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Type I Interferon and Lymphangiogenesis in the HSV-1 Infected Cornea – Are they Beneficial to the Host?

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Abstract

Herpes simplex virus type 1 (HSV-1) is a highly successful pathogen that can result in significant human morbidity. Within the cornea, it was thought the initial recognition of the pathogen was through Toll-like receptors expressed on/in resident cells that then elicit pro-inflammatory cytokine production, activation of anti-viral pathways, and recruitment of leukocytes. However, our lab has uncovered a novel, TLR-independent innate sensor that supersedes TLR induction of anti-viral pathways following HSV-1 infection. In addition, we have also found HSV-1 induces the genesis of lymphatic vessels into the cornea proper by a mechanism independent of TLRs and unique in the field of neovascularization. This review will focus on these two innate immune events during acute HSV-1 infection of the cornea.

1. Introduction

From an immune perspective as it relates to microbial pathogens, the eye is an extraordinary tissue that must maintain a balance between the host immune response and clearance of the insulting organism with preservation of the visual axis. Collateral damage as a result of an inflammatory response to infection can have debilitating consequences on vision. Upon infection with one common ocular pathogen, herpes simplex virus (HSV)-1, a range of pathologies within the cornea are presented by the human host including the relatively benign epithelial keratitis to the more severe stromal necrotizing keratitis. The corneal pathology that manifests as a result of infection is due to the cytopathic effect of the virus on the corneal epithelial cells, the inflammatory response to the virus typically observed as a result of episodic recurrence of latent virus, and the neovascularization that can occur in the normally avascular cornea. To more fully understand the pathogenic process, it is important to identify and characterize those molecules that link the initial recognition of the virus to the innate immune response with the anticipated result that one can uncouple anti-viral resistance from inflammation and angiogenesis.

One of the most important endogenous cytokines produced locally within the cornea following HSV-1 infection is interferon (IFN)- α . This cytokine has previously been linked to anti-viral resistance against corneal HSV-1 infection using neutralizing antibody in a mouse model (Su et al., 1990; Hendricks et al., 1991) or in transgenic mice expressing IFN- α under a glial fibrillary acidic protein promoter (Carr et al., 1998). In the above-referenced studies, HSV-1 replication and spread was restricted due to local expression of IFN- α within

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the cornea and/or trigeminal ganglion. However, the intervention in human HSV-1 keratitis patients using exogenous IFN- α has been controversial with reports of efficacious and non-efficacious results (Sundmacher et al., 1976; Coster et al., 1977; Minkovitz et al., 1995). The mixed outcomes may be due to the amount of IFN- α applied, the severity of the infection, or the timing of the application relative to the stage of infection (i.e., reactivation). Taken together, the results do point to a central role type I IFN (including IFN- α and - β) has in the control of HSV-1 infection in the cornea. As a consequence, the mechanism that drives type I IFN expression in the cornea is crucial in the early defense against HSV-1 and a topic of this review.

Not only is IFN- α coupled to innate anti-viral defense but it is also associated with the development of a Th1 response (Brickman et al., 1993; Farrar et al., 2000) defined by CD4⁺ and CD8⁺ T cell cytokine profiles. Whereas CD4⁺ T cells are not thought to significantly contribute to viral clearance in the cornea at early time points post infection, they are central to the development of stromal keratitis including neovascularization in mice (Hendricks RL et al., 1992; Niemialtowski and Rouse, 1992; Hendricks RL, 1997). Furthermore, CD4⁺ and CD8⁺ T cells are a source of vascular endothelial growth factor (VEGF), one of several pro-angiogenic factors that drive hem- and lymph-angiogenesis (Freeman et al., 1995; Conrady et al., 2012b). Consequently, the development of T cells during the transition from innate to the adaptive immune response along with resident and innate myeloid-derived immune cells play a significant role in neovascularization of the cornea in response to HSV-1. In the current article, we will present our results and those of others in exploring these events generated over the past several years using a mouse model and conclude with the challenges that lie ahead in the development of a realistic and sustainable therapy for the human patient.

2. Role of Innate Immune Sensors in Limiting Ocular HSV-1 Disease

A. Toll-like receptors and the discovery of innate sensors

Ubiquitously expressed toll-like receptors (TLRs) and the innate immune response to pathogen-associated molecular sequences have gained significant notoriety since their identification in the mid 1990's (Lemaitre et al., 1996; Medzhitov et al., 1997). TLR activation has been associated with vaccine development, autoimmunity, host-pathogen responses, adaptive immunity, and cancer (Dabbagh et al., 2003; Hedayat et al., 2011; Fuertes et al., 2012; Sasai and Yamamoto, 2013). Eleven of the thirteen known TLRs signal through the adaptor protein myeloid differentiation primary response gene 88 (MyD88) with TLR-3 and TLR-4 being the exceptions (Conrady et al., 2010; Oldenburg et al., 2012). TLR-3 signals through the TIR-domain-containing adaptor-inducing interferon- β (Trif), while TLR-4 can activate both Trif and MyD88. These adapter proteins then drive downstream type I interferon (IFN) and inflammatory chemokine production in a multi-step process (Conrady et al., 2010). In tissues such as the brain, TLRs are the principal driver responsible for activating innate immunity to select pathogens such as in the case of the double-stranded DNA virus, HSV-1 (Conrady et al., 2013b; Zhang et al., 2007). A loss of the specific sensor (TLR-3) in mice and adult humans results in a significant increase in susceptibility to HSV-1 infection of the brain known as herpes simplex encephalitis (Conrady et al., 2013b; Zhang et al., 2007). Thus, the importance of TLRs in pathogen immune responses is quite evident. However, TLRs involved in ocular host defense have been reviewed extensively elsewhere (Pearlman et al., 2013) and are not the focus of this review.

