

To the Graduate Council:

I am submitting herewith a dissertation written by Pranita Pragnyadipita Sarangi entitled "Induction and Regulation of Herpetic Stromal Keratitis." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Barry T. Rouse, Major Professor

We have read this dissertation
and recommend its acceptance:

Robert N. Moore

Steven J Kennel

Mark Y. Sangster

Accepted for the Council:

Carolyn R Hodges

Vice Provost and
Dean of Graduate School

(Original signatures are on file with official student records)

INDUCTION AND REGULATION OF HERPETIC STROMAL KERATITIS

A Dissertation Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Pranita Pragnyadipta Sarangi

May 2008

DEDICATION

This dissertation is dedicated to my father, who made me feel the strength of knowledge and gave me the passion of learning and to my mother who taught me the art of being versatile and was always besides me from the beginning.

ACKNOWLEDGEMENTS

My sincere appreciation goes to my PhD mentor, Dr. Barry T. Rouse, who has served as a father figure to me over the last four and half years and has helped and supported me in achieving my scientific goals. Additionally, I would like to extend my profound thanks to the members of my committee, Dr. Robert N. Moore, Dr. Stephen J Kennel, and Dr. Mark Y. Sangster, for all their time and needful guidance. I also thank my collaborator Dr. Evelyn Kurt-Jones for her kind advice and suggestions.

I express my thanks to all the members of my lab for providing a stimulating and fun environment to learn and grow. Specifically, I would like to thank Bumseok Kim and Sharvan Shehrawat, Susmit Suvas and, Seng Ryong Woo for their help and time in my projects. It has been a great pleasure to interact with the other lab members as well including, Uday Kumaraguru, Partha Biswas, Kaustuv Banerjee, Luiz Rodrigues, Christopher Dean Pack, Ahmet Kursat Azkur, Daisy Vanitha, Felix Toka, Amol Suryawanshi, Jason A. Burchett and Stacy.

Finally, I express my deepest gratitude to my parents Mr. Nisamani Sarangi, and Mrs. Pratimarani Sarangi and my sisters Amruta and Sukanti who were always besides me and motivated me to come this far. I am eternally grateful for their everlasting support and faith in all my strength and capability. I extend my heartily thanks to my dearest husband Dr. Kiran S. Ambatipudi for his advice and loving support in each and every step which I took in my life. I thank him for teaching me that even the biggest task can be accomplished very easily if it is done one step at a time.

ABSTRACT

Herpetic stromal keratitis (HSK) is an immunopathological and tissue destructive corneal lesion caused by herpes simplex virus (HSV) infection, which induces an intense inflammatory response and finally leads to blindness. Accumulating evidence using the murine model has shown that Th-1 phenotype CD4⁺ T cells orchestrating the inflammation mainly contribute to the immunopathological reaction in HSV-1 infected cornea. However, prior to CD4⁺ T cell infiltration into corneal lesions, various innate immune cells recruit and produce numerous inflammatory and angiogenic molecules into the corneal stroma those in turn drive the corneal immunopathology.

The first part (Part I) of this dissertation focuses on the understanding of HSV-1 induced immunoinflammatory processes in the cornea and trigeminal ganglia including the secondary lymphoid tissues. The next three parts (Part II-IV) focus on different inflammatory and anti-inflammatory mechanisms that are activated following virus infection in the cornea. Results in Part II evaluate the role of various Toll-like receptors (TLRs) in driving the early inflammatory events that occur in the HSV infected corneas. Thus, this part demonstrates that mainly TLR2 and to a lesser extent TLR9 ligand activity of HSV is important for driving SK pathology. Results of the third section show that HSV infection results in the up regulation of IL-23 that is needed for the survival of pathogenic Th-17 cells. The data show that mice lacking IL-23 were more susceptible to SK lesions and that the heightened lesion severity was attributed to higher IL-12 production and Th1 immune response in such animals. The fourth section describes the relative role of

IL-10 and natural regulatory T cells (nTregs) as two independent anti-inflammatory mechanisms that are needed for controlling HSK lesions.

In this study, experiments were designed to understand the mechanisms involved in the induction and regulation of immunopathology in HSK and modulation of such processes could be useful in designing therapeutic for HSK.

TABLE OF CONTENTS

Part	Page
I. Background and Overview	1
Clinical Background.....	2
Murine Herpetic SK model.....	5
Conclusion.....	13
List of references.....	15
Appendix.....	31
 II. Innate recognition network driving the HSV induced corneal immunopathology: Role of Toll Pathway in the early inflammatory events in Stromal Keratitis	 34
Abstract.....	35
Introduction.....	36
Materials and Methods.....	38
Results.....	42
Discussion.....	49
List of references.....	55
Appendix.....	62
 III. Enhanced viral immunoinflammatory lesions in mice lacking IL-23 responses	 79

Abstract.....	80
Introduction.....	81
Materials and Methods.....	82
Results.....	87
Discussion.....	91
List of references.....	95
Appendix.....	101
IV. IL-10 and natural regulatory T cells: Two independent anti inflammatory mechanisms in HSV induced ocular immunopathology	114
Abstract.....	115
Introduction.....	116
Materials and Methods.....	118
Results.....	123
Discussion.....	129
List of references.....	134
Appendix.....	141
VITA.....	163

LIST OF FIGURES

Part	Figure	Page
I	Figure 1. Principal cellular events in herpetic SK pathogenesis.....	32
	Figure 2. Schematic representation of the early events in SK cornea.....	33
II	Figure 1. Induced TLR ligands in the HSV infected cornea	63
	Figure 2. Reduced SK lesions and neovascularization in TLR2 ^{-/-} , TLR2/4d ^{-/-} , and MyD88 ^{-/-} mice as compared to TLR9 ^{-/-} , TLR4 ^{-/-} and WT mice.....	65
	Figure 3. Compromised inflammatory cell influx into the cornea of TLR2 ^{-/-} , TLR9 ^{-/-} , TLR2/4d ^{-/-} , and MyD88 ^{-/-} mice as compared to TLR4 ^{-/-} and WT mice at 48 h p.i.....	67
	Figure 4. Compromised cell infiltration into the TG of TLR2 ^{-/-} and MyD88 ^{-/-} mice as compared to TLR4 ^{-/-} and WT mice.....	69
	Figure 5. Reduced levels of cytokines and angiogenic factor in the corneas of TLR2 ^{-/-} and MyD88 ^{-/-} mice as compared to TLR4 ^{-/-} and WT mice.....	71

	Figure 6. Increased expression of proinflammatory cytokines and reduced levels of IL-10 in TLR4-/- animals as compared to WT.....	73
	Figure 7. Reduced expression of COX-2 in TLR2-/- animals as compared to WT.....	75
III	Figure 1. Expression of IL-23p19 mRNA and protein in the corneas and lymphoid organs after HSV-1 ocular infection.....	102
	Figure 2. IL-23p19-/- mice have higher SK lesion severity, incidence, ocular angiogenesis, and enhanced viral clearance.....	104
	Figure 3. Increased Th1 type immune responses in the spleen of IL-23p19-/- mice after HSV-1 infection.....	106
	Figure 4. Increased population and number of IL-12 expressing cells in the lymphoid organs of p19-/- mice after HSV-1 infection.....	108
	Figure 5. Increased Th1 type immune responses in the SK corneas of p19-/- mice.....	100
	Figure 6. Decreased Th17 immune response in p19-/- mice after HSV-1 infection.....	112

IV	Figure 1. Increased SK lesions in the absence of IL-10 and nTregs.....	142
	Figure 2. Increased inflammation in the corneas of mice deficient in IL-10 and nTregs.....	144
	Figure 3. Increased number of CD4 ⁺ IFN- γ ⁺ T cells in the corneas of mice deficient in IL-10 and nTregs.....	147
	Figure 4. Increased number of IFN- γ ⁺ and TNF- α ⁺ CD4 T cells in the secondary lymphoid tissues of IL-10 ^{-/-} mice as compared to nTregs depleted and WT mice.....	149
	Figure 5. Increased number of SSIEFARL peptide specific CD8 ⁺ IFN- γ ⁺ T cells in the secondary lymphoid tissues of mice deficient in nTregs as compared to IL-10 ^{-/-} and WT.....	151
	Figure 6. Increased expression of inflammatory mediators in the corneas and lymphoid tissues of mice deficient in IL-10 and nTregs.....	153
	Figure 7. Increased SK severity in the absence of nTregs in the IL-10 ^{-/-} animals.....	155

Figure 8. nTregs from both IL-10 ^{-/-} and WT animals can equally suppress the proliferation and cytokine production from conventional T cell isolated from WT animals.....	157
--	-----

Figure 9. A. Tregs do not produce IL-10 but can influence the IL-10 production from other cell types in the lymphoid tissues.....	160
--	-----

LIST OF TABLES

Part	Tables	Page
II	Table. 1. Percentage of mortality in various groups by day 15 p.i.....	76
	Table. 2. Virus titer in log ₁₀ pfu/ml in TG and brain at different time points p.i.....	77
	Table. 3. Virus titer in log ₁₀ pfu/ml in the cornea at different time points p.i.....	78
IV	Table. 1 Phenotype of IL-10 producing cells in various tissues.....	162

ABBREVIATIONS

APC.....	Antigen presenting cell
CCL2, 5, 20.....	Chemokine (C-C motif) ligand 2, 5, 20
CD.....	Cluster of differentiation
CTL.....	Cytotoxic T lymphocyte
COX-2.....	Cyclooxygenase-2
CXCR2.....	CXC chemokines receptor 2
CXCL3, 8.....	CXC chemokine ligand 3, 8
FGF.....	Fibroblast growth factor
HSV-1.....	Herpes simplex virus 1
HSK.....	Herpetic Stromal Keratitis
ICAM-1.....	intercellular adhesion molecule 1
IFN- α , γ	Interferon alpha, gamma
IL-1, 2, 6, 12, 17, 18, 23...	Interleukin 1, 2, 6, 12, 17, 18, 23
IL-18 BP.....	Interleukin 18 binding protein
IP-10.....	Interferon inducible protein 10
KC.....	Keratinocyte-derived chemokine
MCP-1.....	Monocyte chemoattractant protein 1
MIP-1, 2.....	Macrophage inflammatory protein 1, 2
MMP-9.....	Matrix metalloproteinase-9
NF κ B.....	Nuclear factor- kappa B
PECAM.....	Platelet endothelial cell adhesion molecule

PFU.....	Plaque forming unit
PGE2.....	Prostaglandin E2
PMN.....	Polymorphonuclear leukocyte
PAMP.....	Pathogen Associated Molecular Patterns
siRNA.....	small interfering RNA
SK.....	Stromal Keratitis
TGF- β	Transforming growth factor beta
TIMP.....	Tissue inhibitors of metalloproteinases
TLR 2, 4, 9.....	Toll like receptor 2, 4, 9
TNF- α	Tumor necrosis factor alpha
VEGF.....	Vascular endothelial growth factor
VLA4.....	Very late antigen 4
Treg.....	Regulatory T cells

Part-I

Background and overview

CLINICAL BACKGROUND

Herpes simplex virus (HSV) induced eye diseases are one of the commonest causes of unilateral infectious blindness in the western world [1]. Epidemiological studies in the United states reveal that, 400,000 persons are affected, with 20,000 new cases occurring annually with the incidence ranging from 4.1- 20.7 cases/100 000 patients per year [2]. Results from surveys conducted by the National Health and Nutrition Examination between 1976 and 1980 (NHANES II) and again between 1988 and 1994 (NHANES III) showed a 30% increase in HSV-2 specific antibodies within the studied timeframe in the United States [3]. In contrast the age specific seroprevalence of HSV-1 has decreased in the industrial countries with an exception to several developing countries [3]. Ocular lesions are mostly caused by HSV type 1 and very rarely by HSV type 2 infections. However, 80% of neonatal herpes infection is caused by HSV-2 although both strains can be involved.

