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TARGETING EARLY EVENTS IN HERPETIC STROMAL KERATITIS

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TARGETING EARLY EVENTS IN HERPETIC STROMAL KERATITIS

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DEDICATION

I dedicate this dissertation to my most important project of life, my own family: Nicolas, Victoria, Agustina and Ana.

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ABSTRACT

Herpetic stromal keratitis [HSK] is an immunoinflammatory corneal lesion caused by Herpes simplex virus type 1 [HSV] infection. SK is usually the consequence of virus reactivation from latency in the trigeminal ganglion. Studies in animal models have revealed that SK lesions are orchestrated mainly by CD4+T cells that infiltrate the corneal stroma. However, prior to this immunoinflammatory phase, multiple events occur that set the stage for subsequent pathology. These include production of cytokines and chemokines, infiltration of innate immune cells and neovascularization of the avascular cornea.

The first part of this dissertation reviews literature regarding the current understanding of human and murine SK pathogenesis, critical events and treatments. In chapter I we describe the role of Robo 4 [R4] receptor on the development of neovascularization [CV] in SK. We found that compared to wild type animals [WT], mice lacking R4 due to R4 gene knockout [R4 KO], had increased CV after HSV corneal infection. In addition, the administration of soluble extracellular domain of R4 [sR4] reduced angiogenesis in HSV infected WT mice.

The chapter II of this dissertation evaluates the role of Nod like receptor 3 [NLRP3] in driving the early inflammatory events that occur in HSV infected corneas. We found that compared to WT animals, mice lacking NLRP3 due to NLRP3 gene knockout [$NLRP3^{-/-}$], had early onset of the disease and more severe SK lesions after HSV corneal infection.

In this thesis, experiments were designed to explore molecular and cellular events that occur during the early stages in SK. These results allowed us to uncover a new pathway implicated in angiogenesis as well as new pathogen recognition receptor involved in inflammation. Our findings serve as guidelines for future development of more efficient prophylactic and therapeutic strategies.

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ABBREVIATIONS

Ahr	aryl hydrocarbon receptor
AIM2	absent in melanoma 2
APA	aminopeptidase A
APN	aminopeptidase N
ASC	apoptosis associated like protein
b.i.	before infection
BMDC	bone marrow derived cells
BMT	bone marrow transplant
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
CV	corneal neovascularization
CXCL	CXC chemokine ligand
d.	Day
DC	dendritic cell
DLN	draining lymph node
FoxP3	forkhead box P3
HIN	HIN domain-containing protein
hr.	Hour
HSP	heat shock proteins
HSV	herpes simplex virus
i.p.	intra peritoneal
IFI16	interferon-inducible protein 16

IFNγ	interferon gamma
IL	Interleukin
KC	keratinocyte chemoattractant
KO	knock out
LPR	Leu- Pro-Arg
LT-α	lymphotoxin alpha
MAb	monoclonal antibody
MabT25	tumor necrosis factor receptor superfamily member 25 agonistic antibody
MDM2	mouse doble minute 2 homolog
MDSC	myeloid derived suppressor cells
MIP-2	macrophage inflammatory protein 2
MiRNA	micro RNA
NK	natural killer
NLRC4	NLR family, CARD domain containing 4
NLRP	nod like receptor protein
PAMP	pathogen molecular pattern
pi.	post infection
PYD	pyrin domain
Q-RT-PCR	Quantitative real time Polymerase Chain Reaction
R4	robo 4
RIG-1	retinoic acid-inducible gene 1
S/c	Subconjunctival
SHIP1	SH2-containing inositol-5'-phosphatase 1

SiRNA	silencing RNA
SK	herpetic stromal keratitis
SLIT2	slit homolog 2
sR4	soluble Robo 4
SRC	sarcoma family kinases
sVEGFR1	soluble vascular endothelial growth factor
sVR1	soluble form vascular endothelial receptor 1
T	t cells
TCDD	2, 3, 7, 8- Tetrachlorodibenzo-p-dioxin
TGFβ	transforming growth factor b
Th	t helper cells
TIM-3	t cell immunoglobulin and mucin
TIMP-1	tissue inhibitor of metalloproteinases
TLR	Toll like receptor
TNFRSF25	tumor necrosis factor receptor superfamily member 25
Treg	t regulatory cells
UNC5β	uncoordinated homolog 5 β
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WT	wild type

BACKGROUND AND OVERVIEW

HERPES SIMPLEX VIRUS INFECTION

Infectious keratitis in humans is almost always caused by the type I strain of herpes simplex virus (HSV) [1]. It is usually acquired by close contact with persons with clinical or subclinical infections on the face, oral mucosa, or sometimes the genital mucosa [1]. On rare occasions, the virus is suspected to be transmitted by corneal transplantation [2]. The initial infection usually involves active viral replication in corneal epithelial cells that die and need to be replaced. Primary ocular infection is probably the result of direct inoculation of the surface of the eye or adnexa, but in atopic and immune compromised individuals it can be due to autoinoculation of active HSV infections elsewhere on the body, such as a cold sore. Herpetic stromal keratitis (SK), an immune mediated disease of the relatively acellular collagen matrix of the cornea, rarely occurs during primary ocular HSV infection. Most of the time SK is triggered by reactivation of latent HSV infection of neurons in the trigeminal ganglion, with subsequent axonal transport of viral proteins and/or infectious virus into the corneal stroma [3].

Active viral replication in the corneal epithelium proceeds for a few days and the clinical consequences can be reduced by 2-3 days with anti-viral therapy. Therapy does not markedly reduce the duration of viral detection and treatments are only assumed to be effective since untreated controls are rarely available for comparison. Unfortunately, controlling the lesion fails to remove the virus from the body and life-long latency is invariably established in neurons of the trigeminal ganglion. During latency, no replication competent virus is produced and were this situation to remain without interruption, all would be well. Alas, periodically, and perhaps continuously in some cases, a few latently infected neurons are induced by a variety of stress stimuli to reactivate a productive cycle and new virus passes back to peripheral sites where lesions were formerly present [4]. These recurrences may be subclinical or clinically evident, but repeated episodes can result in chronic inflammatory disease in the corneal stroma[5]. About 20% of patients with epithelial keratitis develop the stromal form of HSV corneal disease which is far more likely to compromise vision and more difficult to control therapeutically than epithelial keratitis

[3]. Some patients with SK require prolonged, even indefinite treatment with anti-inflammatory drugs and usually the virus is not present in lesions after the initial stages. Indeed, there is a strong suspicion and some evidence that SK can develop into an auto-immune inflammatory process [6].

For ethical and practical reasons it is difficult to manipulate and understand the role of the many events involved in human SK. In consequence, to assemble the steps involved in pathogenesis it is necessary to use a variety of animal model systems. None of the models are perfect, particularly to mimic the consequences of viral reactivation from latency. The most convenient model to use is primary infection of the mouse cornea with appropriate strains of HSV. Inflammatory lesions occur in the stroma and these resemble SK lesions observed in humans [7]. The mouse model is extremely unreliable to achieve lesions following exposure to virus reactivation stimuli [8, 9]. The rabbit model is better for this circumstance although the many disadvantages of working with rabbits to study inflammation and immunology make their use problematic.

THE TWO CRITICAL EVENTS IN SK: INFLAMMATION AND CORNEAL NEOVASCULARIZATION

The pathogenesis of herpetic SK includes two main events, the early and late immune response to the virus which results in inflammation, and the development of blood vessels in the transparent cornea. The following section will describe in detail both events and the different measures that have been used to reduce inflammation as well as to control corneal neovascularization (CV). For didactic purposes we describe both events separately however, during the development of the disease, inflammation and CV occurs simultaneously and one is the consequence of the other. In consequence, among the treatments that will be described, those drugs that affect primarily inflammation indirectly will affect CV and vice versa.

INNATE AND ADAPTIVE IMMUNE RESPONSE

Following infection, replication commences in epithelial cells and this lasts for 5 to 6 days after which replicating virus is usually no longer detectable in the eye (Figure 1.1). Normally, little or no replicating virus reaches deeper layers in the eye such as the corneal stroma[7]. However, in immunocompromised animals virus does reach deeper tissues and also passes to the brain to cause encephalitis, a usually lethal lesion which in mice might be in part immunopathological [10]. Replication invariably occurs too in the trigeminal ganglion where latency is permanently established [11].

Virus infection triggers numerous signaling events many of which are driven by pathogen activating molecular pattern (PAMP) properties of the virus. These include at least 3 TLR ligands (TLR-2, TLR-3 and TLR-9) all of which activate the innate immune system in some way [12-14]. Curiously, humans without TLR-3 responsiveness show markedly increased susceptibility to HSV infection [12]. It is not clear how and where the virus exerts its TLR ligand stimulating effects. Likely possibilities include virus budding from infected epithelial cells, cell contents released by killed cells, or perhaps extracellular free virus.

While TLRs are the most well known PRR activated by HSV, there are other PRRs that were shown to be activated in vitro by HSV. These include NLRP3 and IFI16/p204 (IFI16 mouse homolog IFI204)[15, 16], two families of PRRs that upon exposure to their ligands form large molecular complexes called inflammasomes[17]. Inflammasomes are characterized by the assemble of (i) a sensor protein that recognizes the trigger (ii) an adaptor molecule known as an apoptosis associated like protein (ASC) and (iii) an effector protein called pro-caspase 1[18](Figure 1.2). According to the sensor protein that inflammasomes contain they are classified into two families, NLR family (nucleotide finding and oligomerization domain (Nod) and leucine rich-repeat-containing) that contain NLRP1, NLRP3, NLRC4 and NLRP6 and NLRP12[19] or PYHIN domain family (Pyrin domain (PYD) and HIN domain-containing) which includes AIM2 and IFI16[20] (Figure 1.2). The formation of this cytoplasmic complex results in

activation of pro-caspase 1 into caspase 1 which in turn induces the maturation of IL-1β and IL-18 into their bioactive forms. As well, activation of the inflammasome can also result in pyroptosis[21]. In addition, while NLRP3 is localized in the cytoplasm and can be activated by a wide range of stimuli, IFI16 is localized in the nucleus and relocalize to the cytoplasm upon stimulation and it is considered a true receptor since it engages double stranded DNA ligands directly[16,22]. The knowledge about inflammasomes and HSV is based exclusively to date on in vitro studies. In vivo experiments are underway to reveal the relevance of these PRRs.

Apart from the secretion of cytokines as a consequence of PRRs activation, there are other products generated (such as TLR ligands, HSPs and some cytokines) that act in a paracrine fashion to induce uninfected cells to produce and secrete numerous factors that participate in pathogenesis. These include the critical proinflammatory cytokines IL-6, IL-1 and IL-17A[7]. Chemokines such as CCL2, CXCL1 and CXCL2 are also produced and these serve to attract inflammatory cells of many types into the corneal stroma from blood vessels present at the ocular limbus [23, 24]. Inflammatory cells are evident by 24 hr pi and these include natural killer cells (NK), γτT cells, dendritic cells (DC), macrophages and most prominently neutrophils [25-28] (Figure 1.1). Notably, there are few if any lymphocytes especially those that are reactive with viral antigens [29]. We remain uncertain as to the role of the various recruited cells, but it is known that they serve to abort the infection[30]. The DCs that invade the eye are likely involved in transporting viral antigen to the draining lymph node and initiating an adaptive immune response. Some DC subtypes and macrophages could be a source of lesion modulating molecules such interferons, transforming growth factor beta (TGFβ) and IL-10.

The stromal lesions that occur in the eye represent immunopathological events that are orchestrated by T cells (Figure 1.3). Accordingly, T cells (primarily CD4+) become evident in the stroma by seven days pi. and this infiltration peaks around 14-21 days pi. [7] (Figure 1.4). Stromal lesions do not occur in animals that lack T cells, but lesion-producing competence can be restored with adoptive transfers of T cells, with CD4+T the most disease producing [31, 32]. It is conceivable that the entrance of T

cells, which are presumably corresponding to chemokines such as CCL2, CCL5 and CXCL10 generated in the stroma, is facilitated by the new quite leaky vascular bed that expands from the limbus. A major unresolved issue is to define the antigen specificity of the CD4+T cells involved in orchestrating SK. Unfortunately, appropriate peptides or tetramer reagents to identify T cell specificity have not been used and are not readily available. Viral antigen specificity is suspected to be one of the targets for T cells, but activated T cells of other specificities are present in lesions and these are hypothesized to participate [33]. Our group, for example, have observed that TCR transgenic mice on a RAG background, where almost 100% of the T cells are reactive to a peptide that has no known cross-reactivity to HSV, can be responsible for lesions that appear identical to those seen in normal infected mice [34]. In fact we have advocated that the environment of the inflamed cornea may serve to stimulate T cells of many specificities providing they gain access to the cornea. We believe that these bystanders can be stimulated to generate pro-inflammatory components that further contribute to SK. However, these bystander activation ideas are not uniformly accepted.

An alternative concept is that SK lesions are the consequence of autoantigens being unmasked in the cornea and these drive autoreactive cells to mediate an autoimmune inflammatory reaction [6]. An extension of this idea is that the virus itself may act as a molecular mimic for some of these autoantigens [35]. This notion has attracted advocates [35], but the concept has never been independently confirmed and has been experimentally refuted [34].

It is a curious and unexplained fact that by far the most frequent T cell type in SK lesions is CD4+T cell. However these T cells can be of many subtypes in terms of function. Early on the major subtypes are Th1 cells along with FoxP3+ regulatory T cells [36, 37]. In the later stages, Th17 cells become more evident, although these never outnumber the Th1 cells [38]. By the time Th17 cells become prominent, CV is quite extensive and it is possible that some cytokines generated from Th17 cells contribute directly or indirectly to the angiogenic process[39]. Murine SK lesions usually do not resolve spontaneously,

particularly if the lesions are severe. However it is conceivable that resolution could be achieved with novel therapies and this is an active area of investigation.

PREVENTING AND CONTROLLING INFLAMMATION

As described in previous sections, the main orchestrators of SK are CD4+T cells. The different approaches that have been used to diminish the infiltration of such cells include reducing the number of T cells, reducing inflammatory mediators or emphasizing/ increasing the number of regulatory T cells (Treg). The description that follows briefly discusses the several previous and current approaches evaluated so far to control corneal inflammation in the mouse. Table 1.1 and figure 1.5 resumes the regimen of administration and mechanisms of action, respectively.

Stopping and inhibiting T cells: Several approaches have been used to reduce the number of inflammatory T cells in cornea. One such measure is destroying activated T cells through the administration of galectins, a family of endogenous carbohydrate binding proteins expressed by activated but not naïve cells. Administration of galectin-9 reduced SK severity by inducing apoptosis of effectors T cells[40]. Such effect is known to be achieved through binding of galectin-9 to TIM-3 (T cell immunoglobulin and mucin-3) receptor, a member of the T cell immunoglobulin and mucin family of proteins[41].

Reduction of SK severity also occurred when administering galectin-1[42]. The effects of galectin-1 on immune and inflammatory cells are likely to be due to the binding and cross-linking of this protein to cell-surface glycoproteins such as CD45, CD43, and CD7 [43]. Another potential way to inhibit T cells in SK and in turn reduce the severity of this disease is targeting T cell transcription factors[44, 45]. TCDD is a drug that activates the cytosolic transcription factor AhR involved in inducing apoptosis of activated T

cells[46, 47]. A single administration of this drug was used successfully to suppress the severity of ocular immunoinflammatory lesions caused by HSV[48].

Targeting Lymphotoxin-α (LT-α) is another way to reduce the function of T cells[49, 50]. LT-α is a cytokine produced by lymphocytes that upon binding to its receptor, which are on the cell surface of Th1 and Th17 cells, can induce the production of inflammatory cytokines and chemokines[49, 50]. Administration of anti-LTα mAb was shown to reduce the severity of SK lesions[51]. Finally another mean to achieve such effect was modulating the expression of miRNAs, for example miRNA 155. It has been reported that this miRNA targets two molecules (SHIP1 and IFNγ receptor α-chain) involved in Th1 differentiation[52]. Administration of antagomir 155 reduced SK lesions in mice through inhibition of T cells function[52].

Changing the subset balance to favor Treg:Treg play an important role in protecting the host from ocular immunopathology and expanding their numbers can result in diminished SK[53]. A novel approach to increase this group of cells takes advantage of the fact that Foxp3+ regulatory T cells, but less so other naïve T cell subsets, constitutively express high levels of the TNF receptor superfamily member 25 (TNFRSF25)[54]. Stimulation of TNFR25 on Treg with an agonist was an effective means of reducing the severity of subsequent SK lesions[55]. However, since TNFR25 receptor was also present on activated T cells, when the therapy was administer at day 6 pi., activated T cells were increased as well. Combining TNFR25 to increased Treg, with galectin-9 (drug mentioned in the previous section) to reduce T cells, resulted in a successful therapy to control SK immunoinflammatory lesion[55].

Other drugs previously mentioned that are known to expand Treg or change the balance towards Treg include galectin-9 and TCDD, respectively[55].

Reducing mediators of inflammation: During the inflammatory process, signaling pathways and cellular mechanisms are activated. However, the host has his own mediators and mechanisms to switch off inflammation. Resolvin E1, a drug derived from polyunsaturated omega-3 fatty acid, represents one of

such mediators involved in the resolution of inflammation[56]. Thus, topical administration of Resolvin E markedly reduced inflammation and the SK lesions in the murine model[56].

Other measures to reduce the cellular infiltration in the cornea and in turn improve SK, include targeting chemokines. CXCL10 is a potent chemoattractant for activated T cells and NK cells[57, 58], monocytes[59], and neutrophils [60]. Administration of anti-CXCL10 has shown to reduce the inflammatory response in the cornea[61]. As well, neutralization of MIP-1α, chemokine known to activate granulocytes, has shown to have beneficial effects on SK[62].

Finally, inhibition of other inflammatory mediators such as IL-1β or metaloproteinases (MMPs) has been used to treat SK and are in included in table 1.1. and figure 1.5.

CORNEAL NEOVASCULARIZATION (CV)

As previously mentioned, another main event that occurs during the development of SK is CV. It is evident as early as 1 day after corneal infection with the process continuing to advance over a 2 to 3 week period [63] (Figure 1.1). New blood vessels sprouting from the normal vasculature at the limbus can be seen in the eyes of Balb/c animals in the very early stages of keratitis. These continue to develop in mice and can expand eventually from the entire limbus to almost reach the central cornea and interfere with vision (Figure 1.6). New blood vessels of pathological angiogenesis are leakier than normal vessels and permit the escape of inflammatory cells into tissues that contribute to vision impairment (Figure 1.7). In the initial stages of CV, blood vessels can be inhibited with treatments such as anti-vascular endothelial growth factor (VEGF) mAb, although ghost vessels remain after treatment and these can become patent again under certain circumstances such as an episode of viral reactivation (Margolis TP, personal communication, 2003). The extent of CV is a rare occurrence in human SK. As discussed subsequently, the source of stimuli that drives this CV likely changes with time, with non-immune and innate immune events dominating initially and the adaptive immune system critically involved in later stages [5].

In the mouse, CV is likely driven by numerous angiogenic factors and facilitators with such molecules having a variety of cellular sources. Moreover, this source may change during the course of lesion pathogenesis [64]. Most studies on CV have focused on VEGF-A and it is evident that a variety of approaches which impair the production or response to VEGF-A can markedly inhibit the extent of CV [65]. Some have advocated that a significant source of the VEGF could be the virus infected epithelial cells [66]. Such infected cells can produce VEGF, at least for a brief time, but the virus rapidly shuts off host cell mRNA and protein synthesis making it doubtful if the infected cell represents a relevant source of the VEGF responsible even for initial angiogenesis[67].

Instead, additional sources are likely to be more consequential for driving CV. The first is a physiological source present in uninfected eyes of all species. The molecule is present, but is prevented from causing angiogenesis since it is bound to a soluble form of one of its receptors, sVEGFR1 [68]. The virus infection also inhibits the synthesis of sVEGFR1 more than VEGF itself [64]. This will change the balance of the two molecules and will release some VEGF to cause angiogenesis. Moreover, early inflammatory entrants, such as neutrophils, contain proteases that readily cause sVEGFR1, but not VEGF, to lose its function [64]. The outcome is also more VEGF to mediate angiogenesis.

