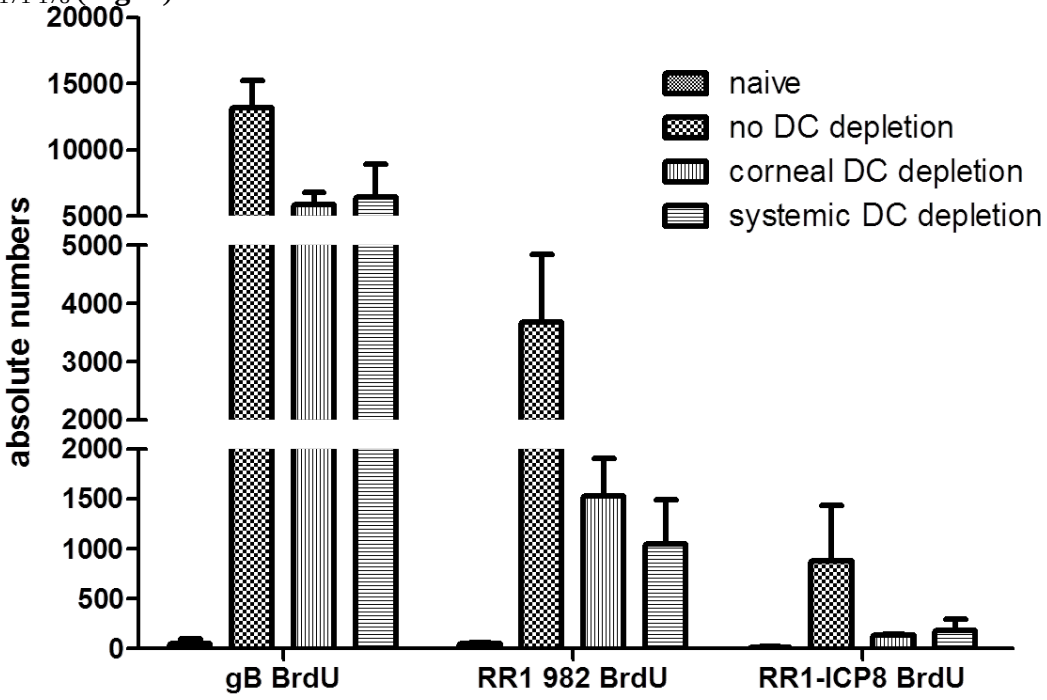

Figure 8. Expansion of subdominant CD8$^+$ T cells is dependent on infiltrating corneal DCs

C57Bl/6 CD11c-DTR bone marrow chimeras were given local (subconjunctival) or systemic (intraperitoneal) treatments of DT at -2, +1, and +4 dpi (as indicated by unfilled arrows) to deplete cornea-derived DCs (corneal DC depletion) or both cornea-derived DC and DLN-resident DC (systemic DC depletion) up to 7 dpi, or PBS (no DC depletion). Chimeras received i.p. injections of 1 mg BrdU 4 hours before excision of DLN at 7 dpi. Single cell suspensions of DLN from C57Bl/6 chimeras were stained with antibodies to CD8 and MHC class I tetramers containing the HSV-1 glycoprotein B $(gB)_{498\text{-}505}$, ribonucleotide reductase 1 $(RR1)_{882\text{-}829,}$ $RR1_{982\text{-}989}$, or infected cell protein 8 $(ICP8)_{171\text{-}178}$ epitopes. Bar graphs show mean ± SEM absolute numbers of proliferating subdominant ($BrdU^+$) $CD8^+$ T cells per DLN. The *p* values for group differences were analyzed by a one-way ANOVA with Bonferroni posttests [no DC depletion vs corneal DC depletion and no DC depletion vs systemic DC depletion are $p < 0.05$, corneal DC depletion vs systemic DC depletion is not significant]. Data are representative of three experiments, with five mice per group.

5.7 CORNEA-INFILTRATING DC ARE CRITICAL FOR POPULATION OF THE TRIGEMINAL GANGLIA WITH $CD8^+$ T CELLS AND ARE CRITICAL TO PREVENT LETHAL INFECTIONS

When corneal DC depleted and systemic DC depleted chimeras were allowed to live past 7 dpi, they develop signs of encephalitis. This is presumably due to a significant reduction of $CD8^+$ T cells able to control the virus in the trigeminal ganglia (**Fig. 9A**). Mice that are depleted of cornea-infiltrating DC and are systemically depleted of DC succumb to death at about 9 to 10 dpi (**Fig. 9B**).

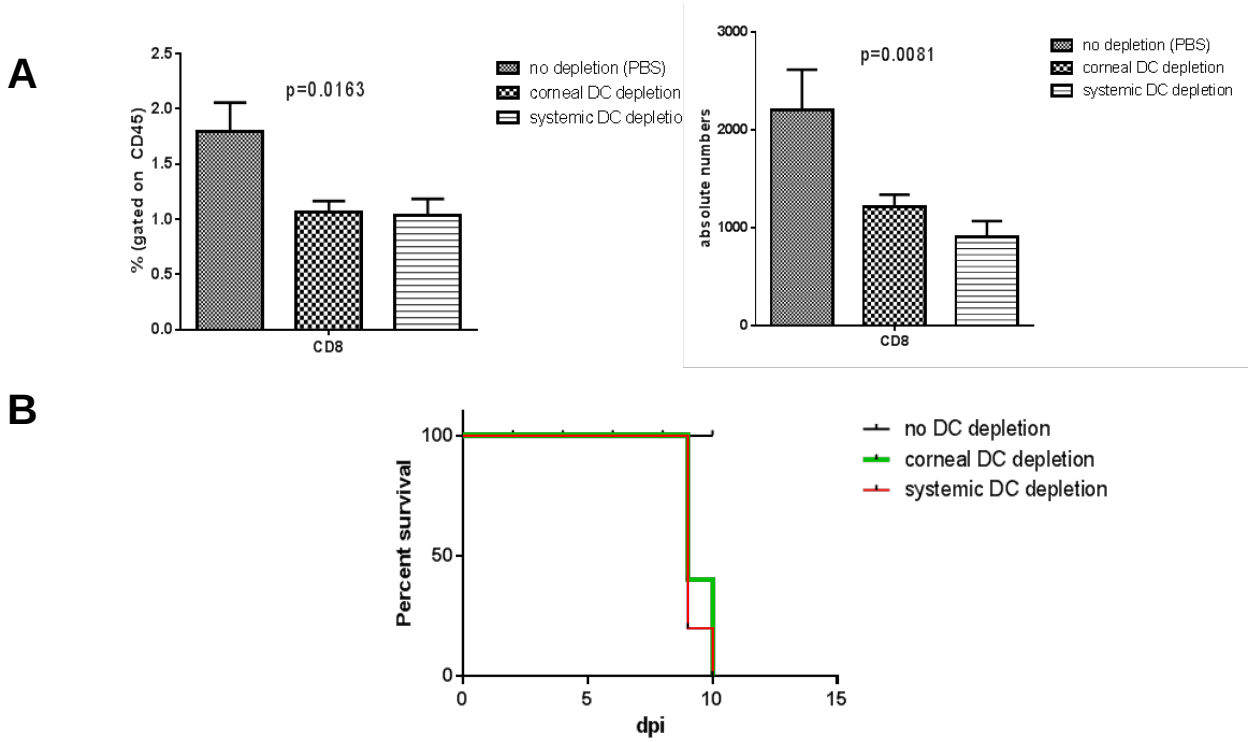

Figure 9. Infiltrating corneal DCs influence the repopulation of $CD8^+$ T cells in the trigeminal ganglia after infection and prevent lethal infections.

BALB/c CD11c-DTR bone marrow chimeras were given local (subconjunctival), or systemic (intraperitoneal) treatments of DT at -2, +1, and +4 dpi to deplete cornea-derived DC (corneal DC depletion) or both cornea-derived and DLN-resident DC (systemic DC depletion) up to 7 dpi, or PBS (no DC depletion). A) The trigeminal ganglia (TG) of treated or non-treated chimeras were excised, treated with collagenase to yield a single cell suspension, and stained with antibodies to CD3 and CD8 to measure the numbers of $CD8^+$ T cells in the TG. Bar graphs show mean ± SEM absolute numbers of $CD8^+$ T cells per TG. The *p* values for group differences were analyzed by a one-way ANOVA with Bonferroni posttests [no DC depletion vs corneal DC depletion and no DC depletion vs systemic DC depletion are $p < 0.05$, corneal DC depletion vs systemic DC depletion is not significant]. Data are representative of three experiments, with five mice per group. B) Chimeras treated or not treated with DT as described were followed for survival. Corneal DC depleted and systemic DC depleted mice succumbed to encephalitis at 9-10 dpi.

5.8 CORNEA-INFILTRATING DC MIGRATE TO THE DRAINING LYMPH NODES AFTER HSV-1 INFECTION

We tracked antigen-presenting cells that migrated to the DLN from the cornea using 1% FITC, as described in Materials and Methods. At steady state, there are about 800 cells that travel from each cornea to its respective DLN. In comparison to the naïve cornea, FITC painting of the infected cornea 16 hours before harvest at 3 dpi showed migration of about 200 more dendritic cells from the cornea to the DLN (**Fig. 10**).

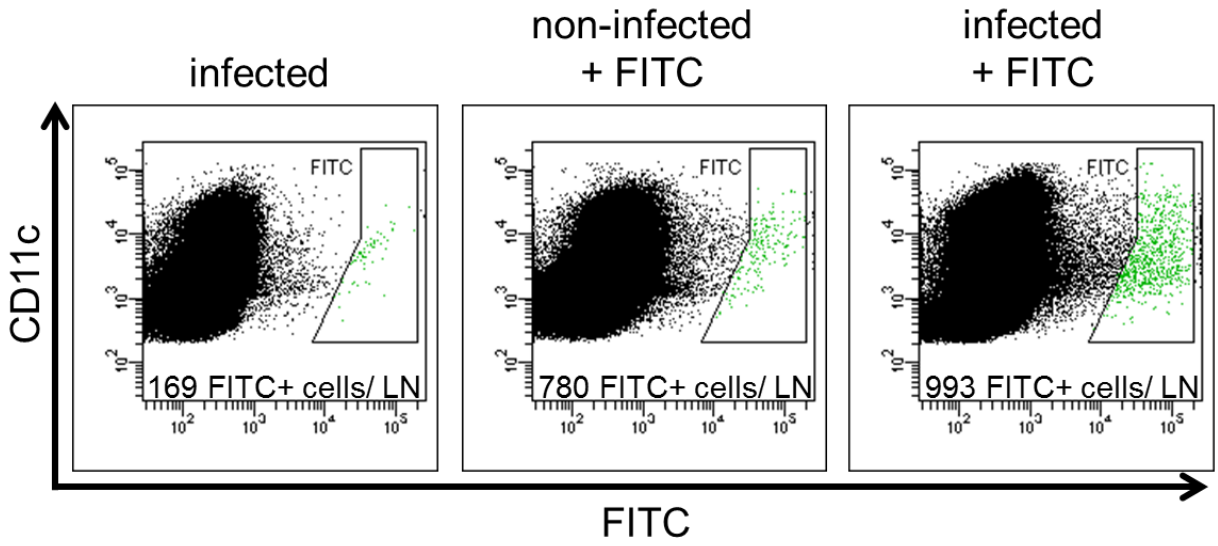

Figure 10. Infiltrating corneal DCs migrate to the DLN.

Wild type BALB/c mice were infected with HSV-1 and at 2 dpi, 1% fluorescein isothiocyanate (FITC) was applied onto their corneas. The DLN of these mice were excised at 3 dpi, treated with collagenase to yield a single cell suspension, enriched for $CD11c^+$ cells by negative isolation, stained with antibodies to CD11c, and analyzed for FITC uptake by $CD11c^+$ cells through flow cytometry. Representative flow cytometry plots demonstrate the $CD11c^+$ cells migrating to the DLN ($FITC^+$) in naïve mice that received FITC, and increased numbers of $CD11c^+$ $FITC^+$ cells migrating to the DLN in HSV-infected mice. Absolute numbers of $CD11c^+$ $FITC^+$ cells are shown for each group. Data are representative of three experiments, with 10-20 lymph nodes per group.

5.9 CORNEA-RESIDENT DENDRITIC CELLS EXPRESS CD86 AND UPREGULATE CD80 AFTER INFECTION

The aforementioned findings are consistent with the idea that cornea-infiltrating DC are more capable at presenting antigen to $CD8^+$ T cells than do DLN-resident DC. Initial activation of a naïve T cell occurs upon interaction of TCR with a specific peptide presented by MHC molecules and additional signals from costimulation and inflammation are required for an effective T cell response. To characterize the differences between cornea-infiltrating and DLN-resident DC, we decided to analyze the expression of the costimulatory molecules CD80 and CD86 in WT BALB/c mice. As illustrated in **Fig. 11**, cornea-infiltrating DC only express CD80 after infection. CD86, on the other hand, is expressed by cornea-resident DC in the naïve cornea, and continue expressing CD86 after infection (**Fig. 11**).

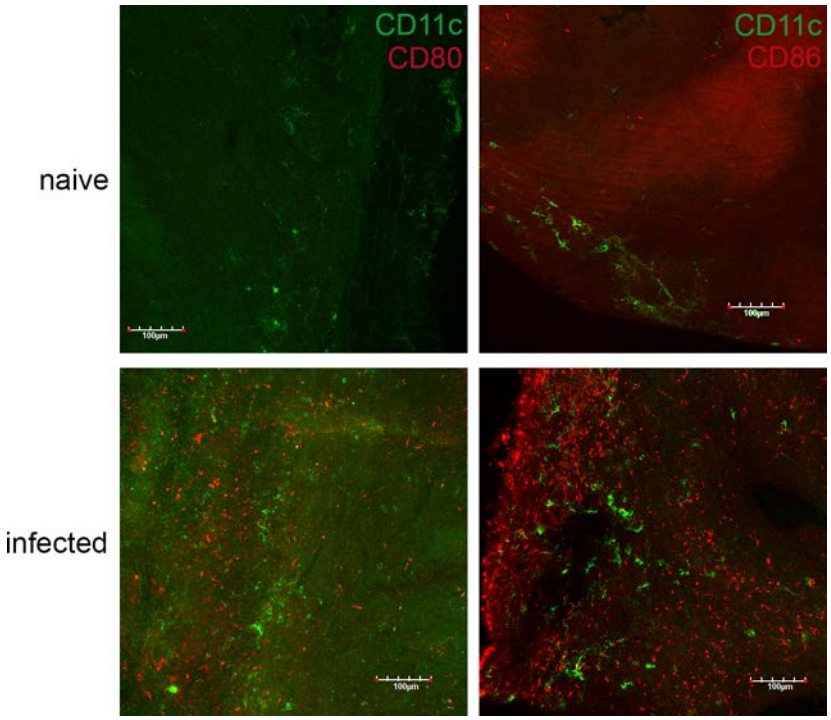

Figure 11. Costimulation of dendritic cells in the HSV-infected cornea.

BALB/c CD11c-DTR bone marrow chimeras were infected with HSV-1 and at 1 dpi, their corneas were excised, along with uninfected cornea controls, stained with antibodies to CD80 or CD86, fixed, and mounted for confocal microscopy imaging. Representative images taken of the peripheral corneas of uninfected mice demonstrate $CD11c^+$ cells expressing CD86, but not CD80. Representative images taken of the peripheral corneas of infected mice show $CD11c^+$ cells expressing CD80 or CD86. Images are representative of three independent experiments, with three mice per group.

5.10 CORNEA-INFILTRATING DC HAVE GREATER EXPRESSION OF COSTIMULATORY MOLECULES COMPARED TO DLN-RESIDENT DC

To track if cornea-infiltrating DC maintain expression of these molecules after migration to the DLNs, cornea-infiltrating DC were labeled with 1% FITC as described in Materials and Methods, and their migration into the DLN and expression of costimulatory molecules were assessed. In naïve mice, DLN-resident DC (DC not labeled with FITC) express low levels of CD40, CD70, CD80, CD86, and MHC II (**Fig. 12**). After infection, the MFI of these molecules when measured for DLN-resident DC do not change significantly. Moreover, cornea-infiltrating DCs express more CD40, CD70, CD80, CD86, and MHC II per cell than DLN-resident DC. These results suggest that preferential upregulation of costimulation in cornea-infiltrating DC compared to DLN-resident DC may allow cornea DCs to stimulate $CD8^+$ T cell expansion.