Classically, the life cycle of HSV in the host is divided into four stages namely, entry, spread, establishment of latency and reactivation. After the primary infection of the skin or mucosal surfaces with HSV, it spreads to the neuronal cell bodies where latency is established [1]. It is currently believed that the virus reactivation is kept in check by host immune cells in the sensory ganglion [4]. The psychological and physical stresses that result in temporary impairment of the immune system facilitate the reactivation of HSV and subsequent release of the virus to the periphery [5]. Most ocular HSV infections result in acute epithelial keratitis that can be controlled with several antiviral drugs [6]. Twenty percent of the patients infected ocularly with HSV develop, a chronic vision impairing stromal lesion known as herpetic stromal keratitis (HSK). In humans,

approximately 50% of HSK occurs as a consequence of virus reactivation from the trigeminal ganglion and only ~2% of such cases result directly from primary HSV infection [1]. The chances of developing SK are three times greater following a recurring herpetic keratitis than following a primary infection [1].

Symptoms of HSK lesions includes, corneal necrosis and ulceration, stromal edema and neovascularization [7]. These lesions are shown to occur mainly as a result of T cell mediated immunopathological responses in the cornea whose pathogenesis are poorly understood. Forty to ninety percent of the patients with stromal keratitis may develop disciform keratitis (corneal endothelium is the main site of damage), while the rest of the patients may develop stromal opacities [1]. If not treated properly, it may lead to corneal scarring and ulcerations warranting corneal transplantation. Apart from stromal keratitis, HSV infection could also result in viral retinitis and/or encephalitis in both immunosuppressive and immunocompetent individuals [1]. In some cases, infection with HSV-1 or 2 have also been reported to be associated with the acute retinal necrosis syndrome [8].

Presently the therapeutic agents used for treating acute keratitis include the administration of antivirals and corticosteroids and non-steroidal anti-inflammatory drugs such as Cyclooxygenase-2 (COX-2) blockers [7, 9, 10]. Prolonged use of antivirals was shown to result in the development of resistance particularly in immunocompromised individuals [11, 12]. Similarly use of corticosteroids might give rise to numerous adverse effects [13, 14] and in some patients it may exacerbate HSK lesions due to enhanced HSV-1 replication [15]. Animal studies in the mouse model of HSK have demonstrated that cytokine and

chemokine blockers such as the IL-1 receptor antagonist are effective [16]. As neovascularization is an essential event for HSK pathogenesis, siRNA against VEGF was shown to be effective in reducing HSK lesions in the mouse [17]. Furthermore, mouse model studies also support the effectiveness of regulatory T cell therapy in controlling HSK immunopathology [18].

In addition to topical and systemic administration of above mentioned drugs, several prophylactic vaccination strategies also are being developed to increase the cell mediated protection against HSV. Plasmid DNA encoding viral antigens such as gD and /or gB [19-22] and live virus mutants such as vhs⁻/ICP8⁻ HSV strain have been shown to reduce the incidence of HSK [23-25]. They also reduced ocular viral shedding and raise the level of neutralizing antibodies in infected animals [25]. Subunit vaccines (gB2+gD2) are also shown to be useful in controlling recurrent shedding and incidence of HSK in latently infected rabbits [26]. Although several clinical trials are being conducted for different HSV vaccines, such as gB2/gD2 by Chiron, gD2 MPL by Glaxo SmithKline (GSK) for HSV-2, with many more in use, only one therapeutic vaccine for herpes simplex keratitis in human has been reported [27]. Effective vaccines against HSV-2 could even help reduce the incidence of neonatal ocular herpes. Approaches such as intranasal/subconjunctival/topical routes of vaccination [20, 26], addition of specific cytokines and chemokines [22, 28] to vaccine or use of an appropriate immune modifier like CpG [29, 30] are under investigation to increase the efficacy of vaccines against HSV. As the antivirals fail to prevent establishment of latency, designing an effective vaccine is important to prevent recurrent ocular diseases.

Thus further studies of immune mechanisms involved in the neurological surveillance and its use in therapeutic vaccine designing is important.

MURINE HERPETIC SK MODEL

Study of the pathogenesis of human HSK from clinical observations, occasional biopsy samples and transplant material is difficult. In the earlier literature, most of the studies on HSK were performed on rabbits but currently the murine model is mostly used to understand SK pathogenesis. Primary ocular HSV infection in mice results in a stromal inflammatory response resembling human SK. Several strains of mice (BALB/c, CAL-20, 129/SVEV, C57BL/6) and HSV-1 (RE, KOS, McRae and F) strain are employed for HSK studies [7].

Primary infection of immunocompetent mice with HSV-1RE results in the initial replication of virus in the corneal epithelium for 5-6 days with minimal involvement of the stromal or other anterior segment tissues. Viral gene expression as judged by mRNA are undetectable after 7-8 days p.i., however, viral DNA could be detected in the cornea till day 20 p.i. At this initial replication phase, the clinical SK lesions and other signs of inflammation are barely visible. However, new blood vessels sprouting from the limbus and histologically inflammatory events are evident within few hours of infection (Fig .1) (All figures and tables are located in the appendices). This early stage of lesion development is usually referred to as preclinical phase of SK.

Inflammatory cells

Toll-like receptors expressed on innate immune cells form a critical component of host surveillance system. Triggering of these pattern recognition receptors activates the transcription factor NF κ B that causes the induction of cytokines and

chemokines in response to the pathogen associated molecular patterns (PAMP) [31]. This initial induction of inflammatory genes could lead to the development of immunopathological lesions [31]. Components of HSV have been shown to possess TLR2 and -9 ligand activity [32, 33]. Human and mice corneas were shown to express different functional TLRs and there is accumulating evidence that suggests TLRs could be involved in the HSK pathogenesis [34-36]. Virus infection in the corneal epithelium, probably by its PAMP activity, initiates several humoral and cellular immune responses that together are responsible for clearing the infection [31]. As summarized in Fig 1., early inflammatory response that is evident in the underlying stroma mainly consisted of the PMNs [37]. This infiltration starts as early as 18 hr post infection (p.i.) peaking at around 48 hr and then declines by 5 days p.i [37]. This initial pattern of PMN influx correlates with the presence of replicating virus and thought to be involved in controlling virus spread [37, 38]. Thus, PMN depletion with specific monoclonal antibodies (mAb) before infection results in delayed virus clearance from the cornea. Although it is not known how the PMNs infiltrating to the stroma could clear the virus from the epithelium, however, it has been proposed that TNF- α , nitric oxide and other reactive oxygen metabolites could be involved in this process. Degranulation of PMNs results in the release of inflammatory mediators such as IL-1 β , TNF- α , IL-12, preformed vascular endothelial growth factor (VEGF) and tissue degrading enzyme metalloproteinase-9 (MMP-9) required to break down the stromal matrix which are critical to drive the subsequent tissue destructive events that ultimately lead to HSK [39-42] (Fig. 2).

Although the early inflammatory phase of ocular HSV infection is dominated by PMNs, other cells such as macrophages [43], dendritic cells (DC) [44, 45] natural killer (NK) cells [46, 47] and $\gamma\delta$ T cells [48] were shown to participate in virus clearance and act as a source of cytokines and chemokines in the infected cornea [43, 46]. Macrophages also act as a source of angiogenic factors VEGF, FGF as well as angiogenic CXC chemokine [49]. In addition to these cells, Langerhans DCs also invade the corneal tissues most likely from the conjunctiva [44, 45]. Apart from acting as a source of cytokines, their major role is to carry the viral antigens to the draining lymph nodes and by which they accelerate the immunopathogenesis of HSK by influencing the generation of an adaptive immune response. But recent studies in skin infection model question the role of Langerhans DCs in antigen presentation and suggest that special subsets of migratory DCs could be involved in carrying the viral antigen to the local draining lymph nodes [50, 51].

Cytokines and Chemokines

Multiple cytokines and chemokine transcripts are expressed early after HSV infection (around 6 hr p.i). Most prominent are IL-1 and IL-6 those are shown to be critical to initiate the subsequent cascade of inflammatory events [52, 53]. Although after infection, most of the host genes are turned off [54], infected epithelial cells can still act as the initial source of IL-1 and IL-6 [55-57]. TLR2 and to a lesser extent TLR9 ligand activity of HSV and other endogenous TLR ligands could play critical role in initiating this cytokine and chemokine release [35]. Accordingly, mice lacking TLR2 and the adapter molecule MyD88 had reduced levels of IL-1, IL-6 and MIP-2 in their corneas post infection [35]. Cytokines

released from the infected cells as a result of stimulation through PAMPs could drive the expression of other key mediators from uninfected cells. Thus, IL-1 can trigger IL-6 production from uninfected cells [59] which is known to trigger macrophage inflammatory protein (MIP-2) (CXCL8), a critical chemo attractant for PMNs [60, 61]. Initial IL-1 production could trigger the upregulation of COX-2 that could help in the synthesis of proinflammatory prostanoides such as prostaglandin E₂ (PGE₂) [10]. Even TLR ligand activity were shown to be involved in the expression of COX-2 in the infiltrating inflammatory cells but it is not yet clear whether it is a direct effect or caused by the cytokines released through TLR stimulation [35]. Another important cytokine that is upregulated in HSV infected cornea is TNF- α [62, 63]. Recent studies show that mice unable to produce TNF- α have higher viral titers in the cornea and are more susceptible to lesion development [62].