An additional source of the VEGF could be even more instrumental in driving pathological angiogenesis. Our group noted some time ago that IL-6, initially produced by infected cells, could cause nearby cells to produce VEGF [69]. Similarly, viral DNA because of its TLR-9 ligand activity could also cause uninfected cells to produce VEGF [70]. More recently, the cytokine IL-17A, produced initially by γδ T cells that are rapidly recruited to the cornea in response to HSV infection, was also shown to drive VEGF-A production from uninfected cells [71]. These amplifying paracrine effects of infection, rather than direct effects of virus replication, would seem to be far more relevant as inducers of the VEGF and perhaps additional angiogenic factors (eg. fibroblast growth factor and Angiopoietin 1) that drive CV. Such ideas, however, need to be formally proven.

Replication of HSV is a relatively short event in the mouse yet pathological angiogenesis is usually a progressive process proceeding well beyond the time when virus has been removed from the eye. The explanation for the prolonged CV remains unresolved but a viable hypothesis is that the lesion orchestrating proinflammatory T cells contribute indirectly once they arrive in the cornea. Such cells release a variety of cytokines and chemokines that together act as direct or indirect stimulators of angiogenic factor production. For example, release of the chemokines CXCL1 and CXCL2 recruits neutrophils to the stroma that themselves contain and release VEGF-A[72]. The neutrophils also produce a number of proteases that can act to degrade the corneal matrix so facilitating angiogenesis [72]. The same molecules can further breakdown any residual sVEGFR1 releasing any bound to VEGF to mediate angiogenesis [64]. In fact, MMP inhibitors can be used to diminish the levels of CV induced by HSV infection [64]. Additionally, HSV infected MMP-9 knockout mice show diminished CV compared to wild type animals [72].

As seen, during CV development proangiogenic pathways that stimulate VEGF effect are activated. However, the host has its own antiangiogenic mechanisms to reduce the effects of VEGF. So far the only one studied is the interaction between SLIT2/Robo4, which counteracts the VEGF signaling. During angiogenesis, the balance between angiogenic and antiangiogenic factors is tipped toward angiogenic molecules. Thus more research is needed to understand and try to enhance the host's antiangiogenic pathways.

PREVENTING AND CONTROLLING CV

As seen, CV plays a critical role during SK pathogenesis and its inhibition can help control disease severity. Numerous approaches to limit the extent of CV have been explored most of which so far have targeted the production, availability or signaling of VEGF. Most often CV can be markedly reduced if the anti-VEGF strategy is begun early after infection, but the therapies are much less successful when

commenced after CV is well established. The description that follows briefly discusses the several previous and ongoing approaches evaluated so far to control CV in the mouse. The regimen of administration and mechanisms of action of the drugs used are listed in table 1.2 and figure 1.5, respectively.

***Limiting availability of VEGF*:** Different approaches can limit the availability of VEGF during pathological angiogenesis. One way is to use a mAb to VEGF (Avastin) that was developed to inhibit angiogenesis in some human tumors [73]. Curiously, avastin (and its more purified product lucensis) appears to be more effective to control aberrant angiogenesis in the human retina (wet macular degeneration) than it is against tumor angiogenesis in the cancers it was developed to control. Avastin works weakly against mouse CV [74], but the more appropriate reagent that is specific to mouse VEGF has yet to be evaluated.

A second approach to limit VEGF availability is to use recombinant soluble VEGF receptor 1 (sVEGFR1), a fusion protein also called the VEGF trap. This reagent is used clinically to counteract aberrant CV in the retina [75] and our group has demonstrated that murine sVEGFR1 can be used to inhibit CV induced by HSV infection [64]. Accordingly, the local administration of recombinant mouse sVEGFR1 to infected eyes resulted in significantly reduced levels of CV, although frequent treatment was necessary. Of interest, as mentioned later, procedures that inhibit CV such as neutralizing IL-17A acts in part by changing the balance between VEGF and VEGFR1 emphasizing the latter [38] (Table 1.2) (Figure 1.5).

***Inhibiting the signals delivered by VEGF*:** Of the 5 VEGF family members, the mainly involved in CV is VEGF-A. This molecule signals vascular endothelial cells in blood vessels at the limbus by binding to specific receptors, VEGFR1 and VEGFR2, with the last the most important [76]. Interrupting VEGF interaction with its receptors using a gene silencing approach (siRNA) given topically was shown to be an effective way of inhibiting CV, at least when used early after HSV infection [65]. Other approaches to

counteract VEGF/VEGFR2 signaling are either more convenient to use, or more effective especially when used during the ongoing CV process. One such approach is to counteract downstream signaling effects of receptor binding. SRC kinase inhibitor drugs achieve that objective [74]. The approach can also simultaneously inhibit the signaling by several other angiogenic factor receptors. A disadvantage of the approach is that more than once daily administration is needed at least with drugs tested so far [74].

A potentially more powerful antiangiogenic approach is to modulate the expression of miRNAs involved in the angiogenic activity of VEGF. One such target for evaluation may be miR-132 recently shown by the Cheresh group as an activator of pathological angiogenesis in some tumors [77]. Recently, we assessed the role of miR-132 in the CV caused by HSV infection[78]. We showed that HSV infection caused the upregulation of miR-132 expression. This effect was initially the consequence of IL-6 and IL-17A driven upregulation of miR-132 in vascular endothelial cells[79]. In later stages, the miR-132 upregulation may result from the products released from invading inflammatory cells[79]. The upregulated miR-132 acted to modulate the signaling response to VEGF/VEGFR2 interaction on vascular endothelial cells. The outcome was a blunting of RAS Gap which normally restrains the cells from proliferating and participating in angiogenesis [77]. Apparently inhibiting miR-132 also acts to restrain retinal angiogenesis and this effect does not result in the compensatory angiogenic mechanisms that usually substitute for VEGF signaling when VEGF is inhibited [80]. miRNA modulation represents a promising therapy because treatment can be administered infrequently. Moreover our preliminary results show that miR-132 modulation in the later phases of CV is a more effective means of controlling the extent of CV than other approaches used so far.

An additional way to counteract CV could be to enhance blood vessel endothelial cell signals which negatively regulate VEGF signaling [81]. Our lab recently reported one such regulatory molecule. SLIT2 was thought to bind to its endothelial Robo4 receptor (R4) that in turn blunted VEGF signaling in eyes after HSV infection [78]. We observed that the majority of the blood vessel endothelial cells isolated from infected corneas expressed the R4 receptor while levels of SLIT2 were minimal during SK.

Accordingly, provision of additional SLIT2 protein in infected mice reduced CV and this inhibitory effect was mediated by reduced VEGF signaling downstream products Arf 6 and Rac 1. We further evaluate the function of R4 in HSV induced CV.

CONCLUSION

Herpetic SK is a chronic immunoinflammatory lesion in the cornea with a complex pathogenesis. Understanding molecular and cellular events that are involved in the development of SK pathogenesis is of interest so as to develop new strategies to the SK treatment and potential cure. As discussed previously cytokine targeted to reduce CV have been the most studied and those include means to reduce or inhibit VEGF signaling. The study described in the first chapter of this dissertation further evaluates the effect of R4 on the levels of CV and SK. In this study mice unable to produce R4 because of gene knockout (R4 KO) developed significantly higher CV after HSV ocular infection than did infected wild type (WT) control. We revealed that this host pathway that counteracts VEGF signaling can be enhanced by administration of soluble R4 (sR4). While this therapy was effective, it was not practical since it was not durable and required frequent administration. Another point of interest is the problem of inducing vascular retraction once the blood vessels are already formed. The last part of this thesis discusses different options to potentially increase sR4 durability and efficacy, and potential means to remove new pathological blood vessels from the cornea.

In the second chapter of this thesis the role of NLRP3 inflammasome on the pathogenesis of SK is evaluated. We discovered not only that NLRP3 in involved in the pathogenesis of SK but also that its absence increases in severity SK lesions. The last chapter of this thesis discusses new lines of research that can be generated after this very first study.

As seen, SK is a multifactorial disease and the proper treatment involves understanding all these complex events and generating therapies that target more than one of the factors involved.

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APPENDIX

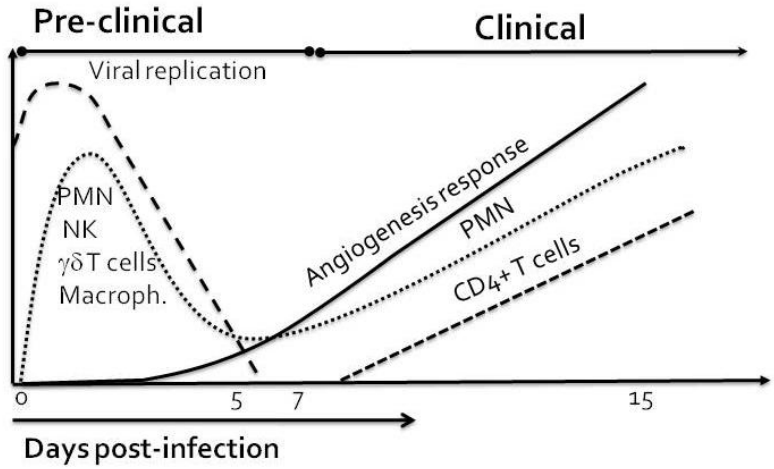

FIGURE 1.1- PRINCIPAL EVENTS IN HSV INDUCED CORNEAL SK PATHOGENESIS. Following HSV infection of the cornea, replicating virus is detectable in the cornea until day 5-7 pi. Neutrophils infiltrate the cornea in a biphasic influx peaking first on day 2 pi. and then around day 11 pi. Development of new blood vessels from existing limbal vessels starts as early as day 1 pi. Influx of pathogenic CD4+ T lymphocytes occurs in the clinical phase around 7-9 days pi.

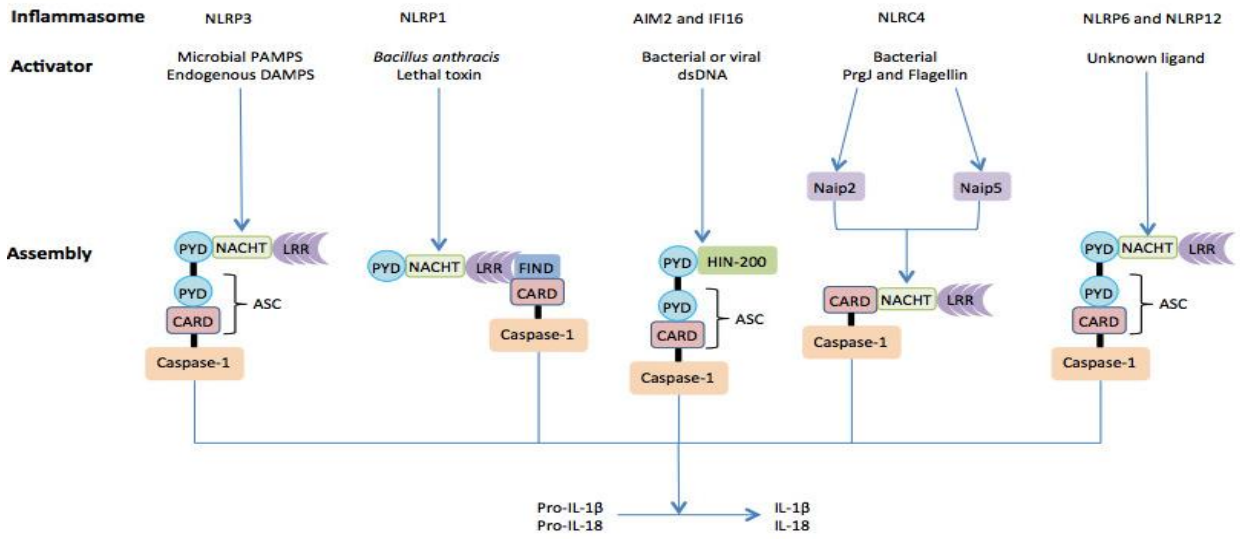

FIGURE 1.2- DIFFERENT INFLAMMASOMES AND ACTIVATORS. http://www.biomedcentral.com/1471-2369/15/21/figure/F2

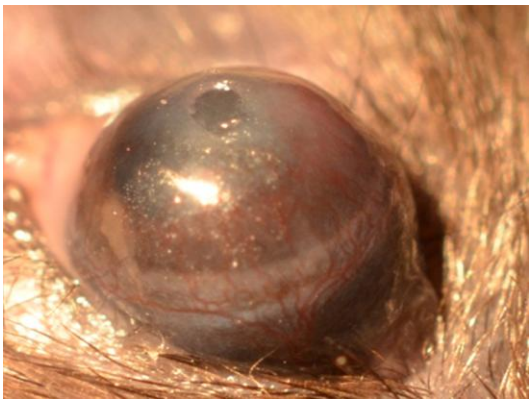

FIGURE 1.3- EYE OF B6 MOUSE SHOWING CORNEAL OPACITY, HAZE, CORNEAL ULCER AND CV AT DAY 15 PI.

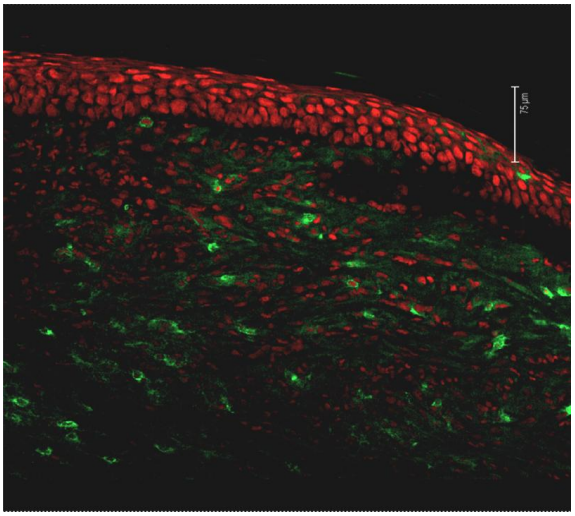

FIGURE 1.4- IMMUNOFLUORESCENCE STAINING OF CD4 +T CELLS (GREEN) IN CORNEAL TISSUE SECTION AT DAY 15 PI.

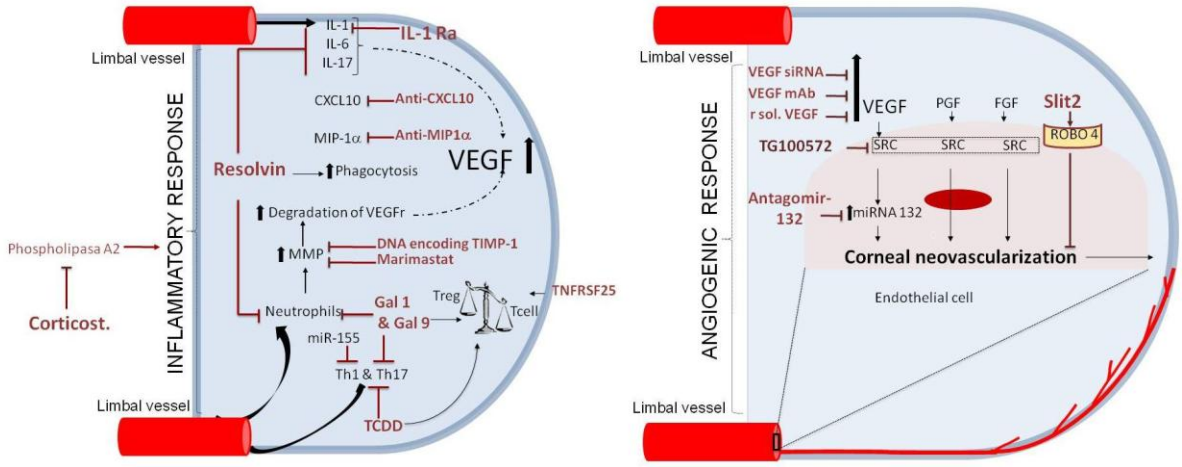

FIGURE 1.5- DRUGS USED TO REDUCE INFLAMMATION AND ANGIOGENESIS IN THE CORNEA

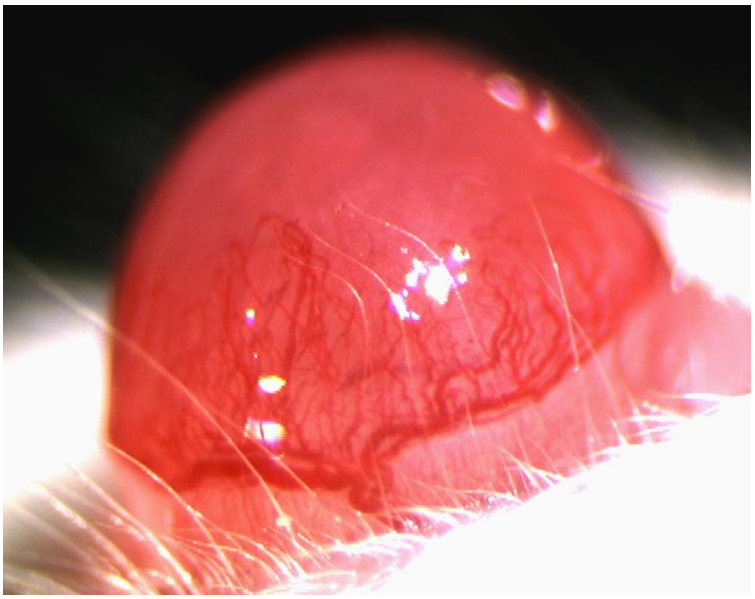

FIGURE 1.6- EYE OF BALB/C MOUSE SHOWING CV AT DAY 15 PI.

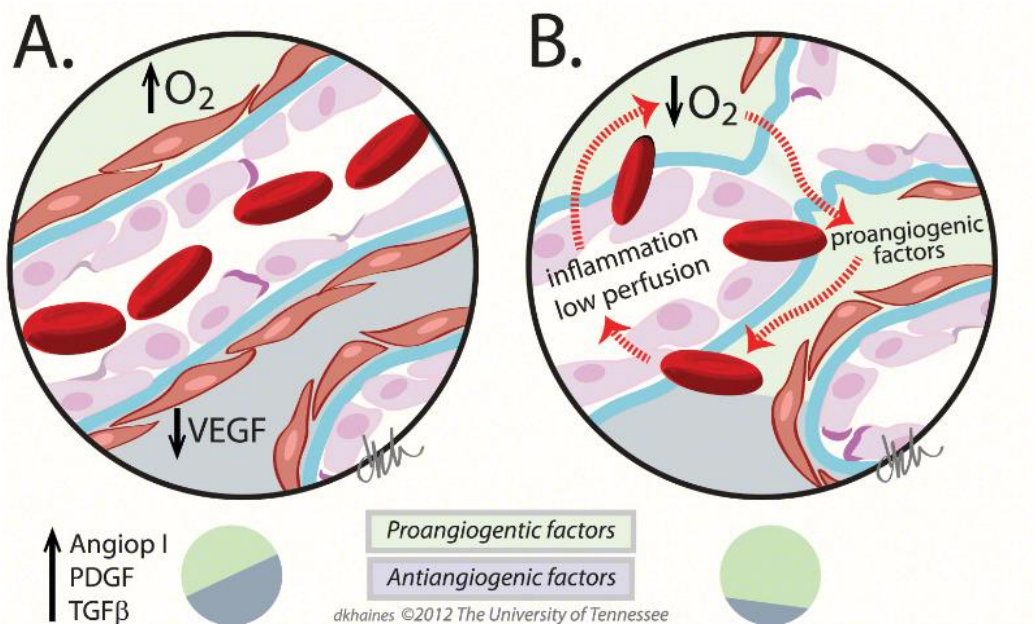

FIGURE 1.7- OUTCOME OF PHYSIOLOGICAL AND PATHOLOGICAL ANGIOGENESIS A. Physiological angiogenesis. New blood vessel are formed. As blood flows, there are increased levels of oxygen, reduced levels of VEGF and the process reaches resolution. **B. Pathological angiogenesis.** Pathological blood vessels are tortuous and leaky. As there is low perfusion, there are low levels of oxygen, increased levels of proangiogenic factors and the process does not reach resolution.