Despite the importance of TLRs, it quickly became apparent from the work of others using *Caenorhabditis elegans* and knockout mouse models that there were innate responses governed by sensors other than TLRs operating through adaptor proteins independent of those employed by TLRs (Couillault et al., 2004; Ishii et al., 2006). Within the last decade,

many of these receptors have been subsequently identified and shown to play a crucial role in the innate immune system in response to microbial cytosolic DNA and RNA sequences/motifs. Their downstream effect is to prohibit viral and bacterial replication and spread by inducing the production of various immune-derived molecules (Sharma and Fitzgerald, 2011). While much remains unknown, there are over 30 nucleic acid sensors identified that recognize viral or bacterial DNA/RNA and influence the host response to infection. In the sections below, we will highlight features for some of the known sensors and then focus on one particular innate DNA sensor expressed within corneal epithelial cells that is critical in the establishment of early host resistance to HSV-1.

B. The Non-TLR nucleic acid sensors

Innate redundancy is much more extensive than previously thought with recently identified RNA and DNA sensors that can respond with exquisite fidelity similar to TLRs. For example, host immune cells, specifically macrophages, are equipped with many specialized non-TLR and TLR innate sensors that respond to pathogenic RNA or DNA to elicit IL-1 β , various type I and III IFN subtypes, and/or pro-inflammatory cytokines (Sharma and Fitzgerald, 2011). However, the precise role of each sensor is currently unknown. Nevertheless, ongoing studies illustrate these proteins as crucial players in the innate immune repertoire in response to invading pathogens by directly inhibiting pathogen replication and/or facilitating a pro-inflammatory state that guides other leukocyte populations to the site of infection in order to contain subsequent spread (Barbalat et al., 2011; Rathinam et al., 2010). There are at least fifteen described intracellular sensors that have been implicated in host surveillance of cytosolic viral DNA alone of which several specifically induce type I IFN expression (Paludan and Bowie, 2013). They include the DNA-dependent activator of IFN-regulatory factor (DAI) (Takaoka et al., 2007), RNA polymerase III (Chiu et al., 2009), several inflammasome sensors (Hornung et al., 2009), leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) (Yang et al., 2010), DExDc family of helicases [DHX9, DHX36, and DDX41] (Kim et al., 2010; Zhang et al., 2011b), LSm14A (Li et al., 2012), IFN-inducible protein (IFI)-16 (Unterholzner et al., 2010), Ku70 (Zhang et al., 2011a) and the most recently identified, cyclic GMP-AMP synthase (Sun et al., 2012).

While the number of intracellular innate sensors is extensive and ever expanding, their function in pathogen recognition is most easily simplified as being responsive to either DNA or RNA. Moreover, most of the innate sensors previously mentioned have been identified and defined in isolated mouse and human cell cultures begging the question as to their role, if any, *in vivo* during pathogen invasion of host tissue. This became most evident with the discovery of DAI *in vitro* and in subsequent knockdown experiments using cells residing within the central nervous system that showed DAI was required in the anti-viral response to HSV-1 (Furr et al., 2011; Takaoka et al., 2007). However, this enhanced susceptibility was not recapitulated *in vivo* (Ishii et al., 2008). Additionally, several of the sensors have only recently been identified and lack confirmation in innate sensing. Current ongoing and future research using knockout mouse and genetic linkage approaches should delineate the contribution of each sensor in innate immunity in mice and the human host.

i. DNA sensors—Most of these aforementioned DNA sensors initiate type I IFN production in response to viral infection by way of IFN regulatory factor (IRF) nuclear translocation; however, Ku70 and the group of inflammasome sensors are the currently known exceptions (Rathinam et al., 2010; Zhang et al., 2011a). The viral inflammasome sensors consist of absent in melanoma (AIM)2 and the nucleotide-binding oligomerization domain (NOD)-like receptors that are known to drive interleukin (IL)-1 β production and activation by way of caspase-1 activity (Hornung et al., 2009). The other sensor, Ku70, has

been shown to initiate IRF-7 signaling and translocation to induce the production of IFN- λ 1, a type III IFN that induces a type I IFN-like response in certain cell types (Zhang et al., 2011a; Zhou et al., 2007). While LRRFIP1 uses a novel β -catenin mechanism not shared with other DNA sensors (Yang et al., 2010), DDX41, IFI-16, LSm14A, cyclic GMP-AMP synthase, and potentially DAI utilize a common pathway to induce IFN production. These sentinel signals converge to initiate signaling through a common adaptor protein, stimulator of IFN genes [STING, also known as MITA, MPYS, or ERIS] (Barber, 2011). STING then facilitates phosphorylation of IRF by TANK binding kinase (TBK)-1, subsequent dimerization and nuclear translocation of the specific IRF species to drive type I IFN production (Ishikawa et al., 2009; Lin et al., 1998; Tanaka and Chen, 2012; Zhong et al., 2008) (Figure 1). The end product from these signaling cascades, IFN- α/β , is responsible for binding in an autocrine and paracrine manner to the type I IFN receptor (CD118) on nucleated cell surfaces. Receptor stimulation leads to the activation of critical downstream anti-viral pathways (oligoadenylate synthetase, protein kinase R, and RNase L) that are responsible for inhibiting further viral replication within the host cell (Hertzog et al., 2013). Therefore, STING is an important central adaptor protein for cytosolic DNA sensing and as such, is involved in HSV-1 “sensing” (Paludan and Bowie, 2013). STING has also been found to detect viruses or cytosolic nucleic acid initiating the phosphorylation of STAT6 by TBK-1 resulting in anti-viral gene expression (Chen et al., 2011).

While crucial in host defense against invading viral pathogens, type I IFN is likely an underlying cause of several autoimmune disorders and as such, its production is tightly regulated during homeostasis (Theofilopoulos et al., 2005). The regulation of type I IFN is due in part to the 3'-5' DNA exonuclease Trex1 (3'-repair exonuclease 1) that acts as a negative regulator of the STING pathway by rapidly degrading nicked DNA to prevent an innate response to host DNA and subsequent development of autoimmune disorders (Yang et al., 2007). Viruses such as human immunodeficiency virus utilize the host Trex1 protein's ability to degrade cytosolic DNA to minimize the accumulation of viral DNA during the replication cycle as a means to avoid detection by host DNA sensors (Yan et al., 2010). To further highlight the importance of DNA exonucleases in regulating activating signals of type I IFN production, a deficiency in DNase II, another exonuclease family member, is embryologically lethal in mice due to the rapid accumulation of host cytosolic DNA resulting in large amounts of IFN- β (Yoshida et al., 2005). We hypothesize that this process is facilitated by cytosolic DNA sensors although this remains untested. Thus, the activation and regulation of STING signaling and IFN- α/β production is an area of intense interest in viral immunity and has a noticeable contribution within the cornea as a major DNA sensor sentinel against HSV-1 challenge as discussed below.