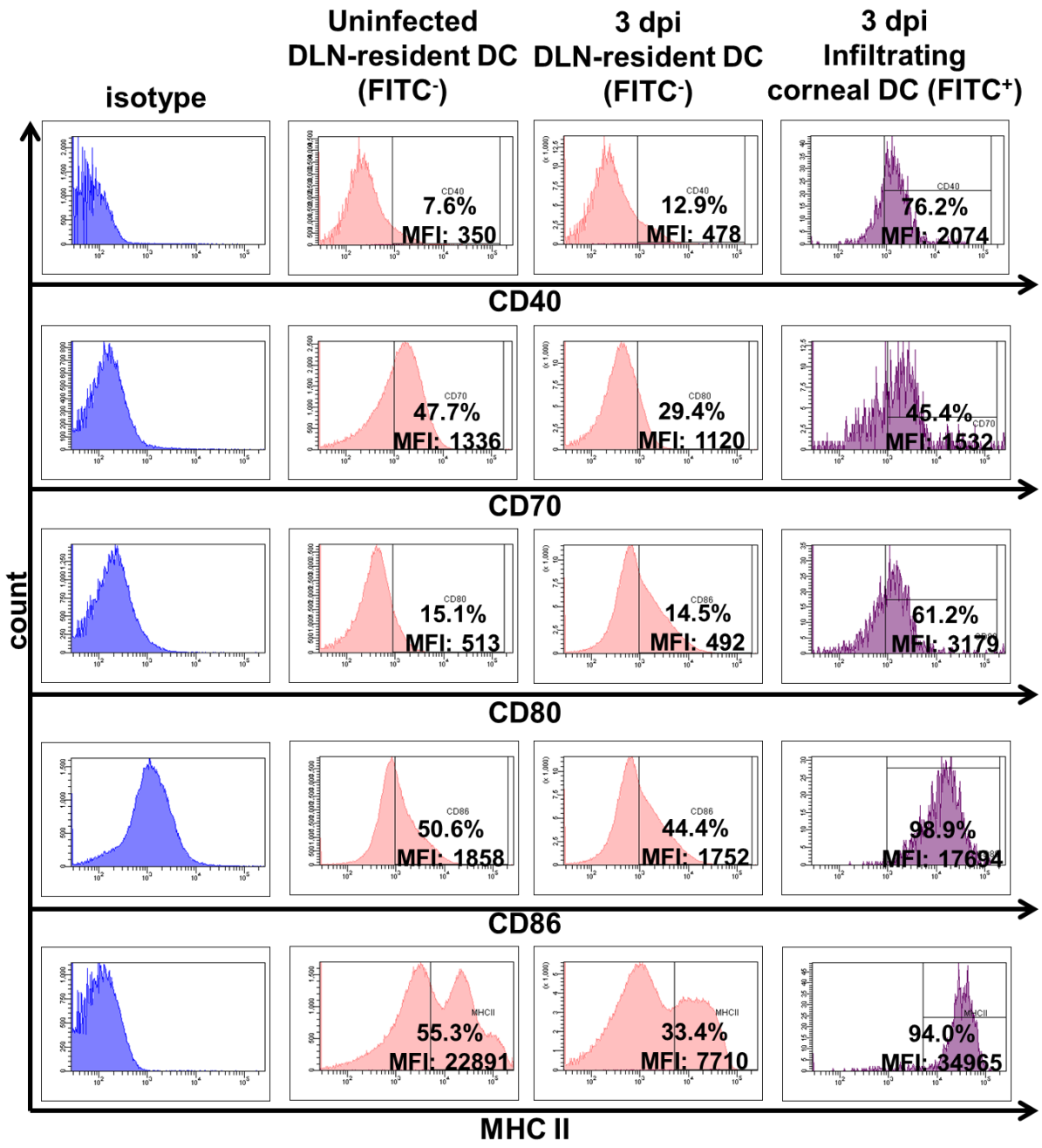

Figure 12. Infiltrating corneal DCs have greater levels of costimulation in the DLN compared to DLN-resident DCs.

Wild type BALB/c mice were infected with HSV-1 and at 2 dpi, 1% fluorescein isothiocyanate (FITC) was applied onto their corneas. The DLN of these mice were excised at 3 dpi, treated with collagenase to yield a single cell suspension, enriched for $CD11c^+$ cells by negative isolation, stained with antibodies to CD11c, CD40, CD70, CD80, CD86, and MHC II. Representative flow cytometry plots show a greater frequency and mean fluorescence intensity

(MFI) of costimulatory molecules expressed by $CD11c^+$ $FITC^+$ cells in HSV-infected mice compared to $CD11c^+$ $FITC^+$ cells in naïve mice**.** Data are representative of three experiments, with 10-20 lymph nodes per group.

5.11 CD86 IS NOT REQUIRED FOR THE EXPANSION OF $CD8^+$ T CELLS IN THE DLN AFTER HSV-1 OCULAR INFECTION

One of the molecules highly expressed by cornea-infiltrating DC compared to DLN-resident DC is CD86 (**Fig. 12**). To test the importance of CD86 interactions, mice were treated with anti-CD86 antibody (clone GL-1) 2 days before and 1 day after infection. At 3 dpi, there was no significant difference between the percentage and absolute numbers of $BrdU^+$ $CD8^+$ T cells (**Fig. 13**). Consequently, there was no significant difference in absolute numbers of $CD8^+$ T cells, thus indicating that CD86 does not play a critical role in the generation of $CD8^+$ T cells in the DLN after HSV-1 infection.

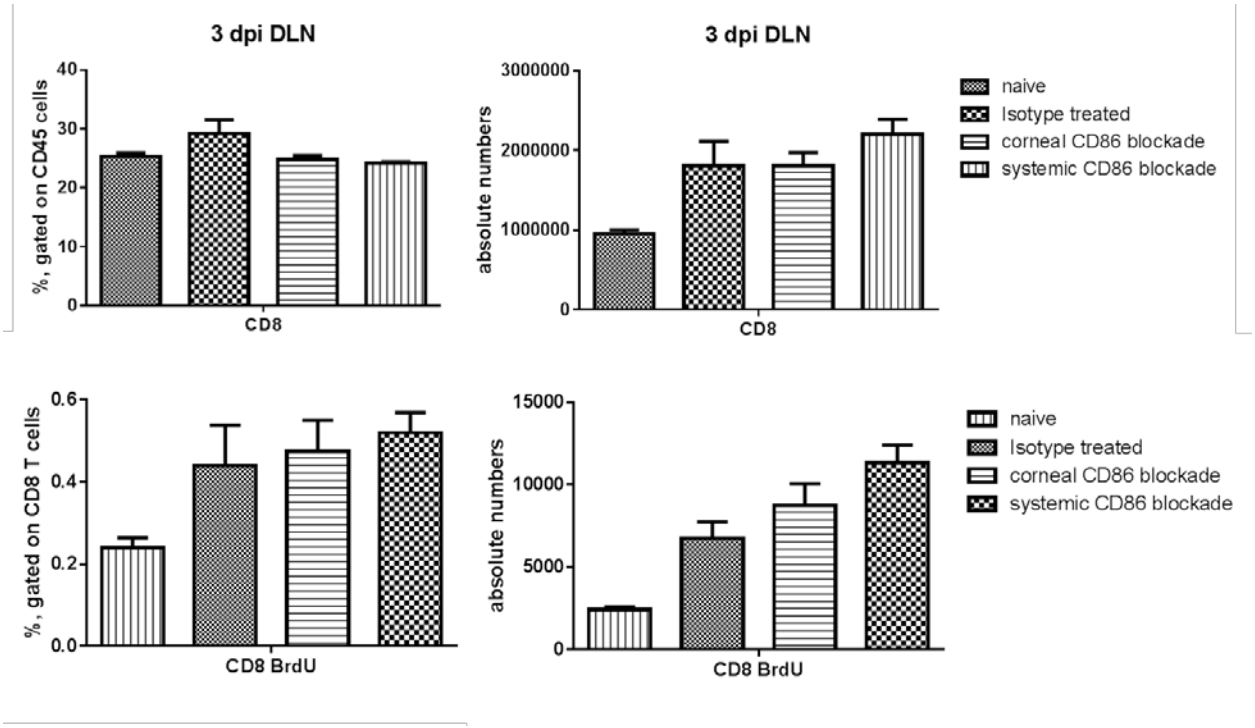

Figure 13. CD86 interactions are not necessary for $CD8^+$ T cell expansion in the DLN.

Wild type BALB/c mice were infected with HSV-1 and received 200 ug anti-CD86 (clone GL1) antibody intraperitoneally (systemic CD86 blockade), 20 ug anti-CD86 antibody subconjunctivally (local CD86 blockade), or treated with the corresponding isotype antibody, at -2, and +1 dpi. Mice received 1 mg BrdU intraperitoneally at 3 dpi. Four hours after, DLN were excised, processed to yield a single cell suspension, and stained with antibodies to CD3, CD8, and BrdU. Bar graphs show mean ± SEM percentage or absolute numbers of proliferating ($BrdU^+$) $CD8^+$ T cells per lymph node. The *p* values for group differences were analyzed by a one-way ANOVA with Bonferroni posttests [no DC depletion vs corneal DC depletion, no DC depletion vs systemic DC depletion, and corneal DC depletion vs systemic DC depletion are not significant]. Data are representative of three experiments, with five mice per group.

5.12 CD154 IS NOT REQUIRED FOR THE EXPANSION OF $CD8^+$ T CELLS IN THE DLN AFTER HSV-1 OCULAR INFECTION

CD40, an important costimulatory molecule with roles in viral immunity, is known to be expressed on B cells, professional antigen-presenting cells and binds CD154 or CD40L, which is expressed generally on T cells. It is also one of the costimulatory molecules highly expressed by cornea-infiltrating DC compared to DLN-resident DC (**Fig. 12**). To test the importance of CD40 interactions, mice were treated locally or systemically with anti-CD154 antibody (clone MR-1) i.p. at -1, +2, and +5 dpi. At 7 dpi, there was no significant difference in the percentage and absolute numbers of $CD8^+$ T cells after corneal or systemic CD154 blockade (**Fig. 14A**). However, a significant reduction was observed between the percentage and absolute numbers of proliferating $BrdU^+$ $CD8^+$ T cells in the lymph nodes of systemically blocked mice (**Fig. 14B**), implying that either there is a greater rate of death in these $CD8^+$ T cells or that egress of these cells from the lymph nodes is happening very quickly. To measure the frequency of dying cells, Caspatag PanCaspase kit was used. No significant difference was observed in the percentage and absolute numbers of $CD8^+$ T cells that were labeled positive with Caspatag (**Fig. 14C**), suggesting that after CD154 blockade, $CD8^+$ T cells leave the lymph nodes instantly.

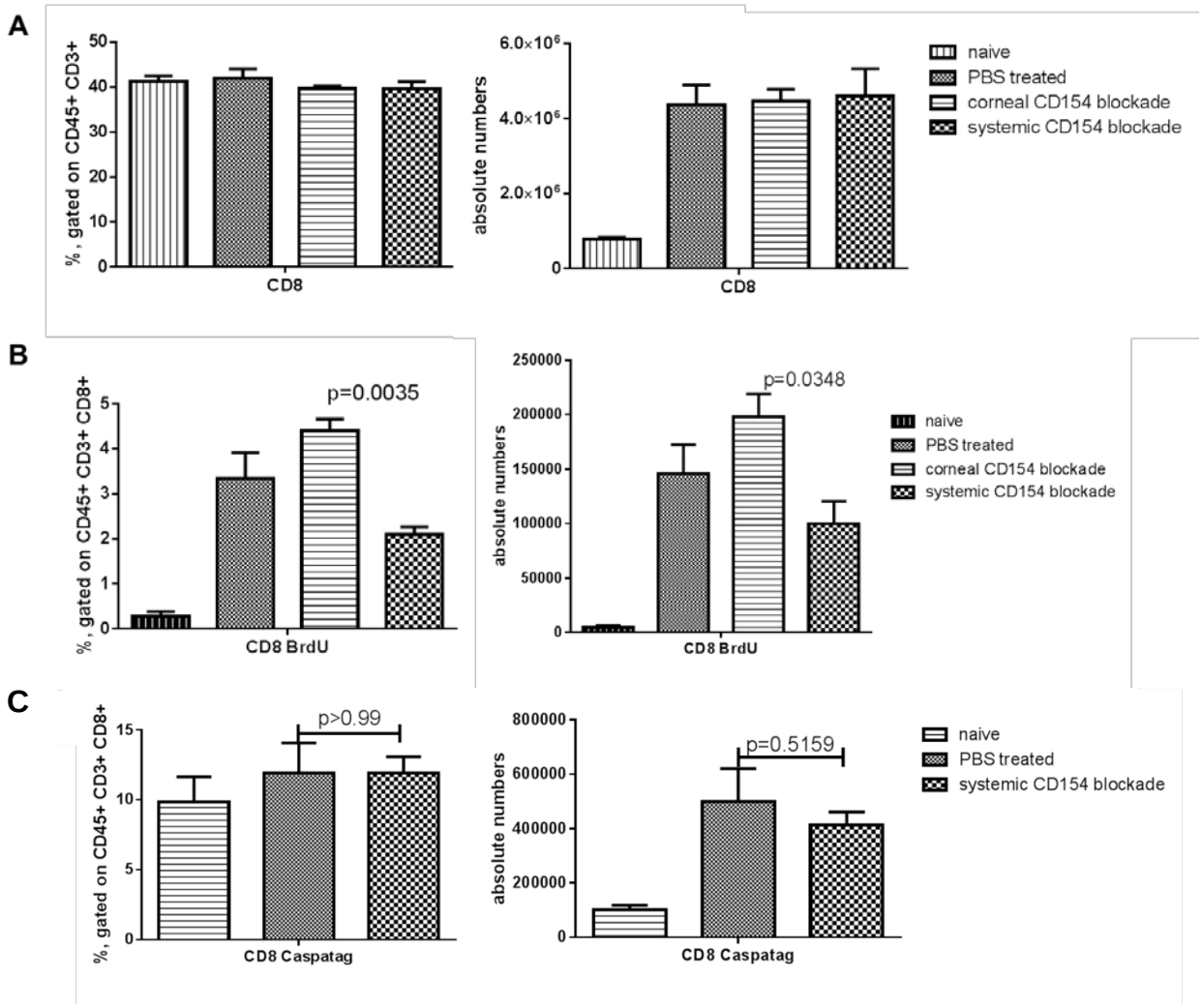

Figure 14. CD154 (CD40L) interactions are not necessary for CD8$^+$ T cell expansion in the DLN.

Wild type BALB/c mice were infected with HSV-1 and received 300 ug anti-CD40L (clone MR-1) antibody intraperitoneally (systemic CD154 blockade), 30 ug anti-CD40L antibody subconjunctivally (local CD154 blockade), or treated with the corresponding isotype antibody at -1, +2, and +5 dpi. Mice received 1 mg BrdU intraperitoneally at 7 dpi. Four hours after, DLN were excised, processed to yield a single cell suspension, and stained with antibodies to CD3, CD8, and BrdU. Bar graphs show A) mean ± SEM absolute numbers of proliferating (BrdU$^+$) CD8$^+$ T cells per lymph node, B) mean ± SEM absolute numbers of CD8$^+$ T cells, and C) mean ± SEM absolute numbers of Caspatag$^+$ CD8$^+$ T cells. The *p* values for group differences were analyzed by a one-way ANOVA with Bonferroni posttests [no DC depletion vs corneal DC depletion, no DC depletion vs systemic

DC depletion, and corneal DC depletion vs systemic DC depletion are not significant]. Data are representative of three experiments, with five mice per group.

5.13 DISCUSSION

The cornea constitutes an immune privileged mucosal surface that was previously thought to be due to the absence of antigen presenting cells. However, recent studies have shown that the normal mouse cornea possesses a population of $CD11c^+$ $CD11b^-$ DCs and $F4/80^+$ $CD11b^+$ macrophages (107, 110), but their purpose has yet to be determined. Our group has previously shown that dendritic cells indirectly control HSV-1 replication in the cornea by influencing natural killer cell migration into the central cornea where viral lesions are located (115). However, the role of corneal dendritic cells in initiating the adaptive immune response against HSV-1 is not defined.