In addition to the above list of cytokines, another cytokine that is pivotal for the pathogenesis of SK is IL-12, but the trigger for early IL-12 release is yet to be identified [64, 65]. The PMNs, macrophages and Langerhans DCs those infiltrating into the cornea are the likely sources of IL-12 [40, 66]. One of the critical downstream events of IL-12 is the production of IFN- γ from cells like macrophage, NK cells and neutrophils that express IL-12 receptor on their surface. Previous reports show the role of STAT-4 signaling pathway in the production of IFN- γ following IL-12 stimulation [67]. IFN- γ is shown to play several important roles in HSK pathogenesis and those include, aiding PMN influx by up regulating PECAM-1 (Platelet cell adhesion molecule-1) [68], and upregulation of ICAM-1 on the corneal epithelium [69], endothelium and keratocytes needed for

extravasation of inflammatory cells. It also upregulates MHC-II on antigen presenting cells (APCs) those are involved in the induction of pathogenic CD4⁺ T cells in the draining lymph nodes [70, 71]. In addition, IFN- γ was also shown to modulate HSK lesions by inducing angiostatic chemokines such as IP-10 (CXCL10) [72].

Another cytokine that has currently received much attention in CD4⁺ T cell mediated immunopathology and autoimmune diseases is IL-17 [73]. IL-17 is produced by CD4⁺ T cells (Th17 cells) and was shown to influence the infiltration of PMNs by up regulating IL-6 and IL-8 [74]. Recent studies suggest that IL-17 could influence the early neutrophil influx into the HSV infected corneas and, thus, could have a role in HSK pathogenesis [75]. Recently, the ability of CD4⁺ T cells to produce IL-17 was shown to be regulated by MyD88 dependant TLR pathway [76]. This issue needs to be investigated in HSK. IL-23 that it is essential for the maintenance of Th17 cells was shown to be upregulated post ocular HSV infection [77]. According to the recent findings, apart from maintaining Th17 producers, IL-23 could be involved in balancing the production of IL-12 in the system. Thus, mice lacking the IL-23 produced more IL-12 and IFN- γ and showed heightened susceptibility to HSK [77].

Apart from the proinflammatory cytokines, HSV infection also induces several anti-inflammatory cytokines such as IL-10 and TGF- β in the infected corneas. IL-10 was shown to be important in minimizing SK lesion severity. Thus mice deficient in IL-10 gene demonstrate a more susceptible phenotype as compared to the IL-10 sufficient animals [78]. Additionally, a higher level of IL-

10 was also observed in the infected corneas during the resolution phase of SK [79].

Along with several cytokines, ocular infection with HSV also upregulated several chemokines such as MIP-2, KC (CXCL3), MCP-1 (CCL-2), RANTES (CCL-5), IP-10, MIP-3 α (CCL20) [80-82]. These may aid in the influx of inflammatory cells into the corneal stroma. Among these MIP-2 and KC were shown to be predominant and are critical for the early PMN infiltration [60]. Induction stimuli for these chemokines seem to be the proinflammatory cytokines released as a result of virus replication in the corneal epithelium. Corneal epithelial and stromal cells were shown to produce CCL20 following the stimulation with TNF- α and IL-1 β [82]. In addition TLR ligand activity of the HSV could also be involved in the induction of these chemokines [35]. Another group of chemokines expressed in the late preclinical phase are MIP-1 α (CCL3) and lymphotactin (XCL1) [80]. Although the stimuli for the upregulation of these chemokines are not known, these chemokines are pivotal for the infiltration of CD4⁺ T cells in the clinical phase of the HSK lesions.

Neovascularization

One of the necessary steps in the immunopathology of HSK is the development of new blood vessels post infection in normally avascular corneas [83] as the newly formed blood vessels could help in the infiltration of inflammatory cells. New blood vessels start sprouting from the limbal vessels by day 1 p.i. reaching the central cornea by day 20 p.i. [84]. Inflammatory cells escape from these highly permeable blood vessels that result in corneal haze and vision impairment. Ocular HSV infection results in the upregulation of several angiogenic factors such as

VEGF, bFGF, MMP-9 and E-L-R motif containing chemokines [42, 80, 84, 85]. VEGF mediated angiogenesis is a prominent and early event in HSK with VEGF transcripts detectable by 12 hr p.i. and protein by 24 hr p.i. [84]. Uninfected corneal epithelial cells and stromal cells act as the initial source of VEGF and subsequently, it is released by the infiltrating inflammatory cells such as PMNs and macrophages [84]. Evidence suggests that proinflammatory cytokines generated early after infection such as IL-1 and IL-6 could stimulate the production of VEGF [86-88]. Additionally, HSV DNA, rich in CpG motif can induce IL-1 and IL-6 with its TLR9 ligand activity that in turn could induce VEGF production [89]. Another critical factor involved in the process of neovascularization is the collagenase MMP-9 [42]. PMNs and corneal epithelial cells are shown to produce MMP-9 in response to inflammatory cytokines such as IL-1 and TNF- α [90]. Apart from cytokines, chemokines with E-L-R motifs such as MIP-2 are also known to induce ocular angiogenesis [91]. While several proinflammatory cytokines and chemokines were shown to induce corneal neovascularization, IL-12 and IL-18 are shown to inhibit angiogenesis by downregulation of VEGF production [72, 92]. Inhibition of angiogenesis by using siRNA against MMP-9 and VEGF were shown to reduce corneal neovascularization and HSK lesion severity in a mouse model [17]. Thus, modulation of angiogenic factors could prove to be beneficial in the therapy of SK.

Clinical Stromal Keratitis

Soon after the early inflammatory events including the clearance of replicating virus and viral antigens, around day 6 and 7 p.i., lymphocytes and other inflammatory cells infiltrate into the corneal stroma leading to the development of

clinically evident SK lesion [93]. Interestingly, although at other inflammatory sites, such as TG, higher frequencies of CD8⁺ T cells are observed but in infected corneas CD4⁺ T cells greatly outnumber the CD8⁺ T cells [94]. Most of the cells infiltrating into cornea express the critical homing marker VLA-4 and chemokine receptor CCR5 (MIP-1 α) [58, 95]. The range of antigens that are recognized by these infiltrating CD4⁺ T cells still remain unknown and the role of viral antigens in driving virus specific CD4⁺ T cells in HSK needs further investigation. Interestingly, in TCR transgenic animals with SCID/ RAG background that could not recognize viral antigens, it is possible to induce lesions with a replication competent virus [96, 97]. These observations suggest that probably the inflammatory events following HSV infection could result in unmasking of the corneal autoantigens [2]. Few other observations also suggest that virus specific CD4⁺ T cells may crossreact with corneal antigens and could contribute to HSK pathogenesis. Support for this hypothesis comes from Cantor's lab where they showed that the UL-6 protein from HSV acts as a molecular mimic for a corneal autopeptide [98]. The role of molecular mimicry still needs further confirmation. Alternately, bystander activation of T cells in a non TCR mediated manner by the inflammatory molecules produced as a result of ocular HSV infection could explain HSK pathogenesis. Although not explained mechanistically, the observation that it is possible to induce SK in the mouse whose T cells are genetically incapable of recognizing viral antigen could support the involvement of bystander mechanisms [99, 100]. In this system cells dying of HSV infection were responsible for inducing cytokine production [100, 101].

Another subset of T lymphocytes that infiltrate the cornea is the CD4⁺CD25⁺Foxp3⁺ Treg cells. In addition to other mechanisms such as the cytokine IL-10 [102], Treg are shown to be involved in controlling the ongoing inflammatory processes in the cornea [95]. In addition, these cells might help in the resolution of the clinical lesions. Studies suggest that depletion of Treg from the mice prior to infection results in more severe SK lesions [95]. Different mechanisms involved in the suppressive activity of Treg are release of cytokines IL-10, IL-35 and TGF- β , direct contact with effectors and APCs etc. [102, 103], but the mechanism and the site of Treg activity in HSK is controversial and need further investigations. The fact that even in IL-10^{-/-} animals have functional Tregs and that depletion of Tregs in IL-10^{-/-} animals could increase the lesion severity suggests that Tregs and IL-10 are two independent anti-inflammatory mechanisms in HSK [104].

CONCLUSION

Currently several therapeutic and prophylactic drugs are being used for the treatment of SK. Discovery of new molecular participants in the pathogenesis of SK will show new targets for designing better therapeutic drugs. Study in mouse model shows a role of TLR2 and TLR9 signaling in HSK. Even in human HSK corneas, changes in the expression levels of several TLRs are being noticed [35, 36]. What is not yet known is under physiological condition how such a small amount of virus could trigger the TLRs. Better understanding of the signaling processes, induction of critical inflammatory molecules and their appropriate modulation could help in finding cure for SK. Similarly, anti-inflammatory mechanisms such as induction of IL-10 and regulatory T cell could be beneficial in

the prevention as well as resolution of SK lesions. Therefore, several drugs and reagents such as FTY720, retinoic acid, galactin-9 [105-107] those are capable of inducing regulatory T cells or IL-10 could be useful therapeutic strategies to suppress SK lesion severity. These approaches could be used as single or in combination with other existing therapies for better result and less side effect.

LIST OF REFERENCES

- [1] J.S. Pepose, D.A. Leib, P.M. Stuart, D.L. Easty, Herpes Simplex Virus Diseases: Anterior segment of the Eye, J.S. Pepose, G.N. Holland, K.R. Wilhelmus (Eds.), Mosby, St. Louis, 1996, pp. 905-932.
- [2] P.S. Biswas, B.T. Rouse, Early events in HSV keratitis--setting the stage for a blinding disease, *Microbes Infect* 7 (2005) 799-810.
- [3] J.S. Pepose, T.L. Keadle, L.A. Morrison, Ocular herpes simplex: changing epidemiology, emerging disease patterns, and the potential of vaccine prevention and therapy, *Am J Ophthalmol* 141 (2006) 547-557.
- [4] B.S. Sheridan, J.E. Knickelbein, R.L. Hendricks, CD8 T cells and latent herpes simplex virus type 1: keeping the peace in sensory ganglia, *Expert Opin Biol Ther* 7 (2007) 1323-1331.
- [5] M.L. Freeman, B.S. Sheridan, R.H. Bonneau, R.L. Hendricks, Psychological stress compromises CD8+ T cell control of latent herpes simplex virus type 1 infections, *J Immunol* 179 (2007) 322-328.
- [6] H.E. Kaufman, Can we prevent recurrences of herpes infections without antiviral drugs? The Weisenfeld Lecture, *Invest Ophthalmol Vis Sci* 43 (2002) 1325-1329.
- [7] S. Deshpande, K. Banerjee, P.S. Biswas, B.T. Rouse, Herpetic eye disease: immunopathogenesis and therapeutic measures, *Expert Rev Mol Med* 6 (2004) 1-14.
- [8] S.S. Atherton, Acute retinal necrosis: insights into pathogenesis from the mouse model, *Herpes* 8 (2001) 69-73.
- [9] R.J. Whitley, B. Roizman, Herpes simplex virus infections, *Lancet* 357 (2001) 1513-1518.