TABLE 1.1- DRUGS USED TO REDUCE INFLAMMATION IN THE MURINE MODEL

		Drug	Route & frequency of administration	Beginning of therapy	Reference
Stopping & Inhibiting T cells		Anti-Lymphotoxin–α	i.p. 3 times a week	6 d. pi.	[51]
		Antagomir miRNA-155	S/c every other day	1 d pi.or 5 d. pi.	[52]
	Changing the subset balance to favor Treg	Galectin-1	i.p. daily S/c every other day	3 d. pi. or 6 d. pi. 6 d. pi.	[42]
		Galectin-9	i.p. every other day S/c daily	4 d. pi. or 8 d. pi.	[40]
		TCDD	i.p. single administration	5 d. pi.	[48]
		MAbT25	i.p. single administration	2 bi. or 8 hs. pi.	[55]
Reducing mediators of Inflammation		Resolvin E1	i.p. daily	6 d. pi. or 1 d. pi.	[56]
		Anti-CXCL10	i.p. 3 times	0 d., 2d. & 5 d. pi.	[61]
		Anti-MIP1-α	i.p. every 4 d. and S/c every other day	8 pi.	[62]
		Marimastat	S/c every other d.	1 d. pi. or 5 d. pi.	[64]
		(rh) IL-1 ra	Combination of infusion pump implantation and S/c every other d.	Infusion pump: 1 d. pi. & S/c every other day	[82]
		Plasmid DNA encoding murine TIMP-1	Intraocular twice	3&6 d. pi.	[72]
Combinatorial treatment		Galectin 9 & MAbT25	i.p. every other day i.p. single dose	6 pi. 6 d. pi.	[55]

i.v.: intravenous; S/c: subconjunctival; i.p.: intraperitoneal; pi.: post-infection; b.i.: before infection

TABLE 1.2- DRUGS TO REDUCE CORNEAL CV IN THE MURINE MODEL

	Drug	Route & frequency of administration	Beginning of therapy	Reference
Drugs that limit the	SiRNA	i.v. twice S/c twice	1&3 d. pi. 1&3 d. pi.	65
	mAb to VEGF (Avastin)	i.p. daily	6 d. pi.	74
	sVEGFR1 (VEGF-trap)	S/c. every other d.	1 d. pi. 5 d. pi. 7 d. pi.	64
Drugs inhibiting the signal	Src Kinase inhibitor TG100801	i.p. daily i.p. daily Topical drop Topical drop	1 d. pi. 6 d. pi. 1 d. pi. 6 d. pi.	74
	Recombinant SLIT-2	S/c every other d.	1 d. pi. 7 d. pi.	78
	Antagomir-miR-132	S/c every other d.	1 d. pi. 7 d. pi. 10 d pi.	79

i.v.: intravenous; S/c: subconjunctival; i.p.: intraperitoneal; pi.: post-infection; b.i.: before infection

CHAPTER 1

ROBO4 COUNTERACTS ANGIOGENESIS IN HERPETIC STROMAL KERATITIS

ABSTRACT

The cornea is a complex tissue that must preserve its transparency to maintain optimal vision. However, in some circumstances, damage to the eye can result in neovascularization that impairs vision. This outcome can occur when herpes simplex virus type 1 (HSV) causes the immunoinflammatory lesion stromal keratitis (SK). Potentially useful measures to control the severity of SK are to target angiogenesis which with herpetic SK invariably involves VEGF. One such way to control angiogenesis involves the endothelial receptor Robo4 (R4), which upon interaction with another protein activates an antiangiogenic pathway that counteracts VEGF downstream signaling. In this study we show that mice unable to produce R4 because of gene knockout (R4 KO)developed significantly higher angiogenesis after HSV ocular infection than did infected wild type (WT) controls. Moreover, providing additional soluble R4 (sR4) protein by subconjunctival administration to R4 KO HSV infected mice substantially rescued the WT phenotype. Finally, administration of sR4 to WT HSV infected mice diminished the extent of corneal angiogenesis compared to WT control animals. Our results indicate that sR4 could represent a useful therapeutic tool to counteract corneal angiogenesis and help control the severity of SK.

INTRODUCTION

The cornea needs to be transparent to allow transmission of incident light so as to achieve optimal vision. While the cornea has different mechanisms to maintain its transparency, certain injuries can result in corneal opacification and impaired vision[1, 2]. Such is the case with stromal keratitis (SK), a lesion that can follow corneal infection with herpes simplex virus (HSV), which in humans is usually the consequence of repeated viral reactivation of latent infection in the peripheral nervous system[3]. SK involves multiple events one of which is the formation of new blood vessels into the normally avascular cornea. Accordingly, diminishing the extent of corneal neovascularization (CV) represents a useful approach to therapy[4]. The main target so far investigated has been the principal stimulus for angiogenesis, vascular endothelial growth factor (VEGF) and its receptors. These treatment approaches have included the use of recombinant soluble VEGFR1, a fusion protein also called the VEGF trap[5]; recombinant humanized monoclonal antibody known as Bevacizumab[6]; VEGF and VEGF receptor silencing RNAs[7]; SRC kinase inhibitors[6] and the inhibition of some miRNAs[8]. An alternative approach that could control CV, is to exploit the mechanisms the host itself has to limit the extent of VEGF induced angiogenesis. This mechanism uses the Robo4 (R4) receptor, a member of the axon guidance receptor family which is expressed on angiogenic endothelial cells[9, 10]. Upon interaction with its ligand, R4 generates a negative signal in the cell that diminishes the VEGF response [11-14]. In support of this, when R4 is absent because of gene knockout, mice may develop accelerated angiogenesis in tissues such as the retina[11]. Currently any role for R4 at modulating HSV induced angiogenesis in the cornea has not been reported.

In the present report, we evaluate the role of R4 signaling in an ocular model of CV and inflammation that follows primary ocular infection with HSV. We demonstrate that mice lacking R4, because of gene knockout, develop more severe corneal angiogenesis compared to WT mice. Moreover, administration of soluble R4 (sR4) to R4 KO HSV infected mice substantially rescued the WT phenotype. In addition, provision of sR4 by subconjunctival administration to WT infected animals significantly

diminished the extent of corneal angiogenesis. It is possible that the outcome observed in R4 KO mice, was due to the interaction of sR4 with the vascular specific axon receptor uncoordinated homolog 5β (UNC5β), however further research is needed to verify this issue. Since the results suggest that the administration of sR4 promotes the activation of an antiangiogenic pathway, this approach may represent a valuable therapeutic tool to control corneal angiogenesis related to HSV induced SK.

MATERIALS AND METHODS

Mice: Female 6-8 wks old C57BL/6 mice were purchased from Harlan Sprague Dawley Inc. (Indianapolis, Indiana, USA). Robo4 knockout (R4 KO) mice were the kind gift of Christopher A Jones (University of Utah). The animals were housed in American Association of Laboratory Animal Care-approved facilities at the University of Tennessee, Knoxville. All investigations followed guidelines of the institutional animal care and use committee.

Virus: HSV strain RE Tumpey was propagated in Vero cell monolayers (ATCC no: CCL81). Virus was grown in Vero cell monolayers (American Type Culture Collection, Manassas, VA), titrated, and stored in aliquots at –80˚C until used.

Corneal HSV infection and scoring: Corneal infections of mice were performed under deep anesthesia. The mice were lightly scarified on their corneas with a 27-gauge needle and a 3µl drop containing HSV RE was applied to one eye. When experiments included R4 KO mice the animals were infected with 8 x 10^3 mice PFU of HSV. When experiments included only WT mice, animals were infected with 10^4 PFU of HSV. The SK lesion severity and angiogenesis in the eyes of mice were examined by slit-lamp biomicroscopy (Kowa Company, Nagoya, Japan). The scoring system was as follows: 0, normal cornea; +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity but iris

visible; +4, opaque cornea and corneal ulcer; +5, corneal rupture and necrotizing keratitis [5]. The severity of angiogenesis was recorded as described previously [15]. According to this system, a grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.5 mm toward the corneal center. The score of the four quadrants of the eye were then summed to derive the neovessel index (range 0–16) for each eye at a given time point.

Subconjunctival Injections: Subconjunctival injections were performed as previously reported [16]. Briefly, these injections were performed using a 2-cm, 32-gauge needle and syringe (Hamilton, Reno, NV) to penetrate into the subconjunctival space.

Murine Treatment with soluble Robo4 (sR4): WT and R4 KO mice were ocularly infected with HSV RE Tumpey. WT mice were treated with PBS vehicle (WT control). R4 KO mice were separated into two groups, one of which was treated with sR4 (R4 KO treated) and the other with vehicle (R4 KO control). sR4 was kindly donated by Ryan Watts (Genentech Inc.). The treatment started 2 days pi., with additional daily doses until day 14 pi. In another experiments WT mice were infected with HSV RE Tumpey and were divided in two groups, one of which received vehicle (control) (PBS) and the other one sR4 (treated). Both treatments were administered daily from day 2 pi. to day 14 pi. These animals were carefully followed for the progression of angiogenesis and SK development.

Flow Cytometry: Corneal single cell suspensions were prepared following Liberase digestion of corneas collected at day 15 pi. Aliquots of the above single-cell suspensions were stained for CD4-FITC, CD45-PerCP, CD31-allophycocyanin, CD11b-PE and Ly6G-Pacific blue cell surface markers (All from BD Biosciences Pharmigen) for 30 minutes on ice. Thereafter, cells were washed twice and resuspended in 1% para-formaldehyde. The stained samples were acquired FACS LSR (BD Biosciences) and the data were analyzed using the FlowJo software.

Quantification of mRNA expression levels by quantitative real time PCR (Q-RT-PCR): Total mRNA was isolated from corneal cells using TRIzol LS reagent (Invitrogen). For RNA, cDNA was made with 500 ng of RNA using oligo (dT) primer and ImProm-II™ Reverse Transcription System (Promega). TaqMan gene expression assays (IL-6, IL-1β, CXCL-1) were purchased from Applied Biosystems and were used to quantify mRNAs using a 7500 Fast Real-Time PCR System (Applied Biosystems). The expression levels of the target genes were normalized to β-actin and with the ΔCT method, and relative quantification between control and infected mice was performed using the $(2^{-\Delta\Delta CT})$ *1000 formula.

Western Blot Analysis: The corneal cells were lysed and total protein in the supernatants was quantified using BCA protein assay kit (Thermo scientific, Waltman, MA). Samples were denatured in Laemmli buffer and resolved by SDS-PAGE and proteins were transferred onto a PVDF membrane. The membrane was blocked with 5% milk in Tris-buffered saline with Tween 20 at room temperature for 1 hour and subjected to incubation with specific primary and secondary antibodies. Proteins bands were visualized using chemiluminiscent HRP substrate (Millipore, Billerica, MA). After keeping in stripping buffer for 10 minutes, the membrane was re-probed using anti β-actin antibody. The antibodies used were as follows: anti Phospho-Src (Tyr416), anti Src (C-20), anti β-actin (AC 74). Protein concentration was determined relative to β actin and quantified using Image J software.

Statistics: The statistical significance between two groups was determined using unpaired one-tailed student's t test. When data did not show normal distribution, the Matt- Whitney test was used. One-way ANOVA with Tukey's multiple comparison tests was used to calculate the level of significance of the experiments with more than two groups to compare. When $P \leq 0.001$ (***), $P \leq 0.01$ (**), $P \leq 0.05$ (*) were considered as significant and results were expressed as mean ± SEM. For all statistical analysis, GraphPad Prism software was used.

RESULTS

Inhibition of R4 pathway increases angiogenesis and SK lesions

To evaluate the role of R4 in HSV induced SK, the outcome of infection was compared over a 15 day time period in ocularly infected R4 KO and WT mice. While in R4 KO mice SK lesions started to be evident from day 9 pi. onward as in WT mice but angiogenesis and SK scores were significantly higher in R4 KO mice and peaked at day 15 pi. ($p=0.001$, for both scores) (Figure 2.1 A-B). Examination of histological sections at day 15 pi. also showed increased lesion severity in the R4 KO animals compared to WT sections (Figure 2.1 C). In independent experiments of the same design, corneal tissues were collected from both groups on day 15 pi. and collagen digested to recover cells for FACS analysis. The numbers of CD31+ corneal endothelial cells (blood vessels) and SK lesion inducing CD4+ T cells increased around 2 fold for both cell types ($p=0.04$ and $p=0.02$, respectively) (Figure 2.1 A-B). Additionally neutrophils were increased by approximately 3 fold ($p=0.02$) (Figure 2.2 C). Finally, at the same time point, pools of corneas from WT and R4 KO mice were collected for the measurement by Q-RT-PCR of proinflammatory cytokines. These included IL-1β, IL-6, and KC all of which were significantly increased in R4 KO compared to WT mice ($p=0.01$, $p=0.002$ and $p=0.04$, respectively) (Figure 2.2 D). In conclusion, these results show that R4 plays a role to limit the extent of angiogenesis that follows HSV infection.

Administration of soluble R4 protein reduces angiogenesis and SK score in R4 KO mice

To determine if the provision of the soluble extracellular domain of R4 (sR4) could reduce the increased vascularization seen in R4 KO mice, WT and two groups of R4 KO animals were ocularly infected with HSV. At day 2 pi., while one group of R4 KO mice received daily administration of 10 μg

sR4 until day 14 pi. (Figure 2.3 A), the other two control groups (R4 KO control and WT control) received daily administration of PBS vehicle during the same time frame. Clinical evaluation revealed that compared to R4 KO animals, sR4 treated R4 KO mice developed reduced clinical angiogenesis and SK scores ($p<0.05$), declining to levels similar to that observed in WT animals (Figure 2.3 B). The clinical observations were also supported by histopathology. Increased cellular infiltration was evident in R4 KO mice without treatment compared to WT and sR4 treated R4 KO animals (Figure 2.3 C-D). Taken together, these results show that the administration of sR4 in the R4 KO acted to diminish the extent of lesions to those observed in WT animals, further demonstrating that Robo4 is a molecule that acts to constrain HSV induced corneal angiogenesis.

Administration of sR4 diminishes angiogenesis and SK

In order to further evaluate the effect of sR4 in the progression of HSV-induced angiogenesis, WT animals were treated subconjunctival with sR4 or vehicle starting on day 2 pi. and repeated daily until day 14 pi. (Figure 2.4 A). As shown in Figure 4 B, sR4 treatment caused reduced levels of angiogenesis and SK development with the maximum reduction observed at the higher dose used for treatment (SK score control:4 vs SK score treated: 2-3, angiogenesis score control:14 vs angiogenesis score control: to 8-10). That the sR4 treatment was effective at reducing lesions was also evident clinically in the histological sections (Figure 2.4 C-E). The extent of vascularization and inflammatory ocular reaction were compared by sacrificing treated and control animals at day 15 pi., following collagenase digestion and recovering corneal cells for FACS analysis. As is evident, the number of $CD31^+$ endothelial cells were reduced around 3 fold in animals treated with sR4 compared to the controls ($p=0.02$) (Figure 2.5 A). In addition CD4+, and neutrophils were reduced around 3 and 2.5 fold respectively, ($p=0.02$ and $p=0.01$) in treated compared with control mice (Figure 2.5 B-C). Finally, pools of 6 corneas HSV infected sR4 treated and non treated were collected at day 15 pi. and processed to prepare RNA. As is evident in figure 2.5 D,

animals treated with sR4 showed reduced of IL-1β, IL-6 and KC transcripts compared with infected controls (p=0.02, p=0.01 and p=0.01, respectively). Thus, taken together our results demonstrate that sR4 is a useful protein to modulate the extent of angiogenesis as well as the consequent immunopathology that follows HSV infection.

Increased angiogenesis in R4 KO mice is due to increased in VEGF signaling

It is known that VEGF signaling is dependent on SRC[17]. To explain the increased vascularization in R4 KO compared to WT mice, we measured the extent of SRC phosphorylation (pSRC). For such purpose both groups of animals were HSV ocularly infected and the corneas were collected at day 15 pi. and analyzed by Western blotting. In addition, WT and R4 KO naïve corneas were used as controls. The results show that whereas pSRC was undetectable in naïve corneal lysates, it was evident in WT and R4 corneas. However, R4 KO corneas presented increased levels of pSRC suggesting that this step in VEGF signaling was increased in R4 KO mice (Figure 2.6 A).

DISCUSSION

This communication explores the role of the vascular molecule R4 in controlling the magnitude of the neovascular response to ocular infection with HSV. We demonstrate that R4 serves to limit the extent of new blood vessel development and the severity of SK lesions following HSV infection. Evidence for such a function of R4 came from two sets of observations. Firstly animals unable to express R4 because of gene knockout developed earlier and more intense neovascular responses, and secondly the subconjunctival administration of a soluble form of the receptor (sR4) resulted in less severe CV and SK in WT infected animals. These observations could mean that targeting R4 could represent a useful therapeutic tool to counteract corneal angiogenesis and help control the severity of SK.

Several years ago CV was demonstrated to be a critical step in the pathogenesis of the blinding immunoinflammatory reaction to HSV infection in the eye[18, 19]. Multiple angiogenic factors were implicated as involved in causing the CV, with VEGF considered to be the principal angiogenic agonist[20, 21]. Therapeutic management of CV which included targeting VEGF[6, 7], its receptors[5] or some downstream signaling events[6, 8, 22], have shown various levels of efficacy. This report adds an alternative approach which represents the host's own mechanism to limit the extent of pathological CV. Accordingly, R4 was initially shown to control the extent of endothelial migration in a non infectious induced model of retinal and choroidal vascular diseases. These initial studies showed that animals unable to express R4 because of gene knockout developed heightened levels of angiogenesis[11] . We used a similar approach with an infectious model of corneal disease and showed that animals lacking R4 expression developed heightened CV responses after HSV infection as well as more severe SK. R4 expression is known to stabilize the vasculature by inhibiting processes stimulated by VEGF[23]. Given that R4 absence leads to leaky blood vessels that allow the escape of inflammatory substances[24, 25], the elevated SK scores together with the increased cellular infiltration seen in R4 KO animals were anticipated consequences of increased neovascularization. In addition, since the elevated CV responses in R4 KO animals could largely be restored to levels observed in normal infected animals by the early local provision of the soluble form of R4, it further suggested that the extent of angiogenesis is regulated by this molecule. Of interest we could also show that the extent of CV in WT infected animals could be diminished when sR4 was administered to the subconjunctival site. Our results are in accordance with Sutching et al., who using the mouse subcutaneous sponge angiogenesis model, by administration of sR4 could reduce vessel development compared to control treatment[26]. However, in our case the administration of sR4 was not shown to be practical for reasons further discussed subsequently.

One issue that remains currently unclear is if R4 acts as a receptor or as a ligand. It was previously shown that SLIT2 was the ligand of R4, by adding R4 expressing cells to supernant with myc-tagged Slit-2 transfected cells or in ELISA assays[27]. However, Sutching et al. results were the one which

initially shed doubt as to whether SLIT2 is the actual ligand of R4[26]. In fact, they could demonstrate by co-immunoprecipitation and Biocore binding assays that SLIT2 does not bind to R4[26]. If SLIT2 binds to R4, the administration of sR4 would have sequestered SLIT2, preventing it from interacting with Robo4 receptor and resulting in more angiogenesis. However, as mentioned before, in Sutching's and our system the administration of sR4 to WT infected mice reduced the pathological angiogenesis compared to control animals. Later, Eichman's group reconfirmed that SLIT2 was not the ligand of R4 and proposed that even though R4 is a receptor; its extracellular domains interacted with the vascular-specific axon guidance receptor uncoordinated 5 homolog β, UNC5β[28]. The latter interaction was confirmed by surface plasmon resonance. Moreover, using the corneal pocket assays with VEGF implants, it was shown that treatment of mice with anti-R4 and anti-UNC5β resulted in corneal hypervascularization[28]. Previously we also supported the hypothesis that SLIT2 bind to R4[22]. However in light of the current evidences, and the increased UNC5β transcripts detected after HSV infection (data not shown) we favor the hypothesis in which R4 acts more as a ligand than a receptor. Thus conceivable a more effective approach to exploit the host own ways of controlling the extent of angiogenesis would be to target UNC5β instead of the R4. Such studies are currently underway in our laboratory.

Despite the successful results obtained when administered sR4 to reduced HSV induced angiogenesis, for this approach to function, it was necessary to administer sR4 soon after infection. Delaying the therapy to later time points (even when given on day 6 pi. before notable CV becomes evident) had no therapeutic effect. Thus, targeting R4 does represent an approach that is effective but from a therapeutic perspective, administering sR4 would be inadequate in clinical settings. It will be necessary to explore ways of prolonging the administration of the reagent perhaps by using an expression vector system or focus instead on the signaling consequences of R4 manipulation. The signaling events have been shown to involve one or more SRC kinases, which in our study was shown to be elevated in R4 KO and less so in WT animals. Further studies are also needed to compare the efficacy of targeting R4 itself or the events it triggers at various stages of infection for their effect on HSV induced angiogenesis.

In addition, since SK is a multifactorial disease, combining sR4 with other approaches that target CV and/or inflammation would be another possible way to delay its administration.

In conclusion we have demonstrated that sR4 administration is a mean to enhance the antiangiogenic host feedback mechanism to reduce HSV induced angiogenesis and subsequent immunopathology. Even though the mechanism is still under investigation, the results obtained suggest that R4 binds to an endothelial receptor to counteract VEGF signaling. Such receptor could be UNC5β. This is the first report that evaluates the relevance of sR4 in an infectious disease that involves pathological angiogenesis. Further research is underway to understand its mechanism and further develop a more practical therapy.