We examined the roles of cornea-resident DC, cornea-infiltrating, and DLN-resident DC. We addressed this by utilizing CD11c-DTR-EGFP bone marrow chimeras to ablate the dendritic cells at different time points locally in the cornea or systemically to deplete cornea- and DLN-resident DC during HSV-1 ocular infection. A subconjunctival injection of diphtheria toxin afforded us to deplete corneal DC without ablating DC in the DLN (**Fig. 3**).

The presence of dendritic cells in the corneal epithelium expressing Langerin in naïve mice has been reported by Hattori, et al (113), and our group has found $CD11c^+$ cells in the naïve corneal epithelium, some expressing langerin. After local DT treatment, we deplete all $CD11c^+$ cells, which include the $CD11c^+$ $langerin^+$ cells in the cornea. Unlike the dendritic cell subsets in the skin which include Langerhans cells in the epidermis and dermal dendritic cells, there is no

counterpart for dermal dendritic cells in the cornea. Thus, the roles of these DC subpopulations may be different from what has been characterized in other mucosal tissue (223, 475).

Using this approach, we depleted cornea-resident DC by giving chimeras DT 2 days before infection, and our results demonstrated that in contrast to previous studies (223), steady-state tissue – resident DC in the cornea are not required in priming of $CD8^+$ T cells. Diphtheria toxin only allows depletion in this system for 3 days and DC reconstitutes the tissue soon afterwards (**Fig. 4**). DC egress from the site of infection to the DLN is amplified after HSV-1 infection (214-216), but in studies by Eidsmo, et al., productively infected DCs were not identified in the DLN (217). The lack of a role for cornea-resident dendritic cells may be because of its lack of migration capabilities. In vivo imaging studies have shown that DC in the central and peripheral cornea are generally non-motile but change morphology upon stimulation (476). It is also a possibility that a lytic virus such as HSV-1 can render directly infected tissue-resident DC in the cornea unable to participate in T cell priming (205, 208, 221, 477). Therefore, our data showing cornea-infiltrating DCs presenting HSV-1 antigen to $CD8^+$ T cells in the DLN reflect antigen presentation by DC that are not directly infected by the virus, but by those that acquire viral antigen from infected epithelial cells or infected DC in the cornea.

Presumably, during infection, monocytes come in and differentiate into DC that can take up antigen and generate a robust T cell response in the DLN even with depletion of cornea-resident DC (**Fig. 5**). Thus in the absence of cornea-resident DC, cornea-infiltrating DC are able to assume this role. Extending the depletion to 3 dpi show an abrogation of the absolute numbers of $CD8^+$ T cells that were undergoing proliferation in mice that were depleted of DC systemically or depleted of DC locally, compared to non-DC depleted mice (**Fig. 6A**). This indicates that corneal-infiltrating DC are sufficient to cause expansion in $CD8^+$ T cells and we

see the same effect when systemic DT treatment depletes both corneal DC and DLN-resident DC. However, when mice were allowed to live after DC depletion from -2 dpi to 3 dpi, $CD8^+$ T cell proliferation recovered to the same numbers as the non-depleted mice at 7 dpi, thus suggesting that cornea-infiltrating DC migrating after 3 dpi or DC resident to the DLN were able to stimulate the $CD8^+$ T cells (**Fig. 6B**).

Local DC depletion from -2 to 7 dpi to determine if cornea-infiltrating DC or DLN-resident DC are responsible for $CD8^+$ T cell proliferation in the DLN after 3 dpi showed considerable reduction of proliferating $CD8^+$ T cells, and the same significant reduction is seen when DC are systemically ablated continuously both in BALB/c (**Fig. 7A**) and C57Bl/6 (**Fig. 7B and 7C**) chimeras. This decrease is seen in both immunodominant and subdominant $CD8^+$ T cells (**Fig. 8**). However, the absolute numbers of proliferating $CD8^+$ T cells in cornea DC depleted or systemic DC depleted mice are still significantly higher than that of naïve mice, suggesting the presence of other antigen presenting cells in the DLN that are able to stimulate these $CD8^+$ T cells. Furthermore, the data also indicate that dendritic cells, presumably monocyte-derived, infiltrate the cornea after infection, migrate to the DLN and are necessary in $CD8^+$ T cell expansion in the DLN after 3 dpi. In contrast, a recent study by Iijima, et al. demonstrated that monocyte-derived DC in the vaginal submucosa were found to be dispensable in priming of naïve $CD4^+$ T cells but are more necessary in regulating cytokine production from infiltrating effector T cells at the infection site (475) while tissue – resident dendritic cells were required to generate the initial $CD4^+$ and $CD8^+$ T cell responses (223). This contrast in the roles of DC reflects the differences in the mucosal tissues at hand. The number of dendritic cells in the corneal epithelium is minor compared to that of the vaginal mucosa and therefore there is a

possibility that most of them will be infected, thus leaving none of the tissue – resident DC able to prime naïve T cells.

Dendritic cells or monocytes differentiating into DCs enter the cornea after infection. Uptake of viral antigens from dying infected epithelial and cells by cornea-infiltrating DC may provide a means for a greater number of dendritic cells that can present antigen to naïve cognate T cells in the DLN. It may be viewed as a scheme to outdo the immune evasion mechanisms by HSV. Transfer of antigens to uninfected DCs undermines the ability of the virus to downregulate the expression and peptide loading of MHC I molecules by infected cells.

Mice that were depleted of corneal DC and DC systemically developed signs of encephalitis such as the presence of ruffled fur, slightly hunched back, limb paralysis, and lethargy, at about 7 dpi, and had to be sacrificed by day 10 post infection. $CD8^+$ T cells are necessary in protection against HSV-1, and there may be impairment in function or number of $CD8^+$ T cells generated to migrate to the trigeminal ganglia to prevent virus spread to the brain (**Fig. 9**).

The results above demonstrate that $CD8^+$ T cells may require cornea-infiltrating DC to carry and deliver antigen. What makes the DC derived from the cornea different? We could not sort for migratory dendritic cells in the DLN coming from the cornea because of their small numbers (**Fig. 10**) and therefore could not use them for T cell stimulation assays. T cell costimulation is an important event after TCR triggering as it provides a requirement for T cell activation. Thus upon looking at their costimulatory phenotype, DC in the cornea begin to express costimulatory molecules CD80 and CD86 after infection (**Fig. 11**). Moreover, through FITC tracking (**Fig. 10**), a greater expression per cell is observed for MHCII, CD40, CD70,

CD80, and CD86 in cornea-infiltrating DC, compared to that of the DLN-resident DC (**Fig. 12**), presumably enabling these DC to stimulate naïve $CD4^+$ and $CD8^+$ T cells more readily.

To determine which costimulatory molecule on infiltrating corneal DCs is critical, antibodies to block costimulation were utilized. Local or systemic neutralization of costimulatory interactions between CD154 and CD40 (**Fig. 14**), as well as CD86 and CD28 (**Fig. 13**), did not influence the generation of $CD8^+$ T cells in the DLN. Early studies indicate that CD28 and CD40 are important costimulators for immune responses against herpes viruses (413, 421). Previously, our lab has shown that CD154/CD40 interactions are not relevant in generating $CD8^+$ and $CD4^+$ T cells in the DLN (387). It is possible that CD80, another ligand for CD28, may compensate when CD86 is impaired. It is also possible that other costimulatory molecules, such as CD70, ICOSL, and 4-1BBL (478), may make up for the lack of CD154 and CD86. Another alternative theory is that it is a combination of these molecules that is important and should be expressed by cornea – infiltrating dendritic cells to activate $CD8^+$ T cells. A reagent such as CTLA4-Ig that targets CD28 itself may be important to determine if both CD86 and CD80 are necessary.

In summary, our findings demonstrate that in this model, cornea-infiltrating dendritic cells, and not the tissue-resident DC in the cornea, are necessary in priming of naïve $CD8^+$ T cells. The ability of cornea-infiltrating DC to induce proliferation may be in part due to its high expression of the costimulatory molecules CD40, CD70, CD80, and CD86. We also show that the contribution of tissue-resident vs tissue – infiltrating dendritic cells in modulating antiviral immunity depends on the tissue of concern and on what kinds of DC subpopulations are present.

6.0 THE ROLE OF DENDRITIC CELLS IN THE EXPANSION OF $CD4^+$ T CELLS IN THE DRAINING LYMPH NODES AND IN THE PROGRESSION OF HERPES STROMAL KERATITIS

Various infection models have shown the necessity of different subpopulations of dendritic cells in activation of antigen-specific T cells. Whether cornea-resident dendritic cells are important for HSV-specific $CD4^+$ T cell proliferation during priming in the lymph nodes is not clear. Although cornea-infiltrating DCs appear to be more important than DLN-resident DC in generating $CD8^+$ T cells, it might not necessarily be the case for $CD4^+$ T cells.

After infiltrating corneal DC come in during infection, a second upsurge of dendritic cells enter murine corneas around 7 dpi that coincides with the onset of HSK. Besides activating $CD4^+$ T cells in the DLN, cornea-infiltrating DC may be a source of stimulation for $CD4^+$ T cells that access the cornea during disease. Greater numbers of dendritic cells in the cornea is linked with greater HSK opacity. Additionally, blocking B7 costimulation locally in the cornea hampers disease severity and occurrence. Taken as a whole, these studies imply a role for dendritic cells in the progression of herpes stromal keratitis.

6.1 CORNEA-RESIDENT DC ARE NOT ESSENTIAL FOR $CD4^+$ T CELL EXPANSION IN THE DLN

The previously described depletion strategy was used to determine the requirement for dendritic cells resident to the cornea (DC present in the cornea before infection) in expanding $CD4^+$ T cells in the draining lymph nodes after HSV-1 infection, by subconjunctival or intraperitoneal injection of DT 2 days before infection. To measure proliferation of $CD4^+$ T cells, chimeras received 1 mg BrdU 4 hours before harvest of draining lymph nodes for flow cytometric analysis at 3 dpi. At 3 dpi, there was no significant difference among the absolute numbers of $BrdU^+$ $CD4^+$ T cells in corneal DC – depleted, systemic DC – depleted, and non – DC depleted chimeras (**Fig. 15A**). Since proliferation was only measured for 4 hours, and differences in $CD4^+$ T cell expansion might be more evident if expansion was measured for a longer period, chimeras then received 1 mg BrdU upon infection and everyday up until 3 dpi. Dendritic cells in these mice were depleted locally in the cornea or systemically at -2 dpi. Draining lymph nodes were then harvested at an earlier point (3 dpi) to measure proliferation of $CD4^+$ T cells. At 3 dpi, there was no significant difference among the absolute numbers of expanding $CD4^+$ T cells in mice that were corneal DC – depleted, systemic DC-depleted, or not depleted (**Fig. 15B**). Additionally, at 7 dpi, there was no significant difference in proliferating $CD4^+$ T cell numbers after corneal DC depletion before infection (**Fig. 15C**). This indicates that DC resident to the cornea before infection are not important in the expansion of $CD4^+$ T cells in the draining lymph nodes after HSV-1 infection.

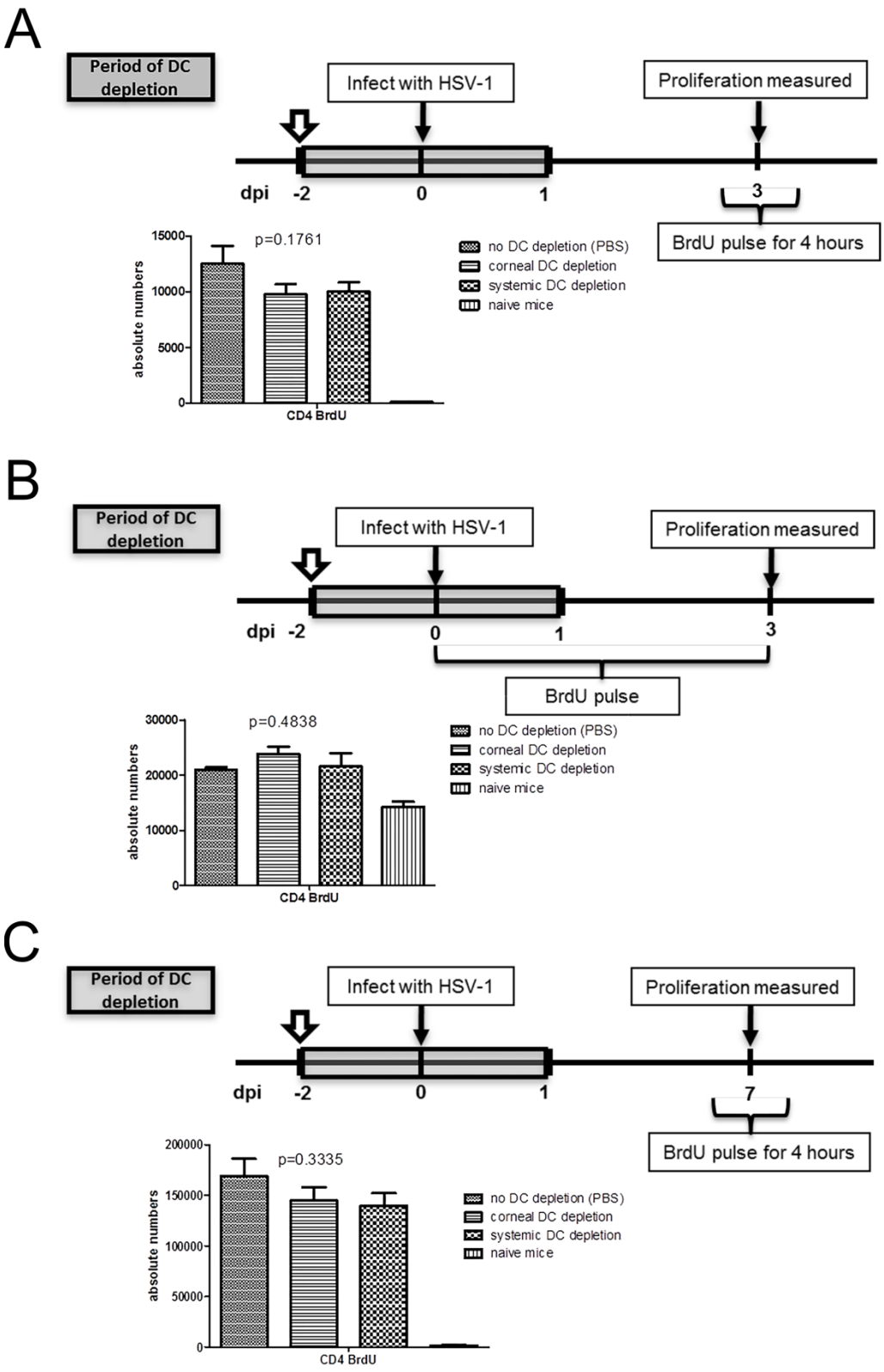

Figure 15. Cornea-resident DCs are not necessary for $CD4^+$ T cell expansion.

BALB/c CD11c-DTR bone marrow chimeras were given subconjunctival injections of 30 ng DT (corneal DC depletion), intraperitoneal injections of 150 ng DT (systemic DC depletion), or PBS (no DC depletion). Chimeras received a single treatment of DT 2 days before HSV-1 infection (as indicated by unfilled arrows) to deplete cornea – resident DCs locally or to deplete both cornea – resident and DLN – resident DCs systemically, and DCs that migrate into the tissue at 1 dpi. Chimeras received i.p. injections of 1 mg bromodeoxyuridine (BrdU) at indicated times. DLN were excised at 3 or 7 dpi and processed to yield single cell suspensions. DLN cells were stained with antibodies to CD3, CD4, and BrdU to measure $CD4^+$ T cell proliferation by flow cytometry. Depletion of cornea – resident DCs does not influence $CD4^+$ T cell proliferation when measured at 3 dpi using A) a 4 hour BrdU pulse or B) 3 day BrdU pulse, or C) when measured at 7 dpi using a 4 hour BrdU pulse. The *p* values for group differences were analyzed using a one-way ANOVA with Bonferroni posttests (all comparisons between groups are not significant). Data are representative of three experiments, with five mice per group.

6.2 CORNEA-RESIDENT DC ARE NOT ESSENTIAL FOR HSK PROGRESSION

Not surprisingly, when corneal DC – depleted, systemic DC – depleted, and non – depleted DC chimeras were allowed to develop HSK, all of these mice progressed to having disease at the same kinetics (**Fig. 16**).