ii. RNA sensors—Innate recognition of pathogenic DNA is not the only method employed by the host in surveillance of cells. Non-host RNA sequences are recognized by retinoic acid inducible gene (RIG)-I (Yoneyama et al., 2004), melanoma differentiation-associated gene 5 [MDA5] (Kato et al., 2006), and LSm14A, a sensor which appears to have a role in recognition of both foreign RNA and DNA (Li et al., 2012). Similar to that of several DNA sensors, MDA5 and RIG-I respond to distinct pathogens/sequences/motifs yet share a common downstream adaptor protein, MAVS [Mitochondrial antiviral signaling protein; also known as VISA, Cardif, and IPS-1], to activate phosphorylation and nuclear translocation of IRF. LSm14A appears to utilize the RIG-I pathway to facilitate activation of innate immunity in response to RNA motifs (Li et al., 2012). The end result is that of IFN production (Kato et al., 2006; Kawai et al., 2005; Seth et al., 2005) [Figure 1]. An isolated report has suggested that MDA5 and RIG-I may have some role in DNA recognition as well but the results lack clear confirmation by other laboratories and thus remain controversial (Choi et al., 2009). In addition to the more clearly defined historical roles of TLRs, nucleic acid sensors play an integral role in the activation of the innate immune response to bacterial

pathogens including activators of the inflammasome and IL-1 β : NOD1 and NOD2 (Chamaillard et al., 2003; Cooney et al., 2010), NOD-like receptors (NLR) [such as NLRP3 and NLRC4 (Broz et al., 2010)], AIM2 (Rathinam et al., 2010) and those that drive the production of IFN [RIG-I (Monroe et al., 2009), IFI-16, LRRFIP1]. While most commonly viewed as an adaptor protein, STING may play some role as a *bona fide* sensor in response to bacterial cyclic-di-GMP (Burdette et al., 2011; McWhirter et al., 2009). Currently untested, cyclic GMP-AMP synthase has been shown to activate IFN production in response to transfected DNA (Sun et al., 2012).

In addition to their previously mentioned role in IFN secretion, DNA and RNA sensors are responsible for activating NF- κ B pathways. The activation of these cascades results in the production of cytokines and chemokines that facilitate the recruitment of leukocytes to the site of infection. Relative to the eye, pro-inflammatory cytokine production and leukocyte influx can have devastating consequences on the visual axis (Niederhorn et al., 2011). In the next section, we will focus on one DNA sensor, IFI-16/p204 and the critical role it plays in orchestrating the innate immune response in the cornea to HSV-1 infection during the first 48-72 hr following virus challenge.

C. The innate sensor IFI-16/p204 and type I IFN are critical to control ocular HSV-1 disease

Our laboratory has been interested in the host immune response to viral infection in the cornea, specifically the interplay between HSV-1 and type I IFN. As such, we became interested in the underlying mechanism of innate control of the virus due to the significant morbidity associated with herpetic infections of the cornea. This morbidity is most striking in the clinical setting where a significant increase in the incidence of blindness due to corneal opacity from permanent scarring is clearly evident, and a severe reduction in efficacy of corneal transplants in patients with documented HSV-1 infections of the eye is well known (Larkin, 1998). As such, HSV-1 is the leading cause of infectious corneal blindness in the developed world (Conrady et al., 2010) and consequently the most studied viral infection of the eye. This statistic is underscored by the frequency by which patients within our own cornea clinic present with ocular pathology as a result of HSV-1 infection which currently holds between 20-25% of all patients within this specialty clinic at the Dean McGee Eye Institute.

As a result of earlier work indicating type I IFN was essential in the control of HSV-1 infection in the eye of mice (Su et al., 1990; Hendricks et al., 1991; Noisakran and Carr, 2000) along with mixed results in the human patient population (Sundmacher et al., 1976; Coster et al., 1977; Minkovitz and Pepose 1995), this area of study is now undergoing a renaissance due to newly discovered pathways and nucleic acid sensors highlighted above. We hypothesize that specific compounds could be used in addition to anti-viral reagents (e.g., acyclovir) to limit virus replication in the cornea by activating only critical innate responses all while limiting inflammation in such a sensitive tissue. Consequently, mapping the innate immune response to HSV-1 has become a crucial aspect of our research in an attempt to segregate the beneficial from detrimental host immune response to ocular HSV-1 infection.

Innate regulation of HSV-1 is critical to host morbidity and mortality by reducing virus replication and spread from the primary site of infection. Our laboratory and others have previously shown that in the absence of type I IFN signaling (IFN A1 receptor knockout, CD118^{-/-}), mice are highly susceptible to viral dissemination and death emphasizing the importance of this anti-viral cytokine in HSV-1 defense (Conrady et al., 2011a; Conrady et al., 2009; Leib et al., 1999; Luker et al., 2003). Ongoing work in our laboratory has confirmed the importance of type I IFN during the acute infection of the cornea with HSV-1. We have found the loss of a functional type I IFN pathway results in significantly more

infectious virus and a more pronounced inflammatory response and ocular pathology within the cornea (Bryant-Hudson et al., under review). Uncontrolled viral replication within the cornea enhances viral spread into the trigeminal ganglia, brainstem, and brain causing 100% mortality within five or six days post infection (Conrady et al., 2011b; Conrady et al., 2013b). Based on these findings we surmised that IFN- α/β is critical in the control of HSV-1 replication and spread within the cornea and as a consequence, limits tissue pathology and inflammation. We hypothesized from this basic understanding that if the processes that led to the activation of type I IFN production were better understood, it could aid in the development of adjunct therapy or an elusive HSV-1 vaccine ultimately leading to a better clinical outcome of those diagnosed with herpetic keratitis. As such, the role and identification of the innate sensor(s) responsible for initiating the immune response to HSV-1 in the cornea was and is of significant interest to our laboratory. Previous studies have shown *in vitro* that the addition of TLR-3 (poly I: C) and TLR-9 agonists (HSV DNA) activated IFN- β production in human corneal epithelial cells (Hayashi et al., 2006; Kumar et al., 2006). However, no study had identified the sensor responsible for type I IFN production *in vivo* or even the specific IFN subtypes produced during HSV infection of corneal epithelial tissue when we embarked on the mission to identify the innate sensor of HSV-1 in the cornea.