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APPENDIX

FIGURE 2.1- ROBO4 DEFICIENT MICE ARE MORE SUSCEPTIBLE TO HSV INFECTION. WT and R4 KO mice were infected with HSV RE. (A) SK lesion and angiogenesis severity were significantly increased in R4 KO mice compared to WT mice on day 15 pi. (B) Representative eye photos show increased SK lesion and angiogenesis severity in R4 KO compared to WT mice (C) Eyes were processed for cryo-sections on day 15 pi. Hematoxilyn and eosin staining was carried out on 6 μm sections, and pictures were taken at 40x magnification. The sections show decreased cellular infiltration in R4 KO mice compared to WT mice. Data are representative of three independent experiments and show mean values ± SEM (n = 15 mice/group). ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$. Statistical levels of significance were analyzed by t test.

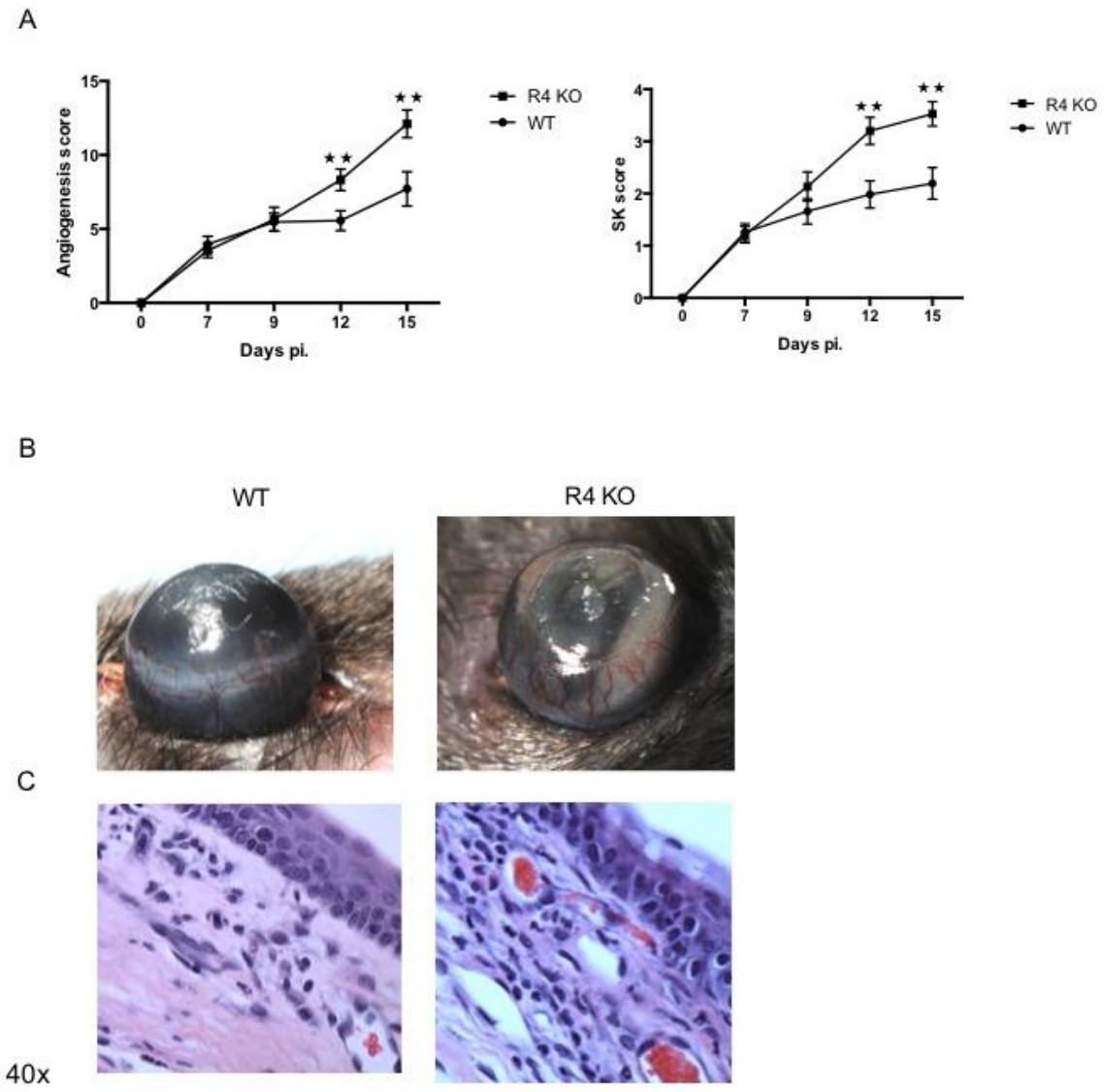

Figure 2.1 continued

FIGURE 2.2- ROBO4 DEFICIENT MICE PRESENT MORE INFLAMMATION AND VASCULARIZATION. R4 KO and WT mice were infected with HSV RE and at day 15 pi. corneas were collected and pooled for analysis by flow cytometry or Q-RT-PCR. The frequency and total cell number per cornea for (A) endothelial cells (CD31+ CD45- cells), (B) CD4+ T cells (CD4+CD45+) and (C) neutrophils (Ly6G+ CD11b+ gated on total CD45+ cells) show significant increase in R4 KO mice. Data are a combination of 3 independent experiments and show mean values ± SEM (n = 7 and each sample is representative of 2 corneas). ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$. Statistical levels of significance were analyzed by *t* test. (E) Relative fold change in mRNA expression of IL-1β, IL-6 and CXCL-1 was examined and compared between WT and Robo4 KO mice on day 15 pi. by Q-RT-PCR. Data represent means ± SEM from two different independent experiments (n = 3 and each sample is representative of 5 corneas). ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$. Statistical levels of significance were analyzed by *t* test.

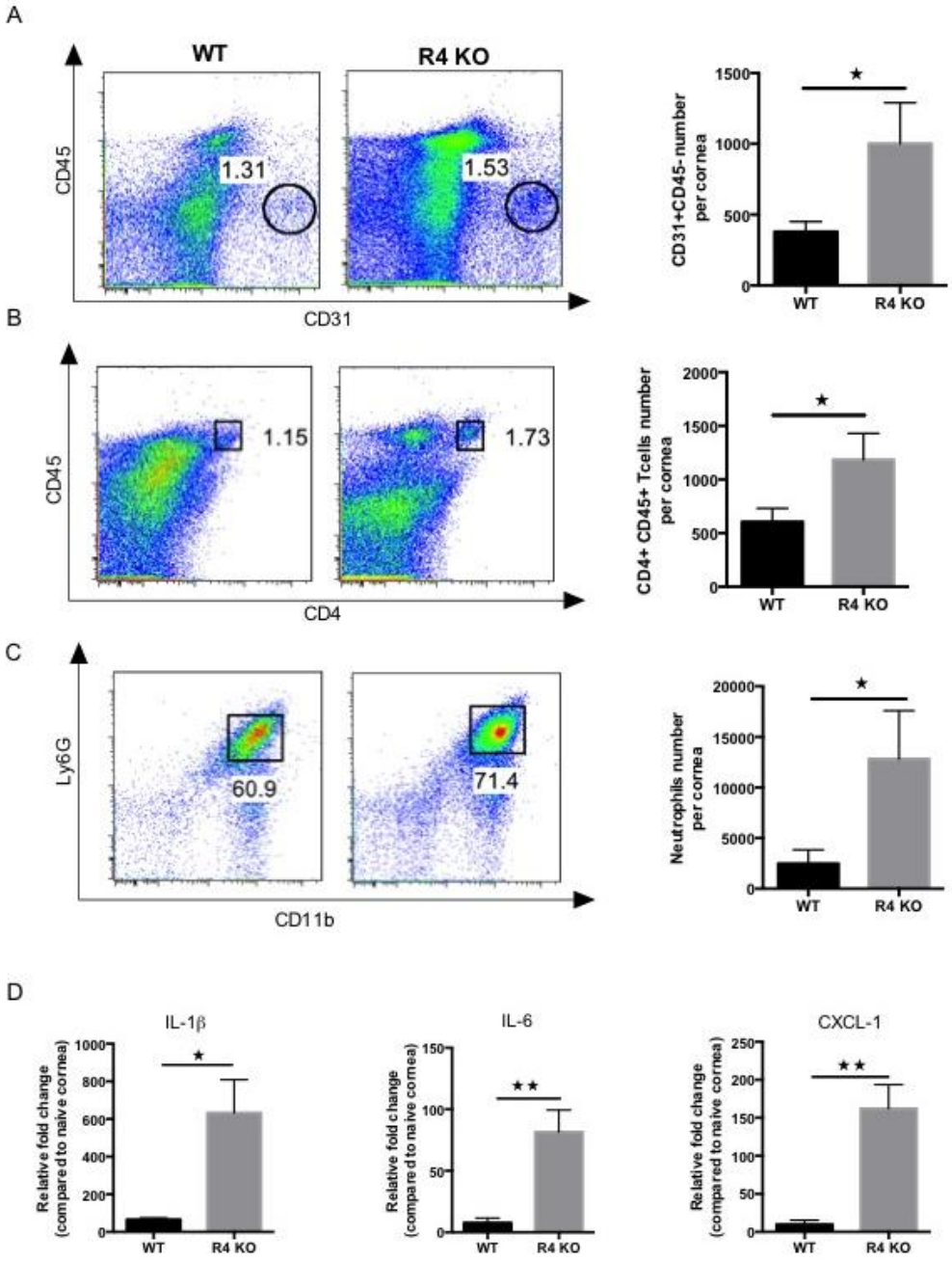

Figure 2.2 continued

FIGURE 2.3- ADMINISTRATION OF SOLUBLE ROBO4 (SR4) SHIFTS THE ROBO4 KNOCKOUT TO THE WT PHENOTYPE. WT and Robo4 knockout mice (R4 KO) were infected with HSV RE. (A) R4 KO mice received either sR4 (R4 KO treated w/ sR4) or vehicle (R4 KO Control) from 2 to 14 days pi. WT mice were included and received vehicle (WT Control) under the same regimen previously stated. (B) SK lesions and angiogenesis severity was decreased in R4 KO mice treated with sR4. (C) Representative eye photos show that R4 KO mice treated with sR4 do not develop the severe phenotype that R4 KO control animals do (D) Hematoxylin and eosin staining was carried out on 6-μm sections, and pictures were taken 40x magnification. Representative eye sections show decreased cellular infiltration in R4 KO treated w/ sR4 and WT control compared to R4 KO control mice. Data are representative of two independent experiments and show mean values ± SEM (n = 12 mice/group). ***$p \leq 0.001$, **$p \leq 0.01$,*$p \leq 0.05$. Statistical levels of significance were analyzed by one-way ANOVA test with Tuckey's post hoc test settings.

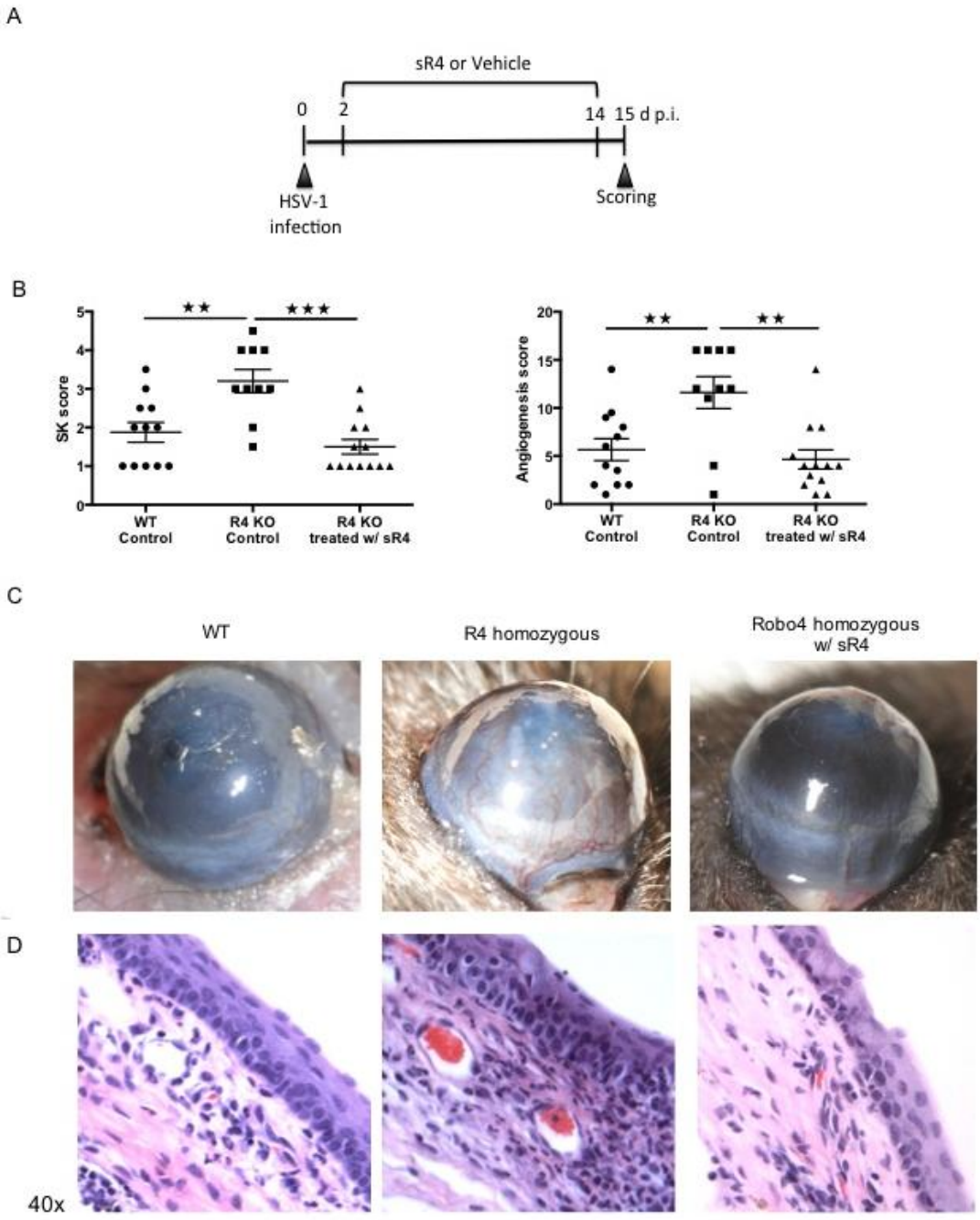

Figure 2.3 continued

FIGURE 2.4- PREVENTIVE ADMINISTRATION OF SOLUBLE ROBO4 (SR4) REDUCES LESION SEVERITY IN HSV INFECTED MICE. WT mice were infected with HSV RE and treated with sR4 or vehicle (PBS) (A) The sR4 treatment was given to HSV infected mice as shown (B) Dose dependent inhibition of angiogenesis scores after sR4 treatment. Data represents means ± SEM from three different experiments (n = 10) (C) sR4 treatment regimen resulted in SK and angiogenesis scores reduction. Data are representative of three independent experiments and show mean values ± SEM (n = 10 mice/group). ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$. Statistical levels of significance were analyzed by *t* test. (D) Representative eye photos show decreased SK lesion and angiogenesis severity in sR4 treated mice compared to control mice (E) Eyes were processed for cryo-sections on day 15 pi. Hematoxilyn and eosin staining was carried out on 6 μm sections, and pictures were taken at 40x magnification. The sections show decreased cellular infiltration in mice treated with sR4 compared to control mice.

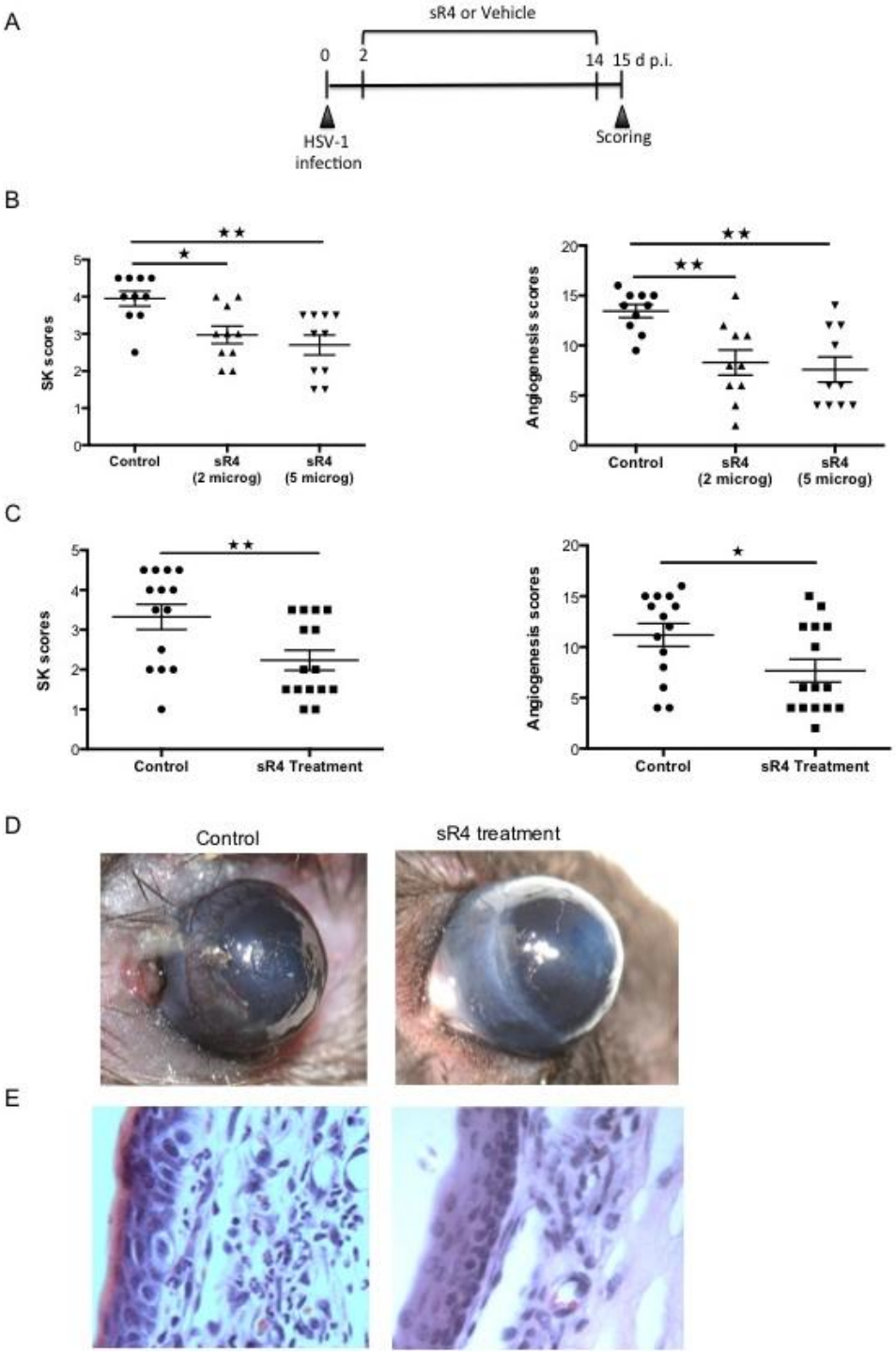

Figure 2.4 continued

FIGURE 2.5- PROVISION OF SR4 REDUCES CORNEAL INFLAMMATION AND ANGIOGENESIS. WT mice were infected with HSV RE and treated with sR4 of vehicle (PBS) from day 2 pi. to 14 pi. At day 15 pi. corneas were collected and pooled for analysis by flow cytometry or Q-RT-PCR. The frequency and total cell number per cornea for (A) endothelial cells (CD31+ CD45- cells), (B) CD4+ T cells (CD4+CD45+) and (C) neutrophils (Ly6G+ CD11b+ gated on total CD45+ cells) show significant decrease in sR4 treated mice compared to WT mice. Data are a combination of 3 independent experiments and show mean values ± SEM (n = 7 and each sample is representative of 2 corneas). ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$. Statistical levels of significance were analyzed by *t* test. (D) Relative fold change in mRNA expression of IL-1β, IL-6 and CXCL-1 was examined and compared between sR4 treated and control mice on day 15 pi. by Q-RT-PCR. Data represent means ± SEM from three different independent experiments (n = 2 and each sample is representative of 6 corneas). ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$. Statistical levels of significance were analyzed by *t* test.