- [10] P.S. Biswas, K. Banerjee, B. Kim, P.R. Kinchington, B.T. Rouse, Role of inflammatory cytokine-induced cyclooxygenase 2 in the ocular immunopathologic disease herpetic stromal keratitis, *J Virol* 79 (2005) 10589-10600.
- [11] Y. Chen, C. Scieux, V. Garrait, G. Socie, V. Rocha, J.M. Molina, D. Thouvenot, F. Morfin, L. Hocqueloux, L. Garderet, H. Esperou, F. Selimi, A. Devergie, G. Leleu, M. Aymard, F. Morinet, E. Gluckman, P. Ribaud, Resistant herpes simplex virus type 1 infection: an emerging concern after allogeneic stem cell transplantation, *Clin Infect Dis* 31 (2000) 927-935.
- [12] S. Biswas, L. Jennens, H.J. Field, Single amino acid substitutions in the HSV-1 helicase protein that confer resistance to the helicase-primase inhibitor BAY 57-1293 are associated with increased or decreased virus growth characteristics in tissue culture, *Arch Virol* 152 (2007) 1489-1500.
- [13] L. Kolbe, A.M. Kligman, V. Schreiner, T. Stoudemayer, Corticosteroid-induced atrophy and barrier impairment measured by non-invasive methods in human skin, *Skin Res Technol* 7 (2001) 73-77.
- [14] R. Dahl, Systemic side effects of inhaled corticosteroids in patients with asthma, *Respir Med* 100 (2006) 1307-1317.
- [15] B.I. Weinstein, J. Schwartz, G.G. Gordon, M.O. Dominguez, S. Varma, M.W. Dunn, A.L. Southren, Characterization of a glucocorticoid receptor and the direct effect of dexamethasone on herpes simplex virus infection of rabbit corneal cells in culture, *Invest Ophthalmol Vis Sci* 23 (1982) 651-659.
- [16] P.S. Biswas, K. Banerjee, M. Zheng, B.T. Rouse, Counteracting corneal immunoinflammatory lesion with interleukin-1 receptor antagonist protein, *J Leukoc Biol* 76 (2004) 868-875.

- [17] B. Kim, Q. Tang, P.S. Biswas, J. Xu, R.M. Schiffelers, F.Y. Xie, A.M. Ansari, P.V. Scaria, M.C. Woodle, P. Lu, B.T. Rouse, Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor pathway genes: therapeutic strategy for herpetic stromal keratitis, *Am J Pathol* 165 (2004) 2177-2185.
- [18] S. Sehrawat, S. Suvas, B.T. Rouse, In vitro generated antigen specific CD4⁺CD25⁺Foxp3⁺ regulatory T cells control the severity of virus induced immunoinflammatory lesion, *Immunology*, Miami, 2007.
- [19] E. Caselli, P.G. Balboni, C. Incorvaia, R. Argnani, F. Parmeggiani, E. Cassai, R. Manservigi, Local and systemic inoculation of DNA or protein gB1s-based vaccines induce a protective immunity against rabbit ocular HSV-1 infection, *Vaccine* 19 (2000) 1225-1231.
- [20] S.K. Eo, M. Gierynska, A.A. Kamar, B.T. Rouse, Prime-boost immunization with DNA vaccine: mucosal route of administration changes the rules, *J Immunol* 166 (2001) 5473-5479.
- [21] T. Inoue, Y. Inoue, K. Hayashi, Y. Shimomura, Y. Fujisawa, A. Aono, Y. Tano, Effect of herpes simplex virus-1 gD or gD-IL-2 DNA vaccine on herpetic keratitis, *Cornea* 21 (2002) S79-85.
- [22] T. Inoue, Y. Inoue, K. Hayashi, A. Yoshida, K. Nishida, Y. Shimomura, Y. Fujisawa, A. Aono, Y. Tano, Topical administration of HSV gD-IL-2 DNA is highly protective against murine herpetic stromal keratitis, *Cornea* 21 (2002) 106-110.
- [23] B.J. Geiss, T.J. Smith, D.A. Leib, L.A. Morrison, Disruption of virion host shutoff activity improves the immunogenicity and protective capacity of a

replication-incompetent herpes simplex virus type 1 vaccine strain, J Virol 74 (2000) 11137-11144.

[24] T.L. Keadle, L.A. Morrison, J.L. Morris, J.S. Pepose, P.M. Stuart, Therapeutic immunization with a virion host shutoff-defective, replication-incompetent herpes simplex virus type 1 strain limits recurrent herpetic ocular infection, J Virol 76 (2002) 3615-3625.

[25] Y. Osorio, H. Ghiasi, Recombinant herpes simplex virus type 1 (HSV-1) codelivering interleukin-12p35 as a molecular adjuvant enhances the protective immune response against ocular HSV-1 challenge, J Virol 79 (2005) 3297-3308.

[26] A.B. Nesburn, S. Slanina, R.L. Burke, H. Ghiasi, S. Bahri, S.L. Wechsler, Local periocular vaccination protects against eye disease more effectively than systemic vaccination following primary ocular herpes simplex virus infection in rabbits, J Virol 72 (1998) 7715-7721.

[27] P. Pivetti-Pezzi, M. Accorinti, R.A. Colabelli-Gisoldi, M.P. Pirraglia, M.C. Sirianni, Herpes simplex virus vaccine in recurrent herpetic ocular infection, Cornea 18 (1999) 47-51.

[28] M. Daheshia, N. Kuklin, E. Manickan, S. Chun, B.T. Rouse, Immune induction and modulation by topical ocular administration of plasmid DNA encoding antigens and cytokines, Vaccine 16 (1998) 1103-1110.

[29] W.S. Gallichan, R.N. Woolstencroft, T. Guarasci, M.J. McCluskie, H.L. Davis, K.L. Rosenthal, Intranasal immunization with CpG oligodeoxynucleotides as an adjuvant dramatically increases IgA and protection against herpes simplex virus-2 in the genital tract, J Immunol 166 (2001) 3451-3457.

- [30] A.B. Nesburn, T.V. Ramos, X. Zhu, H. Asgarzadeh, V. Nguyen, L. BenMohamed, Local and systemic B cell and Th1 responses induced following ocular mucosal delivery of multiple epitopes of herpes simplex virus type 1 glycoprotein D together with cytosine-phosphate-guanine adjuvant, *Vaccine* 23 (2005) 873-883.
- [31] R.W. Finberg, J.P. Wang, E.A. Kurt-Jones, Toll like receptors and viruses, *Rev Med Virol* 17 (2007) 35-43.
- [32] E.A. Kurt-Jones, M. Chan, S. Zhou, J. Wang, G. Reed, R. Bronson, M.M. Arnold, D.M. Knipe, R.W. Finberg, Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis, *Proc Natl Acad Sci U S A* 101 (2004) 1315-1320.
- [33] A. Krug, G.D. Luker, W. Barchet, D.A. Leib, S. Akira, M. Colonna, Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9, *Blood* 103 (2004) 1433-1437.
- [34] A.C. Johnson, F.P. Heinzel, E. Diaconu, Y. Sun, A.G. Hise, D. Golenbock, J.H. Lass, E. Pearlman, Activation of toll-like receptor (TLR)2, TLR4, and TLR9 in the mammalian cornea induces MyD88-dependent corneal inflammation, *Invest Ophthalmol Vis Sci* 46 (2005) 589-595.
- [35] P.P. Sarangi, B. Kim, E. Kurt-Jones, B.T. Rouse, Innate recognition network driving herpes simplex virus-induced corneal immunopathology: role of the toll pathway in early inflammatory events in stromal keratitis, *J Virol* 81 (2007) 11128-11138.
- [36] X. Jin, Q. Qin, W. Chen, J. Qu, Expression of toll-like receptors in the healthy and herpes simplex virus-infected cornea, *Cornea* 26 (2007) 847-852.

- [37] J. Thomas, S. Gangappa, S. Kanangat, B.T. Rouse, On the essential involvement of neutrophils in the immunopathologic disease: herpetic stromal keratitis, *J Immunol* 158 (1997) 1383-1391.
- [38] T.M. Tumpey, S.H. Chen, J.E. Oakes, R.N. Lausch, Neutrophil-mediated suppression of virus replication after herpes simplex virus type 1 infection of the murine cornea, *J Virol* 70 (1996) 898-904.
- [39] S.K. Mistry, M. Zheng, B.T. Rouse, S.M. Morris, Jr., Induction of arginases I and II in cornea during herpes simplex virus infection, *Virus Res* 73 (2001) 177-182.
- [40] M. Daheshia, S. Kanangat, B.T. Rouse, Production of key molecules by ocular neutrophils early after herpetic infection of the cornea, *Exp Eye Res* 67 (1998) 619-624.
- [41] P. Scapini, F. Calzetti, M.A. Cassatella, On the detection of neutrophil-derived vascular endothelial growth factor (VEGF), *J Immunol Methods* 232 (1999) 121-129.
- [42] S. Lee, M. Zheng, B. Kim, B.T. Rouse, Role of matrix metalloproteinase-9 in angiogenesis caused by ocular infection with herpes simplex virus, *J Clin Invest* 110 (2002) 1105-1111.
- [43] D. Bauer, S. Mrzyk, N. van Rooijen, K.P. Steuhl, A. Heiligenhaus, Macrophage-depletion influences the course of murine HSV-1 keratitis, *Curr Eye Res* 20 (2000) 45-53.
- [44] M.J. Jager, S. Atherton, D. Bradley, J.W. Streilein, Herpetic stromal keratitis in mice: less reversibility in the presence of Langerhans cells in the central cornea, *Curr Eye Res* 10 Suppl (1991) 69-73.