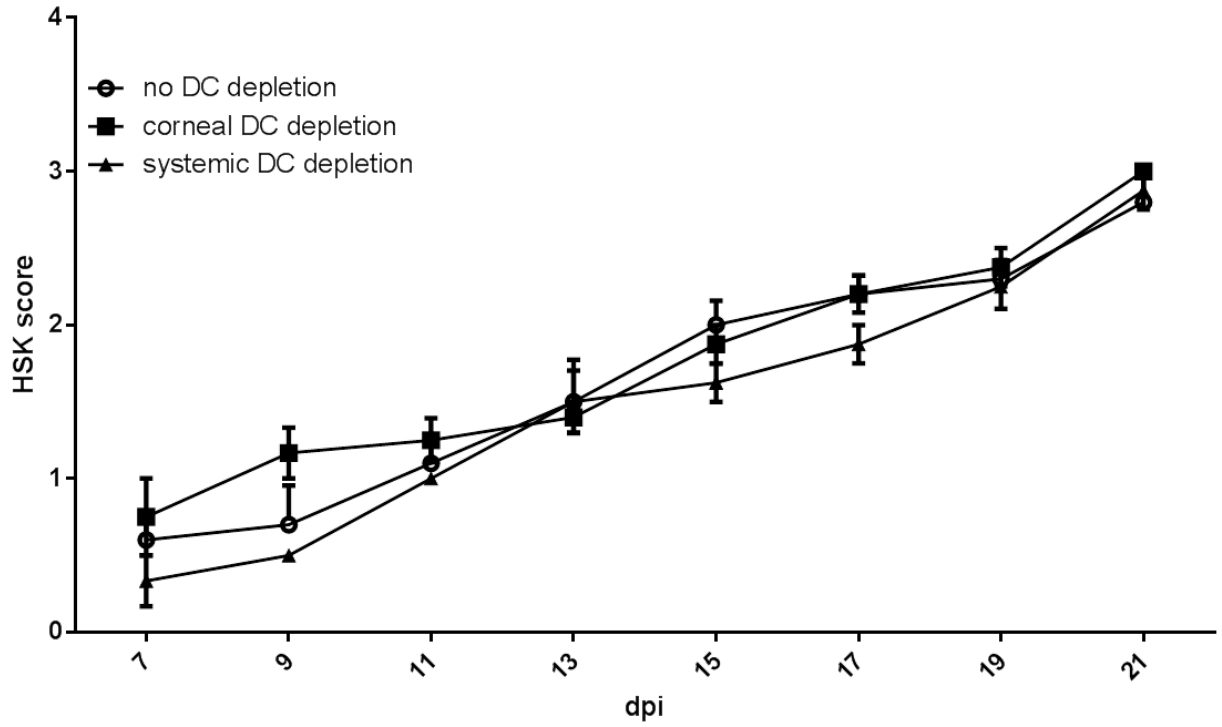

Figure 16. Depletion of cornea-resident DC does not influence HSK progression.

BALB/c CD11c-DTR bone marrow chimeras were given subconjunctival injections of 30 ng DT (corneal DC depletion), intraperitoneal injections of 150 ng DT (systemic DC depletion), or PBS (no DC depletion), at -2 dpi to deplete cornea-resident DCs locally or to deplete both cornea-resident and DLN-resident DCs systemically, and DCs that migrate into the tissue at 1 dpi. Mice were observed for disease progression and recorded as mean ± SEM HSK severity.

6.3 CORNEA-INFILTRATING DC AND DLN-RESIDENT DC BOTH CONTRIBUTE TO THE EARLY EXPANSION OF $CD4^+$ T CELLS IN THE DLN

After DT treatment of CD11c-DTR chimeras once, dendritic cells start to reconstitute tissue after 3 days (**Fig. 4**) (115). Dendritic cells that migrate into the cornea after infection could be more important than cornea-resident DC in $CD4^+$ T cell expansion in the DLN so chimeras received DT at -2 and +1 dpi to prolong depletion of dendritic cells locally or systemically up until 3 dpi. Chimeras received 1 mg BrdU 4 hours before harvest at 3 dpi and BrdU incorporation of $CD4^+$ T cells was measured through flow cytometry. At 3 dpi, absolute numbers of $BrdU^+$ $CD4^+$ T cells of corneal DC – depleted mice was reduced to less than half compared to that of mice with intact DC. Absolute numbers of $BrdU^+$ $CD4^+$ T cells of systemic DC – depleted mice on the other hand, was ablated completely. These values were significantly reduced compared to that of the corneal DC – depleted mice and was comparable to that of the naïve mice. This suggested that there is a need for infiltrating corneal DC in expanding $CD4^+$ T cells, but since the $CD4^+$ T cell response gets totally abrogated after systemic DC depletion, there is also a role for DC resident to the DLN in contributing to these responses (**Fig. 17A**).

To determine what would transpire if DCs are allowed to reconstitute the tissue after DC depletion up until 4 dpi, the draining lymph nodes of these mice were taken out at 7 dpi. At 7 dpi, $BrdU^+$ $CD4^+$ T cells in the DLN of mice that were either corneal DC – depleted, systemic DC – depleted recovered to the levels of non-DC-depleted mice (**Fig. 17B**).

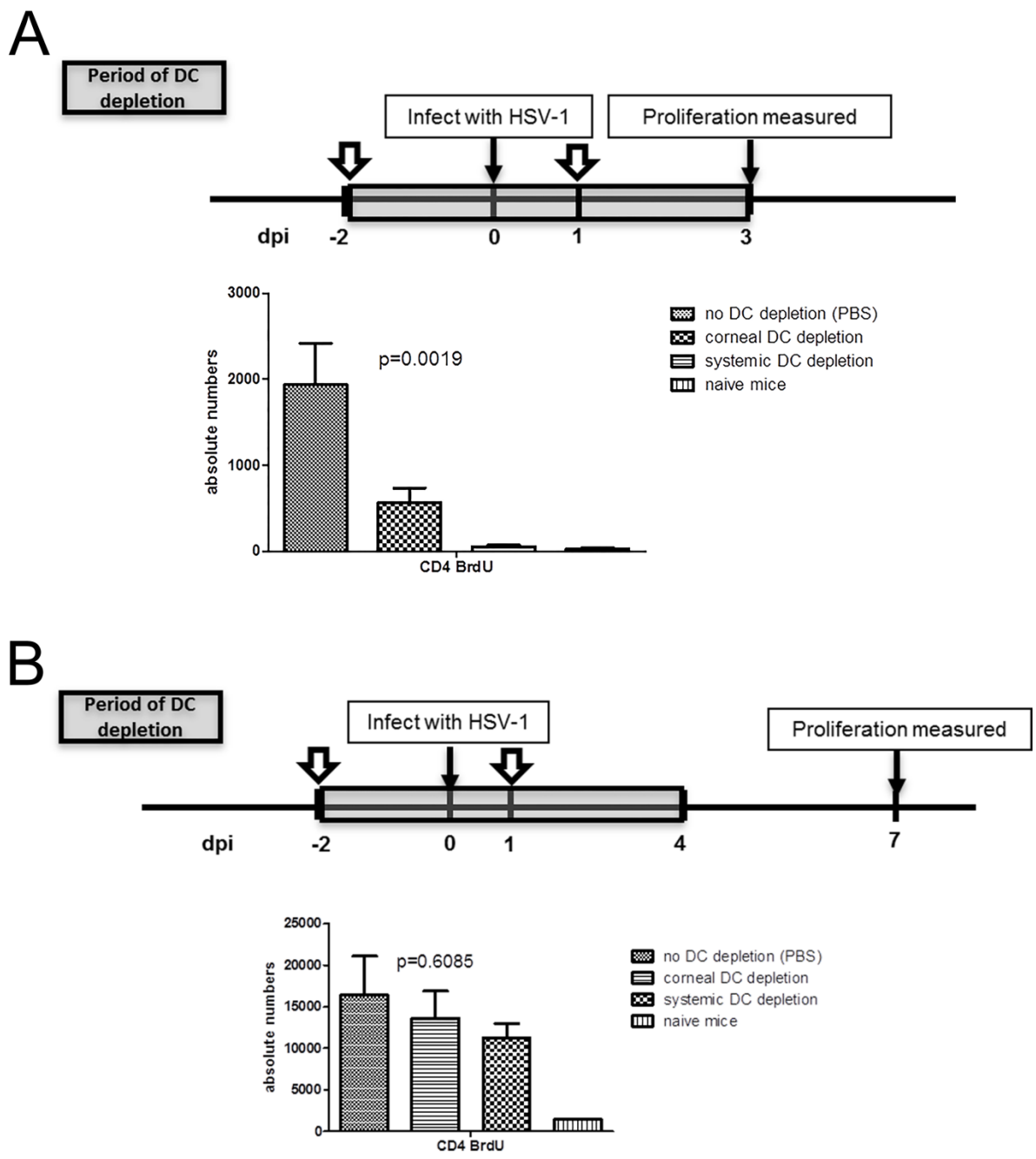

Figure 17. Both cornea-infiltrating DC and DLN-resident DC play a role in CD4$^+$ T cell expansion in the DLN at 3 dpi.

BALB/c CD11c-DTR bone marrow chimeras were given local (subconjunctival) or systemic (intraperitoneal) DT treatments at -2 and +1 dpi (as indicated by unfilled arrows) to deplete infiltrating corneal DCs (corneal DC depletion) or both infiltrating corneal DCs and DLN-resident DCs (systemic DC depletion) up to 4 dpi, or PBS (no DC depletion). Chimeras received i.p. injections of 1 mg BrdU 4 hours before excision of DLN. Single cell

suspensions of DLN were stained with antibodies to CD3, CD4 and BrdU to measure $CD4^+$ T cell proliferation by flow cytometry. Bar graphs show mean ± SEM of absolute number of proliferating ($BrdU^+$) $CD4^+$ T cells per DLN. A) $CD4^+$ T cell proliferation was significantly reduced by corneal DC depletion and abrogated by systemic DC depletion. B) Corneal or systemic DC depletion up to 4 dpi had no impact on $CD4^+$ T cell proliferation measured at 7 dpi. The *p* values for group differences were determined using a one-way ANOVA with Bonferroni posttests [(A) no DC depletion versus corneal DC depletion, no DC depletion versus systemic DC depletion, and corneal DC depletion versus systemic DC depletion are $p < 0.05$, (B) no depletion versus systemic DC depletion, no depletion versus corneal DC depletion, and systemic DC depletion versus corneal DC depletion are not significant]. Data are representative of three experiments, with five mice per group.

6.4 THE ABSENCE OF DENDRITIC CELLS UP UNTIL 4 DPI DOES NOT INFLUENCE THE PROGRESSION OF HSK

Since proliferation of $CD4^+$ T cells in the chimeras recovered after local or systemic DC depletion until 4 dpi, when they were followed up for disease, it was not surprising that there was no significant difference found in HSK progression among the different groups (**Fig. 18**).

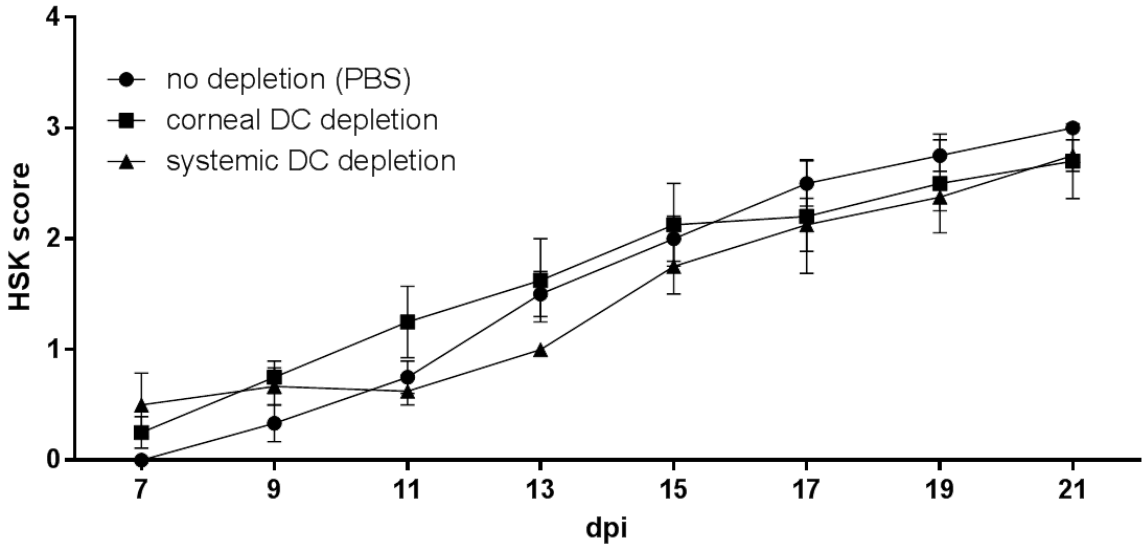

Figure 18. Depleting DC from the cornea and/or the DLN does not affect HSK progression.

BALB/c CD11c-DTR bone marrow chimeras were given local (subconjunctival) or systemic (intraperitoneal) DT treatments at -2 and +1 dpi to deplete cornea – infiltrating DCs (corneal DC depletion) or both cornea – infiltrating DCs and DLN – resident DCs (systemic DC depletion) up to 4 dpi or PBS (no DC depletion). Mice were observed for disease progression and recorded as mean ± SEM HSK severity. Data are representative of three experiments, with five mice per group.

6.5 ANTIGEN APPLIED TO THE CORNEA DRAINS TO THE LYMPH NODES EVEN IN THE ABSENCE OF CORNEA DENDRITIC CELLS

How do DLN-resident DC acquire antigen even in the absence of cornea-infiltrating DC? We hypothesized that antigen can passively drain through the lymphatics even without cornea-infiltrating DC migrating to the DLN. To serve as a surrogate antigen, ovalbumin labelled with Alexa Fluor 546 was topically introduced onto the corneas of CD11c-DTR chimeric mice at the time of infection. These mice were either corneal DC-depleted, systemic DC-depleted, or kept

their DC intact. At 1 dpi, DLN were harvested, cut in half, stained with an antibody to CD11b, and mounted whole for imaging through confocal microscopy. Images of non – depleted mice show that OVA is acquired by $CD11c^+$ dendritic cells in the DLN, and in the absence of cornea – infiltrating dendritic cells, OVA still gains access to the DLN and is taken up by dendritic cells. In the absence of dendritic cells systemically, OVA still drained to the DLN (**Fig. 19**).