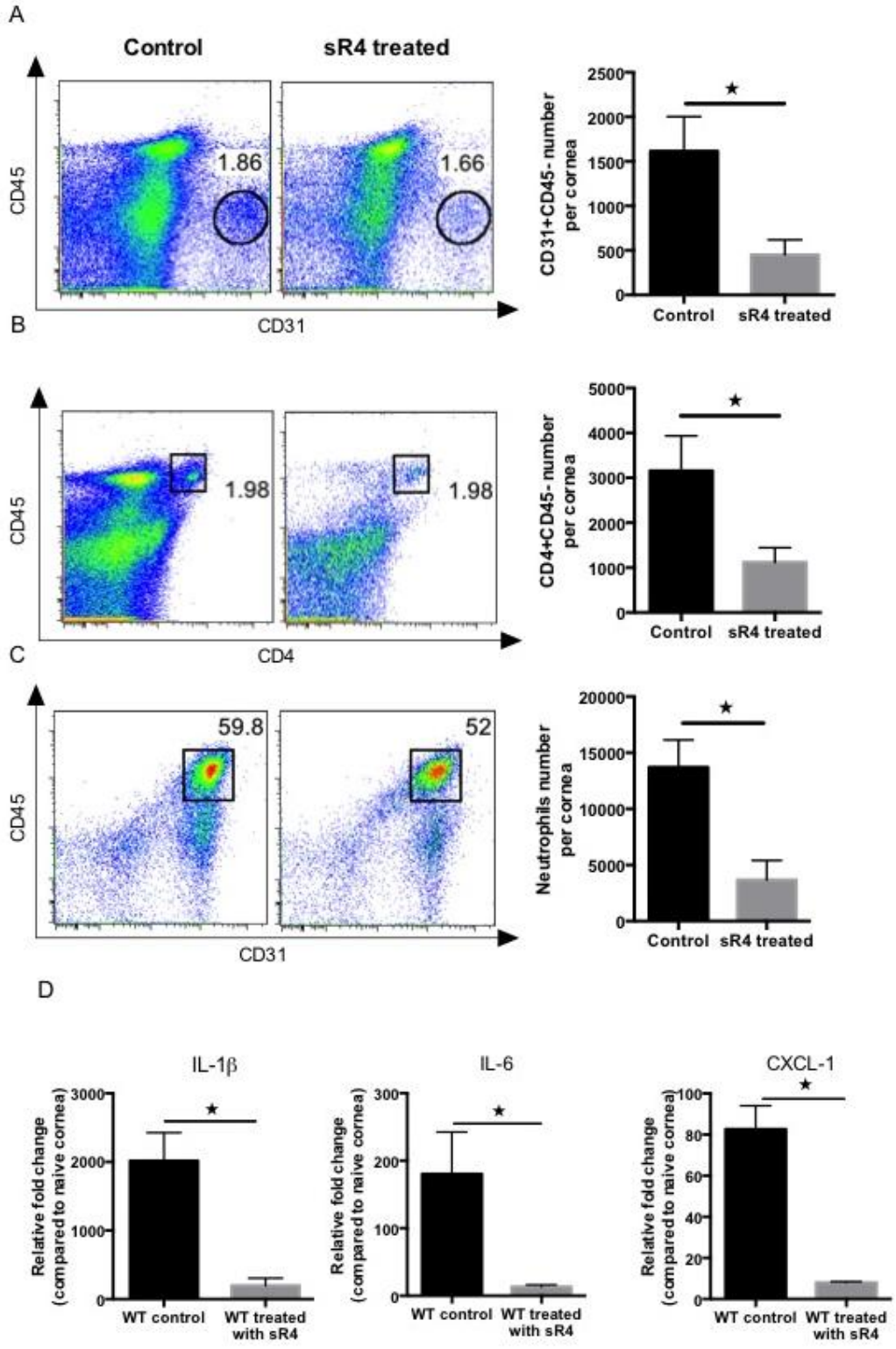

Figure 2.5 continued

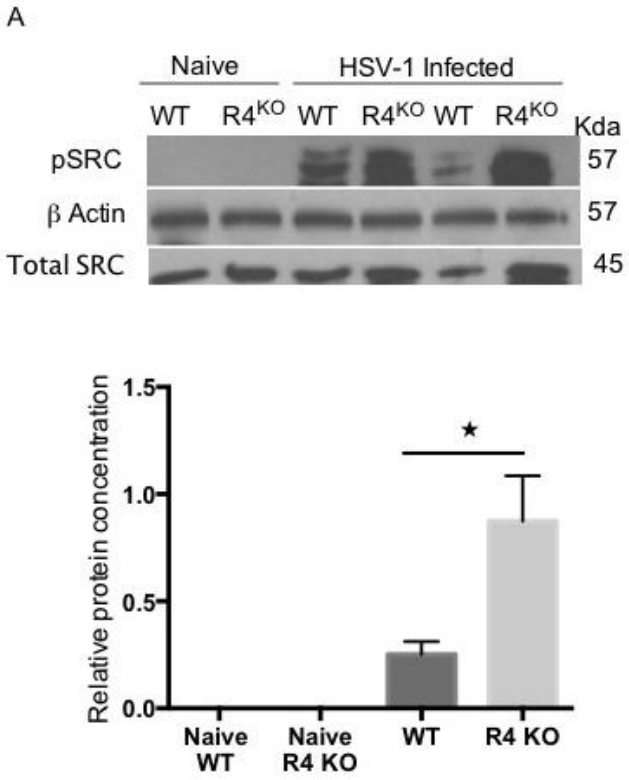

FIGURE 2.6- INCREASED LESION SEVERITY IN ROBO4 KO MICE IS DUE TO INCREASED VEGF SIGNALING. WT and R4 KO mice were infected with HSV RE in one eye and corneas were collected and pooled for analysis by WB. (A) Robo4 KO animals have increased VEGF signaling showed by increased SRC phosphorylation at day 15 pi. (n = 2 and each sample is representative of 6 corneas).

CHAPTER 2

THE BENEFICIAL ROLE OF NLRP3 IN HERPETIC STROMAL KERATITIS

ABSTRACT

Herpes simplex 1 (HSV) infection of the eye can be a cause of blindness with lesions largely attributable to inflammatory events that include components of both adaptive and innate immunity. Several innate immune responses are triggered by HSV but it is unclear how such innate events relate to the subsequent inflammatory process. In this study we compared the immunological responses to HSV ocular infection of mice unable to express NLRP3 due to gene knockout ($NLRP3^{-/-}$) with that of wild type (WT) mice. $NLRP3^{-/-}$ mice developed more severe and earlier SK lesions and a higher angiogenesis scores than did infected WT animals. In addition $NLRP3^{-/-}$ mice generated an increased early immune response with heightened chemokines and cytokines, including IL-1β and IL-18, and elevated recruitment of neutrophils. Increased numbers of CD4+T cells were seen at later stages of the disease in $NLRP3^{-/-}$ animals. Reduction of neutrophils prevented the early onset of the disease in $NLRP3^{-/-}$ animals and lowered levels of bioactive IL-1β, although not of bioactive IL-18. In conclusion, our results indicate that NLRP3 has a regulatory and beneficial role in herpetic SK pathogenesis.

INTRODUCTION

The outcome of a viral infection depends on many host factors as well as properties of the virus itself. In some instances, damage to tissues is largely the consequence of immunoinflammatory events set off by an infection. An example is herpetic stromal keratitis (SK) which is characterized by a blinding ocular lesion[1, 2]. Studies in animal models indicate that SK represents an immune inflammatory event orchestrated mainly by CD4+ T cells, but the early events induced by the infection that result in the development of overt SK are not fully understood[1-3]. Prominent events of the early response to HSV ocular infection include invasion by innate immune cells, expression of proinflammatory cytokines, particularly IL-1β and IL-6, as well as the involvement of several chemokines, angiogenic factors and neuropeptides[4-8]. Control of early events is considered important since their modulation represents a potentially useful approach for therapy. One unresolved issue is how the infection itself triggers innate recognition events. Although a critical role for TLR activation by the virus has been demonstrated [9-11], other mechanisms are likely involved and have not been fully investigated.

One likely mechanism so far poorly explored is that the infection may serve to activate one or more members of the inflammasome family. There is evidence that some viruses may activate inflammasomes such as NLRP3 in immunological cells that respond to the infection, such as macrophages and dendritic cells [12-16]. The activation of NLRP3 occurs by a wide range of molecules including self-derived, environmentally-derived and pathogen-derived activators. Therefore, as various stimuli with divergent structures and biochemical properties activate the NLRP3 inflammasome, it is proposed that a common cellular event elicited by the different stimuli, serves as the activating signal for the NLRP3 inflammasome[17-24]. These results in the formation of the biochemical complex known as the NLRP3 inflammasome, which recruits and activates pro-caspase 1, which acts in IL-1β and IL-18 into their bioactive forms, and concurrently acts to initiate the process of pyroptosis[25, 26].This situation has been advocated for influenza virus infection where the viral M2 ion channel induces H+ imbalance in

infected cells resulting in NLRP3 inflammasome activation[27]. Additionally adenovirus activates NLRP3 through the disruption of lysosomal membranes and the release of cathepsin B into the cytoplasm[28, 29]. With regard to HSV, in vitro results have shown that infection of human foreskin fibroblasts results in activation of IFI16/204 and NLRP3 inflammasomes [30], but it is not clear if similar events occur in vivo. Many groups have shown that mice lacking NLRP3 have defective inflammatory responses to a range of situations, which include autoimmunity, metabolic diseases as well as immunoinflammatory lesions[24, 31-34]. With the exception of influenza, few studies have evaluated the role of NLRP3 during in vivo responses to viral infections. Moreover with influenza some groups showed that compared to WT animals, NLRP3$^{-/-}$mice were more susceptible [19,35], others have reported contradictory results[36].

It is not known if NLRP3 is activated and serves to influence the outcome of infection with HSV. We investigate this issue in an SK model system where lesions are largely the consequence of an immunoinflammatory process[19, 29]. In this study, we compared the responses to HSV ocular infection in mice that lack the expression of NLRP3 because of gene knockout with WT animals. Surprisingly, NLRP3$^{-/-}$ mice manifested an early onset and more severe SK lesions and angiogenesis development than in WT animals. This was associated with significantly increased early neutrophil infiltration into the eyes, heightened cytokines and chemokines, including cleaved IL-1β, IL-18, and elevated T cell numbers compared to WT mice. Reduction of the early neutrophil infiltration prevented the early onset of the disease and reduced the levels of cleaved IL-1β in NLRP3$^{-/-}$ animals. Our study indicates that NLRP3 has an immunoregulatory function in SK pathogenesis modulating the early immune response after HSV infection.

MATERIALS AND METHODS

Mice: Female 6-8 wks old C57 BL/6 mice were purchased from Harlan Sprague Dawley Inc. (Indianapolis, Indiana, USA). NLRP3 knockout (NLRP3 KO) mice were the kind gift of Gabriel Nuñez

(University of Michigan). The animals were housed in American Association of Laboratory Animal Care-approved facilities at the University of Tennessee, Knoxville. All investigations followed guidelines of the institutional animal care and use committee.

Virus: HSV strain RE Tumpey was propagated in Vero cell monolayers (ATCC no: CCL81). Virus was grown in Vero cell monolayers (American Type Culture Collection, Manassas, VA), titrated, and stored in aliquots at –80˚C until used. Corneal infections of mice were performed under deep anesthesia. The mice were lightly scarified on their corneas with a 27-gauge needle, and a 3 µl drop containing 10^4 PFU of HSV RE was applied to one eye. Scratched animals were used as controls. These mice were monitored for the development of SK lesions. The SK lesion severity and angiogenesis in the eyes of mice were examined by slit-lamp biomicroscopy (Kowa Company, Nagoya, Japan). The scoring system was as follows: 0, normal cornea; +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity but iris visible; +4, opaque cornea and corneal ulcer; +5, corneal rupture and necrotizing keratitis [37]. The severity of angiogenesis was recorded as described previously [38]. According to this system, a grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.5 mm toward the corneal center. The score of the four quadrants of the eye were then summed to derive the neovessel index (range 0–16) for each eye at a given time point.

Histopathology: Eyes from isotype and treated mice were extirpated on day 15 pi. and snap frozen in OCT compound (Miles; Elkart, IN, USA). Six-micron-thick sections were cut and air dried in a desiccation box. Staining was performed with hematoxylin and eosin (Richard Allen Scientific; Kalamazoo, MI, USA).

ELISA: Corneal samples were pooled groupwise (4-5 corneas per sample) and collected in PBS with anti-protease cocktail. After homogenization of the sample using a tissue homogenizer (Pellet Pestle

mortar; Kontes), the concentrations of bioactive IL-1β, IL-18 and VEGF were measured by sandwich ELISA kits from eBioscience as per the manufacturers' instructions (Mouse IL-1β ELISA Ready-SET-Go!®; Mouse IL-18 Platinum ELISA; Mouse VEGF-A Platinum ELISA).

Quantification of mRNA expression levels by quantitative real time PCR (Q-RT-PCR): Total mRNA was isolated from corneal and lymph node cells using mirVana miRNA Isolation Kit (Ambion). For RNA, cDNA was made with 500 ng of RNA using oligo (dT) primer and ImProm-II™ Reverse Transcription System (Promega). TaqMan gene expression assays (IL-6, IL-17, MIP-2, KC, TNF-α, IL-12, proteinase 3, elastase, cathepsin) were purchased from Applied Biosystems and were used to quantify mRNAs using a 7500 Fast Real-Time PCR System (Applied Biosystems). The expression levels of the target genes were normalized to β-actin and with the ΔCT method, and relative quantification between control and infected mice was performed using the ($2^{-\Delta\Delta CT}$) *1000 formula.

Virus recovery and titration: Corneas were extracted at day 2, 4, 7 and 10 pi. and placed on ice sterile 2 ml straight-wall ground-glass tissue homogenizers (Wheaton) with media and homogenized. Homogenates were centrifuged (2,250 g at 4°C) for 5 min, place on ice and immediately plated titrations were performed by a standard plaque assay as described previously[39]. Titers were calculated as $\log^{10}$ pfu/ml per a standard protocol[40].

Flowcytometry

Cell preparation: Single cell suspensions were prepared from cornea and cervical DLN of mice at different time points pi. Corneas were excised, pooled group wise, and digested with 60 U/ml Liberase (Roche Diagnostics) for 35 minutes at 37°C in a humified atmosphere of 5% CO_2. After incubation, the corneas were disrupted by grinding with a syringe plunger on a cell strainer and a single-cell suspension was made in complete RPMI 1640 medium.

Staining for flow cytometry: The single cell suspensions obtained from corneas and DLN were stained for different cell surface molecules for FACS. All steps were performed at 4°C. A total of 1×10^6 cells were stained with the respective antibodies for 30 min on ice. Finally, the cells were washed three times and re-suspended in 1% *para*-formaldehyde. The stained samples were acquired with FACS LSR (BD Biosciences) and the data were analyzed using the FlowJo software. For corneas, total cell numbers were calculated by acquiring the totality of the sample and taking in consideration total number of corneas in the sample. The antibodies used include anti-CD4 Percp, anti-CD45 APC cy7, anti-CD11b Pecp, anti-Ly6G Pacific blue, anti-NK PE, anti-γδT cell FITC, anti-F4/80 PE. anti-CD4 APC, anti-CD45 Percp, anti- CD11b PE, anti- Ly6G FITC, F4/80 FITC, or anti-CD3 APC for 30 minutes on ice.

To enumerate the number of IFN-γ and IL-17 producing CD4+ T cells, intracellular cytokine staining was performed as previously described[41]. In brief, 10^6 freshly isolated cells from lymph node and corneas were cultured in U bottom 96 well plates. Cells were left unstimulated or stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng) and ionomycin (500ng) for 4h in the presence of brefeldin A (10 µg/ml). Subsequently, cell surface staining was performed, followed by intracellular cytokine staining using a Cytofix/Cytoperm kit (BD Pharmingen) in accordance with the manufacturer's recommendations. The antibodies used were anti-IFN-γ APC and anti-IL-17 PE. The fixed cells were resuspended in 1% para-formaldehyde. The stained samples were acquired with a FACS Calibur (BD biosciences), and the data were analyzed using the FlowJo software.

FLICA assay: Active caspase 1 was detected using a fluorescent inhibitor of caspases (FLICA, Immunochemistry Technologies, Bloomington, MN, USA), according to the manufacturer's instructions. Briefly, single cell suspensions were prepared from pools of infected corneas previously collagen digested and stained for anti-CD45. Then 10 µl of a 30×FLICA solution was added. The culture plates were covered with aluminum foil and incubated 1 h at 37°C in 5% CO_2. Following incubation, the cells were washed with wash buffer. At the end the samples were labeled with cell Propidium Iodide (PI) (Molecular

Probes) and acquired with a FACS LSR (BD biosciences). The data were analyzed using the FlowJo software.

Murine treatment with soluble anti-Ly6G: WT and two groups of NLRP3$^{-/-}$ mice were ocularly infected with HSV RE Tumpey. Only one of the groups of NLRP3$^{-/-}$ mice were treated with 50 μg/kg of anti-mouse Ly6G mAb (clone 1A8; BioXcell, West Lebanon, NH) intraperitoneally from day -1 to day 6 pi. Animals in control group, NLRP3$^{-/-}$ and WT mice, were given isotype control (IgG2b) Ab (LTF-2; BioXcell) following the same regimen. All experiments were repeated two times.

Statistics: The statistical significance between two groups was determined using unpaired one-tailed student's t test. When data did not show normal distribution, the Matt- Whitney was used. One-way ANOVA with Tukey's multiple comparison tests was used to calculate the level of significance of the experiments with more than two groups to compare. When $P \leq 0.001$ (***), $P \leq 0.01$ (**), $P \leq 0.05$ (*) were considered as significant and results were expressed as mean ± SEM. For all statistical analysis, GraphPad Prism software was used.

RESULTS

NLRP3 deficiency induces an early onset and more severe disease

To investigate the potential role of NLRP3 in the pathogenesis of SK, the outcome of HSV ocular infection was compared in WT animals and mice lacking NLRP3 because of gene knockout. In addition, at day 7 and 15 pi. eyes were collected and histological sections examined for the level of inflammation. Since herpetic SK is an immunoinflammatory lesion and reports with some other immunoinflammatory

diseases indicate that NLRP3$^{-/-}$ express higher resistance than WT animals, we anticipated that NLRP3$^{-/-}$ mice would be more refractory to SK. Unexpectedly, NLRP3$^{-/-}$ mice developed more severe SK lesions than WT mice (Figure 3.1 A-B). Usually SK lesions in WT animals start to be manifested at about day 9 pi., but lesions were evident in NLRP3$^{-/-}$ mice as soon as day 6 pi. ($p<0.05$) (Figure 3.1 A-B). Consistently, HSV infected NLRP3$^{-/-}$ mice exhibited increased cellular infiltration as could be seen in tissue sections compared to HSV infected WT mice (Figure 3.1 B). At day 15 pi., whereas both NLRP3$^{-/-}$ and WT mice showed clinically and histologically evident corneal lesions, both parameters were exacerbated in NLRP3$^{-/-}$ animals ($p=0.001$) (Figure 3.1 B). Alongside infected and naive animals, scratched control samples were also included in the analyses. None showed lesions and were more or less identical to naïve controls (Figure 3.1 B). These experiments demonstrate that in the absence of NLRP3, HSV infection generates an earlier onset and more severe manifestations of ocular lesions.

HSV induced IL-1β and IL-18 levels are NLRP3 independent

One of the consequences of inflammasome signaling involves the inflammasome dependent secretion of IL-1β and IL-18[17]. These cytokines are known to be synthesized as proproteins without significant biological activity until caspase 1 cleaves them into their bioactive forms for subsequent release[24, 42]. To examine the presence of IL-1β and IL-18 in our model, WT and NLRP3$^{-/-}$ animals were ocularly infected with HSV and at day 2 and 15 pi. corneas were collected for the measurement of such cytokines by an ELISA assay that detects the bioactive form of both cytokines. Surprisingly, IL-1β and IL-18 were expressed in NLRP3$^{-/-}$ corneas (Figure 3.2 A-B). Comparing both groups of animals, IL-18 levels were similar at all time points, but IL-1β levels showed some differences. IL-1β levels were increased at all time points and significantly different at day 7 pi. Next, to explore the levels of bioactive caspase 1 in both groups of animals, HSV infected WT and NLRP3$^{-/-}$ corneas were collected at day 2 pi.

and caspase 1 was measured using a fluorescent inhibitor of caspases (FLICA assay). As shown in figure 3 C NLRP3$^{-/-}$ and WT corneas expressed similar high levels of bioactive caspase 1 in CD45+ corneal cells. Taken together these results indicate that upon HSV infection, maturation of caspase 1 and subsequent increased levels of IL-1β and IL-18 can occur independently of NLRP3.