In a series of *in vivo* studies, we found IFN- α was secreted by virally-infected epithelial cells and those adjacent, non-infected epithelial cells in response to HSV-1 (Conrady et al., 2011b). Furthermore, TLR signaling *in vivo* (using mice deficient in MyD88 and Trif adaptor proteins) was not necessary to elicit the type I IFN or CCL2 expression required to contain viral replication within the cornea thereby eliminating any contribution of TLRs to these critical soluble factors within the first 48-72 hr post infection. These results are consistent with a previous observation by our laboratory in which TLR9-deficient mice did not show a significant loss in the expression of CCL2 in the cornea in response to HSV-1 challenge (Wuest et al., 2006). We then began to survey other potential candidates that were responsible for the induction of type I IFN and CCL2 production. After eliminating several likely candidates, most notably DAI and RIG-I, our search focused on IFI-16, a DNA sensor found to be localized within epithelial tissue and a protein of interest in prostate cancer (Wei et al., 2003; Xin et al., 2003).

Consistent with previous studies, we identified expression of IFI-16 and the mouse homologue, p204, within corneal epithelial cells localized predominantly perinuclear but also within the nucleus and cytosol of the cell (Conrady et al., 2012a). Targeting expression of this DNA sensor using an siRNA approach in mice *in vivo* and *in vitro* using immortalized human corneal epithelial cells resulted in a loss of viral containment, IRF-3 nuclear translocation, and production of both IFN- α and CCL2 (Conrady et al., 2012a). This response was specific to DNA viruses because viral containment *in vivo* of the negative sense RNA virus, vesicular stomatitis virus (VSV), required IFN signals but was not dependent on p204, in that targeting p204 expression by siRNA did not increase susceptibility of mice to VSV replication (Conrady et al., 2012a). Furthermore, CCL2 production was linked to IFN- α expression by way of chimeric mice in which corneal resident cells unresponsive to type I IFN signals were unable to produce the chemokine. In addition, topical application of recombinant IFN alone to cell cultures or host tissue resulted in the expression of CCL2. The novel regulation of CCL2 by IFN was the direct result of IFN-stimulated transcription factors binding to the upstream promoter of CCL2 to drive expression of the chemokine (Conrady et al., 2013a). CCL2, in turn, was responsible for the recruitment of invading inflammatory monocytes expressing the anti-viral inducible nitric oxide synthase (iNOS) that along with type I IFN suppresses local HSV-1 replication. Our observations excluded neutrophils as a contributor to HSV-1 resistance during the initial stages of acute infection. Moreover, these findings were substantiated in both CCL2- and

STING- deficient mice in which a loss of either protein resulted in a compromised innate immune response due to the loss of IFN- α production and/or inflammatory monocyte recruitment (Conrady et al., 2012a; Conrady et al., 2013a).

In summary, we identified IFI-16/p204 as the predominant innate sensor responsible for HSV-1 surveillance of the cornea by activating type I IFN production through a STING-IRF-3-dependent cascade all while recruiting infiltrating inflammatory monocytes by way of a type I IFN-dependent CCL2 mechanism (Conrady et al., 2012a; Conrady et al., 2013a) (Figure 3). A loss of any of these key factors (CCL2, inflammatory monocytes, STING, or IFN- α/β) resulted in a loss of HSV-1 containment (Conrady et al., 2012a; Conrady et al., 2013a). These findings are supported, in part, by studies expressing the importance of iNOS in inhibiting viral replication and spread and by ongoing experiments in our laboratory highlighting the importance of IFN- α/β production in ocular defense against HSV-1 (Croen, 1993; Karupiah et al., 1993; Bryant-Hudson et al., *under review*). However, these results need to be reconciled with a previous study in which virus within the tear film of IRF-3 deficient mice was similar to that of WT controls (Menachery et al., 2010). This conundrum could be at least partially explained by the reduced sensitivity of tear film to predict total viral content within the corneal tissue as compared to whole tissue viral titers used within our studies (Conrady et al., 2012a; Conrady et al., 2013a; Menachery et al., 2010; Wuest et al., 2006). Furthermore, our studies were performed *in vivo* but did not utilize IFI-16/p204 knockout mice. We employed siRNA delivery to corneal tissue and as such, further research using IFI-16/p204 knockout mice would strengthen our findings (Conrady et al., 2012a; Conrady et al., 2013a). Lastly, IFI-16/p204 is a sensor found within host nuclei and likely traffics to the cytosol to interact with STING. In our studies, we were able to partially abrogate p204-regulated innate immunity with anti-p204 antibody following subconjunctival application in mice in which direct access to the cytosol was observed (Conrady et al., 2012a). It was also possible to identify p204 expression in the cytosol of epithelial cells. However, our results did not identify the location of p204 recognition of HSV DNA, nuclear versus cytosol. Recent evidence is emerging to support a cytosolic role of IFI-16/p204 in HSV-1 recognition. In macrophages, digested viral capsids are released into the cytosol where HSV-1 or cytomegalovirus DNA interacts with IFI-16 to activate IFN production (Horan et al., 2013). However, this is in opposition to a report in which trafficking of IFI-16 to the cytosol was not detected at any point during HSV-1 infection (Orzalli et al., 2012). It was also of interest to note the immediate early gene-encoded viral protein, ICP0 targets degradation of IFI-16 in a proteasome-dependent manner (Orzalli et al., 2012). Likewise, innate recognition of another herpesvirus, Kaposi sarcoma-associated herpes virus or HHV-8, is mediated within the host nucleus to activate a novel role of IFI-16, the inflammasome (Kerur et al., 2011). Thus, the exact location of IFI-16 recognition of viral DNA remains controversial and will require further evaluation before definitive conclusions can be drawn.