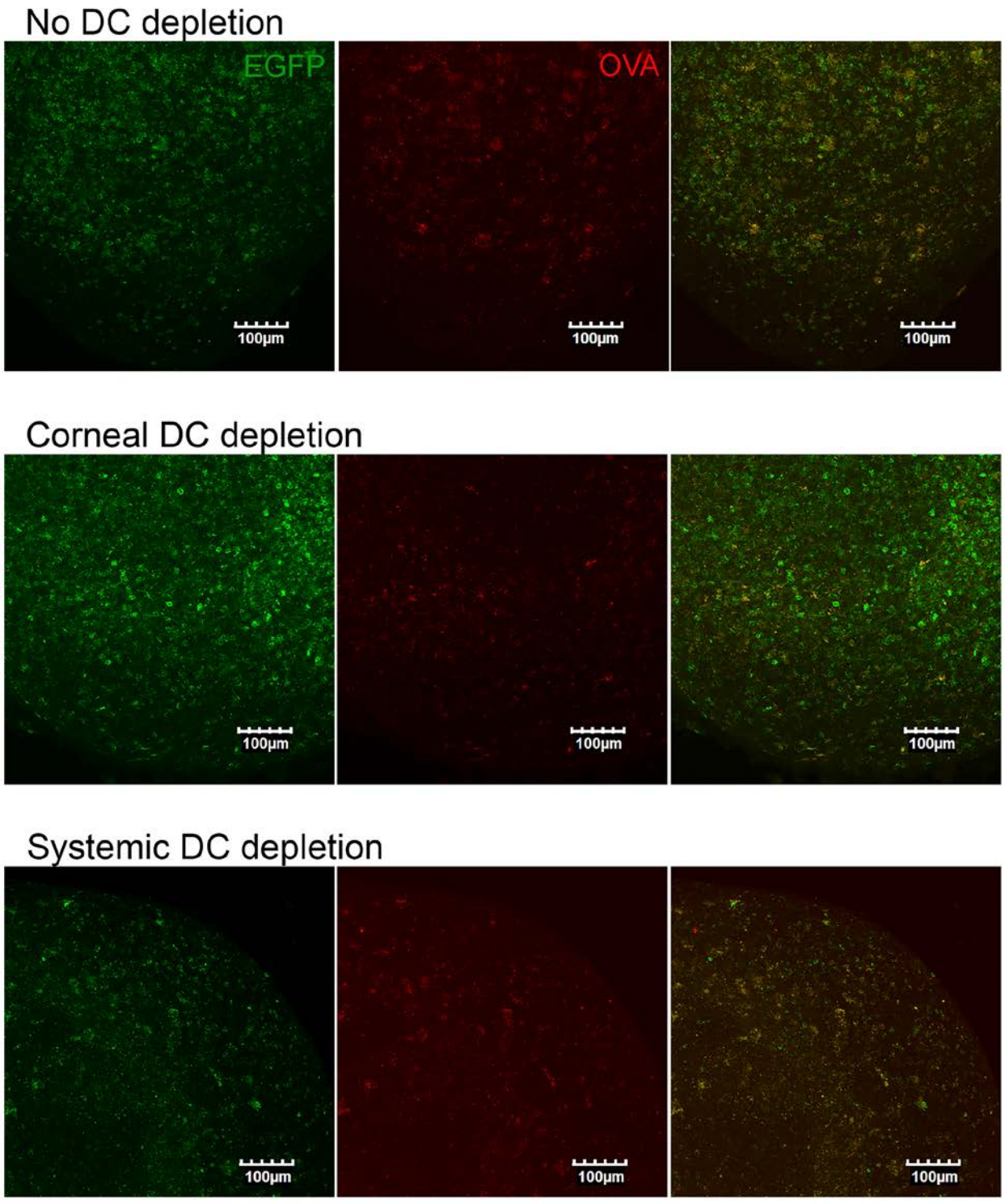

Figure 19. OVA antigen drains to the lymph nodes with or without DC from the cornea.

BALB/c CD11c-DTR bone marrow chimeras given PBS (no DC depletion), local (subconjunctival), or systemic (intraperitoneal) DT treatments at -2 dpi to deplete cornea-infiltrating DCs (corneal DC depletion) or both cornea-infiltrating DCs and DLN-resident DCs (systemic DC depletion) up to 1 dpi or PBS (no DC depletion). Corneas were infected with HSV-1 and Alexa Fluor 594-conjugated ovalbumin (OVA-AF594) was applied onto the corneas. At 1 dpi, DLNs were excised, cut in half, fixed with 1% paraformaldehyde and mounted for confocal microscopy imaging of DCs distinguished by EGFP expression from the CD11c promoter (green) and AF594-tagged OVA (red). Compressed images of z-stacks in the *xy* plane of lymph nodes are representative of three experiments, three to five mice per group.

6.6 BEYOND 3 DPI, $CD4^+$ T CELL EXPANSION IN THE DLN IS MORE DEPENDENT ON CORNEA-INFILTRATING DC THAN DLN-RESIDENT DC

We demonstrated that absolute numbers of proliferating $CD4^+$ T cells recover at 7 dpi after depleting cornea-infiltrating DC only or both cornea-infiltrating DC and DLN-resident DC from -2 to 4 dpi. This indicated that after 4 dpi, either cornea-infiltrating DC start to come into the cornea again and then migrate to the DLN to present antigen to $CD4^+$ T cells, or that DLN-resident DC are now capable of antigen presentation. To test this possibility, CD11c – DTR chimeras received a continuous treatment of DT either locally or systemically to deplete DC up until 7 dpi. Mice received 1 mg BrdU 4 hours before harvest of DLN at 7 dpi. At 7 dpi, there is a significant difference between the absolute numbers of proliferating $CD4^+$ T cells in corneal DC – depleted mice and that of the non-DC – depleted mice. There is also a significant reduction in $BrdU^+$ $CD4^+$ T cell numbers in systemic DC-depleted mice compared to that of the non-DC – depleted mice. The $BrdU^+$ $CD4^+$ T cell numbers were comparable however, between corneal DC – depleted and systemic DC-depleted mice, indicating that contrary to what was seen at 3 dpi,

there seems to be a greater involvement for cornea-infiltrating DC than for DLN-resident DC in $CD4^+$ T cell expansion at 7 dpi. Proliferating $CD4^+$ T cell numbers are not completely abrogated in corneal DC-depleted mice or systemic DC-depleted mice, suggesting another kind of antigen – presenting cell in the DLN may play a role in $CD4^+$ T cell expansion (**Fig. 20A**).

Recently, a C57BL/6 $CD4^+$ T cell epitope was identified on HSV-1 glycoprotein D ($gD_{290-302}$) (479), the depletion of corneal DC was repeated in C57BL/6 CD11c-DTR bone marrow chimeric mice and the expansion of HSV-specific $CD4^+$ T cells in the DLN was measured using MHC II multimers containing the gD epitope. The HSV-specific $CD4^+$ T cell expansion in C57BL/6 mice was reduced by 55%, comparable to the 53% reduction in expansion of the overall $CD4^+$ T cells in BALB/c mice (**Fig. 20B**).

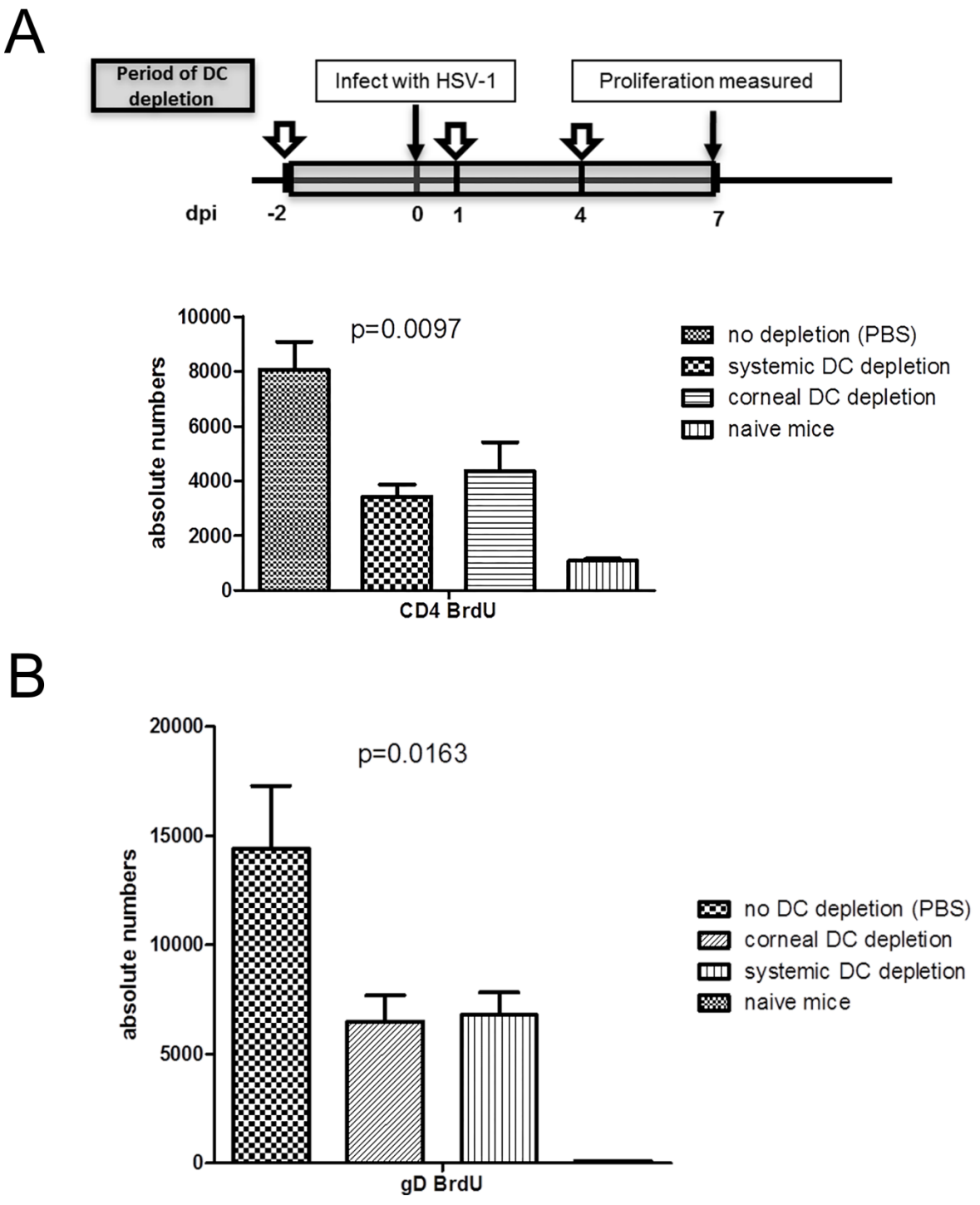

Figure 20. DC migrating from the cornea are the main DC responsible for expansion of $CD4^+$ T cells at 7 dpi.

BALB/c or C57Bl/6 CD11c-DTR chimeras were given local (subconjunctival) or systemic (intraperitoneal) treatments of DT (as indicated by unfilled arrows) at -2, +1, and +4 dpi to deplete cornea-derived (corneal DC depletion) or cornea-derived and DLN (systemic DC depletion) DCs up to 7 dpi, or PBS (no DC depletion). Mice received i.p. injections of 1 mg BrdU 4 hours before excision of DLN. A) Single cell suspensions of DLN from BALB/c chimeras were stained with antibodies to CD3, CD4, and BrdU to measure $CD4^+$ T cell proliferation by flow cytometry. B) Single cell suspensions of DLN from C57Bl/6 chimeras were stained with MHC II multimers containing the HSV-1 glycoprotein D $(gD)_{290-302}$ epitope. Bar graphs show mean ± SEM absolute number of proliferating ($BrdU^+$) $CD4^+$ T cells per lymph node in BALB/c chimeras (A) or proliferating ($BrdU^+$) gD-specific $CD4^+$ T cells per lymph node in C57Bl/6 chimeras. The *p* values for group differences were analyzed by using a one-way ANOVA with Bonferroni posttests (no DC depletion versus corneal DC depletion and no DC depletion versus systemic DC depletion are $p < 0.05$, corneal DC depletion versus systemic DC depletion is not significant). Data are representative of three experiments, with five mice per group.

6.7 CORNEA DENDRITIC CELLS ARE NOT REQUIRED FOR RE – STIMULATION OF $CD4^+$ T CELLS IN THE CORNEA DURING HSK

Mice infected with HSV-1 in their corneas start to develop an immunopathological disease mediated by $CD4^+$ T cells around 7 dpi. A massive infiltration of immune cells such as $CD4^+$ T cells, neutrophils, dendritic cells, and monocytes occurs during HSK onset. Our previous study using UV-B irradiation to locally deplete corneal DC suggested a requisite role for corneal DC in re-stimulating the effector $CD4^+$ T cells that mediate HSK in infected corneas (384). Since UV-B could affect cells other than DC, we re-evaluated this role for corneal DC using DT treatment to deplete only DC and evaluated its effect on HSK progression. Intraperitoneal DT treatment was performed to deplete corneal DC without the possible confounding mechanical effect of subconjunctival injections.

To determine if dendritic cells that infiltrate the cornea are necessary in activating the $CD4^+$ T cells that orchestrate the disease, DC were depleted from CD11c – DTR chimeras by continuous systemic treatment with DT from 7 dpi up until 21 dpi. Corneas were excised from DT treated and mock treated mice at 10, 15, and 21 dpi, the cells were dispersed with collagenase, and analyzed by flow cytometry for expression of various cell lineage markers. Representative flow plots and bar graphs show complete depletion of $CD11c^+$ $EGFP^+$ cells in the corneas of these mice (**Fig. 21A**). When these mice were scored for disease, chimeras systemically depleted of DC developed HSK at the same rate as the non-DC depleted mice, indicating that cornea-infiltrating DC during HSK may not be important for disease progression (**Fig. 21B**).

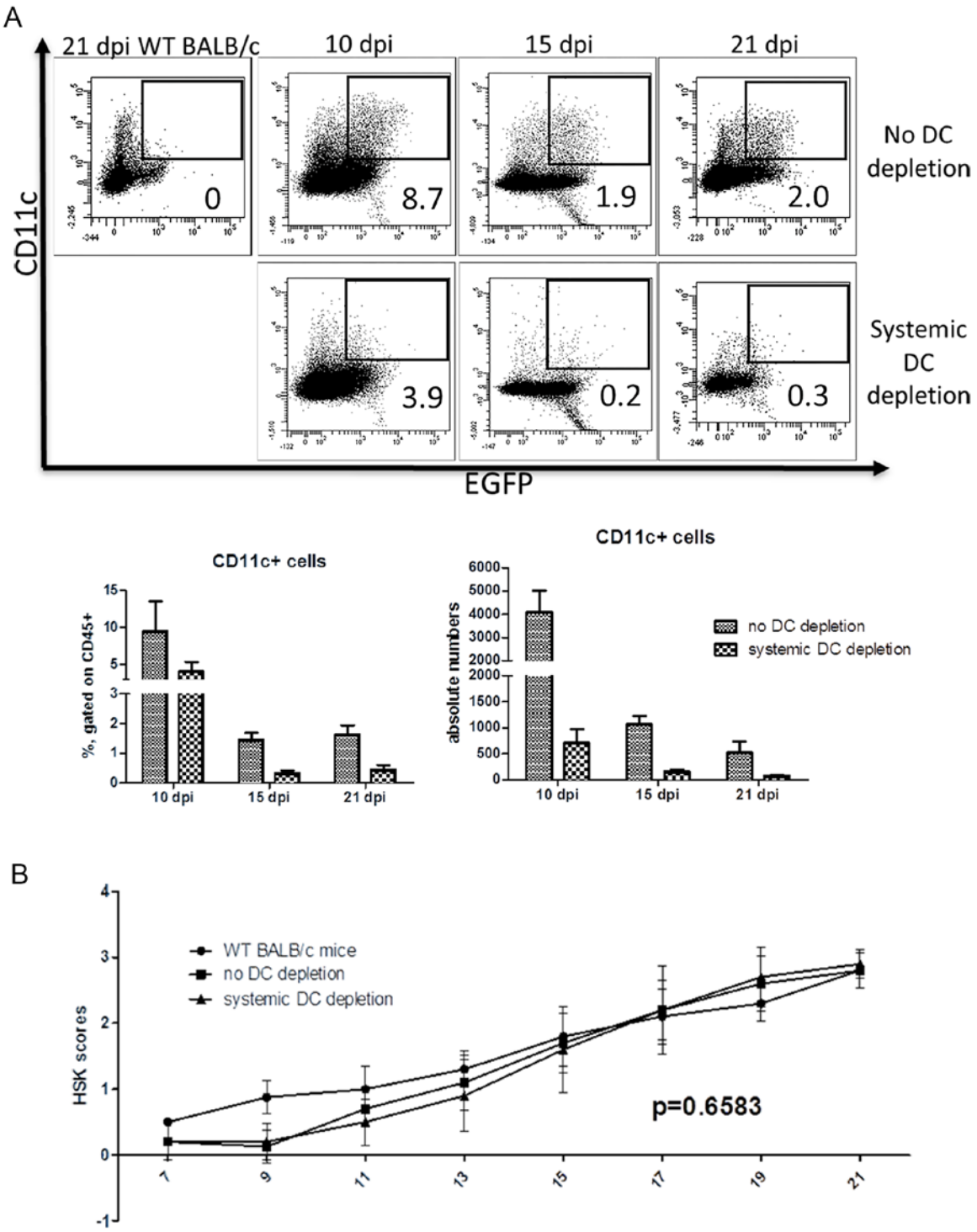

Figure 21. Dendritic cells present in the cornea during HSK are not necessary for disease progression.