NLRP3 absence amplifies the proinflammatory cytokine and chemokine response

To assess the effect of NLRP3 on the production of proinflammatory cytokines and chemokines production in SK, mRNA was prepared from HSV infected NLRP3$^{-/-}$ and WT corneal extracts on day 2 and day 15 pi. Subsequently IL-6, MIP-2, IL-17, IL-12, TNF-α and KC were measured by taqman Q-RT-PCR. As shown in figure 3.3 A, by day 2 pi., almost all cytokine levels were significantly increased in NLRP3$^{-/-}$ mice compared to WT mice (1.5-5 fold higher) ($p \leq 0.05$). However, at day 15 pi., while IL-6, IL-12, MIP-2 and TNF-α were increased in NLRP3$^{-/-}$ compared to WT animals (around 2 to 5 fold higher) ($p \leq 0.05$), there were no significant differences in IL-17 and KC levels between the two groups. In addition, pools of corneas were collected at day 2 and 15 pi. for measurement of VEGF by ELISA. As shown in figure 3.3 B VEGF was significantly increased at both time points by around 2 and 4 fold in NLRP3$^{-/-}$ compared to WT animals. These data indicate that the deficiency of NLRP3 results in early increase of proinflammatory and proangiogenic cytokines and chemokines in response to HSV infection.

Effect of NLRP3 on viral clearance

To compare viral titers between NLRP3$^{-/-}$ and WT animals corneas were extracted at day 2, 4, 7 and 10 pi. and viral titers were detected by plaque assay. The results revealed that NLRP3$^{-/-}$ animals presented 4 and 2 fold higher viral titers at 2 and 4 days pi. respectively (Figure 3.4). However, by day 7 pi. both

groups of animals cleared the virus. These data suggest that animals unable to respond with NLRP3, have slightly increase viral titers at early stages of the disease. However, while the viral titers were statistically significant, such differences were not considered biologically meaningful.

NLRP3 deficiency increases dramatically the influx of neutrophils at early stages

To evaluate the extent of neutrophil infiltration, pools of corneas from NLRP3$^{-/-}$ and WT animals were collected at day 2, 7 and 15 pi. After collagen digestion, the pools of corneas were processed to quantify neutrophils by FACS. At day 2 and 7 pi. neutrophil infiltration was around 2 and 10 fold higher in NLRP3$^{-/-}$ compared to WT animals ($p \leq 0.05$), respectively (Figure 3.5 A-B). At day 15 pi., even though NLRP3$^{-/-}$ animals had higher number of neutrophils infiltrating the cornea, there were no significant differences when compared to WT mice (Figure 3.5 A-B). The results indicate that in the absence of NLRP3, the infiltration of neutrophils continues to increase from early time points until the peak of the disease.

Loss of NLRP3 leads to increased Th1 and Th17 cell responses

Since NLRP3$^{-/-}$ mice developed clinical SK lesions as soon as 7 days pi. the level of CD4+, CD4+Th1+ and CD4+Th17+ corneal and DLN infiltration was examined at day 7 and 15 days pi. Number of cells of several phenotypes were measured on pools of 4 corneas previously collagen digested and DLN by FACS. At day 7 pi., NLRP3$^{-/-}$ mice showed around 2 to 4 fold increased CD4+, CD4+Th1+ and CD4+Th17+ corneal cell numbers compared to WT animals ($p \leq 0.05$) (Figure 3.6 A). Additionally, at day 15 pi., CD4+T cells and Th1 cells increased around 1.5 fold in NLRP3$^{-/-}$ corneas compared to WT corneas, but there was no difference in the Th17 response (Figure 3.7 A). Examination of DLN cell number revealed that at day 7 pi. NLRP3$^{-/-}$ animals showed increased CD4+, Th1+ and Th17 cells

compared to WT mice (Figure 3.6 B) (around 1.5 fold increase). Finally, at day 15 pi. the numbers of CD4+, CD4+Th1+ and CD4+Th17+ cells were increased in NLRP3$^{-/-}$ DLN compared to WT DLN, respectively (around 1.5 to 2.5 fold increase) (Figure 3.7 B). In accordance with the severe disease seen in NLRP3$^{-/-}$ mice, these results provide evidence that as early as 7 days pi. and at day 15 pi. the major orchestrators of this disease, CD4+T cells, were significantly increased in NLRP3$^{-/-}$ compared to WT mice. These experiments indicate that mice unable to activate NLRP3 after HSV ocular infection generate a more intense adaptive immune response compared to WT animals. This is probably the consequence of the amplified immune response that occurs during early stages of the disease in NLRP3$^{-/-}$ mice.

Neutrophil reduction prevents the early onset of the SK

Previously we showed that NLRP3$^{-/-}$ animals presented high levels of IL-1β and IL-18 despite the absence of NLRP3. However, it has been demonstrated that in neutrophil-mediated inflammatory responses, neutrophil-derived serine proteases can also cleave pro-IL-1β and pro-IL-18. To explore the neutrophil-derived serine proteases expression, pools of 5 corneas were collected at day 2 pi. from NLRP3$^{-/-}$ and WT animals. Corneas from both groups were processed for the extraction of mRNA and quantification of the aforementioned proteases. Exposure to HSV significantly increased the neutrophil proteinase 3 and cathepsin b by around 3 fold more in NLRP3$^{-/-}$ compared to WT animals (Figure 3.8 A-C). Even though elastase levels were increased in NLRP3$^{-/-}$ animals, the differences between groups were not significant.

Neutrophils together with CD4+T are known to be the main cells driving the immunopathogenesis of SK [43]. However, the early infiltration of neutrophils is usually non-clinically evident. To evaluate if the early infiltration of neutrophils was the main cause of the early onset of disease in knockout mice, NLRP3$^{-/-}$ animals were depleted of neutrophils using anti-Ly6G (Clone 1A8; BioXcell) and NLRP3$^{-/-}$ and WT animals received isotype control (IgG2b) Ab (LTF-2; BioXcell) from day -1 to day 6 pi. (Figure 3.9

A). To confirm neutrophil reduction, histopathology and neutrophil count by FACS was used. The results showed that the treatment effectively reduced neutrophil infiltration (Figure 3.9 B-C). Comparing NLRP3$^{-/-}$ treated animals with NLRP3$^{-/-}$ control animals, the SK scores were reduced by approximately 40%. Accordingly antil-Ly6G treatment in NLRP3$^{-/-}$ animals did not lead to an early onset of the disease (Figure 3.9 D). In addition, administration of anti-Ly6G decreased IL-1β levels by approximately 4 fold in NLRP3$^{-/-}$ treated compared to NLRP3$^{-/-}$ control animals ($p \leq 0.05$) but IL-18 did not change significantly ($p \geq 0.05$) (Figure 3.9 E-F). This data suggest that the infiltration of neutrophils play an important role driving the early manifestation of the disease in NLRP3$^{-/-}$ animals. In addition, the increased levels of IL-1β in NLRP3$^{-/-}$ animals could be neutrophil dependent.

DISCUSSION

Ocular infection with HSV sets off an array of events that succeed in clearing virus from the cornea but the tissue is damaged by a CD4+T-cell-orchestrated chronic inflammatory lesion that impairs vision[1]. An unresolved issue is how early events during infection relate to the subsequent immunoinflammatory SK lesions. In this report, we determined if the NLRP3 inflammasome participates in the early response to HSV by comparing the outcome of infection in WT with animals that lack NLRP3 function because of gene knockout. Unexpectedly, we observed that without NLRP3 animals developed more severe lesions than did intact animals. Along with more severe clinical lesions, infiltrates of CD4+T cells and neutrophils were higher as were the levels of proinflammatory chemokines and cytokines, including IL-1β and IL-18. The heightened lesions in NLRP3$^{-/-}$ mice appeared dependent on neutrophils since removal of such cells lessened lesion severity. Our results indicate that NLRP3 may be playing a modulatory role acting in some way to diminish the severity of lesions in ocularly HSV infected animals.

Few studies have evaluated the role of NLRP3 in the pathogenesis of a viral infection[19, 35, 36]. Most past reports have focused on immunoinflammatory[34, 44, 45], autoimmune diseases[31-34, 46] and some types of cancers[47] and have shown that NLRP3 helps mediate inflammatory effects which are markedly reduced if NLRP3 is absent or its function inhibited. Previous studies on the role of NLRP3 in influenza have revealed a confused phenotype. While some advocate that NLRP3 plays a proinflammatory role which is beneficial for viral clearance and animal survival[19, 35], others show that the presence or absence of NLRP3 does not influence the final outcome of the disease[36]. However, in diseases such as colitis and fungal infection NLRP3 was shown to have beneficial roles[18, 48-52]. In colitis the absence of NLRP3 reduced the levels of IL-18, which was shown to be critically involved in the maintenance of intestinal homeostasis[50]. With the fungal infection, the explanation for more severe lesions in the absence of NLRP3 was attributed to the impaired IL-1β response[18, 48-53]. Our study is the first to our knowledge to evaluate any role for NLRP3 during an in vivo infection with HSV. However, studies in vitro with HSV had indicated that activation of NLRP3 and IFI16/204 inflammasomes occurs with the subsequent maturation of the IL-1β response[29, 30].The exact component of the virus that activates NLRP3 it is not known, but Chandran et al suggested that NLRP3 could be acting indirectly following activation by causing the production of reactive oxygen species during HSV infection and that another inflammasome IFI16/204 could also be involved in the HSV response[30]. Our observation that in the absence of NLRP3 an inflammatory response followed by SK occurs means that NLRP3 inflammasome formation is not an essential event responsible for the early inflammatory response in the eye to HSV. Conceivable other recognition systems are mainly involved such as the known TLR ligand activity of the virus itself[54] or involvement of additional inflammasomes. In support of the latter possibility we could show in preliminary studies that mice lacking ASC, the adaptor molecule for the assemble of many inflammasomes, had a less severe response to HSV than WT animals. Further studies are needed to evaluate the involvement of additional inflammasomes such as the aforementioned IFI16/204.

Although our studies argue that NLRP3 may not be critical for driving the early inflammatory response to HSV, they do make the point that some form of recognition must be occurring since lesions were more severe in the absence of NLRP3. We also observed that the more severe phenotype depended on the presence of neutrophils since when these cells were depleted, the more severe phenotype in NLRP3$^{-/-}$ was eliminated. Although it was not clear what generated the heightened IL-1β responses, this molecule could have been acting in a positive feedback loop with neutrophils. Thus, it is known that IL-1 signaling through one of its receptors results in the activation of the transcription factors NF-kB and AP-1[56]. These in turn cause the expression of vascular adhesion molecules and induction of chemokines such as KC and MIP-2, known to increase neutrophils infiltration[56]. While caspase 1 is the master regulator of IL-1β, inflammatory mediators such as neutrophil-derived proteases can also mature IL-1β [57, 58]. Since the neutrophil-derived proteases, proteinase and cathepsin-G were shown to increase in NLRP3$^{-/-}$ animal it is likely that neutrophils also contributed in the maturation of IL-1β (Figure 10).

The most fascinating and yet unexplained aspect of our study was to determine why the SK lesions in NLRP3$^{-/-}$ animals were more severe than in animals with functional NLRP3. Initially we suspected it might be the consequence of less ability of NLRP3$^{-/-}$ animals to control viral infection giving rise to an inflammatory reaction that was more severe. However, there was a minimal effect on viral titers but these marginal increased levels observed in NLRP3$^{-/-}$ animals were not judged to be biologically significant. In addition both WT and NLRP3$^{-/-}$ cleared virus in the same time frame. Another likely explanation could relate to the balance of the T cell response with less regulatory T cell induction in NLRP3$^{-/-}$ animals. However we could not detect any significant difference in the effector to regulatory T cell relationship between NLRP3$^{-/-}$ and WT infected animals. We also thought to compare levels of inhibitory cytokines such as IL-10 and TFG-β made by NLRP3$^{-/-}$ and WT animals, but our results did not support such hypothesis. Other explanation could include that NLRP3 influences the levels of lipid mediators. We are currently evaluating such hypothesis by recording the differential expression of resolvins in NLRP3$^{-/-}$ compared to WT animals, expecting NLRP3$^{-/-}$ animals having lessened resolvins that WT animals.

Ultimately, our results show that the inflammatory milieu created early after infection plays an important role in HSV-induced ocular lesions and an important participant in modulating this environment is NLRP3. To our knowledge, this is the first study that evaluates the role of NLRP3 in the pathogenesis of SK and the data suggest that NLRP3 regulates the innate immune response effect during HSV ocular infection. Future experiments are underway to determine the involvement of other inflammasomes and their role in disease pathogenesis.

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APPENDIX

FIGURE 3.1- NLRP3$^{-/-}$ ANIMALS HAVE AN EARLY ONSET AND MORE SEVERE DISEASE. C57BL/6 (WT) and NLRP3$^{-/-}$ animals were scarified and infected with HSV (HSV infected) and scarified but uninfected (Scratched control). The disease progression was analyzed throughout time in a blinded manner using a scale described in materials and methods. (A) The progression of SK and angiogenesis lesion severity was significantly increased in the NLRP3$^{-/-}$ compared with WT mice. (B) Representative eye photos show increased SK lesions and angiogenesis development in NLRP3$^{-/-}$ mice compared to WT mice on day 7 and 15 pi. Those eyes were processed for cryo-sections and hematoxylin and eosin staining was carried out on 6-μm sections. Histopathogy pictures were taken at 40x microscope augmentation. Data are representative of 3 independent experiments and show mean values ± SEM (n=8 mice/group). $P \leq 0.001$(***), $P \leq 0.01$(**), $P \leq 0.05$(*).

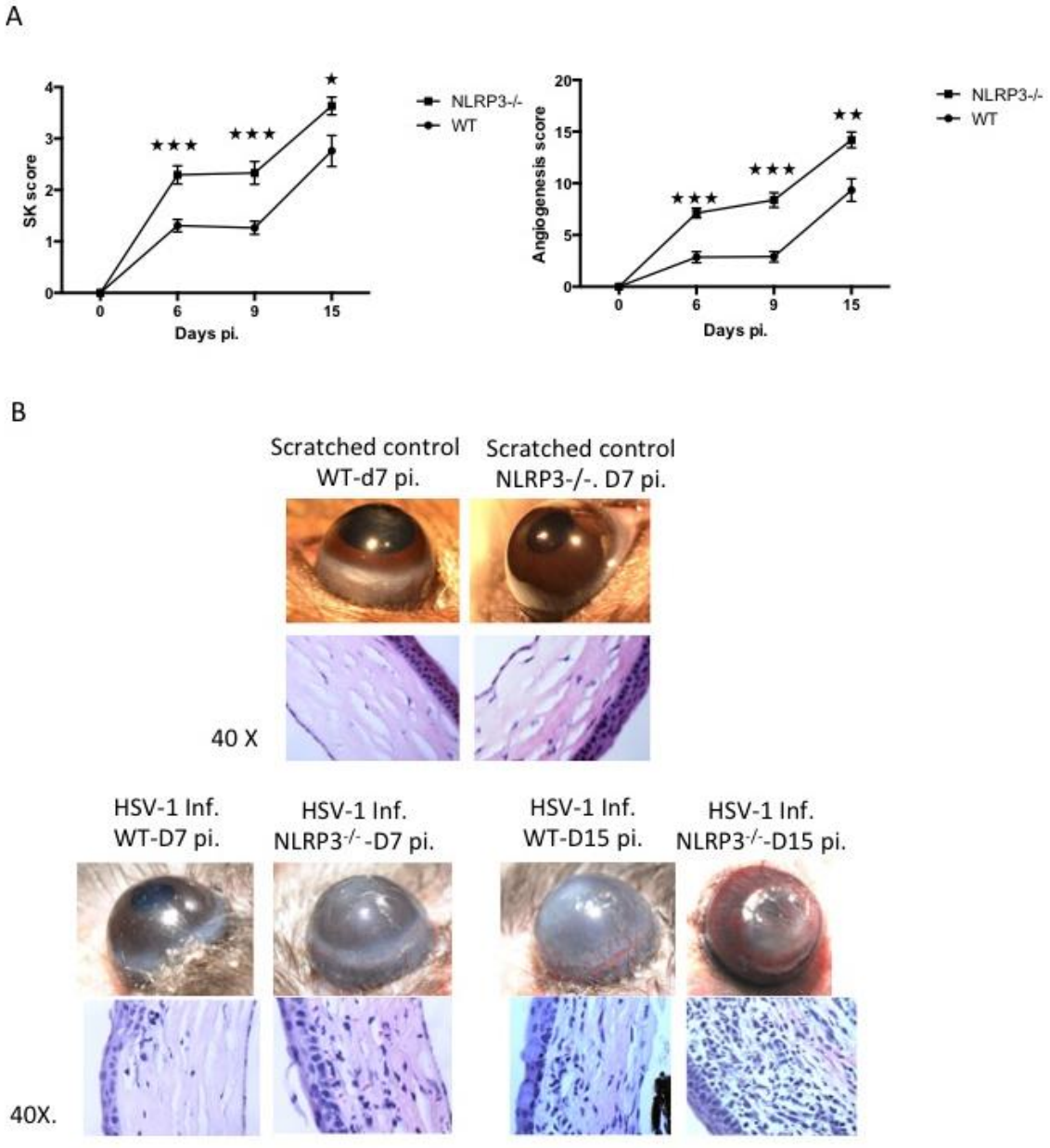


Figure 3.1 continued

FIGURE 3.2- MICE LACKING NLRP3 PRESENT BIOACTIVE LEVELS OF IL-1β AND IL-18 FOLLOWING OCULAR CHALLENGE WITH HSV. C57BL/6 (WT) and NLRP3$^{-/-}$ mice were infected with HSV and corneal samples were processed to measure IL-1β and IL-18 by ELISA and activated caspase 1 by FLICA assay **(A)** Quantification of bioactive IL-1β at different time points. At day 7 pi. NLRP3$^{-/-}$ mice had significantly increased levels of bioactive IL-1β **(B)** Quantification of bioactive IL-18 protein at different time points. Bioactive IL-18 was similar between NLRP3$^{-/-}$ and WT animals **(C)** Representative histogram of FLICA+ cells gated on total CD45+ PI- cells infiltrated in the corneas of WT and NLRP3$^{-/-}$ animals. Spleen of Naïve mice was used as an isotype control. Data are representative of 3 independent experiments and show mean values ± SEM (n = 3 and each sample is representative of 5 corneas). $P \leq 0.001$(***), $P \leq 0.01$(**), $P \leq 0.05$(*).