D. Future Directions

The eye is in a unique anatomical position due to its constant interaction with the external environment, and as such, is vulnerable to invasion by pathogenic organisms. The anterior segment of the eye is equipped with mechanisms to reduce pathogen adherence to corneal tissue thereby decreasing the frequency of invasion. They include the eyelids that act as a mechanical deterrent, constant epithelial cell shedding, and the tear film which is composed of several immunological active substances to constantly flush the eye (Klotz et al., 2000). In addition, the microbiome of the conjunctiva may also influence the local immune response via low level activation of TLRs (Dong et al., 2011). However once a pathogen has gained access to the outer eye by circumventing these measures, the cornea must balance initiating an inflammatory response that can be detrimental to the overall tissue architecture

and clarity with a quiescent state. To partially alleviate unnecessary inflammation, the host adaptation of intracellular expression of TLRs in ocular epithelial tissue is a unique way to restrict innate recognition to only invading pathogens (Ueta et al., 2004). Due to the distinct nature of the eye, it is difficult to suggest innate immune responses found in other tissues and cell types are responsible for host recognition of the pathogen within ocular tissue. Consequently, much within the eye is still not clear and thus, a better understanding of host-pathogen responses is essential to tailor therapies to reduce the morbidity and, in some cases such as orbital cellulitis, mortality associated with infections of the eye. This last point is especially true in attempting to marriage observations in the mouse model to that in the human host. Specifically, our studies detailed above were entirely conducted over a short time course during acute infection as a means to carefully control the conditions under investigation. In contrast, the human patient typically presents in clinic during bouts of reactivation of latent virus and may have a rich history of intervention with acyclovir and anti-inflammatory reagents (e.g., prednisolone) that would likely skew the innate immune profile prior to examination. Consequently, new *ex vivo* models such as the organotypic culture model of human cornea may be of use in assessing the innate (i.e. resident cell) immune response to HSV-1 infection in the absence of infiltrating leukocytes (Alekseev et al., 2012).

In summary, the establishment of a defining role of non-TLR sensors in viral surveillance and specifically, IFI-16 responses to HSV-1 infection of epithelial cells of the cornea is coming to fruition. In defining the mechanism of action of these sensors, the field of innate immunity will be more adept in addressing questions such as what sensor(s) is required and in what cell type to maximize the host response in containing the infection and reducing collateral damage to the tissue. At which point this work will become translational with potential to aid in adjunct therapy or vaccine development to the specific pathogen of choice remains unanswered. Furthermore, innate redundancy in some situations may be irrelevant as our laboratory and others are now showing that despite expression of multiple sensors in a given tissue there is a central sensor responsible for the initial innate immune response. This is most evident in the acute immune response to HSV-1 in which IFI-16 and TLR-3 regulate much of the response in the cornea and brain, respectively (Conrady et al., 2012a; Conrady et al., 2013b; Zhang et al., 2007).

While IFI16/p204 recognition of HSV-1 and induction of type I IFN is required to control the initial acute ocular infection, a remarkable pathology is set into motion during acute infection that continues to become more pronounced following the clearance of virus, corneal neovascularization. The next portion of the article will discuss HSV-1-induced neovascularization with an emphasis on the genesis of lymphatic vessels in the cornea proper.

3. Role of corneal neovascularization in ocular herpetic keratitis

The vascular system is made up of two components with blood vessels providing tissues with oxygen and nutrients and lymphatic vessels draining extravasated fluids and macromolecules from the periphery and returning them to the blood circulation (Karpanen and Alitalo, 2008). Neovascularization of tissues can include the development of blood vessels (hemangiogenesis) and/or lymphatic vessels (lymphangiogenesis). Corneal neovascularization is known to occur during wound healing, tumorigenesis, and infection. Each of these conditions can result in inflammation of the cornea and the subsequent recruitment of immune cells and increased expression of inflammatory cytokines. Identification and characterization of corneal hemangiogenesis has been extensive. However, studies regarding corneal lymphangiogenesis are relatively new and have been

spurred on by the identification of markers specific for lymphatic vessels such as LYVE-1 and podoplanin (Cursiefen et al., 2002).

A. Initiation of corneal lymphangiogenesis

The normal cornea exists in a state of angiogenic privilege through the expression of anti-angiogenic factors and decoy receptors for pro-angiogenic factors (Ambati et al., 2006; Cursiefen et al., 2011). This privilege is lost following inflammation and the expression of pro-angiogenic factors such as vascular endothelial growth factors (VEGF-A/C/D) by resident epithelial cells, fibroblasts, and infiltrating macrophages (Bock et al., 2013). In addition to VEGFs, lymphangiogenesis can be stimulated by a variety of factors including hepatocyte growth factor, insulin-like growth factor, platelet-derived growth factor, and fibroblast growth factor (Karpanen and Alitalo, 2008). However, the contribution of these factors to corneal lymphangiogenesis is still unknown. The binding of VEGF-C/D to VEGFR3 strongly induces sprouting, migration, and proliferation of lymphatic endothelium, whereas VEGF-A/VEGFR2 signaling strongly influences blood vessel development (Karpanen and Alitalo, 2008).

B. Wound healing and transplantation models

Studies utilizing the murine model of suture induced inflammatory corneal neovascularization have revealed new blood and lymphatic vessels develop from the pre-existing vasculature located in the limbus region surrounding the cornea. The new vessels develop quickly, within 24 hours of suture placement, and continue to progress towards the suture with lymphatic vessels extending farther into the central cornea earlier than blood vessels (Cursiefen et al., 2006). Over time the newly developed vessels begin to regress with lymphatic vessels preceding blood vessels (Cursiefen et al., 2006). This regression is most likely due to the decreased presence of pro-angiogenic factors within the cornea. Blood vessels are believed to be more susceptible to regression when not covered by pericytes, such as newly formed capillaries (Bock et al., 2013). Such observations are not limited to murine models. Immunohistochemical analysis of human corneas revealed the presence of LYVE-1⁺ lymphatic vessels associated with blood vessels occurring during the early stages of corneal neovascularization (Cursiefen et al., 2002).