- [45] M.J. Jager, D. Bradley, S. Atherton, J.W. Streilein, Presence of Langerhans cells in the central cornea linked to the development of ocular herpes in mice, *Exp Eye Res* 54 (1992) 835-841.
- [46] R.R. Tamesis, E.M. Messmer, B.A. Rice, J.E. Dutt, C.S. Foster, The role of natural killer cells in the development of herpes simplex virus type 1 induced stromal keratitis in mice, *Eye* 8 (Pt 3) (1994) 298-306.
- [47] D.M. Bouley, S. Kanangat, B.T. Rouse, The role of the innate immune system in the reconstituted SCID mouse model of herpetic stromal keratitis, *Clin Immunol Immunopathol* 80 (1996) 23-30.
- [48] T. Inoue, Y. Inoue, R. Kosaki, K. Nishida, Y. Shimomura, Y. Tano, K. Hayashi, Immunohistological study of infiltrated cells and cytokines in murine herpetic keratitis, *Acta Ophthalmol Scand* 79 (2001) 484-487.
- [49] K. Banerjee, B.T. Rouse, Immunopathological aspects of HSV Infection, *Human Herpes Viruses Biology, Therapy, and Immunoprophylaxis*, Cambridge University Press, New York, 2007, pp. 642-655.
- [50] R.S. Allan, C.M. Smith, G.T. Belz, A.L. van Lint, L.M. Wakim, W.R. Heath, F.R. Carbone, Epidermal viral immunity induced by CD8alpha+ dendritic cells but not by Langerhans cells, *Science* 301 (2003) 1925-1928.
- [51] R.S. Allan, J. Waithman, S. Bedoui, C.M. Jones, J.A. Villadangos, Y. Zhan, A.M. Lew, K. Shortman, W.R. Heath, F.R. Carbone, Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming, *Immunity* 25 (2006) 153-162.

- [52] R.N. Lausch, S.H. Chen, T.M. Tumpey, Y.H. Su, J.E. Oakes, Early cytokine synthesis in the excised mouse cornea, *J Interferon Cytokine Res* 16 (1996) 35-40.
- [53] H.F. Staats, R.N. Lausch, Cytokine expression in vivo during murine herpetic stromal keratitis. Effect of protective antibody therapy, *J Immunol* 151 (1993) 277-283.
- [54] A.D. Kwong, J.A. Kruper, N. Frenkel, Herpes simplex virus virion host shutoff function, *J Virol* 62 (1988) 912-921.
- [55] S. Kanangat, J.S. Babu, D.M. Knipe, B.T. Rouse, HSV-1-mediated modulation of cytokine gene expression in a permissive cell line: selective upregulation of IL-6 gene expression, *Virology* 219 (1996) 295-300.
- [56] M.T. Tran, D.A. Dean, R.N. Lausch, J.E. Oakes, Membranes of herpes simplex virus type-1-infected human corneal epithelial cells are not permeabilized to macromolecules and therefore do not release IL-1alpha, *Virology* 244 (1998) 74-78.
- [57] S.R. Paludan, Requirements for the induction of interleukin-6 by herpes simplex virus-infected leukocytes, *J Virol* 75 (2001) 8008-8015.
- [58] T.M. Tumpey, H. Cheng, D.N. Cook, O. Smithies, J.E. Oakes, R.N. Lausch, Absence of macrophage inflammatory protein-1alpha prevents the development of blinding herpes stromal keratitis, *J Virol* 72 (1998) 3705-3710.
- [59] P.S. Biswas, K. Banerjee, B. Kim, B.T. Rouse, Mice transgenic for IL-1 receptor antagonist protein are resistant to herpetic stromal keratitis: possible role for IL-1 in herpetic stromal keratitis pathogenesis, *J Immunol* 172 (2004) 3736-3744.

- [60] X.T. Yan, T.M. Tumpey, S.L. Kunkel, J.E. Oakes, R.N. Lausch, Role of MIP-2 in neutrophil migration and tissue injury in the herpes simplex virus-1-infected cornea, *Invest Ophthalmol Vis Sci* 39 (1998) 1854-1862.
- [61] R.R. Fenton, S. Molesworth-Kenyon, J.E. Oakes, R.N. Lausch, Linkage of IL-6 with neutrophil chemoattractant expression in virus-induced ocular inflammation, *Invest Ophthalmol Vis Sci* 43 (2002) 737-743.
- [62] H. Minagawa, K. Hashimoto, Y. Yanagi, Absence of tumour necrosis factor facilitates primary and recurrent herpes simplex virus-1 infections, *J Gen Virol* 85 (2004) 343-347.
- [63] T.L. Keadle, N. Usui, K.A. Laycock, J.K. Miller, J.S. Pepose, P.M. Stuart, IL-1 and TNF-alpha are important factors in the pathogenesis of murine recurrent herpetic stromal keratitis, *Invest Ophthalmol Vis Sci* 41 (2000) 96-102.
- [64] S. Kanangat, J. Thomas, S. Gangappa, J.S. Babu, B.T. Rouse, Herpes simplex virus type 1-mediated up-regulation of IL-12 (p40) mRNA expression. Implications in immunopathogenesis and protection, *J Immunol* 156 (1996) 1110-1116.
- [65] Y. Osorio, S.L. Wechsler, A.B. Nesburn, H. Ghiasi, Reduced severity of HSV-1-induced corneal scarring in IL-12-deficient mice, *Virus Res* 90 (2002) 317-326.
- [66] U. Kumaraguru, B.T. Rouse, The IL-12 response to herpes simplex virus is mainly a paracrine response of reactive inflammatory cells, *J Leukoc Biol* 72 (2002) 564-570.
- [67] R.J. Lund, Z. Chen, J. Scheinin, R. Lahesmaa, Early target genes of IL-12 and STAT4 signaling in th cells, *J Immunol* 172 (2004) 6775-6782.

- [68] Q. Tang, R.L. Hendricks, Interferon gamma regulates platelet endothelial cell adhesion molecule 1 expression and neutrophil infiltration into herpes simplex virus-infected mouse corneas, *J Exp Med* 184 (1996) 1435-1447.
- [69] J. Yannariello-brown, C.K. Hallberg, H. Haberle, M.M. Brysk, Z. Jiang, J.A. Patel, P.B. Ernst, S.D. Trocme, Cytokine modulation of human corneal epithelial cell ICAM-1 (CD54) expression, *Exp Eye Res* 67 (1998) 383-393.
- [70] J.C. Mester, B.T. Rouse, The mouse model and understanding immunity to herpes simplex virus, *Rev Infect Dis* 13 Suppl 11 (1991) S935-945.
- [71] S.K. Seo, B.M. Gebhardt, H.Y. Lim, S.W. Kang, S. Higaki, E.D. Varnell, J.M. Hill, H.E. Kaufman, B.S. Kwon, Murine keratocytes function as antigen-presenting cells, *Eur J Immunol* 31 (2001) 3318-3328.
- [72] S. Lee, M. Zheng, S. Deshpande, S.K. Eo, T.A. Hamilton, B.T. Rouse, IL-12 suppresses the expression of ocular immunoinflammatory lesions by effects on angiogenesis, *J Leukoc Biol* 71 (2002) 469-476.
- [73] J. Witowski, K. Ksiazek, A. Jorres, Interleukin-17: a mediator of inflammatory responses, *Cell Mol Life Sci* 61 (2004) 567-579.
- [74] J. Maertzdorf, A.D. Osterhaus, G.M. Verjans, IL-17 expression in human herpetic stromal keratitis: modulatory effects on chemokine production by corneal fibroblasts, *J Immunol* 169 (2002) 5897-5903.
- [75] S.J. Molesworth-Kenyon, R. Yin, J.E. Oakes, R.N. Lausch, IL-17 receptor signaling influences virus-induced corneal inflammation, *J Leukoc Biol* 83 (2008) 401-408.
- [76] M. Fukata, K. Breglio, A. Chen, A.S. Vamadevan, T. Goo, D. Hsu, D. Conduah, R. Xu, M.T. Abreu, The myeloid differentiation factor 88 (MyD88) is

required for CD4⁺ T cell effector function in a murine model of inflammatory bowel disease, *J Immunol* 180 (2008) 1886-1894.

[77] B. Kim, P.P. Sarangi, A.K. Azkur, S.D. Kaistha, B.T. Rouse, Enhanced viral immunoinflammatory lesions in mice lacking IL-23 responses, *Microbes and Infection* In press (2007).

[78] X.T. Yan, M. Zhuang, J.E. Oakes, R.N. Lausch, Autocrine action of IL-10 suppresses proinflammatory mediators and inflammation in the HSV-1-infected cornea, *J Leukoc Biol* 69 (2001) 149-157.

[79] J.S. Babu, S. Kanangat, B.T. Rouse, T cell cytokine mRNA expression during the course of the immunopathologic ocular disease herpetic stromal keratitis, *J Immunol* 154 (1995) 4822-4829.

[80] J. Thomas, S. Kanangat, B.T. Rouse, Herpes simplex virus replication-induced expression of chemokines and proinflammatory cytokines in the eye: implications in herpetic stromal keratitis, *J Interferon Cytokine Res* 18 (1998) 681-690.

[81] Y.H. Su, X.T. Yan, J.E. Oakes, R.N. Lausch, Protective antibody therapy is associated with reduced chemokine transcripts in herpes simplex virus type 1 corneal infection, *J Virol* 70 (1996) 1277-1281.

[82] J. Shirane, T. Nakayama, D. Nagakubo, D. Izawa, K. Hieshima, Y. Shimomura, O. Yoshie, Corneal epithelial cells and stromal keratocytes efficiently produce CC chemokine-ligand 20 (CCL20) and attract cells expressing its receptor CCR6 in mouse herpetic stromal keratitis, *Curr Eye Res* 28 (2004) 297-306.

- [83] M. Zheng, M.A. Schwarz, S. Lee, U. Kumaraguru, B.T. Rouse, Control of stromal keratitis by inhibition of neovascularization, *Am J Pathol* 159 (2001) 1021-1029.
- [84] M. Zheng, S. Deshpande, S. Lee, N. Ferrara, B.T. Rouse, Contribution of vascular endothelial growth factor in the neovascularization process during the pathogenesis of herpetic stromal keratitis, *J Virol* 75 (2001) 9828-9835.
- [85] K. Banerjee, P.S. Biswas, B. Kim, S. Lee, B.T. Rouse, CXCR2^{-/-} mice show enhanced susceptibility to herpetic stromal keratitis: a role for IL-6-induced neovascularization, *J Immunol* 172 (2004) 1237-1245.
- [86] P. Salven, K. Hattori, B. Heissig, S. Rafii, Interleukin-1alpha promotes angiogenesis in vivo via VEGFR-2 pathway by inducing inflammatory cell VEGF synthesis and secretion, *FASEB J* 16 (2002) 1471-1473.
- [87] Y. Saijo, M. Tanaka, M. Miki, K. Usui, T. Suzuki, M. Maemondo, X. Hong, R. Tazawa, T. Kikuchi, K. Matsushima, T. Nukiwa, Proinflammatory cytokine IL-1 beta promotes tumor growth of Lewis lung carcinoma by induction of angiogenic factors: in vivo analysis of tumor-stromal interaction, *J Immunol* 169 (2002) 469-475.
- [88] H. Nakahara, J. Song, M. Sugimoto, K. Hagihara, T. Kishimoto, K. Yoshizaki, N. Nishimoto, Anti-interleukin-6 receptor antibody therapy reduces vascular endothelial growth factor production in rheumatoid arthritis, *Arthritis Rheum* 48 (2003) 1521-1529.
- [89] M. Zheng, D.M. Klinman, M. Gierynska, B.T. Rouse, DNA containing CpG motifs induces angiogenesis, *Proc Natl Acad Sci U S A* 99 (2002) 8944-8949.