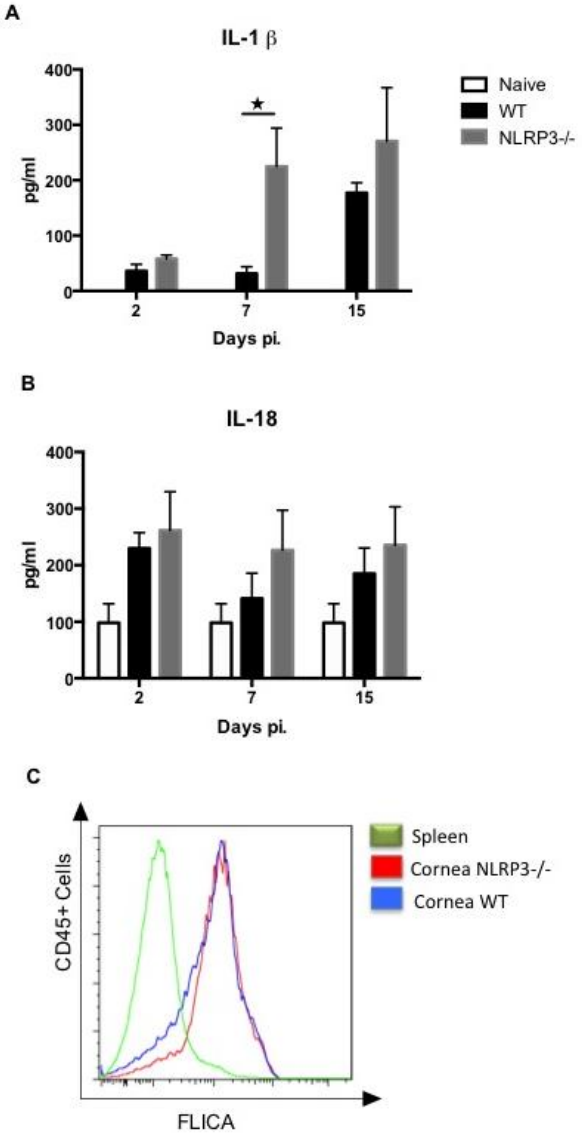
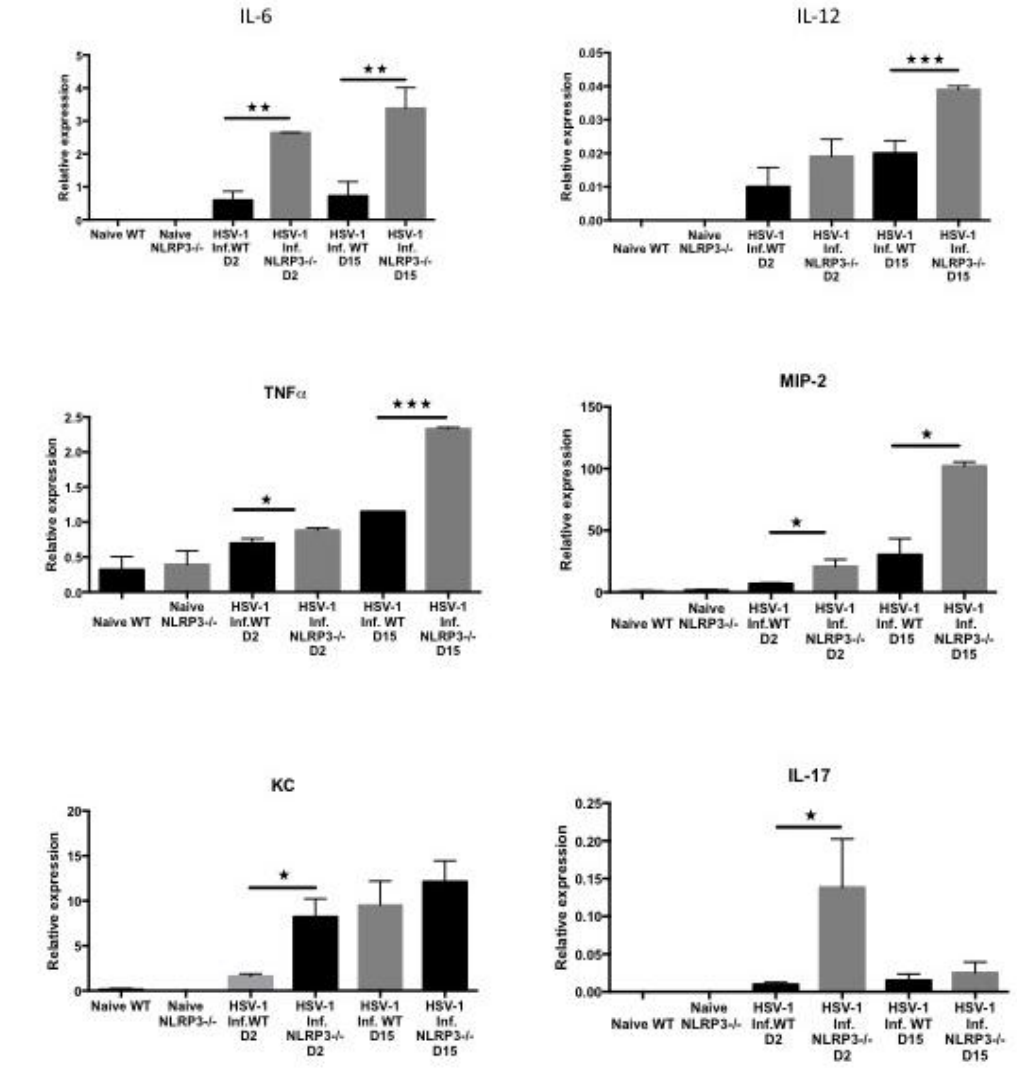


Figure 3.2 continued

FIGURE 3.3- NLRP3 DEFICIENCY INCREASES PROINFLAMMATORY CYTOKINES AND CHEMOKINES C57BL/6 (WT) and NLRP3$^{-/-}$ mice corneas were scarified and infected with HSV. At day 2 and 15 pi. corneas were collected and **(A)** relative fold change in mRNA expression of IL-6, IL-12, IL-17, KC and MIP2 was examined by Q-RT-PCR and compared between both groups **(B)** Quantification of VEGF was measured by ELISA at day 2 and 7 pi. NLRP3$^{-/-}$ had significantly increase levels of VEGF at both time points. Data represent means ± SEM from three different independent experiments (n = 3 and each sample is representative of 5 corneas). $P \leq 0.001$(***), $P \leq 0.01$(**), $P \leq 0.05$(*).



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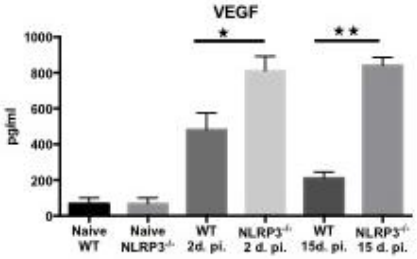

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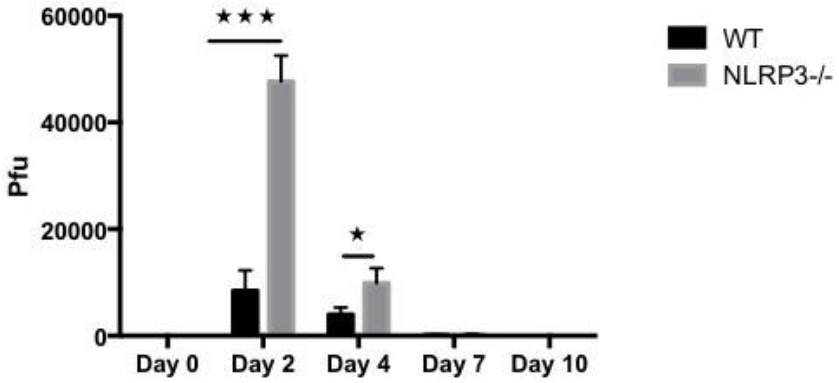

FIGURE 3.4- CORNEAL VIRAL TITERS OF NLRP3-/- VS WT ANIMALS. Corneal tissue was collected on day 0, 2, 7 and 10 pi. and titration was performed by standard plaque assay as described on materials and methods. Titers were calculated as $\log^{10}$pfu/ml. Data are representative of 3 independent experiments and show mean values ± SEM (n = 10 mice/group). $P \leq 0.001$ (***), $P \leq 0.01$ (**), $P \leq 0.05$ (*).

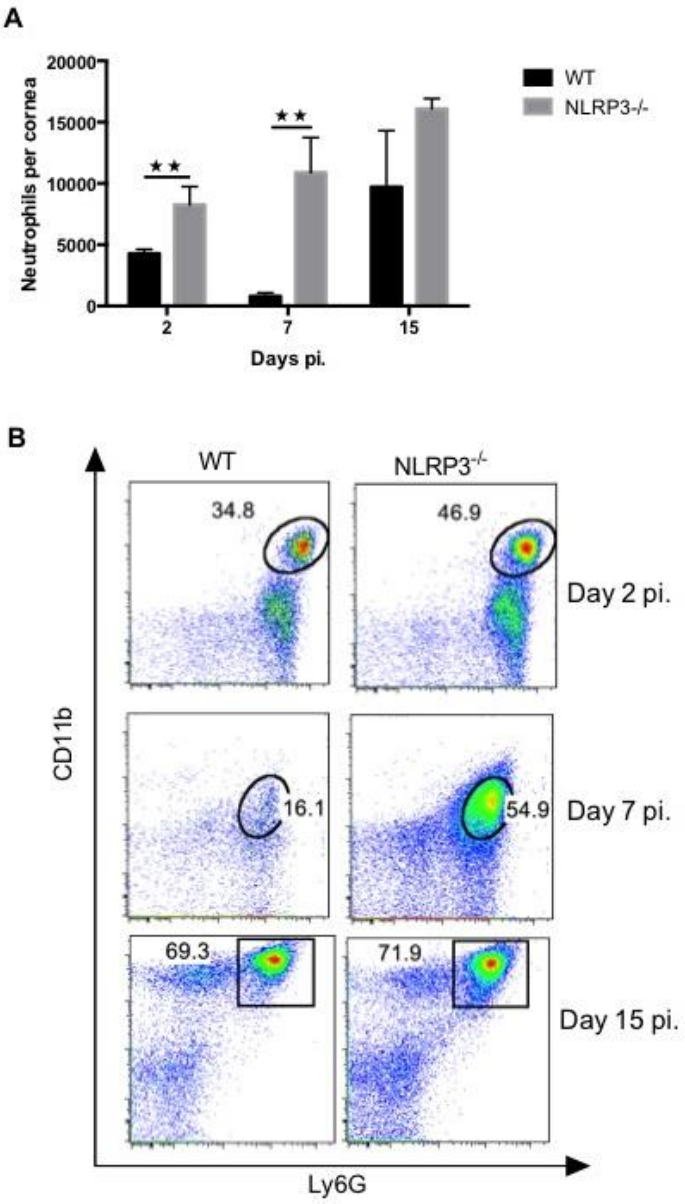

FIGURE 3.5- NEUTROPHIL INFILTRATION KEEPS INCREASING THROUGHOUT THE DISEASE IN HSV INFECTED EYES OF NLRP3-/- ANIMALS. C57BL/6 (WT) and NLRP3$^{-/-}$ mice were scarified and infected with HSV. Corneas were collected at different time points to analyze the neutrophil infiltration throughout the disease. Numbers of total neutrophil infiltration (left) and representative FACS plots and percentages (right) are shown at day 2, 7 and 15 pi. At all time points neutrophil infiltration was significantly increased in NLRP3$^{-/-}$ compared to WT mice.

FIGURE 3.6- NLRP3-/- MICE EXHIBIT INCREASED CORNEAL AND DLN CELLULAR INFILTRATES AT DAY 7 PI. C57BL/6 (WT) and NLRP3$^{-/-}$ animals were infected with HSV. At day 7 pi. corneas and DLN were collected and stimulated with PMA/ionomycin during 4 hours **(A)** Representative FACS plots and percentages (left) and numbers of CD4+ T cells, CD4+ IFN-γ and IL-17 secreting cells from pooled corneas. **(B)** Representative FACS plots and percentages (left) and numbers of total CD4+T cells (right), CD4+ IFN-γ and IL-17from DLN

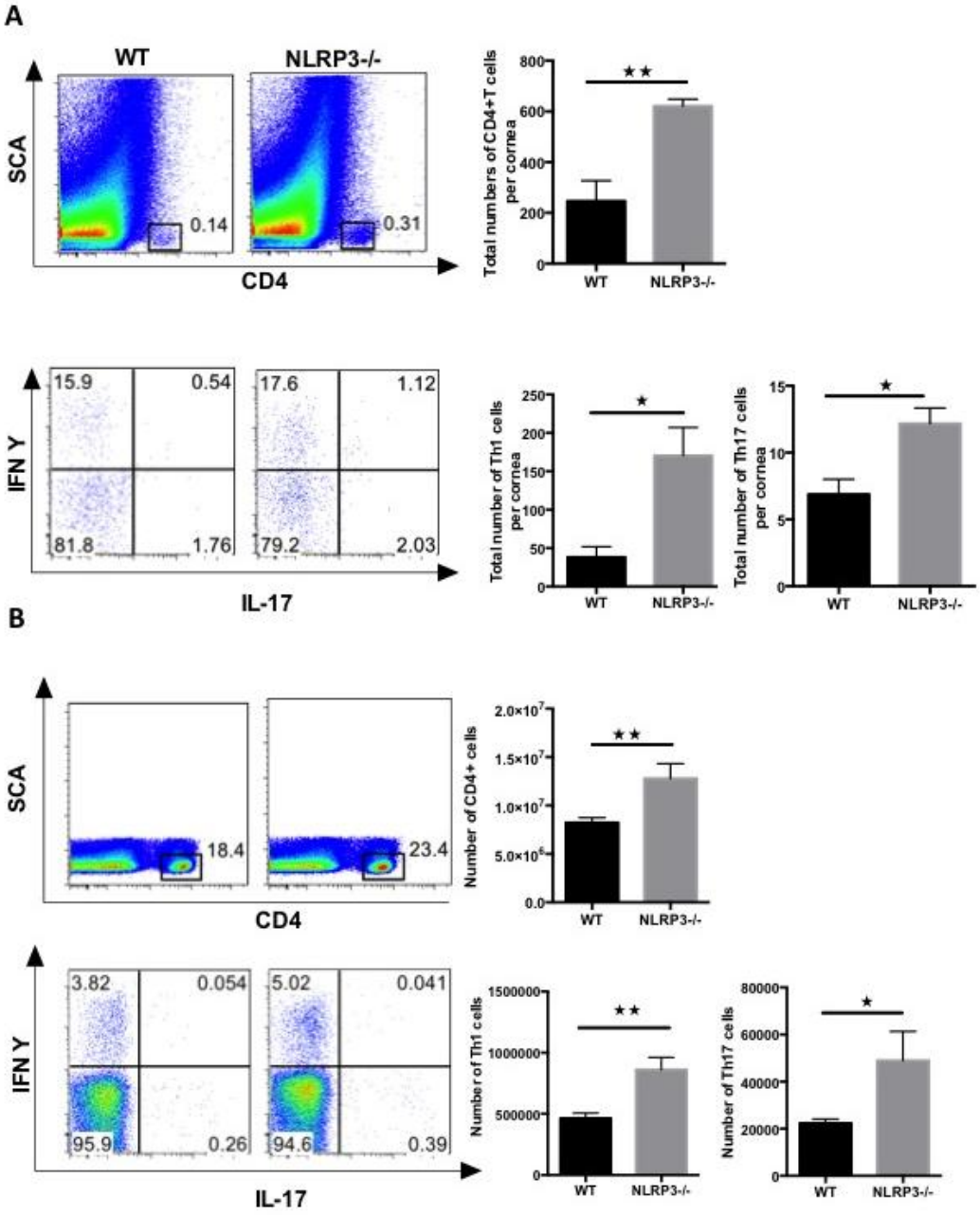

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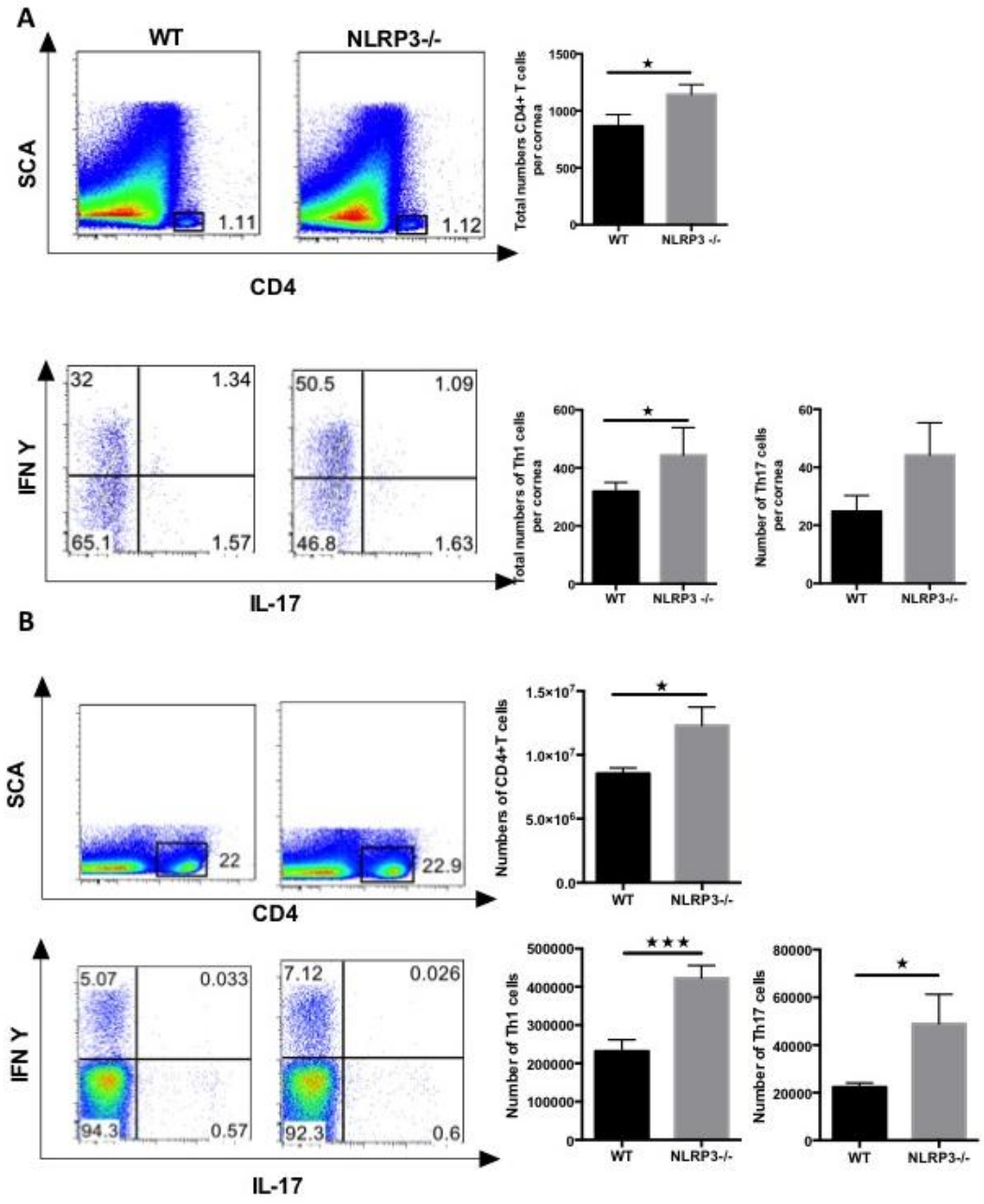
FIGURE 3.7- NLRP3$^{-/-}$ MICE EXHIBIT INCREASED CORNEAL AND DLN CELLULAR INFILTRATES AT DAY 15 PI. C57BL/6 (WT) and NLRP3$^{-/-}$ animals were infected with HSV. At day 15 pi. corneas and DLN were collected and processed for stimulation with PMA/ionomycin during 4 hr. **(A)** Representative FACS plots and percentages and numbers of CD4+ T cells, CD4+ IFN-γ and IL-17 secreting cells from corneas taken at day 15 pi. **(B)** Representative FACS plots and percentages and numbers of total CD4+T cells. CD4+ IFN-γ and IL-17 from DLN at 15 days pi. Data are representative of 3 independent experiments and show mean values ± SEM (n = 8). In the case of corneas each sample is representative of 3 corneas. $P \leq 0.001$(***), $P \leq 0.01$(**), $P \leq 0.05$(*).doi:10.1371/journal.ppat.1002427.g004.