Wildtype and CD11c-DTR BALB/c bone marrow chimeras were infected with HSV-1 and were given intraperitoneal injections of PBS or DT every other day from 7 to 21 dpi. A) Corneas were excised at 10, 15, and 21 dpi; treated with collagenase to yield a single cell suspension; stained with antibodies to CD11c and CD45; and

analyzed by flow cytometry for CD11c expression and EGFP expression from the CD11c promoter. Representative flow plots show $CD11c^+$ $EGFP^+$ cells gated on $CD45^+$ cells. Numbers on flow cytometry plots represent percentage of $CD11c^+$ $EGFP^+$ cells gated on $CD45^+$ cells. B) Mice were observed for disease progression and recorded as mean ± SEM HSK severity. The significance of group differences in HSK severity was determined by calculating the area under the curve for each mouse and then performing an ANOVA with Bonferroni posttests (posttest comparisons of all pairs of groups are not significant). Data are representative of three experiments, with five mice per group.

6.8 DENDRITIC CELL DEPLETION HAD NO EFFECT ON $CD4^+$ T CELL AND NEUTROPHIL INFILTRATE IN THE CORNEA DURING HSK

In line with the HSK data, DC depletion had no effect on the percentage and absolute numbers of infiltrating $CD4^+$ T cells or neutrophils into the cornea (**Fig. 22**).

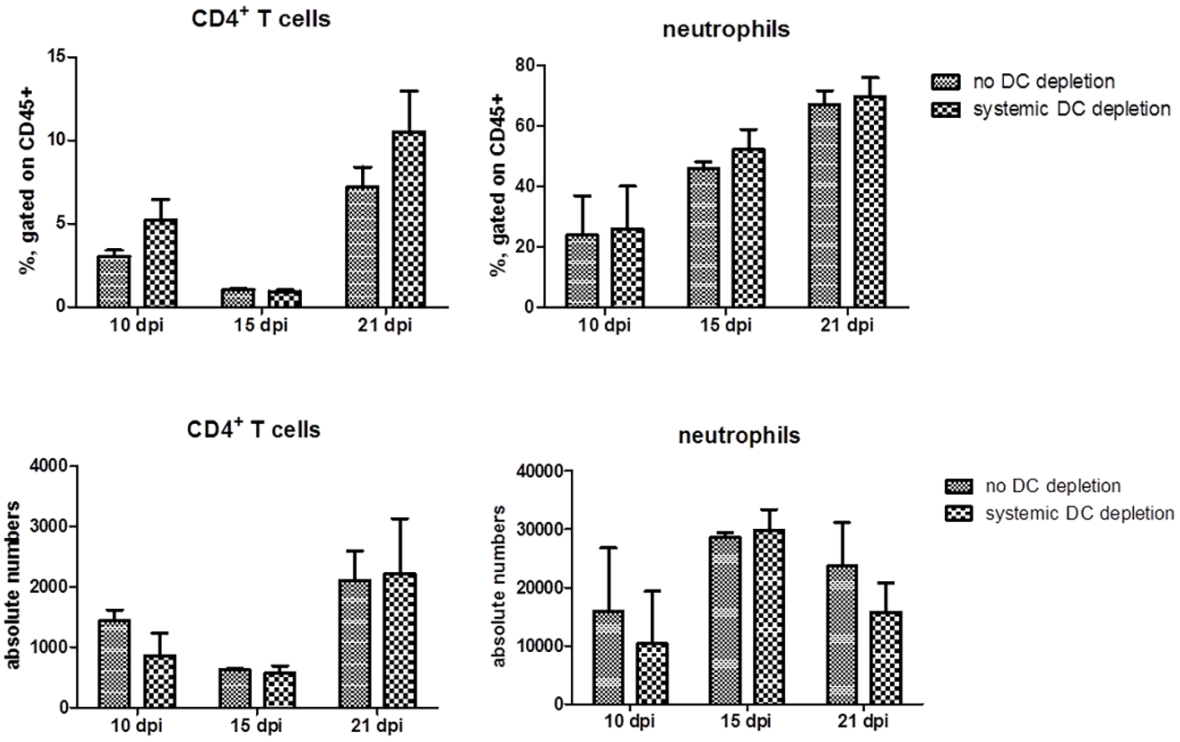

Figure 22. Cellular infiltrate into the cornea is not changed after depletion of DC during HSK.

CD11c-DTR BALB/c bone marrow chimeras were infected with HSV-1 and were given intraperitoneal injections of PBS or DT every other day from 7 to 21 dpi. Corneas were excised at 10, 15, and 21 dpi; treated with collagenase to yield a single cell suspension; stained with antibodies to CD45, CD4, CD11b, and Ly6g; and analyzed by flow cytometry for $CD4^+$ T cells and neutrophils. Bar graphs show mean ± SEM frequency and absolute number of $CD4^+$ T cells and $CD11b^+$ $Ly6g^+$ neutrophils (gated on $CD45^+$ cells). The significance of group differences in number and frequency of leukocyte populations in DC-depleted and non-depleted mice was analyzed by a Student *t* test. Data are representative of three experiments, with five mice per group.

6.9 IN THE ABSENCE OF DENDRITIC CELLS, $CD4^+$ T CELLS MAY ASSOCIATE WITH MHC II POSITIVE CORNEAL EPITHELIAL CELLS OR MACROPHAGES

The lack of an effect of DC depletion in HSK progression might be explained by the presence of other immune cells that are able to activate $CD4^+$ T cells in the absence of dendritic cells. To determine what antigen presenting cells might be present in the cornea that express MHC II, corneas of 10 dpi DC-depleted and non-depleted CD11c-DTR chimeras were excised and stained with markers for immune cells (CD45), macrophages (F4/80), and $CD4^+$ T cells (CD4). $CD4^+$ T cells appear to be interacting with MHC II^+ $F4/80^+$ macrophages and MHC II positive –, CD45 negative cells with location and morphology consistent with epithelial cells in HSV-1 infected corneas of both DC – depleted and non – depleted mice (**Fig. 23**).

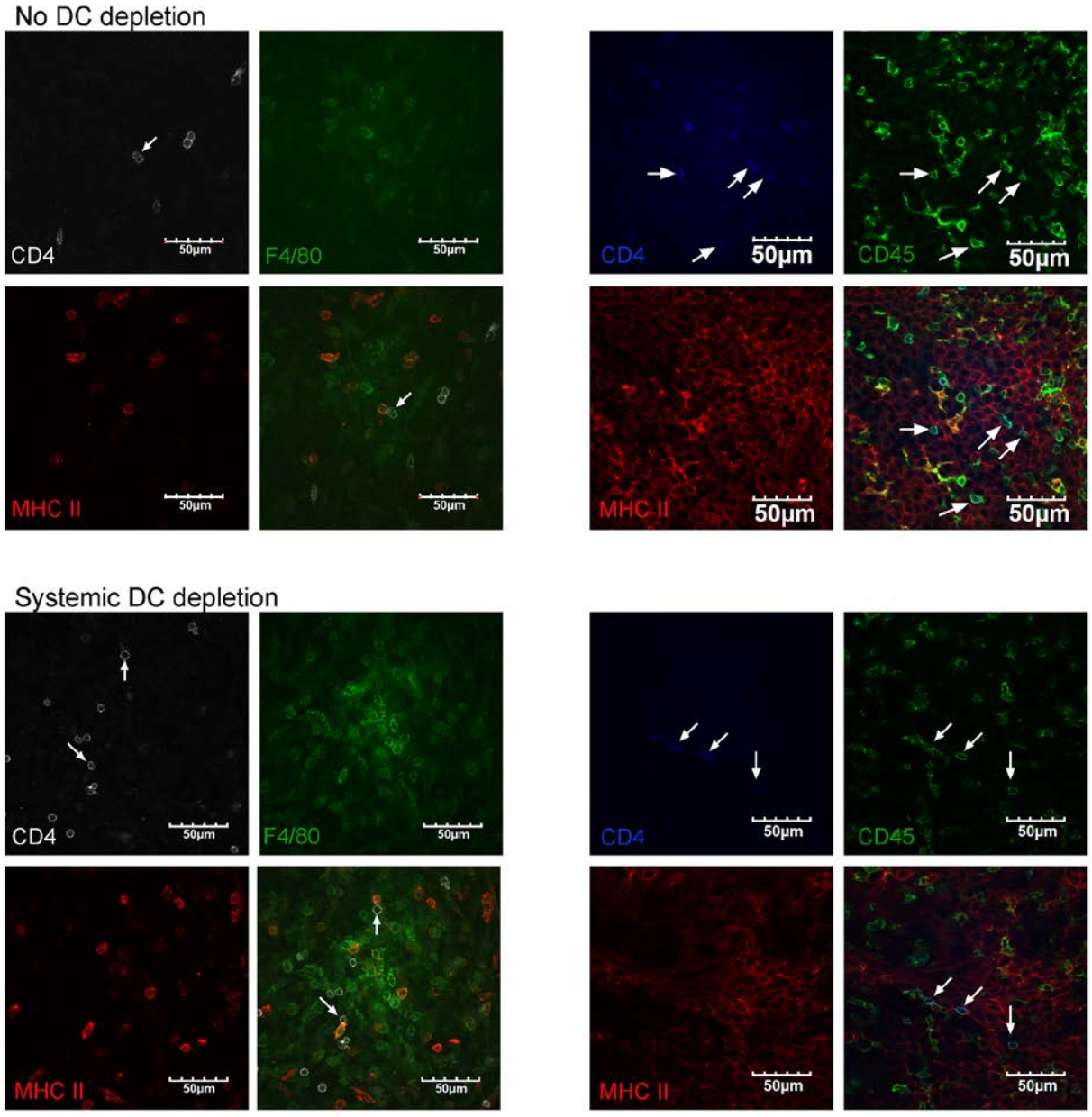

Figure 23. In the absence of infiltrating corneal DCs, $CD4^+$ T cells may interact with epithelial cells and macrophages that express MHC II.

CD11c-DTR BALB/c bone marrow chimeras were infected with HSV-1 and were given intraperitoneal injections of PBS or DT every other day from 7 to 10 dpi. At 10 dpi, corneas were excised from mice with no DC depletion (upper left and right panels) or systemic DC depletion (lower left and right panels). Confocal microscopic images in

the *xy* plane from the periphery of corneal whole mounts depict CD4 (white), MHC II (red), F4/80 (green), and a merged image on the first two columns, or CD45 (green), CD4 (blue), MHC II (red), and a merged image on the last two columns. White arrows identify $CD4^+$ T cells that are closely associated with MHC II^+ macrophages and corneal epithelial cells. All data are representative of three experiments (mean ± SEM of five mice per group).

6.10 DISCUSSION

Determining the contributing roles of cornea-infiltrating DC and DLN-resident DC in the proliferation of $CD4^+$ T cells in the draining lymph nodes after primary infection of the cornea with HSV-1 is possible by a subconjunctival injection of diphtheria toxin into the eye, and this does not affect the DC population in the draining lymph nodes (**Fig. 3**). Our results show that cornea-resident DCs are not necessary in priming of $CD4^+$ T cells in the DLN at 7 dpi (**Fig. 15**), and contrasts with studies with HSV-1 infection of the vaginal submucosa which demonstrate that steady state tissue – resident DC are involved with naïve $CD4^+$ T cell priming (223). The disparity in these observations may be explained by accessibility to antigen. In the studies of Lee, *et al* (223), antigen conjugated to fluorochrome applied onto the vaginal compartment was detected only in the vaginal submucosa and not in the DLN. There was no free flowing antigen acquired from the lymph in the DLN in these mice. However, in our model, antigen applied onto the corneal mucosal surface accessed the DLN after infection (**Fig. 19**). As previously mentioned, another explanation for this could be that DCs resident to the cornea do not have migrating capabilities and therefore cannot be involved in initiating adaptive immune responses in the DLN (21). Also, lytic infections with HSV-1 of dendritic cells can undermine its participation in CTL priming (22-26). It is more likely that antigen presentation to $CD4^+$ T cells is performed by uninfected dendritic cells that take up viral antigen from infected epithelial cells

or dendritic cells from the cornea. Predictably, when chimeras depleted of resident corneal DCs or DCs systemically were allowed to develop HSK, they established disease at the same rate as non – depleted mice as observed from 7 dpi onwards (**Fig. 16**).

We show that dendritic cells come into the cornea after infection, and that in the absence of cornea-resident dendritic cells, cornea-infiltrating DCs can presumably take up viral antigen and present to $CD4^+$ T cells in the DLN. Prolonging the depletion with treatments of diphtheria toxin at -2 and 1 dpi extends local corneal or systemic depletion until 4 dpi, and will affect dendritic cells infiltrating the cornea and other tissues after 1 dpi. Our results demonstrate that local DC depletion reduces absolute numbers of $BrdU^+$ $CD4^+$ T cells to a third of that of the non-depleted mice (**Fig. 17A**). On the other hand, systemic depletion completely abrogates the absolute numbers of $BrdU^+$ $CD4^+$ T cells (**Fig. 17A**), indicating that there is a role for both cornea-infiltrating DCs and DLN-resident DCs in promoting $CD4^+$ T cell responses in the DLN. This trend is also reflected in work by Allenspach, et al. which demonstrated the need for both DC populations: that lymph node – resident DCs trap antigen – specific $CD4^+$ T cells to initiate activation, while DCs migrating from peripheral tissues are necessary to induce proliferation (236). This trend is different from what transpires with $CD8^+$ T cell proliferation after corneal or systemic DC depletion, where there is a greater dependence of $CD8^+$ T cells on DC coming from the cornea in generating $CD8^+$ T cells in the DLN (**Fig. 6A**). There may be a reflection of the differences in activation requirements between $CD4^+$ and $CD8^+$ T cells early in the infection. It is also possible that $CD4^+$ T cells are able to license DLN-resident DCs through CD40-CD154 interactions, thus enabling them to stimulate $CD4^+$ T cells themselves.

However, when chimeras locally or systemically depleted of DC from -2 to 4 dpi were observed at 7 dpi, the absolute numbers of proliferating $CD4^+$ T cells in both DC – depleted

groups recovered to that of the non – depleted chimeras (**Fig. 17B**). This suggested that after 4 dpi, either cornea-infiltrating DC migrating to the DLN are still necessary to stimulate $CD4^+$ T cell responses or that at this time point, DLN-resident DCs are now capable of doing so. Expectedly, when chimeras depleted of cornea-infiltrating DC or DLN-resident DC were observed for disease starting at 7 dpi, they developed disease not unlike the non – depleted chimeras (**Fig. 18**).

Experiments using topical application of OVA-AF594 onto the corneal surface demonstrate that DLN-resident DC are able to acquire antigen in the DLN even when cornea-infiltrating DC are not present. OVA – AF594 gets to the DLN through the lymphatics and are acquired in the DLN by $CD11c^+$ cells (**Fig. 19**).

Both cornea-infiltrating and DLN-resident DC can participate in stimulating $CD4^+$ T cell responses. To establish if $CD4^+$ T cell proliferation beyond 3 to 4 dpi still requires both DC populations, continuous depletion of local corneal-infiltrating DC or DCs all throughout was done up until 7 dpi, and proliferation was measured at 7 dpi. Our results show that after 3 dpi, continuous local DC depletion reduces absolute numbers of $BrdU^+$ $CD4^+$ T cells to half of the non – depleted mice, and is similar to that of the continuous systemically depleted mice (**Fig. 20A**). This is also reflected in tetramer staining done in C57Bl/6 chimeric mice where the $CD4^+$ T cell epitope in the protein gD is known (**Fig. 20B**). This indicates that beyond 4 dpi, cornea-infiltrating DCs migrate to the DLN, and are more important than DLN-resident DC in $CD4^+$ T cell priming. In our murine model of HSV-1 corneal infection, virus starts to get cleared from the epithelium at 4 dpi. Therefore, in the absence of replicating virus in the eye at 4 dpi, cornea-infiltrating DC may be necessary to carry viral antigen to the DLN for antigen presentation, and DLN-resident DC may not obtain any free flowing antigen.

$CD4^+$ T cell responses in both corneal DC depleted mice and systemic DC depleted mice were not reduced completely to the levels of naïve mice, indicating that some other antigen presenting cell may be important in stimulating these responses. Possibly, MHC II – expressing antigen presenting cells such as macrophages or B cells could play a role in priming $CD4^+$ T cell responses against HSV-1 as well. B cells in the DLN can capture and efficiently process antigen for presentation and has been demonstrated in an autoimmunity model (480-482). Likewise, $CD169^+$ macrophages resident to the DLN are capable of cross-presenting antigens to antigen – specific $CD8^+$ T cells as shown in a tumor model (483, 484).