- [90] D.Q. Li, B.L. Lokeshwar, A. Solomon, D. Monroy, Z. Ji, S.C. Pflugfelder, Regulation of MMP-9 production by human corneal epithelial cells, *Exp Eye Res* 73 (2001) 449-459.
- [91] D.A. Arenberg, P.J. Polverini, S.L. Kunkel, A. Shanafelt, J. Hesselgesser, R. Horuk, R.M. Strieter, The role of CXC chemokines in the regulation of angiogenesis in non-small cell lung cancer, *J Leukoc Biol* 62 (1997) 554-562.
- [92] B. Kim, S. Lee, S. Suvas, B.T. Rouse, Application of plasmid DNA encoding IL-18 diminishes development of herpetic stromal keratitis by antiangiogenic effects, *J Immunol* 175 (2005) 509-516.
- [93] S.P. Deshpande, M. Zheng, S. Lee, B.T. Rouse, Mechanisms of pathogenesis in herpetic immunoinflammatory ocular lesions, *Vet Microbiol* 86 (2002) 17-26.
- [94] K. Banerjee, P.S. Biswas, B.T. Rouse, Elucidating the protective and pathologic T cell species in the virus-induced corneal immunoinflammatory condition herpetic stromal keratitis, *J Leukoc Biol* 77 (2005) 24-32.
- [95] S. Suvas, A.K. Azkur, B.S. Kim, U. Kumaraguru, B.T. Rouse, CD4⁺CD25⁺ regulatory T cells control the severity of viral immunoinflammatory lesions, *J Immunol* 172 (2004) 4123-4132.
- [96] S. Gangappa, S.P. Deshpande, B.T. Rouse, Bystander activation of CD4(+) T cells can represent an exclusive means of immunopathology in a virus infection, *Eur J Immunol* 29 (1999) 3674-3682.
- [97] S. Gangappa, S.P. Deshpande, B.T. Rouse, Bystander activation of CD4⁺ T cells accounts for herpetic ocular lesions, *Invest Ophthalmol Vis Sci* 41 (2000) 453-459.

- [98] Z.S. Zhao, F. Granucci, L. Yeh, P.A. Schaffer, H. Cantor, Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection, *Science* 279 (1998) 1344-1347.
- [99] S. Deshpande, M. Zheng, S. Lee, K. Banerjee, S. Gangappa, U. Kumaraguru, B.T. Rouse, Bystander activation involving T lymphocytes in herpetic stromal keratitis, *J Immunol* 167 (2001) 2902-2910.
- [100] K. Banerjee, S. Deshpande, M. Zheng, U. Kumaraguru, S.P. Schoenberger, B.T. Rouse, Herpetic stromal keratitis in the absence of viral antigen recognition, *Cell Immunol* 219 (2002) 108-118.
- [101] S. Gangappa, J.S. Babu, J. Thomas, M. Daheshia, B.T. Rouse, Virus-induced immunoinflammatory lesions in the absence of viral antigen recognition, *J Immunol* 161 (1998) 4289-4300.
- [102] B.T. Rouse, Regulatory T cells in health and disease, *J Intern Med* 262 (2007) 78-95.
- [103] L.W. Collison, C.J. Workman, T.T. Kuo, K. Boyd, Y. Wang, K.M. Vignali, R. Cross, D. Sehy, R.S. Blumberg, D.A. Vignali, The inhibitory cytokine IL-35 contributes to regulatory T-cell function, *Nature* 450 (2007) 566-569.
- [104] P.P. Sarangi, S. Sehrawat, S. Suvas, B. Kim, B.T. Rouse, IL-10 and natural regulatory T cells: two independent anti inflammatory mechanisms in HSV induced ocular immunopathology, *Immunology-2007*, Miami, FL, 2007.
- [105] M. Seki, S. Oomizu, K.M. Sakata, A. Sakata, T. Arikawa, K. Watanabe, K. Ito, K. Takeshita, T. Niki, N. Saita, N. Nishi, A. Yamauchi, S. Katoh, A. Matsukawa, V. Kuchroo, M. Hirashima, Galectin-9 suppresses the generation of

Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis, Clin Immunol (2008).

[106] M.J. Benson, K. Pino-Lagos, M. Roseblatt, R.J. Noelle, All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation, J Exp Med 204 (2007) 1765-1774.

[107] S. Sehrawat, B.T. Rouse, Anti-Inflammatory Effects of FTY720 Against Viral Induced Immunopathology: Role of Drug Induced Conversion of T cells to Become Foxp3⁺ Regulators, Keystone Symposia, Keystone, Colorado, 2008.

APPENDIX

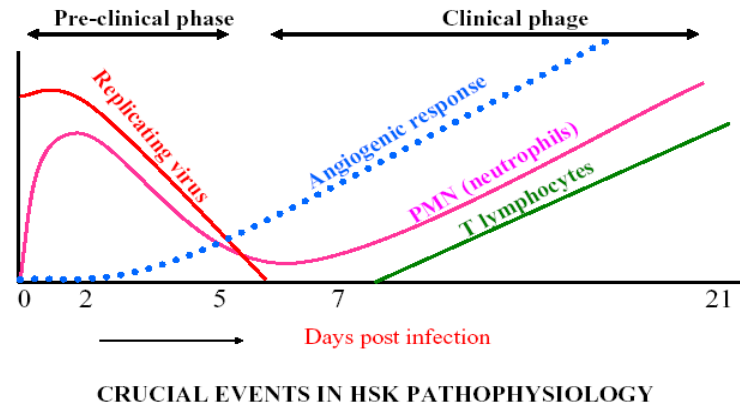


Figure. 1. Principal events in herpetic SK pathogenesis

Following ocular HSV infection replicating virus could be detected in the cornea till 5-7 days p.i. Early inflammatory response in the cornea is dominated by the polymorphonuclear leukocytes (PMN). Infiltration of PMNs into the cornea is characterized by a typical biphasic influx. Angiogenesis or the process of new blood vessel development from the existing limbal vessels starts at 24h p.i. and peaks around 15 days p.i. Influx of pathogenic CD4+ T lymphocytes occurs in the clinical phase around 7-9 days p.i.

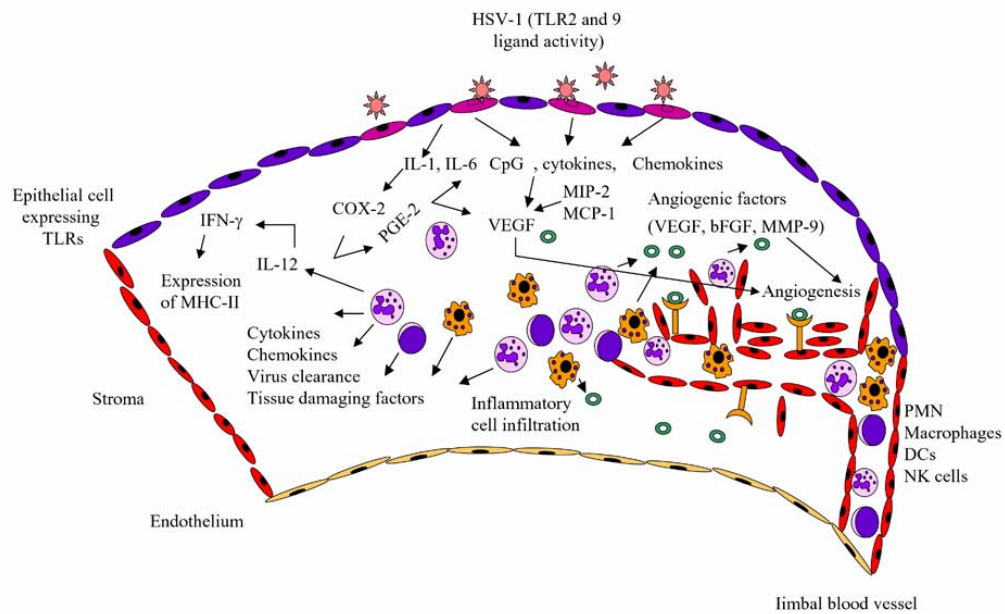


Figure. 2 Schematic representation of the early events in the SK cornea

HSV-1 with its TLR 2 and TLR9 ligand activity induces numerous proinflammatory cytokine and chemokines early after infection of corneal epithelium. These cytokines and chemokines in turn help in the influx of inflammatory cells such as PMNs, macrophages, NK cells and dendritic cells into the infected corneas. Presence of inflammatory cells and the production of more inflammatory mediators and the angiogenic factors help in creating favorable conditions for the subsequent events that lead to the clinical phase. Development of new blood vessels from the exiting limbal vessels also helps in the influx of inflammatory cells into the corneal stroma in the clinical phase of SK.

Part-II

**Innate recognition network driving the HSV
induced corneal immunopathology: role of toll
pathway in the early inflammatory events in
stromal keratitis**

Research described in this chapter is a modified version of an article published in 2007 in *The Journal of Virology* by Pranita P Sarangi, Bumseok Kim, Evelyn Kurt-Jones and Barry T Rouse.

Sarangi PP, Kim B., Kurt-Jones E, Rouse BT. Innate recognition network driving the HSV induced corneal immunopathology: Role of Toll Pathway in the early inflammatory events in Stromal Keratitis. *J Virol.* 2007. Oct; 81(20):11128-3.

Copyright©2007, American Society for Microbiology

In this chapter “our” and “we” refers to me and co-authors. My contribution in the paper includes (1) Selection of the topic (2) Compiling and interpretation of the literature (3) Designing experiments (4) understanding the literature and interpretation of the results (5) providing comprehensible structure to the paper (6) Preparation of graphs, figures and tables (7) Writing and editing

Abstract

Ocular infection with herpes simplex virus (HSV) sets off an array of events that succeed in clearing virus from the cornea but leaves the tissue with a CD4+ T cell orchestrated chronic inflammatory lesion that impairs vision. We demonstrate that Toll-like receptor (TLR) signaling forms a part of the recognition system that induces the syndrome that eventually culminates in immunopathology. Accordingly, in a comparison of the outcome of the infection in wild-type (WT) mice and those lacking TLR function it was apparent that the absence of TLR2, and to a lesser extent TLR9, resulted in significantly diminished lesions. Similarly mice lacking the adapter molecule MyD88 were resistant to lesion development but such animals were also unable to control infection with most succumbing to

lethal encephalitis. The susceptibility of TLR4^{-/-} animals was also evaluated. These animals developed lesions more rapidly that were more severe than in WT animals. We discuss the possible mechanisms by which early recognition of HSV constituents impact on the subsequent development of immunopathological lesions.