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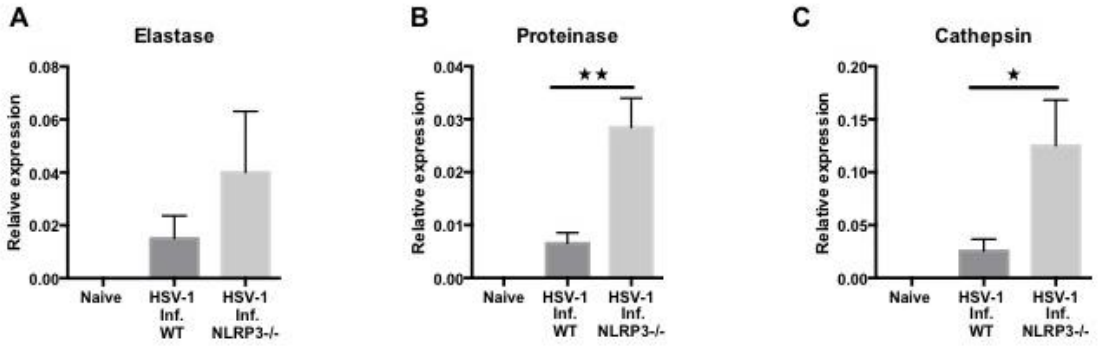
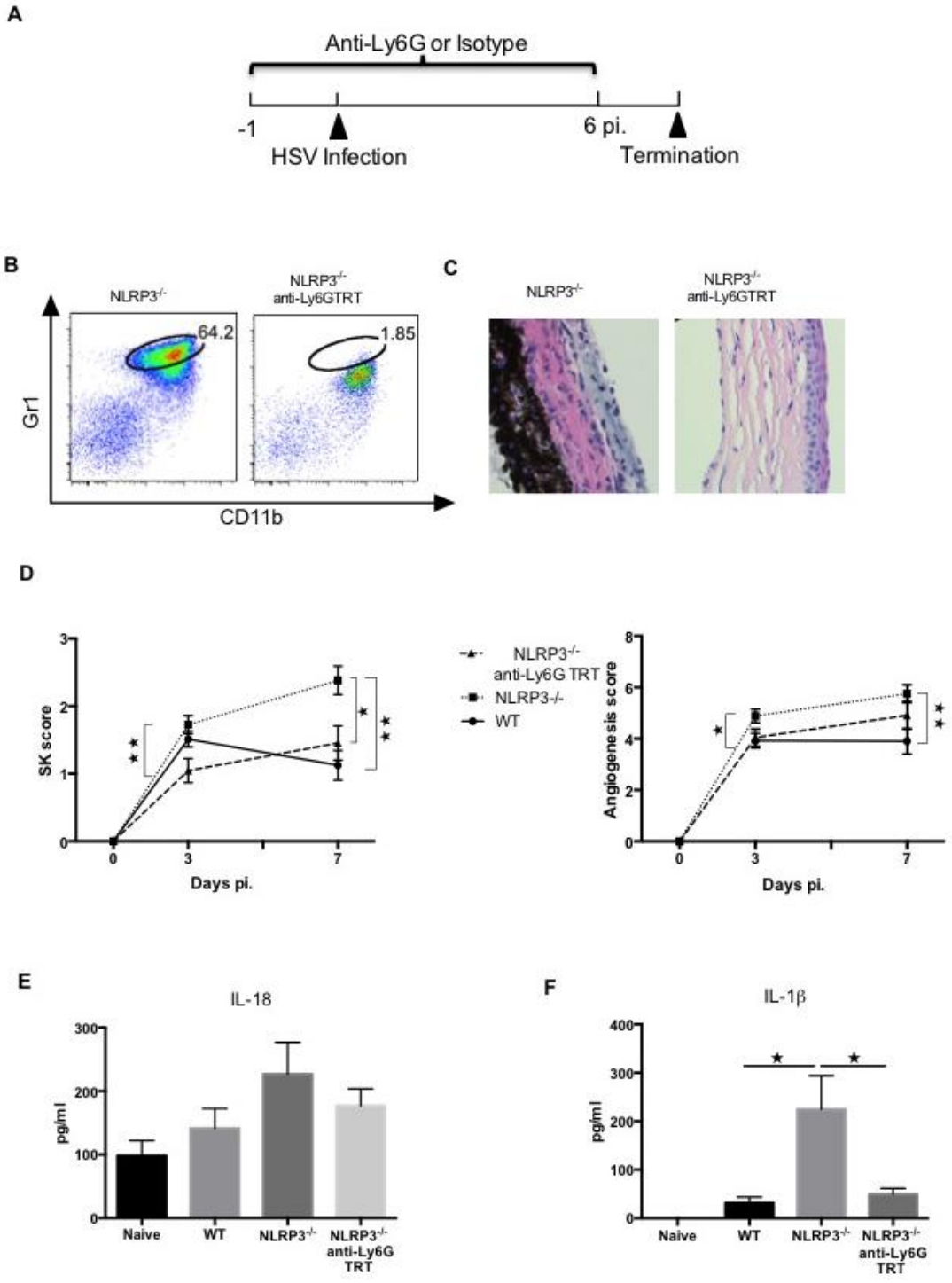


FIGURE 3.8- NLRP3-/- ANIMALS HAVE HIGH LEVELS OF NEUTROPHIL-DERIVED PROTEASES C57BL/6 (WT) and NLRP3$^{-/-}$ mice corneas were scarified and infected with HSV. At day 2 pi. corneas were collected and relative fold change in mRNA expression of **(A-C)** neutrophil elastase **(A)**, proteinase-3 **(B)** and cathepsin **(C)** was examined by Q-RT-PCR and compared between both groups. Data represent means ± SEM from three different independent experiments (n = 3 and each sample is representative of 5 corneas). $P \leq 0.001$(***), $P \leq 0.01$(**), $P \leq 0.05$(*).

FIGURE 3.9- REDUCTION OF THE EARLY NEUTROPHILIC INFILTRATE PREVENTS THE EARLY ONSET OF THE DISEASE IN NLRP3-/- ANIMALS. C57BL/6 (WT) and two groups of NLRP3$^{-/-}$ animals were scarified in the eye and infected with HSV. **(A)** Using mAb against Ly6G from day -1 to day 6 pi., neutrophils were reduced in one group of NLRP3$^{-/-}$ animals (NLRP3$^{-/-}$ TRT). The other two groups, including NLRP3$^{-/-}$ and WT mice, were used as controls (NLRP3$^{-/-}$ control and WT control) and treated with isotype control (IgG2b) Ab from day -1 to day 6 pi. **(B)** Representative FACS plots and percentages of corneas collected at day 7 pi. show that mouse anti-Ly6G was effective reducing neutrophils The progression of SK lesion severity was significantly increased in the NLRP3$^{-/-}$ control mice compared to NLRP3$^{-/-}$ TRT and WT control animals. Kinetics of SK severity is shown. **(C)** Representative histopathological pictures taken at 40x microscope augmentation show that NLRP3$^{-/-}$ TRT animals had less cellular infiltration than NLRP3$^{-/-}$ control mice. **(D)** The progression of SK and angiogenesis was evaluated throughout the disease and both were significantly increased in NLRP3$^{-/-}$ control compared to NLRP3$^{-/-}$ TRT and WT control animals **(E-F)** Quantification of bioactive IL-18 and IL-1β protein at day 7 pi. by ELISA. NLRP3$^{-/-}$ mice treated with anti-Ly6G had similar levels of bioactive IL-18 to NLRP3$^{-/-}$ and WT control. However, bioactive concentrations of IL-1β were reduced in NLRP3$^{-/-}$ TRT compared to NLRP3$^{-/-}$ and WT control. Data are representative of 3 independent experiments and show mean values ± SEM (n=8 mice/group). $P \leq 0.001$(***), $P \leq 0.01$(**), $P \leq 0.05$(*).



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CONCLUSION AND FUTURE DIRECTIONS

Stromal keratitis (SK) is an immunoinflammatory lesion produced by herpes simplex virus type 1 (HSV) infection. Two critical events that result in overt SK include corneal neovascularization (CV) and inflammation. In the normal cornea there are angiogenic factors present, however their function is overridden by antiangiogenic control systems. Such is the case of vascular endothelial growth factor (VEGF)-A which is present in the cornea but its angiogenic activity is impeded by being bound to a soluble form of the VEGF receptor-1 (sVEGFR1)[1]. Under pathological situations the host also activates its own antiangiogenic pathways to reduce angiogenesis. Such a pathway could be represented by signals from endothelial receptor Robo4 (R4), which was initially shown to control the extent of endothelial migration on retinal and choroidal vascular diseases. Accordingly, in the first chapter of this thesis we analyzed for the first time whether or not the R4 pathway played a role in limiting the extent of pathological CV. We first showed that mice lacking R4 because of gene knockout developed heightened CV responses after HSV infection as well as more severe SK. Moreover, administration of soluble R4 (sR4) reduced the angiogenesis in HSV ocularly infected wild type (WT) mice. These results revealed that R4, which likely acts as a ligand of the endothelial receptor uncoordinated homolog 5 β (UNC5β), inhibits the VEGF effects. Even though sR4 was successful, its administration had to be started early after infection and administered frequently, which is a far from an ideal situation in any clinical circumstance. Possible reasons for the limited success could be a very short half-life of sR4 at the site of action and/or impaired penetration into the eye during advance stages of the disease, perhaps due to changes in blood flow and/or local sR4 solubility. Another potential reason for the limited effect of sR4 during the late stages of disease could be its local inactivation, which could occur due to non specific binding to tissue debris. Local tissue pharmacokinetic studies could help to define optimal dosing regimen strategies, so as to maximize the therapeutic potential of reagents for treating HSV induced CV.

Another suitable approach could be designing gene therapies, which include using vectors such as adenovirus or lentivirus to induce the host's endothelial cells to over express R4. This would likely allow

reducing the frequency of administration, resulting in a more successful outcome. Future research should be conducted to evaluate such delivering systems.

It is also conceivable that combination therapy targeting the reduction and/or increase of more than one proangiogenic and/or antiangiogenic factor respectively, would be of more benefit than administering only one treatment modality. Most of the alternatives to reduce CV discovered by our group focused on VEGF. However, there are other factors apart from VEGF that are involved in angiogenesis. Such is the case of Angiopoietin-1, known to be critical for vessel maturation, adhesion, migration, and survival[2, 3]; and Angiopoietin-2, which promotes cell death and disrupts neovascularization[4]. Combining sR4 to reduce the VEGF signaling, together with an agonist of Angiopoietin-1 and an antagonist of Angiopoietin-2 may be a successful combinatorial approach targeting different molecules involved in angiogenesis. Future experiments should be directed to standardize the combination of antiangiogenic reagents and generate a more successful treatment for HSV induced angiogenesis. With regard to sR4, we speculate that using sR4 as part of a combinatorial treatment will allow us to administer it therapeutically, with less frequency and more success.

One issue of relevance is that when treating CV, it would be ideal to reverse the progress of neovascularization. Apart from approaches such as laser surgery, methods for removal of corneal pathological vessels are unknown. Moreover, inactive vessels remain as ghosts that under the correct circumstances can be resurrected into patent vessels. It has previously been seen that vessels undergoing angiogenesis present specific markers that are absent in normal blood vessels. Such markers include integrins[5, 6], certain receptors for vascular growth factors[7, 8] and membrane bound proteinases such as aminopeptidases[9]. It has been reported that aminopeptidase A (APA), a regulator of blood vessel formation, is expressed in angiogenic blood vessels[10]. Moreover, APA null mice have normal development but fail to mount the expected angiogenic responses to hypoxia or growth factors[10]. Using phage display technologies it was shown that the motif CPRECESIC specifically binds to APA, inhibits its enzymatic activity, and suppresses migration and proliferation of vascular endothelial cells[10].

CPRECESIC has been shown to successfully suppress tumor growth in mice through its effects on the vasculature[10]. If, as we anticipate, neovessels following HSV infection express APA, we could use the APA binding peptide motif, CPRECESIC, to reduce the angiogenesis. A potential advantage of using this treatment would be the specificity of CPRECESIC to target only pathological new vessels.

Aminopeptidase N (APN) is another surface protein that is expressed on endothelial cells of angiogenic vessels, and most of the myeloid origin cells including monocytes, macrophages and granulocytes[11, 12]. Evidence suggests that APN is an important receptor targeted by the NGR motif [9, 13]. In addition, it is known that peptides that contain the NGR motifs are useful for delivering cytotoxic drugs or proapoptotic peptides[13, 14]. In the past, coupling of Doxorrubicin or a pro-apoptotic peptide to an integrin binding NGR peptide, yielded compounds that have enhanced antitumor activities and reduced side effects[14]. Thus, it may be possible to target angiogenic blood vessels by attaching reagents to NGR peptides. One potential reagent could be the D-enantiomer $_{D}$(KLAKLAK) sequence peptidomimetic that disrupts mitochondrial membranes upon receptor mediated cell internalization and causes targeted apoptosis. This technology has been used to induce the apoptosis of fat tissue endothelial cells [15]. Thus, if APN is present in corneal neovessels of mice with SK, as we suspect it is, use of this reagent would allow selectively-induced apoptosis of endothelial cells and reversal of formed pathological blood vessels. In addition, if the receptor is expressed on myeloid cells, we would be able to concurrently target inflammation. It is possible that neither APA nor APN are present on corneal blood neovessels, in which case alternate technologies such as phage display would allow identification of specific markers on such cells. Having this information, we could create peptides containing motifs with specific binding capacity for corneal neovessels and attaching D-enantiomer $_{D}$(KLAKLAK) to the peptide may induce apoptosis or inhibitory effects on the corneal vessels.

Another possibility includes use of peptides that carry certain motifs specific for angiogenic receptors such as VEGFR1. Using a combinatorial screening on VEGF-activated endothelial cells, it has been shown that the motif Arg-Pro-Leu $_{(D)}$(LPR) targets VEGF receptor-1[16]. This peptide motif markedly

inhibited neovascularization in three mouse models: matrigel-based assay, functional human/murine blood vessel formation, and retinopathy of prematurity[16]. In addition to its systemic activity, $_{(D)}$(LPR) also inhibited retinal angiogenesis when administered in an eye-drop formulation. Preliminary studies showed that the $_{(D)}$(LPR) peptide could have activity in an experimental tumor-bearing mouse model[16]. Thus, targeting the extracellular domains of VEGF receptors also offers potential for clinical application toward reducing angiogenesis in SK. If proven effective in the SK model, use of $_{(D)}$(LPR) in the eye drop formulation would allow improved quality of life and avoidance of unpleasant, risky, and expensive repetitive injections into the eye.

Finally, there are antiangiogenic reagents yet to be evaluated by us but that have shown promise in reducing angiogenesis in other models. One reagent is Nutlin-3, a mouse double minute 2 homolog (MDM2) inhibitors. Nutlin-3 is a small molecule that upon binding to HIF-1α in the p53 binding domain results in HIF-1α inactivation[17-20] and leads to diminished VEGF transcription. In vitro matrigel plug endothelial cell studies suggested that Nutlin-3 inhibits angiogenesis[21]. In vivo studies using laser-induced choroidal neovascularization, intravitreal administration of Nutlin-3 also reduced neovascularization[22]. In light of previous studies, administration of MDM2 could represent another novel strategy to diminish SK induced angiogenesis.

The second part of the thesis describes the role of NLRP3 inflammasome on the pathogenesis of herpetic SK. Most of the information about inflammasomes and infectious diseases comes from in vitro experiments and have not been verified by in vivo studies. Unexpectedly, mice lacking NLRP3 because of gene knockout presented an amplified innate immune response with elevated levels of cytokines, chemokines and increased infiltration of neutrophils. In fact, reduction of neutrophils prevented the early onset of the disease and reduced the severity of SK lesions at day 7 pi. Accordingly, it is possible that during HSV infection, NLRP3 acts to modulate the effect of other inflammasomes or recognition systems set off by the HSV infection. Preliminary data indicated that mice deficient in ASC, a critical adaptor required for the assembly of many inflammasomes, had less severe SK lesions (data not shown). Even

though further experiments must be done to confirm our preliminary data, our findings indicate that in the absence of ASC, SK lesions are reduced, likely due to the inability of HSV-activated inflammasome assembly. In this case, our hypothesis that the NLRP3 inflammasome is capable of modulating the effects of other activated inflammasomes would be supported. Future research should be directed to elucidate which inflammasomes are activated after HSV infection. NLRP3, AIM2, IFI16 and RIG-1 inflammasomes are currently known to be involved in various viral infections[23]. We can perhaps exclude RIG-1 and AIM2, since RIG-1 is considered to sense RNA viruses[24] and AIM2 was shown by in vitro studies not to sense HSV-1[25]. Consequently, as previously reported[26], the focus should be on NLRP3 and IFI16. However, we do not discard the possibility that some yet unknown inflammasome could also be activated upon HSV infection. Confirming our preliminary data would create an exciting line of research, since HSV ocular infection in the mouse would represent a valuable model to elucidate the crosstalk between inflammasomes.

Recent reports have shown that certain inflammasome components have inflammasome-independent functions[27,28,29]. As in our system we could show a more severe phenotype and inflammation in NLRP3$^{-/-}$ than in ASC$^{-/-}$, it is likely that NLRP3 has an inflammasome-independent function in the HSV ocular model. In order to elucidate this, future studies should be focused on experiments with knockout and double knockout mice lacking various specific components of the NLRP3 inflammasome.

Another point of interest needing clarification is cellular the source of NLRP3. While the majority of cells that express NLRP3 are macrophages, dendritic cells, and neutrophils,[30] it is known that other cell types such epithelial cells can also express NLRP3[31, 32]. It is also likely that stromal cells express NLRP3. In fact our in vitro studies have shown that the MKT cell line increases the expression of NLRP3 after HSV infection (data not published). In addition, it would be useful to determine the contribution of bone marrow derived cells (BMDC) to the phenotype seen in HSV ocularly infected NLRP3$^{-/-}$ mice. It is possible that the phenotype observed in NLRP3$^{-/-}$ animals was due to NLRP3 missing in non-BMDC. To explore this, radiation bone-marrow chimeras could be used. After irradiation

of the recipient mice, four bone marrows can be transplanted (BMT): WT bone marrow transplanted to WT recipient ($BMT^{WT\ to\ WT}$), WT bone marrow transplanted to $NLRP3^{-/-}$ recipient ($BMT^{WT\ to\ NLRP3-/-}$), $NLRP3^{-/-}$ bone marrow transplanted to WT recipient ($BMT^{NLRP3-/-\ to\ WT}$), and $NLRP3^{-/-}$ bone marrow transplanted to $NLRP3^{-/-}$ recipient ($BMT^{NLRP3-/-\ to\ NLRP3-/-}$). In that way it will be possible to know if the increased pathogenesis is because of the BMDC that lack the NLRP3 or some other cell in the eye, such as epithelial corneal cells or stromal cells.

Stimulating macrophages, dendritic cells (DC) and neutrophils with HSV might also evaluate the issue of which innate cells respond to HSV. For example, it is likely that $NLRP3^{-/-}$ macrophages, neutrophils or both are hyperactive secreting more of some cytokine upon HSV stimulation compared to WT cells. However, the experiments with neutrophils could be quite challenging due to the short life of such cells.

It is now known that there are different neutrophils subsets with potential pro and anti-inflammatory roles under both physiological and pathological conditions. It is recognized that different subsets of neutrophils regulate lymphocyte function[33, 34]. Eicosanoids are the primary products released by neutrophils. Specific receptors for these clinically important lipid signals are expressed in several lymphocyte subpopulations[33, 34]. It would be interesting to characterize the different type of neutrophils present in SK lesions of $NLRP3^{-/-}$ and WT animals. It is likely that not only the number but also the subsets of neutrophils present in both groups of animals could differ with the proinflammatory subset increased in $NLRP3^{-/-}$ mice. In turn, it is possible that the proinflammatory factors released by neutrophil subsets may affect lymphocytes resulting in increased CD4+T cells and the severe phenotype observed in $NLRP3^{-/-}$ mice.

While we could not find differences in macrophage number between $NLRP3^{-/-}$ and WT animals, it is possible that the ratio of M1/M2 macrophages[35] is altered, with the M1 subtype prevailing in $NLRP3^{-/-}$ compared to WT animals. Characterization of such macrophage subsets in our model would be of interest to determine if macrophage subtype correlates in some way with our severe phenotype.

Another possibility by which CD4+T cells could be increased in NLRP3$^{-/-}$ animals could be that NLRP3 has a regulatory function on cells such as myeloid derived suppressor cells (MDSC). MDSC are a heterogeneous subset of cells with the capacity to impair T cell function [36-38]. It has been reported that NLRP3 expression promotes accumulation of MDSC in tumor sites [39]. Thus, it is possible to infer that in the absence of NLRP3 expression the accumulation of MDSC is reduced and T cell function increases. Because NLRP3$^{-/-}$ animals have an increased corneal T cell infiltration, as soon as soon as 7 days pi., it is likely that there is a reduced number of MDSC in the cornea. Further research should be done to identify if MDSC are present in HSV infected corneas and determine if there is some relationship between MDSC and NLRP3.

While we did not find differences in the of Tcell/Treg balance (data not shown) between NLRP3$^{-/-}$ and WT animals, another possible explanation for increased T cell number and more severe phenotype in NLRP3$^{-/-}$ mice may be that Treg are less functional in NLRP3$^{-/-}$ mice. Thus, co-culture studies with Tcells and Treg, evaluating the function of Treg may address such a hypothesis.

Finally, another way to investigate the causality of this phenotype in NLRP3$^{-/-}$ animals is comparison of a whole profile gene expression in corneas of HSV infected NLRP3$^{-/-}$ and WT animals at different time points pi. (It would include taking samples every 12 hs during the first 3 days pi.). Conducting early and frequent sampling would allow exploration of the cause effect and not the consequent effect. The same could be done in different immunological cells known to contribute in the pathogenesis of SK, mainly neutrophils, macrophages and CD4+T cells. Sorting of these cells from NLRP3$^{-/-}$ and WT mice and evaluating their genetic, miRNA and non coding long RNA profile would be useful to help determine if there is some downregulated or upregulated pathway that may explain the increased innate immune response. The use of Bio-informatics would be necessary for data analysis and management.

In conclusion, these two studies open new avenues for the development of novel lines of research. As described more studies are needed before closing the chapter on SK pathogenesis research.

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