Cornea transplants have significantly higher success rates compared to other tissues due to the immune privilege of the cornea. However, the success rate for graft survival dramatically decreases in the presence of blood and lymphatic vessels. Lymphatic vessels significantly contribute to graft rejection, more so than blood vessels, by transporting donor antigen to the draining lymph nodes allowing for the initiation of an immune response against the neoantigens (Bock et al., 2013). Advances in the field of tumorigenesis have led to the development of immune modulators such as anti-VEGF-A treatment, often used off-label to treat neovascularization and augment graft survival (Cursiefen et al., 2004a; Cursiefen et al., 2004b).

C. HSV-1 induced corneal neovascularization

Herpes stromal keratitis (HSK) is the most common cause of corneal blindness in the developed world (Liesegang et al., 1989). The genesis of blood vessels into the cornea following HSV-1 infection is well characterized and is a known contributing factor to the development of HSK. Newly developed blood vessels directly impair the transparency of the cornea and also allow antigen transport and subsequent induction of the adaptive immune response. As a consequence, activated T cells are recruited to the anterior segment including the cornea where they too can serve as a source of growth factors. The development of HSK and the contribution of corneal hemangiogenesis has recently been reviewed (Gimenez, et al. 2012; Rowe, et al. 2012) and will only briefly be discussed in this article.

Early work using a mouse model of corneal hemangiogenesis demonstrated the onset of new blood vessel sprouts in response to HSV-1 infection within 24 hr following infection (Zheng et al., 2001a). This blood vessel genesis was associated with VEGF-A expression initially by resident epithelial cells and subsequently by infiltrating leukocytes including neutrophils and macrophages (Zheng et al., 2001a; Lee et al., 2002). Additional soluble factors generated by resident corneal cells or infiltrating leukocytes thought to contribute to hemangiogenesis in response to HSV-1 infection included IL-1 α , IL-6, IL-17A, and matrix metalloproteinase 9 (Lee et al., 2002; Biswas et al., 2004; Suryawanshi et al., 2012). Ironically, blood vessel growth into the cornea proper was found to further develop upon clearance of infectious virus from the cornea suggesting the initial stimulus driving pro-angiogenic factor expression (i.e., replicating virus) is not necessary in the progression of HSK (Zheng et al., 2001a). Such results would be consistent with the human condition in which neovascularization is apparent in the absence of detectable virus.

In contrast to HSV-1-induced corneal hemangiogenesis, the development of lymphatic vessels into the cornea following HSV-1 infection is a new area of study. An analysis of the timing of corneal neovascularization following HSV-1 infection demonstrates lymphangiogenesis precedes hemangiogenesis (Wuest and Carr, 2010). Similar to blood vessels, lymphatic vessels remain beyond the resolution of infection, at least up to day 30 post-infection (Wuest and Carr, 2010). HSV-1 induces the expression of both VEGFR-2 and VEGFR-3 on the newly acquired lymphatic vessels, key receptors that respond to factors that drive angiogenesis (Wuest and Carr, 2010). The use of competitive inhibitors identified the VEGF-A/VEGFR-2 signaling pathway as necessary for HSV-1 induced lymphangiogenesis, which is in contrast to other well-studied models of corneal neovascularization (Wuest and Carr, 2010). Specifically, during corneal wound healing, infiltrating macrophages are believed to be the primary source for pro-lymphangiogenic growth factors such as VEGF-A/C/D. Furthermore, macrophages can trans-differentiate to an endothelial cell type and directly integrate into the lymphatic vessel wall (Maruyama et al., 2005). However, in contrast to this previously described model of inflammatory lymphangiogenesis, HSV-1-induced lymphangiogenesis does not require the presence of macrophages during the early onset of lymphatic vessel growth into the cornea proper (Wuest and Carr, 2010). Consistent with the unique induction process, the predominant initial source of VEGF-A is the infected corneal epithelial cells (Wuest et al., 2011; Wuest and Carr, 2010). Specifically, a Cre-lox system to selectively excise the VEGF-A gene from HSV-1 infected cells was employed to identify the source of initial VEGF-A expression. In this model, VEGF-A floxed mice infected with Cre-expressing HSV-1 (ICP0-Cre SC16) display 75% less corneal VEGF-A protein compared to VEGF-A floxed mice infected with the parental strain of HSV-1 (SC16) (Wuest et al., 2011). As predicted, the 4-fold reduction in VEGF-A dramatically impairs the lymphangiogenic response following infection (Figure 3). Furthermore, it was found that VEGF-A expression occurs independent of TLR signaling and is due to the binding of the HSV-1 encoded immediate-early transcription factor, ICP4, to the promoter of VEGF-A (Wuest et al., 2011).

Previous reports have suggested VEGF-A induced lymphatic vessels may be less functional compared to VEGF-C or VEGF-D induced vessels (Kajiya et al., 2006; Nagy et al., 2002). However, HSV-1-induced lymphatics are capable of transporting soluble antigen to the draining mandibular lymph nodes (MLN) (Wuest and Carr, 2010). To further demonstrate the transport of soluble antigen to the MLN, the lymphatic vessel specific size-exclusion dye FITC-dextran, administered intrastromally could be transported to the MLN of HSV-1 infected mice (Figure 4). However, structural ablation of the ocular lymphatics completely blocked drainage, evident by the lack of detection in the MLN (Figure 4). This dye was not apparent in the cervical lymph nodes at this time indicating the MLN are the primary draining lymph node over the first 5-7 days post infection. These results also suggest factors

generated locally within the cornea (e.g. cytokines and chemokines) may traffic to the MLN via the corneal lymphatics and facilitate the adaptive immune response. However, this hypothesis is untested and is currently under study in our laboratory. The impact of VEGF-A on hemangiogenesis is well appreciated, whereas the strong pro-lymphangiogenic phenotype following HSV-1 infection is surprising. Traditionally, reports have indicated VEGF-C and VEGF-D are the primary cytokines that drive lymphangiogenesis, while the most common isoforms of VEGF-A only lead to weak sprouting and enlargement of lymphatic vessels in a skin model (Karpanen and Alitalo, 2008; Wirzenius et al., 2007). Nevertheless, there has been at least one report of VEGF-A driven lymph node lymphangiogenesis without affecting hemangiogenesis following skin inflammation (Halin et al., 2007).