Introduction

Viruses induce several immediate host reactive events that impact on the long term consequences of infection. For example, ocular infection of mice with herpes simplex virus (HSV) causes a multitude of cytokine, chemokine and cellular changes to occur [1]. Manipulating the expression of many of these affects the severity of the subsequent vision impairing consequences such as neovascularization and chronic inflammation [2-5]. The latter reaction is immunopathological set off initially by infection, but sustained well after replication has ceased and viral antigens are removed [1]. These stromal keratitis (SK) reactions occur in response to HSV in humans and represent the commonest cause of infectious blindness in developed countries [6].

We know that viruses and their components can trigger multiple innate immune protective and inflammatory responses [7]. These include interferon induction, the production of multiple cytokines, and activation of complement as well as innate cellular defenses [7, 8]. Recognition mechanisms that initiate and control innate reactions to viruses remain poorly understood but accumulating evidence indicates that membrane bound cell surface or intracellular toll-like receptors (TLRs) form part of the surveillance system [9]. After the initial observation with respiratory syncytial virus [10], several viruses were shown to

express ligand activities for one or more TLRs [9]. Infection could also result in the release and induction of host molecules such as heat shock proteins that may themselves activate TLRs and so contribute to the innate response [11, 12]. The observation that TLRs participate during HSV infection came from several lines of investigation. Initially purified HSV DNA like synthetic CpG DNA, the ligand for TLR9 [13], was shown to induce ocular neovascularization, an essential step in the pathogenesis of SK [14]. HSV DNA was later confirmed to possess TLR9 ligand activity [15] and studies in knockout mice revealed a role for TLR9 in HSV induced type I interferon production [13]. Other investigations demonstrated that HSV also possesses TLR2 activity [16] with mice lacking TLR2 showing higher resistance to lethal HSV encephalitis than WT animals [16]. This perhaps unexpected observation that brain inflammation and death were reduced in the absence of TLR2 (but virus levels were similar in WT and TLR2^{-/-} mice) indicated that encephalitis, the usual cause of death with HSV infection, was the consequence of the virus acting as a TLR2 ligand driving an inflammatory reaction in the brain [16]. In addition to inflammatory processes, herpetic encephalitis, especially in humans may represent a direct destructive effect of the virus [17] and even in mice neurovirulence appears associated with the success of virus gaining access to and replicating in the CNS [18]. Thus both viral and host components may contribute to disease pathogenesis.

In the present report, we measured the influence of TLR2 and other potential TLR ligand effects of HSV in a corneal scarification disease model where lesion expression is widely agreed to represent a host immunopathological response [1]. In this model, animals lacking lymphocytes fail to develop SK

lesions and in fact die of herpetic encephalitis with an abundance of virus in the brain [19]. We examined the sequence of events that occurred following infection of different TLR knockout mice compared to WT animals. Our results demonstrate that TLR2^{-/-}, MyD88^{-/-} and to a lesser extent TLR9^{-/-}, but not TLR4^{-/-}, mice showed significantly reduced SK lesions upon HSV infection. Similarly, TLR2^{-/-} and MyD88^{-/-} mice also showed reduced neovascularization as compared to other groups. These latter strains also showed marked reduction in early non lymphoid inflammatory infiltrates as well as lower levels of proinflammatory cytokines compared to WT or TLR4^{-/-} animals. In addition, whereas both TLR2^{-/-} and TLR9^{-/-} animals resisted the lethal effects of HSV, most MyD88^{-/-} animals infected with the same dose succumbed to lethal disease. The TLR4^{-/-} animals developed lesions more rapidly that were more severe than in WT animals. We discuss the possible mechanisms by which early recognition of HSV constituents impacts on the subsequent development of immunopathological lesions.

Materials and methods

Animals.

TLR2^{-/-}, TLR4^{-/-}, TLR9^{-/-}, TLR2/4d^{-/-}, MyD88^{-/-} mice were the kind gift of Dr S Akira, (Osaka University, Osaka) and were backbred for more than 10 generations onto the C57BL/6 background. Backbreeding was confirmed by examining a 110 marker panel that distinguishes between C57BL/6 and 129 (Charles River Laboratories). These microsatellite markers are spread across the 19 autosomes and the X chromosome at approximately 15cM intervals. C57BL/6 mice were obtained from Harlan Sprague-Dawley, (Indianapolis IN). All

experimental procedures followed the guidelines the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

Corneal HSV-1 infection and clinical observation

HSV-1 strain RE was used in all procedures. Details of corneal infections of mice under deep anesthesia were described in a previous publication [20]. Animals were infected with a range of virus doses although most of the reported experiments mice were infected with 5×10^3 pfu/eye. The experiments were repeated multiple times especially the comparison of TLR2-/- with control animals. Lesion scores and the extent of angiogenesis were measured at various times post infection as described in detail previously [21, 22].

Histopathology

For histopathologic analysis, eyes from different groups of mice were extirpated at the indicated time point p.i. and snap-frozen in OCT compound (Miles, Elkart, IN). 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).

Flow Cytometry.

For flow cytometry measurement of the infiltrating cells, four corneas or two trigeminal ganglia (TGs) were collected per time point. Corneas or TGs were digested in liberase (Roche Diagnostics Co., Indianapolis, IN) for 45 min at 37°C. Single cell suspension was prepared as described elsewhere [23]. The Fc receptors were blocked with unconjugated anti CD16/32 (BD Pharmingen, San Diego, CA) for 30 min. Samples were incubated with fluorescein isothiocyanate (FITC) labeled anti Gr-1, anti CD11b, anti CD11c (BD Pharmingen), F4/80 (eBioscience),

or anti CD4 (BD Pharmingen), antibody for 30 min. For intracellular staining for (Cyclooxygenase-2) COX-2, goat anti COX-2 and Donkey anti goat IgG were used as primary and secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) respectively. For detection of induced Hsp-70, FITC conjugated Hsp70 (Hsp72) antibody was obtained from Stressgen (Ann Arbor, Michigan). FITC conjugated Rat IgG was used as isotype control for Hsp-70. Intracellular staining for COX-2 and Hsp70 was performed using Fixation/Permeabilization Solution Kit with BD GolgiPlug (BD Bioscience). All samples were collected on a FACScan (BD Biosciences, San Diego, CA), and data were analyzed by using cell Quest 3.1 software (BD Biosciences).

Cytokine and chemokine ELISA of corneal lysate

For preparation of corneal lysates, six corneas per indicated time point were pooled from each group of mice and were processed as described previously [20]. For draining lymph nodes (DLNs), cervical lymph nodes from each mouse were collected and processed. Levels of IL-6, IL-1 β , Macrophage Inflammatory Protein-2 (MIP-2) and Vascular Endothelial Growth Factor (VEGF), IL-10 in corneal lysates and IL-10, IL-6, IFN- γ in DLN lysates were determined by a standard sandwich enzyme-linked immunosorbent assay (ELISA) protocol. Anti IL-6, anti IL-10, anti IFN- γ capture and detection antibodies were purchased from BD Pharmingen. Anti IL-1 β , anti-MIP-2, anti VEGF capture and biotinylated detection antibodies were purchased from R&D Systems (Minneapolis, MN). The color reaction was developed using ABTS (Sigma-Aldrich, St. Louis, MO) and measured with an ELISA reader (Spectramax 340; Molecular Devices, Sunnyvale,

CA) at 405 nm. Quantification was performed with Spectramax ELISA reader software version 1.2.

Reverse Transcription and real time PCR

Total RNA from naïve and infected C57BL/6 corneas and DC2.4 cell line were isolated at different days p.i. by using RNAeasy kit (QIAGEN, RNeasy MinElute Cleanup Kit) and was reverse transcribed by using Murine Leukemia Virus reverse transcriptase (Life Technologies, Bethesda, MD) with oligo (dT) as the primer (Invitrogen, San Diego, CA). All cDNA were divided into aliquots and stored at -20°C until further use. All murine DCs express TLR2, 4 and 9 as did the DC2.4 cell line (Dendritic cell line, obtained from Dr K.L. Rock). PCR was performed in a PTC-100 programmable thermal controller (MJ Research, Cambridge, MA) using Hot start PCR Master mix (Promega, Madison, WI). The primers used were murine GAPDH forward (CATCCTGCACCACCAACTGCTTAG) and reverse (GCCTGCTTCACCACCTTCTTGATG), murine TLR2 forward (AAGAGGAAGCCCAAGAAAGC) and reverse (CGATGGAATCGATGATGTTG), murine TLR4 forward (ACCTGGCTGGTTTACACGTC) and reverse (CTGCCAGAGACATTGCAGAA), murine TLR9 forward (ACTGAGCACCCCTGCTTCTA) and reverse (AGATTAGTCAGCGGCAGGAA).

Real-time PCR was performed using an iQ5 Real-Time PCR Detection System (Bio-Rad). PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems), according to manufacturer's protocol. PCR amplification of housekeeping gene, murine HPRT, was done for each sample as a control for

sample loading and to allow normalization between samples. The primers used were murine HPRT forward (GACCGGTCCCGTCATGC) and reverse (TCATAACCTGGTTCATCATCGC), murine β -defensin 2 (mBD-2) forward (TATGCTGCCTCCTTTTCTCA) and reverse (GACTTCCATGTGCTTCCTTC), murine β -defensin 3 (mBD-3) forward (CTCCTGGTGCTGCTGTCTCCAC) and reverse (AACTCCACAAGTGCCAATCTGA).

Virus recovery and titrations

Eye swabs were taken from infected corneas (four eyes/group) using sterile swabs at the indicated time points. Similarly, brain tissue and TG were taken out from the infected animals at the indicated time points (N=3). The tissues were disrupted by homogenization in 1 ml ground glass grinders (Wheaton) and centrifuged, and the supernatant was used to assay viral titers. and was the titration was performed by standard plaque assay as described previously [20]. Titers were calculated as log₁₀ pfu/ml as per standard protocol [24].

Statistical Analysis

Statistical significance was determined by Student's *t* test. $P < 0.05$ was regarded as a significant difference between the groups.