It was not determined if chimeras that were depleted of DC continuously in the cornea or systemically from -2 to 7 dpi were able to acquire immunopathology because at 9 to 10 dpi, these mice succumbed to death due to encephalitis (**Fig. 9B**). Absolute numbers of proliferating $CD8^+$ T cells was reduced considerably in the DLN of chimeras that were either locally or systemically depleted of DC from -2 to 7 dpi (**Fig. 7 and 9**). Consequently, corneal DC depletion led to fewer $CD8^+$ T cells migrating to the DLN that are able to control virus replication in the trigeminal ganglia (**Fig. 9A**).

The requirement for dendritic cells in herpes stromal keratitis is not clear. HSK is an immunopathological disease mediated by $CD4^+$ T cells and is characterized by stromal edema, leukocytic infiltration, and neovascularization. Previous studies have shown that a higher degree of opacity is related to greater number of infiltrating dendritic cells into the cornea (359, 384). Also, the necessity for costimulation was shown in HSK with experiments that involved blocking interactions between B7.1 and CD28 (385), as well as 4-1BB and 4-1BBL (386). However, other costimulatory molecules such as OX40 (388) and CD40 (387) are not required for HSK. Still,

several studies have shown the importance of dendritic cells or blood – derived dendritic cells in the re-stimulation of T cells at the site of infection in both influenza and HSV models (37-40).

In a more definitive experiment to see if dendritic cells were necessary for $CD4^+$ T cell activation in the cornea, both mice eyes were infected with HSV-1, and antigen presenting cells were depleted from mice by treating one eye with UV-B irradiation, and then the other eye was left untreated (19). The irradiated eye did not develop disease, while the untreated eye did, denoting a role for antigen presenting cells for HSK. On the contrary, when we treated our CD11c-DTR chimeras with DT systemically starting at 7 dpi and up until 21 dpi, $CD11c^+$ cells were depleted in their corneas (**Fig. 21A**), and there was not a significant difference in the incidence and severity of disease in our mice (**Fig. 21B**). Indeed, analyzing the infiltrate in HSK corneas, there is no difference in the frequency and absolute numbers of infiltrating $CD4^+$ T cells and neutrophils between DC depleted and non-depleted mice (**Fig. 22**). In the UV-B irradiation experiments, antigen – presenting cells were detected by their expression of CD205. It is possible that UV-B irradiation depletes other immune cells such as macrophages or monocytes, and was not detected in those experiments. Interestingly, this is contradictory with what Iijima, et al. demonstrated in their model where monocyte – derived dendritic cells are not required for priming, but are necessary for re-stimulating cytokine production by effector $CD4^+$ T cells at the infection site (40).

It is also conceivable that other MHC II expressing cells are able to stimulate $CD4^+$ T cells in the cornea in the absence of dendritic cells. A lot more $CD11b^+$ macrophages or inflammatory monocytes compared to DCs infiltrate the cornea during HSK, and interactions between $CD4^+$ T cells and $F4/80^+$ macrophages may be more important in the progression of the

disease (**Fig. 23**). Additionally, CD45 negative epithelial cells in the cornea express MHC II after infection (**Fig. 23**), and could conceivably interact with $CD4^+$ T cells.

In summary, these studies indicate that cornea-resident dendritic cells are not necessary in initiating an adaptive immune response in the DLN against HSV-1. These findings also establish a requirement for both cornea-infiltrating dendritic cells and DLN-resident dendritic cells in priming a $CD4^+$ T cell response early against HSV-1 infection of the cornea. In the later part of the infection when virus gets cleared in the corneal epithelium, there is a need for cornea-infiltrating dendritic cells to carry antigen to the DLN where it can present to $CD4^+$ T cells. Conversely, DCs are not required in the cornea to re-stimulate $CD4^+$ T cells that infiltrate during HSK.

7.0 SUMMARY AND CONCLUSIONS

It is now well recognized that the mucosal surface of the cornea is resident to dendritic cells. Its presence in the cornea implies a role for these DCs as gatekeepers of the body against pathogen and injury. The recent years has also seen major advances in our understanding of the development and the heterogeneity of these dendritic cells based on their location and function. Contingent on the DC subpopulation and microenvironment, DCs can elicit differential but appropriate immune responses to commensal and pathogenic microbial species, resulting in protection against infectious disease.

The studies outlined here have demonstrated the importance of DCs derived from the cornea in HSV-1 infection. Through the usage of CD11c-DTR mice to ablate dendritic cells, we show that dendritic cells resident to the cornea before HSV-1 ocular infection are not necessary to prime an immune response in the draining lymph nodes of infected mice. Though they are important in orchestrating the early events that enable innate immune cells such as natural killer cells and inflammatory monocytes to clear virus from the cornea (115), they do not contribute to generating HSV-specific $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes.

In its place, the dendritic cells present in the cornea after infection are essential in expanding the T cells in the DLN. In the absence of these cornea-infiltrating DCs, we show an abolition of $CD8^+$ T cell proliferation as early as 3 dpi, as well as later (7 dpi) in the infection. The need for infiltrating DC in initiating proliferation in the DLN, instead of cornea-resident DC

or DLN-resident DC, implies that infiltrating corneal DC are more competent in presenting to $CD8^+$ T cells. Resident corneal DC may be inhibited from stimulating T cells due to death by lytic infection, the inability to migrate, or by failure to upregulate costimulatory molecules due to the virus's immune evasion strategies. Additionally, the costimulatory phenotype of infiltrating corneal DC shows greater levels of expression compared to DC resident to the DLN. However, blocking costimulatory interactions between CD40 and CD40 ligand, as well as between CD86 and CD80, did not influence the generation of $CD4^+$ and $CD8^+$ T cells, suggesting that other molecules such as CD80, OX40 ligand, or ICOS ligand may compensate. This significant reduction in expansion after depletion of infiltrating corneal DC led to a decrease in $CD8^+$ T cells migrating to the trigeminal ganglia. Consequently, mice were not protected against encephalitis and expired at about 9 dpi.

Cornea-infiltrating DCs are critical in expanding $CD4^+$ T cells in the DLN as well. However, we demonstrate that it is a combined effort of both DLN-resident DCs and cornea-infiltrating DCs early in the infection. In the absence of cornea-infiltrating DC, DLN-resident DCs are able to acquire antigen that passively drain through the lymph during infection. Though antigen gains access to DLN-resident DCs, $CD8^+$ T cells solely need DCs coming from the cornea for presentation. This may reflect a disparity between the activation requirements of $CD4^+$ and $CD8^+$ T cells.

Dendritic cells have also been implicated in the disease herpes stromal keratitis (359). The affector part of the immune response that occurs during this $CD4^+$ T cell – mediated immunopathology appears to involve antigen-presenting cells. However, continuous DC ablation starting at disease onset did not influence the severity and frequency of HSK. Further investigation shows that in the absence of dendritic cells in the cornea during disease, epithelial

cells in the cornea, as well as $F4/80^+$ macrophages express MHC II molecules that may be capable of activating $CD4^+$ T cells in the cornea.

Through detecting HSV through pattern recognition receptors, taking up and transporting HSV-derived antigens to the lymph node, and activating naïve HSV-specific T cell responses and activating effector T cells at sites of infection, dendritic cells bridge innate and adaptive immune responses. The need for functional specialization between DC subsets implies a fine-tuning of immune responses based on the kind of pathogen at hand and where it is encountered. Our comprehension of dendritic cells and HSV pertaining to immunity against acute infections has expanded, and recently, so have DC – HSV studies pertaining to HSV reactivation and latency. Immune complications related to HSV such as encephalitis and keratitis is a major concern worldwide, yet there is still no vaccine against herpes (485).

Research is necessary to completely comprehend how the immune system responds to HSV infection, and looking deeper into how the balance and communication between specialized DC subsets is modulated based on context is imperative for an effective antiviral response and maintenance of immune homeostasis.

8.0 FUTURE DIRECTIONS

8.1.1 What distinct specific DC subsets are necessary in priming the $CD4^+$ and $CD8^+$ T cell immune responses?

It is widely accepted that conventional DCs, pDCs, Langerhans cells, and monocyte-derived DCs are developmentally distinct lineages. At steady state, although the dendritic cells in the cornea do not express CD11b, it was recently characterized as $CD103^{low}$ (113). We determined that there is no necessary and sufficient role for cornea-resident DC in the expansion of T cells in the DLN. Additionally, although the specific role of $CD8^+$ lymphoid tissue resident DCs in cross presentation has been demonstrated for different models of HSV-1 infection (214, 244), it has not been established for infections of the corneal mucosal surface.

The CD11c-DTR mouse model has been extensively used to study the functions of DCs in vivo but it has been shown since that other non-DCs express CD11c as well, such as macrophages, activated monocytes, pDCs, and activated T cells (471, 474, 486). Alternate knockout mouse models have been established ever since to look at delineating roles of specific DC subsets.

Langerhans cells resident to the cornea have been identified in the corneal epithelium (113), but the role of these cells has not been established. Employing Langerin - (diphtheria toxin subunit A) DTA mice that have constitutive absence of Langerhans cells (225), can confirm their

importance in priming T cells in the DLN. Alternatively, inducible ablation of Langerhans cells can also be performed in Langerin-DTR mice (487).

A plethora of cytokines and transcription factors regulate the genetic program that determines the specification and differentiation of these diverse DC subsets. Loss of expression of certain transcription factors has been demonstrated to be the principal cause of defects in DCs and other immune cells developmentally (488-490). The roles of each of these different DC categories may be elucidated through the transcriptional regulators that define each individual DC population. Although there have been a lot of transcription factors that have been determined to direct various dendritic cell lineages (reviewed in (491)), we will be focusing on some that may be relevant to our infection model (summarized in Table 2).

The signals that are necessary for DC precursors to differentiate into the CD8α and CD103 lineages are integrated by these transcription factors: IRF8, E4BP4 (E4 promoter binding protein 4), ID2, and BATF3 (basic leucine zipper transcription factor, ATF-like 3).

Table 2. Transcriptional programming of different DC subsets

Transcription factor	Transcription factor family	Function
IRF8	Interferon-regulatory factor	Required for the development of pDCs and $CD8\alpha^+$ and $CD103^+$ cDCs
ID2	Inhibitor of DNA binding family protein containing HLH domains	Required for the development of $CD103^+$ DCs and $CD8\alpha^+$ DCs in the spleen and popliteal LN; not required for mesenteric LN DCs
BATF3	bZip family	Required for establishing $CD103^+$ DC lineage, have impaired development of $CD8\alpha^+$ DCs

E4BP4/NFIL3	PAR-related bZip transcription factor	Required for the development of $CD8\alpha^+$ DCs
IRF4	Interferon-regulatory factor	Required for the development of non-CD8α+ DCs
Zbtb46	Broad complex, Tramtrack, Bric-a-brac, and Zinc finger	Required for the development of all conventional DCs
Mycl1	Basic helix-loop-helix	Required for the development of $CD8\alpha^+$ and $CD103^+$ DCs

IRF8 is a transcription factor that is required for the development of pDCs and conventional DC subsets, particularly the $CD8\alpha^+$ and $CD103^+$ subsets. IRF8-deficient mice have diminished numbers of $CD11c^+$ $CD8^+$ as well as $CD8^-$ DCs that are not able to upregulate costimulatory molecules nor secrete cytokines in the presence of microbial products (492-494). IRF8 also regulates some DC functions, including TLR9 expression and IFNα secretion by pDCs (495), as well as IL-12 production by CD8α DCs (496). $CD4^+$ T cell expansion in the DLN after HSV-1 ocular infection is partly dependent on DLN-resident DC at 3 dpi, while $CD8^+$ T cell expansion relies solely on the presence of cornea-infiltrating DC. If there is a role for the $CD8^+$ DC subset located in the DLN, we may find that after infecting IRF8 knockout mice with HSV-1 in the cornea, $CD4^+$ T cell expansion, and not $CD8^+$ T cell expansion, may be reduced. Interestingly, IRF8 knockout mice do not produce IL-12p40, lack Th1 polarization, and are susceptible to infections with intracellular pathogens (497-501). Therefore we expect IRF8 knockout mice to develop HSK, consistent with studies from our lab which demonstrate that IL-$12p40^{-/-}$ mice establish disease with the same severity as wild type mice (502). Alternatively, the importance of $CD8\alpha^+$ and $CD103^+$ DCs, can be studied in mice without the transcription factor ID2. Loss of ID2 prevents the development of these two DC subsets (503, 504).

The first transcription factor that was demonstrated to regulate the development of $CD8\alpha^+$ DCs is BATF3 (505). It has since been shown to be involved in establishing the $CD103^+$ $CD11b^-$ DC lineage in lymphoid tissues as well (506). Although the absence of Batf3 in knockout mice does not completely abolish CD8α DCs (504, 507, 508), there is a profound impairment in the ability of these mice to act against infections with West Nile virus (505), influenza (509), and Toxoplasma gondii (510). Contrastingly, a lack of susceptibility to Listeria in Batf3 KO mice has been demonstrated and is due to failure of this bacterium to productively infect Batf3-dependent $CD8\alpha^+$ and $CD103^+$ DCs (511). Batf3 KO mice are also defective in cross-priming (512), which, taken with the previous studies, implies that Batf3 is critical for cross presentation of exogenous antigens to $CD8^+$ T cells. Infecting Batf3 KO mice ocularly with HSV-1 will confirm if cross presentation is necessary for T cell priming, as well as if there is a role for $CD8\alpha^+$ DCs.

E4BP4 or NFIL3 is a basic leucine zipper transcription factor that is essential for $CD8\alpha^+$ DC development. $Nfil3^{-/-}$ mice lack $CD8\alpha^+$ DCs in lymphoid tissues, but retain the $CD8\alpha^-$ cDCs and plasmacytoid DC subpopulations. These knockout mice were demonstrated to have defective $CD8^+$ T cell cross priming and impaired IL-12 secretion after TLR3 ligation. Remarkably, dendritic cell development is controlled by NFIL3 through the transcription factor Batf3. Like in $Batf3^{-/-}$ mice, infecting these mice ocularly with HSV-1 will help us determine the requirement for $CD8\alpha^+$ DCs and if there is cross-priming is critical to generate $CD8^+$ T cells after infection (513).

Studies with another interferon related transcription factor, IRF4, have revealed that the loss of this transcription factor leads to a defect in the development of $CD11b^{high}$ $CD8\alpha^-$ conventional DCs (514). Additionally, the spleen of these mice lack $CD4^+$ $CD8\alpha^-$ (also $CD11b^+$) DCs. More recently, it has been demonstrated that IRF4-dependent dendritic cells are necessary

for control of Th2 responses in the context of allergy (515, 516), as well as for IL-17 responses in lung and gut mucosal surfaces (517). The studies above are consistent with the notion that $CD11b^+$ dendritic cells are dedicated to stimulate $CD4^+$ T helper cells, and loss of IRF4 solely only on $CD11c^+$ DCs impairs the configuration of peptide-MHC II complexes and results in insufficient priming of $CD4^+$ T helper cells, but not of $CD8^+$ T cells (518). We have shown that DLN-resident DC can contribute to $CD4^+$ T cell responses early during infection. Which specific DLN-resident DC subpopulation and whether $CD11b^{high}$ DCs are important can be determined by infecting IRF4 KO mice.

Methods such as gene array analyses, that allow comparison of expression levels of various genes between DC subsets and have determined the significance of previously studied DC-related genes such as Batf3 and Id2, have led to the identification of more transcription factors that regulate the differentiation of DCs. Zbtb46, a zinc finger transcription factor, was found to be preferentially expressed on DC precursors and conventional DCs, and not monocytes or other immunes populations through microarray analysis (519, 520). $Ly6c^{hi}$ monocytes upregulate CD11c and MHC II upon infection or upon stimulation with cytokines and TLR ligands in vitro (156, 521, 522). Upon diphtheria toxin treatment in CD11c-DTR mice infected with Listeria monocytogenes, these $Ly6c^{hi}$ $CD11c^{hi}$ cells are depleted (519). However, in mice that encode the diphtheria toxin receptor into the Zbtb46 gene (zDC-DTR mice), it was found that DT treatment did not alter the numbers of $CD11c^+$ $Ly6c^{hi}$ cells. In our studies, infiltrating $CD11c^+$ cells in the cornea express CD11b, and so to further confirm the ascribed roles for infiltrating DCs and resident corneal DCs, zDC-DTR mice can be infected ocularly and depleted of conventional DCs all throughout infection.