While recent studies have identified infected epithelial cells as the source for VEGF-A following HSV-1 infection and subsequently driving lymphangiogenesis during early acute infection, it is not clear what cytokines and cells are influencing lymphangiogenesis once the virus is cleared. VEGF-A protein levels peak at day 1 post-infection, whereas cornea expression of LYVE-1 continues to increase at least to day 30 post-infection (Wuest and Carr, 2010). It is tempting to speculate that after viral clearance infiltrating cells such as neutrophils, macrophages, or activated T cells may provide the necessary signals to further drive lymphangiogenesis. A recent study has shown that CD8⁺ T cells are an appreciable source of VEGF-C by day 7 post HSV-1 infection in an HSV glycoprotein B-specific T cell receptor transgenic mouse model (Conrady et al., 2012b). Moreover, depletion of CD8⁺ T cells during acute HSV-1 infection results in impaired lymphatic vessel development specifically affecting the length by which newly acquired vessels protrude into the central cornea (Conrady et al., 2012b). Consistent with these findings, neutralization of VEGF-C using VEGFR3-Fc results in the blunting of lymphatic vessels entering the cornea proper (Conrady et al., 2012b). We interpret these results to suggest in addition to VEGF-A, given the right circumstances, other sources of pro-lymphangiogenic factors can contribute to the development and maintenance of lymphatic vessels in the cornea proper in response to HSV-1 infection.

HSV-1 induced VEGF-A generated during acute infection could impact the development of an adaptive immune response in the draining lymph node, especially T cell activation that, in turn, can significantly influence the development of HSK. VEGF-A produced by chronically inflamed tissues can drain to the lymph node and induce lymphangiogenesis, possibly increasing the presentation of antigen to naive T cells (Halin et al., 2007). Dendritic cells are believed to be the main antigen-presenting cell responsible for the processing and trafficking of antigen to the draining lymph node during HSV-1 infection; however, VEGF-A can inhibit dendritic cell maturation and function through VEGFR1 signaling (Bedoui et al., 2009; Dikov et al., 2005). Such results are consistent with a number of other immune-evasive properties of HSV-1-encoded gene products. One further consequence of early VEGF-A expression may be the recruitment of VEGF-C expressing macrophages capable of inducing lymphangiogenesis after viral clearance (Cursiefen et al., 2004b). Therefore, our current working model of HSV-1-induced corneal lymphangiogenesis includes a two stage induction and maintenance process (Figure 5). Initially, local induction of virus-induced epithelial cell VEGF-A production signals lymphatic vessels within the limbus to undergo budding and growth toward the source of VEGF-A. As the virus is cleared from the cornea, cells that have subsequently infiltrated (T cells, monocytes, and neutrophils) the cornea and resident non-hematopoietic-derived cells express pro-lymphangiogenic factors in addition to VEGF-A in the maintenance of the newly created vessels. This model has yet to be formally proven and whether such events transpire in the human cornea has not been established.

D. Future Directions

The findings conveyed herein using the murine host demonstrate two key events that transpire within the first 6-12 hr post HSV-1 infection: the activation of the type I IFN pathway and induction of neovascularization through VEGF-A expression. Both pathways are elicited within epithelial cells of the cornea but function independently as neovascularization can occur in the absence of a functional type I IFN pathway (*Bryant-Hudson et al., under review*). Since type I IFN activation is independent of VEGF-A induction of and therefore, neovascularization initiation, in theory it should be possible to segregate the activation of these distinct processes. However, both pathways are interwoven since it is the immediate early gene product of HSV-1, ICP4 that drives VEGF-A production within the infected corneal epithelial cells (Wuest et al., 2011). By reducing the amount of replicating virus, there would be less lytic gene product including ICP4 available to drive VEGF-A expression. In fact, a previous study reported reduced levels of VEGF-A expression corresponds with an attenuation in hemangiogenesis (Zheng et al., 2001b). Along these lines, pharmacological intervention in reducing the expression of VEGF-A during acute HSV-1 infection has been found not only to significantly reduce hemangiogenesis but also attenuate the severity of HSK (Sharma et al., 2011; Rajasagi et al., 2011; Mulik, et al., 2012). Whether a reduction in VEGF-A significantly impacts on lymphangiogenesis after resolution of acute infection has not been formally proven but is actively under investigation by our laboratory.

While control of VEGF-A expression may reduce hemangiogenesis and HSK during the initial bout of acute infection, there is no indication VEGF-A levels modify virus replication, spread, or establishment of latency. This point is most appropriate in the human patient in which acute infection is generally sub-clinical, and episodic reactivation likely drives HSK. Therefore, individuals already afflicted with HSK will require continuous anti-viral and anti-inflammatory drug intervention as per their condition demands. For naive individuals (i.e., HSV-1 seronegative), the development of a vaccine to block the initial infection and establishment of latency is still a valid option. One would imagine this vaccine encompasses a protective barrier principally driven by humoral immunity composed of neutralizing antibodies as opposed to T cell-driven immunity. T cell recruitment into the cornea would likely result in neovascularization as T cells are a source of pro-angiogenic factors in response to HSV-1 infection (Freeman et al., 1995; Conrady et al., 2012b). Alternatively, the vaccine could promote the development of T_{reg} cells that may control inflammation and reduce neovascularization (Veiga-Parga et al., 2011). In addition to considering T cells as a first line of defense against HSV-1 in the cornea of the vaccinated host, T cells are thought to control reactivation of latent HSV-1 within the ganglion and therefore, may be useful in patients latently infected with the virus (Knickelbein et al., 2008).

The extent by which these events transpire in the human host is still relatively undefined. Specifically, very little is known about microbial pathogenesis, inflammation, and neovascularization in the human cornea. The majority of data, to date, is essentially derived from mouse studies or in vitro studies with human cells. A recent report has recorded the incidence of hem- and lymph-angiogenesis from a sample size of 35 corneas from human patients with infectious and non-infectious etiology (Tshionyi et al., 2012). Whereas most of the specimens histologically evaluated did not exhibit neovascularization, some samples possessed CD31⁺/Lyve1⁻ (blood) or CD31⁺Lyve1⁺ (mixed possibly lymphatic and blood)-expressing vessels. Consistent with this observation, preliminary results from our laboratory have found blood and lymphatic vessels co-localized within corneal buttons of some patients presenting with keratitis (e.g., ulcerative keratitis) (Bryant-Hudson and Carr, unpublished observation). In contrast to the human samples, the topography of blood and lymphatic vessels from the corneas of mice infected with HSV-1 is distinct with the CD31⁺ and LYVE-1⁺ vessels in different planes within the tissue (unpublished observation). Additional

studies are warranted to more fully define the existence of vessels relative to etiology (infectious vs non-infectious) and expression of pro- and anti-angiogenic factors especially in the human patient population. Until such studies are conducted, it will be difficult to develop strategies to address corneal neovascularization as it relates to inflammation and infection in the cornea of the human host.

4. Conclusions

The current article describes two host innate immune responses that transpire in the cornea following HSV-1 infection. Both events are rapid occurring within the first 6-12 hrs post infection involving the production of type I IFN and expression of VEGF-A by infected corneal epithelial cells. The relevance of type I IFN production in the control of HSV-1 replication is underscored by gene products encoded by the virus to counter the type I IFN activation process at the level of the innate sensor, IFI-16 (Orzalli et al., 2012), anti-viral effector pathways (Mulvey et al., 2003), and type I IFN receptor-induced Jak/Stat 1 activation (Yokota et al., 2005). Therefore, it is a race between the virus to counter type I IFN activation or induction of anti-viral pathways and the anti-viral environment established by type I IFN expression to shut down virus replication and spread. The consequence of blocking virus replication would undoubtedly suppress lytic gene expression including the gene product ICP4; the virus-encoded protein that drives VEGF-A expression and initiates lymphangiogenesis (Wuest et al., 2011). Based on our unpublished work and that published by others (Zheng et al., 2001a), angiogenesis (blood and lymphatic vessel development) in the cornea as a result of HSV-1 infection is correlative with VEGF-A levels. However, whether one can sufficiently reduce lytic gene expression and by inference, VEGF-A driven neovascularization by type I IFN-inducible, anti-viral pathways in a timely manner is questionable. Moreover, a provocative article recently published suggests a form of “memory response” exists in lymphatic vessel development in the cornea following recurrent inflammation (Kelley et al., 2013). Whether this property is unique for mechanical corneal wounds (e.g., sutures) or also applies to microbial-induced inflammation is unknown. If applicable, this trait of lymphatic vessel memory may be aligned with the pathology that exists in the human patient population that experiences episodic reactivation of latent virus. With appropriate support, a tremendous amount of exciting work awaits investigative teams in the identification of the mechanisms and cells that facilitate neovascularization, and the consequences of neovascularization on the host response to the infection long term.

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Figure 1. RNA and DNA Recognition by Non-TLR Sensors

Pathogen associated DNA and RNA are recognized by many non-TLR sensors. The RNA sensors include RIG-I, MDA5, and LRRFIP1, while the DNA sensors are RNA-PolIII, LRRFIP1, Ku70, DAI, DDX41, IFI-16, and the inflammasome. The RNA sensors, excluding LRRFIP1, signal through a TANK-TBK-1-dependent manner to induce phosphorylation of IRF species as either homo- or hetero-dimers. These phosphorylated complexes then translocate to the nucleus to activate IFN production. Several of the DNA sensors (IFI-16, DAI, and DDX41) signal through a similar sequence but acquire STING as an adaptor protein and subsequent phosphorylation of IRF homodimers by TBK-1 to stimulate IFN production. LRRFIP1 responds to both RNA and DNA in a similar manner and does so by inducing IRF nuclear translocation through a β -catenin- dependent manner. Lastly, the inflammasome is activated to produce IL-1 β in response to pathogenic sequences by innate sensors such as AIM2 and the NOD-like proteins. Orange lightening bolt, pathogenic RNA sequences; orange star burst, pathogenic DNA sequences; P, phosphorylated; cGAS, cyclic GMP-AMP synthase.

Figure 2. Overview of the Host Innate Immune Response to HSV within Corneal Epithelial Tissue

IFI-16/p204 recognition of HSV within the cell drives IFN- α production through an IRF-3-STING-dependent manner. IFN- α then directly activates CCL2 secretion by binding to the CCL2 upstream promoter thereby recruiting inflammatory monocytes to the site of infection. The monocytes further inhibit viral replication and spread by secreting anti-viral compounds such as nitric oxide. In addition, IFN- α also stimulates the activation of potent anti-viral proteins such as oligoadenylate synthetase via RNase L and protein kinase R to block viral replication within host cells.

Figure 3. Loss of HSV-1 induced VEGF-A significantly impairs lymphangiogenesis
VEGF-A floxed C57BL/6 mice were infected with either 10^5 PFU wild-type HSV-1 (SC16) or a recombinant SC16 virus expressing Cre under the ICP0 promoter (ICP0-Cre) resulting in the selective Cre-mediated loss of VEGF-A expression in ICP0-Cre infected cells. At day 5 post-infection corneas were examined for CD31-expressing blood vessels (red) and LYVE-1-expressing lymphatic vessels (green).

Figure 4. Block of lymphatic drainage to mandibular lymph node following ablation of limbal lymphatic vessels in the cornea

The limbal lymphatics of HSV-1 (McKrae) infected mice were thermally ablated on day 4 post-infection at three sites: two lateral and one ventral, representing the principal draining sites for the mouse cornea. Twenty-four hours post-cauterization, FITC-dextran was applied to the cornea. Twenty-four hours later, the mice were exsanguinated, and the mandibular lymph nodes were exposed (arrows) and visualized under white (upper) and fluorescent (lower) light.

Figure 5. Model depicting the induction and maintenance of HSV-1 induced lymphangiogenesis in the cornea

During the first 24-48 hours post-infection, HSV-1 infected epithelial cells are the primary source for VEGF-A, which drives the sprouting of new lymphatic vessels from pre-existing limbal lymphatics (left panel). Infiltrating cells that aid in viral clearance (monocytes and T cells) produce pro-lymphangiogenic factors that may contribute to the maintenance of newly developed lymphatic vessels within the central cornea after the clearance of HSV-1 from the cornea (right panel).