In the same line, the transcription factor Mycl1 is highly expressed in resident $CD8\alpha^+$ and migratory $CD103^+$ conventional DCs, as well as pDCs. Interestingly, the expression of Mycl1 is regulated by IRF8 and GM-CSF. Mice deficient in Mycl1 are not able to prime $CD8^+$ T cells optimally in Listeria and VSV infections (523). If $CD8\alpha^+$ or $CD103^+$ DCs were necessary in priming immune responses to HSV-1 ocular infections, we would see a deficiency in them upon infection of Mycl1-deficient mice.

Severe immunodeficiencies and reduced resistance to various pathogens in humans are a consequence of impairment in these transcription factors. Accordingly, determining the implications of the deficiencies in these pathways in the event of bacterial, fungal, and viral infections are critical to appreciate the underlying immune regulation performed by DCs.

8.1.2 What other markers are expressed by DCs infiltrating the cornea?

Further characterization of DCs infiltrating the cornea is necessary to be able to illustrate what transpires in the cornea during ocular HSV-1 infection. Different strategies to identify the different $CD11c^+$ DCs exist but they fail to separate $CD11b^+$ conventional DCs from $CD11b^+$ monocyte derived DCs. Dendritic cells resident to the cornea do not express CD11b. However, corneal DCs infiltrating after infection express CD11b, indicating their myeloid origin. The molecule Ly6c has been used to tell apart $CD11b^+$ monocyte-derived DCs from $CD11b^+$ conventional DCs (524), but the expression of Ly6c is transient and occurs when monocytes differentiate into DCs.

Recent studies have revealed that staining for CD64 and MAR-1 distinguishes monocyte-derived dendritic cells from conventional DCs (525, 526). CD64 is the high affinity IgG receptor FcγRI typically expressed in macrophages, while MAR-1 is the antibody clone that recognizes

the high affinity IgE receptor FcεRI expressed on mast cells and basophils. Using these two markers, as well as Ly6c, CD103, and CD11c, and other DC tracking techniques (CFSE or FITC labeling) as well as fluorescent viruses, may determine if there are different DC subsets infiltrating the corneas and if there is a DC subpopulation that preferentially gets infected, or presents antigen to T cells in the DLN.

8.1.3 Is migration of cornea – infiltrating DCs carrying antigen necessary in T cell priming?

Evidence from our CD11c-DTR mice has shown that in the absence of resident corneal DCs and infiltrating corneal DCs, proliferating $CD8^+$ T cell responses are abolished at 3 dpi, but there is only about a 50% reduction in $CD4^+$ T cell responses. Freely flowing antigen also gain access to the DLN even without DCs coming from the cornea and this may explain how DLN-resident DCs also participate in priming $CD4^+$ T cells. This suggests that in the case of $CD8^+$ T cells, there is direct presentation of corneal DCs to cytotoxic T lymphocytes, or that DCs from the cornea are necessary to take antigen down to the DLN for DLN-resident DCs to process and present it to $CD8^+$ T cells.

In the presence of inflammation and injury, dendritic cells undergo maturation and upregulate CCR7 expression. CCR7 directs the migration of DCs from peripheral tissue to the DLN through its interactions with its ligands CCL19 and CCL21 (527-529), and consequently, DCs in peripheral tissues of CCR7 KO mice are unable to travel to the DLN. To establish the requirement for DCs migrating from the cornea in carrying viral antigen for presentation to the $CD8^+$ T cells in the DLN, mice deficient in CCR7 can be infected ocularly. If $CD8^+$ T cell responses do not change compared to wild type mice, this will indicate that viral antigen gets to

the DLN, and DLN-resident DCs are able to present to $CD8^+$ T cells. Whether it is through cross-presentation or direct presentation is not easily established, as both live virus and viral antigen can drain to the lymph nodes freely. If the $CD8^+$ T cell responses are lower, then it would suggest that cornea-infiltrating DCs are necessary in taking the antigen to the DLN, and possibly also for presentation to T cells.

Cornea-infiltrating DCs have presumably developed from blood monocytes that enter the cornea after infection. It has been shown that egress of monocytes from the bone marrow (530) and recruitment of monocytes to infected tissue require the chemokine receptor CCR2 (475). Infecting CCR2 knockout mice ocularly will establish if there is a need for DCs derived from inflammatory monocytes that infiltrate the cornea after infection in priming T cell responses in the DLN. Experiments with CCR2 knockout mice will also confirm if there is indeed no role for infiltrating corneal DCs in clearing the virus from the cornea as our lab has shown previously (115). Additional studies with these mice can determine if inflammatory monocytes that infiltrate the cornea during disease contribute to herpes stromal keratitis by re-stimulating $CD4^+$ T cells in the cornea.

8.1.4 What role do DCs play in the trigeminal ganglia during latency?

Dendritic cells that may derive from monocytes infiltrate the trigeminal ganglia as well after HSV-1 ocular infection. It has been shown that stimulation of tissue – resident memory T cells after HSV-1 secondary challenge in the flank is dependent on dendritic cells (531). The role these dendritic cells play in expanding T cells in the TG, contributing to their effector function and survival after HSV-1 ocular infection is not well established.

It is well accepted that antigen presentation to promote effector function to $CD8^+$ T cells in the TG is through HSV-infected neurons. Antigen presentation to $CD4^+$ T cells however, requires antigen-presenting cells such as DCs and macrophages expressing MHC II molecules. Potentially, DCs that enter the TG upregulate MHC II and could phagocytose dying cells that have been infected. They can then present these viral antigens to $CD4^+$ T cells. Utilizing CD11c-DTR mice and depleting DCs in these mice during latency will show if there is loss of antiviral cytokine production in $CD4^+$ and $CD8^+$ T cells that infiltrate the TG, if there is reduced proliferation of these T cells, and if there is increased viral reactivation in these TGs due to diminished T cell effector function.

Other possible functions for DCs in the TG could involve regulating the migration and retention of memory T cells possibly through chemokine production (532). Ablating DCs during latency will establish if there are changes in immune cells infiltrating the TG as well as differences in chemokine transcript that may occur.

8.1.5 What is the role of other costimulatory interactions during priming, latency, and HSK?

We previously described above, how blockade of either CD86 or CD40L interactions did not affect $CD4^+$ or $CD8^+$ T cell responses. CD80 and CD86 typically have overlapping functions during viral infection. When either one of these ligands is absent, virus-specific T cell responses are similar in infections with MHV-68, MCMV, and VSV (412, 413, 419, 420). In vaccinia virus infections however, CD86 is favored over CD80 in generating vaccinia-specific $CD8^+$ T cell responses (533). Thus in our ocular HSV-1 infection model, it may be necessary to block CD80, or to block both CD80 and CD86 functions using a CTLA4-Ig fusion protein to affect the

generation of $CD4^+$ or $CD8^+$ T cells in the DLN. Redundant signaling in costimulation may be significant.

The expression of other costimulatory molecules such as CD70, ICOSL, 4-1BBL, and OX40L on cornea-infiltrating dendritic cells and DLN-resident DCs may need to be studied as well. Utilizing blocking antibodies to interfere with costimulation or infecting knockout mice with HSV-1 may demonstrate the importance of specific costimulatory molecules in generating acute responses against HSV-1 infection.

It is also important to determine if there is only one dendritic cell subset that can provide multiple costimulatory signals. Do different antigen presenting cells provide various costimulatory signals?

It is possible that various factors during viral infection influence which costimulatory receptor/ligand pairs are necessary to enhance virus-specific T cell responses. These parameters include route and dose of infection, tissue tropism, antigen presenting cell subsets in the tissue of concern, rate of virus replication, TLR stimulation, and viral immune evasion. Interestingly, the expression of costimulatory molecules may be found on distinct subsets of APCs that localize at the infection site and migrate to the DLN (227).

The requirement for costimulation during herpes stromal keratitis has not been fully studied. Though the studies presented here have shown that dendritic cells are dispensable during HSK, and other costimulatory interactions such as OX40/OX40L are not necessary (388), the necessity for costimulation in HSK progression has been demonstrated only through B7 (385) and 4-1BB/4-1BBL interactions (386) locally in the cornea. Other costimulatory molecules may be contributing and may be important.

Of great interest as well is whether tissue – resident memory $CD4^+$ and $CD8^+$ T cells that infiltrate the trigeminal ganglia during latency require costimulatory signals for survival and maintenance, as well as for activation upon secondary infection. Blocking costimulatory interactions during latency using antibodies will be able to answer those questions.

With the significance of costimulation for the development of viral immunity being known, it is imperative to define which costimulatory molecules are required for generating protective antiviral T cell responses, contributing to disease and immunopathology, and establishing when their function is necessary. This information is critical for improving vaccine design and efficacy against a diversity of pathogens.

8.1.6 What is the role of other immune cells present in the cornea in the innate and adaptive immune responses in HSV-1 ocular infection?

Immune cells such as macrophages, natural killer cells, and neutrophils have always been considered as effector cells that have little influence in fine-tuning the adaptive immune response. Numerous reports have been challenging that long-held view.

Macrophages resident in the cornea of naïve mice have been identified and located in the corneal stroma (110). Although various functions have been attributed to these macrophages after HSV-1 infection such as restricting virus replication and contributing to delayed hypersensitivity type responses (152), it is not clear if they present and process HSV-1 antigen to contribute to adaptive immune responses after corneal infection. Utilizing local clodronate depletion 2 days before infection in HSV-infected C57Bl/6 mice, and determining if there is expansion of $CD8^+$ and $CD4^+$ T cells in the DLN will prove if cornea-resident macrophages play a role in initiating adaptive immune responses. Extending clodronate depletion after infection

will establish if macrophages that may infiltrate the cornea after infection contribute to T cell proliferation in the DLN.

Alternatively, Macrophage Fas-Induced Apoptosis (MAFIA) mice (534) may be used. In cells of these mice that express the mouse colony-stimulating factor 1 receptor (Csf1r) promoter (such as macrophages and DCs), a transgene was inserted containing a mutant human FK506 binding protein 1A that can bind the drug AP20187. Upon treatment with AP20187, Fas ligand is selectively activated in macrophages and DCs, and these cells proceed to apoptosis. Since we have previously shown that DCs resident to the cornea are not critical in generating $CD4^+$ and $CD8^+$ T cell responses in the DLN, employing these mice and depleting both macrophages and DCs will show if resident corneal macrophages are necessary in expanding T cells in the DLN.

The absence of macrophages resident to the cornea have also been implicated in the development of corneal inflammation, decreased cytokine production, impaired pathogen clearance, and reduced cellular infiltration after fungal and bacterial infections (535-537). In these studies, corneal macrophages were shown to contribute to the recognition of PAMPs that initiated the cascade of innate immune responses necessary to clear pathogen replication. It is possible that macrophages in the cornea can aid in viral recognition after HSV-infection (538).

Natural killer cells infiltrate the cornea after infection and have been shown to be able to control virus replication (539). Though its innate immune functions are well characterized, its involvement in regulating adaptive immune responses has been shown in various models, to stimulate or inhibit T cell responses (540). Both direct interactions between NK cells and T cells, as well as indirect, which involve interactions with dendritic cells and the impact of NK cells on the infected cells and pathogen burden, underlie NK cell modulation of T cell function.

In recent years, the significant role of a two-way crosstalk between natural killer cells and dendritic cells has been clearly elucidated. The results of this interaction lead to development of an efficient innate immune response, through DC-mediated NK cell activation, and to a robust adaptive immune response, through NK-mediated DC maturation. Early NK cell interactions with other leukocytes can have enduring consequences on both quantity and quality of memory T cells, as well as influence the exhaustion of T cells during chronic infections (541).

Activation of NK cells promotes DC maturation through the cytokines TNFα and IFNγ. Both these cytokines enhance the levels of costimulatory molecule expression in DCs and contribute to IL-12 secretion by DCs (542, 543). Moreover, exposure of NK cells to cytokines such as IL-12, IL-18, and IL-15, which are produced by dendritic cells, can promote T cell differentiation, survival, and activation (544, 545). IFNγ produced by NK cells can modulate the differentiation of monocytes into dendritic cells at the infection site (546). Furthermore, DC and NK cell interaction can replace $CD4^+$ T cell helper signals to generate efficient CTL responses (547, 548). Additionally, evidence has been shown that NK cells may support antigen cross presentation of DCs. Dendritic cells are able to take up antigen from dying cells lysed by NK cells and present them in the context of MHC I (549, 550). In a tumor model, it has been shown that activated NK cells recruited to the tumor site promote DC maturation and migration to the LN. These DCs then cross present tumor antigens to $CD8^+$ T cells in the LN. Depletion of NK cells in this system leads to an abrogation of $CD8^+$ T cell priming (551). Impairing NK cell function in the cornea using an anti NK1.1 blocking antibody will help determine if T cell effector function, activation, and expansion is impacted in the DLN as well as in the TG after HSV-1 infection.

The role of neutrophils in regulating T cell function and priming appears to be more complex and less characterized. They infiltrate the corneas of HSV-infected mice as early as 1 dpi (unpublished data), and although their role in virus clearance in the cornea is contentious (115, 159, 552), they may contribute to virus-specific T cell responses at the site of pathogen entry (553, 554). Recently, it was shown in a contact hypersensitivity model that neutrophils are important for DC migration to the lymph nodes and priming of T cells specific to the contact allergen (555). Interestingly, neutrophils have been proven to carry antigen and migrate to the DLN themselves through the aid of CCR7 (556-558) and directly cross prime T cells (559). Establishing a role for neutrophils in the adaptive immune responses against HSV requires depleting neutrophils locally and early in corneal infection and studying proliferation and effector function of antigen-specific T cells in the DLN.

8.1.7 Does the cytokine and chemokine milieu in the DLN change with DC depletion?

We observe a reduction in expansion of both $CD4^+$ and $CD8^+$ T cells upon corneal DC depletion but it is not known if the cytokine production of these T cells were affected by DC ablation. After ablation of DCs in the cornea, T cells from the DLN can be isolated and stimulated with bone marrow-derived DCs pulsed with HSV antigen and intracellular cytokine staining may reveal changes in production of Th1 cytokines such as IFNγ and IL-12, Th17 cytokines such as IL-17 and IL-22, and Th2 cytokines such as IL-10 in $CD4^+$ T cells. The gene expression of these cytokines may also be analyzed by quantitative PCR. There may also be a difference in immune cells infiltrating the DLN from the blood after infection (560).

8.1.8 Do dendritic cells contribute to nerve damage observed in HSV-infected mice and mice that develop HSK?

The murine model of herpes stromal keratitis mirrors a lot of the immunopathological features found in the human disease, but it is much more similar to a more chronic and severe HSK. HSK in humans is characterized by loss of blinking reflex as a consequence of retraction of sensory nerve ends in the corneal epithelium during HSK. Recent studies from our laboratory has shown that a similar retraction occurs in corneal nerves of mice, and that the stromal inflammation occurring during mouse HSK is due to corneal surface exposure and desiccation promoted by corneal nerve damage and loss of blink reflex (561). Preventing desiccation leads to a milder form of the disease. The corneal nerve retraction, loss of blink reflex, and associated pathology was found to be reversible and is influenced by $CD4^+$ T cells. Nerve damage occurs as early as 2 dpi in mice, but it is not known if the dendritic cells infiltrating the cornea after infection contribute to nerve damage and retraction associated with HSK. Depleting DCs after infection and determining if it affects blink reflex and contributes to pathology later in the infection can be established in CD11c-DTR mice.

On a broader scale, the interactions between dendritic cells and corneal nerves remain poorly characterized. In a murine model of diabetes mellitus, it was suggested that contact between DCs and the subbasal nerve plexus of the cornea can trigger nerve damage found in diabetic corneas (562). Important questions include: are dendritic cells located in close apposition to nerves in the cornea? Do cornea dendritic cells respond to corneal nerve damage?

Corneal nerves are also known to secrete neuropeptides such as Substance P and vasoactive intestinal peptide (563-565), and these peptides can possibly influence the recruitment, survival, and activation of dendritic cells (566, 567).

APPENDIX A

LIST OF WORKS

1) **Buela, K. A.** and Hendricks, R. L. Migratory cornea-infiltrating dendritic cells are critical for CD8+ T cell expansion in the draining lymph nodes after HSV-1 infection. *(manuscript in preparation).*

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