Results

Expression of TLRs and TLR ligands in the naive and infected corneas

Since HSV replication in the eye occurs almost exclusively in corneal epithelial cells [1], the expression of TLRs in corneal buttons taken from uninfected eyes was examined by RT-PCR for the presence of TLR2, 4 and 9 mRNA. All could be demonstrated (Fig.1A). Whereas previous reports had shown that both TLR2 and TLR9 activating ligands are expressed by HSV [25],

endogenous TLR ligands might also be induced and released from dead or dying cells following infection [11, 12]. As shown in Fig1B, the TLR4 ligand, inducible Hsp70 [11] could be demonstrated in corneal tissues in samples analyzed at 24 h post HSV infection. Similarly, as shown in Fig1C, both β -defensin2 and β -defensin3 mRNA expression was increased in the infected as compared to the naïve corneas of which and β -defensin3 has been shown to trigger TLR4 [26]. Hence TLR4 as well as previously demonstrated TLR2 and TLR9 ligand effects could be involved in the recognition events following ocular HSV infection.

Disease expression following HSV infection in control and knockout mice

To determine the role of TLRs in HSV pathogenesis in the eye, TLR knockout mice that were fully backbred onto the C57BL/6 background were ocularly infected with different doses of HSV-1 RE and evaluated over a 15-20 day time period to quantify the extent of corneal neovascularization as well as the severity of SK. At infection doses of 2×10^5 pfu/eye or above all groups developed lesions and neovascularization responses that were similar in magnitude. However, significantly different responses in the various groups became evident at infection doses of 2×10^4 and below. Fig. 2 shows the results of 1 of 2 experiments in which the animals were infected with 5×10^3 pfu virus. As is evident, at both days 8 and 15 p.i., both the TLR2^{-/-} and MyD88^{-/-} animals showed significantly diminished lesions as well as neovascularization compared to WT animals. For instance, where as almost 90% of control WT animals developed SK lesions with a severity score of 3 or greater and neovascularization scores of 8 or greater, in TLR2^{-/-} and MyD88^{-/-} animals only around 30% of animals had such severity scores. Moreover at the peak response time (day15 p.i.) the mean

severity scores in TLR2^{-/-} and MyD88^{-/-} were 1.5 ± 0.37 and 1.8 ± 0.4 respectively which were significantly ($p \leq 0.005$) less than those observed in WT mice (3.8 ± 0.26). On average the mean severity of SK in TLR2^{-/-} was 60% reduced over controls at day 8 p.i. and was further reduced to 65% by day 15 p.i. These experiments indicate that animals unable to respond to the TLR2 ligand activity of HSV showed a resistant phenotype. However curiously, both TLR2^{-/-} and MyD88^{-/-} animals often showed quite severe herpetic periocular disease, a lesion seldom seen in the WT animals at the low dose of virus used.

Animals lacking TLR9 also showed more resistance to HSV induced SK and neovascularization than WT animals although differences were modest and only significantly reduced with the 15 day scores (2.7 ± 0.38 compared to 3.8 ± 0.26 , $p=0.03$) (Fig. 2). Most of the TLR9^{-/-} animals also developed herpetic periocular disease but lesions were less intense than those noted in TLR2^{-/-} and MyD88^{-/-} animals.

The pattern of response in TLR4^{-/-} animals revealed a modestly more susceptible phenotype. Thus by day 8 p.i., 30% of TLR4^{-/-} showed level 3 lesions (mean, 2.3 ± 0.13) whereas almost all WT animals lacked lesions at this time point (mean, 1.2 ± 0.15 , $p = 0.004$). The neovascularization score reflected a similar heightened susceptibility of TLR4^{-/-} (mean, 7 ± 0.5) compared to WT animals (mean, 4.5 ± 0.4 , $p=0.009$) (Fig. 2). Curiously, animals lacking both TLR2 and 4 responsiveness showed a resistance phenotype although this was less in magnitude compared to TLR2 or MyD88 knockout animals.

MyD88 is an essential downstream adapter of TLR signaling, particularly for TLR2 and TLR9 responses [27]. TLR4 also signals via MyD88 but can

activate an alternate pathway of MyD88-independent gene expression [28]. Interestingly, although the MyD88^{-/-} animals showed a resistant phenotype to disease expression similar to the TLR2^{-/-} mice, the MyD88^{-/-} animals were far more susceptible to the lethal effects of HSV infection. As shown in table. 1, even at the 5×10^3 pfu dose, up to 70 % of the infected MyD88^{-/-} animals were dying by day15 p.i. although almost 60 % of them showed no signs of clinical disease. The animals were presumed to be dying from viral encephalitis and replicating virus could be demonstrated in the homogenates of brain tissue harvested from moribund MyD88^{-/-} animals (Table. 2). Thus, eliminating signaling through multiple TLRs by knocking out MyD88 reduces local inflammation but may allow enhanced viral replication and/or spread, while eliminating single TLRs, i.e., TLR2 or TLR9, ameliorates local immune pathology but these receptors may be redundant for control of virus replication/spread.

As shown in table. 1, both at 2×10^4 pfu/eye and 5×10^3 pfu/eye of infection, neither TLR2^{-/-} nor TLR9^{-/-} animals were more susceptible to lethal infection as were WT animals. In addition to brain and TGs, virus titers were also measured in eye swabs collected from infected cornea. As shown in table3, in contrast to CNS, virus titers did not increase in the absence of TLRs or MyD88^{-/-} in the corneas and all of the groups could clear the virus efficiently by day7 p.i. Our data confirms the previous finding by others that MyD88 independent antiviral mechanisms can effectively compensate for the absence of MyD88 in a localized HSV-1 infection [13]

Cellular differences between control and knock out animals

Soon after HSV infection of the corneal epithelium, cytokine expression and cellular changes occur [1]. One prominent event is infiltration in the underlying stroma by mainly neutrophils [29]. The stimuli that induce such responses remain to be identified but they appear to include chemokines such as MIP-2 and MCP-1 induced directly or indirectly by HSV infection in the corneal epithelium [1, 30]. Additionally, angiogenic molecules such as VEGF and IL-6 are induced both of which play a role at causing the neovascularization of the cornea [20, 31]. To compare the initial inflammatory response in knockout versus WT control mice, tissue sections were examined. As is evident in Fig. 3, diminished cell infiltrates at 48 h were apparent in TLR2^{-/-}, MyD88^{-/-} as well as in TLR9^{-/-} sections compared to WT controls. In the latter the inflammatory infiltrates had fully accessed the central cornea, but in both TLR2^{-/-} and TLR9^{-/-} the infiltrates were largely confined to the limbal region. Of these two TLR knockouts, the infiltration of cells in TLR2^{-/-} was far less than in TLR9^{-/-} animals. Of interest, the inflammatory events in TLR4^{-/-} animals appeared slightly elevated as compared to WT. This more severe reaction was also more evident by flow cytometric analysis of Gr1⁺ and CD11b⁺ cells in the infected corneas (Fig. 3).

It has been documented by past studies that after ocular infection with HSV, replicating virus could be detected in the TG for 2-7 days p.i. [32]. The presence of virus in the TG induces a chronic inflammatory reaction involving multiple cell types that is responsible for controlling the virus load as well as to restricting the further spread of the virus [32]. Therefore, we examined the cells from the TG of different groups at 48 h and 96 h p.i. by flow cytometry. A

diminished inflammatory lesion was observed in the infected TGs in the TLR2^{-/-} and MyD88^{-/-} as compared to wild type. As shown in Fig. 4, infiltration of Gr1⁺, CD11b⁺, CD11c⁺ and F4/80⁺ cells was reduced in the TLR2^{-/-} and MyD88^{-/-} animals. Interestingly, similar to the cornea, an increased inflammatory cell number was noticed in the TLR4^{-/-} animals as compared to WT controls.

Cytokine and other proinflammatory protein differences between control and knock out animals

A likely explanation for the reduced influx of inflammatory cells and eventual resistance to disease expression in TLR2^{-/-} (and MyD88^{-/-}) mice could be a reduced proinflammatory cytokine/chemokine response to infection. Lysates of corneas collected at 48 h and 96 h p.i. were used to measure the levels of candidate molecules shown in previous studies to correlate with the outcome of ocular HSV infection [1]. These were IL-6, MIP-2, and IL-1 β , VEGF as well as the enzyme COX-2. As shown in Fig. 5, compared to WT, a three- to four-fold reduction in the levels of these proinflammatory mediators was evident in the lysates of TLR2^{-/-} and MyD88^{-/-} corneas at 48 h and 96 h p.i. IL-6 and IL-1 β , produced from virus infected cells were shown to stimulate noninfected resident corneal cells and other inflammatory cells in a paracrine manner to secrete VEGF, a potent angiogenic factor [1]. Interestingly, a marked reduction was noticed in the level of IL-1 β and IL-6 and VEGF in the TLR2^{-/-}, MyD88^{-/-} mice. The TLR9^{-/-} animals had a moderate decrease in inflammatory mediators but this was only evident at the 48 h time point. In contrast, TLR4^{-/-} animals had slightly increased levels (20-30 % elevated) of inflammatory mediators as compared to the WT controls at all time points examined.

In two separate experiments the level of IL-10 induction in TLR4^{-/-} mice was compared to WT animals in both corneal extracts and the cervical DLN at day2 and day5 after infection. As is evident in Fig. 6A, levels of IL-10 in the corneas of TLR4^{-/-} mice were less than observed in WT animals at both time points accounting perhaps for the more severe reactions seen in TLR4^{-/-} animals. Although significant differences were not observed in the levels of IL-10 in DLN at these time points (Fig. 6B), the levels of proinflammatory and Th1 cytokine, IL-6 and IFN- γ were significantly higher ($p \leq 0.05$) in TLR4^{-/-} animals as compared to WT (Fig. 6C and D). Interestingly, no difference was observed in the IL-10 levels in the DLNs of TLR2^{-/-} animals although the levels of IL-6 and IFN- γ were lower in TLR2^{-/-} as compared to WT animals (data not shown).

Previous studies had showed that, HSV infection of the cornea upregulates the expression of COX-2, a critical enzyme for the induction of potential inflammatory mediators such as prostaglandins [33, 34]. Moreover, the administration of a selective COX-2 inhibitor diminished virus induced SK lesions and neovascularization [35]. Recent *in vitro* findings had demonstrated that TLR2 signaling could induce COX-2 in peritoneal macrophages *in vitro* [36-38]. Therefore, we examined the role of TLR2 in HSV induced COX-2. Intracellular staining revealed over 50% reduction in the percentage of COX-2 expressing cells in HSV infected TLR2^{-/-} mice corneas as compared to the WT and TLR4^{-/-} mice (Fig. 7A). In addition, reduced numbers of COX-2⁺Gr1⁺ cells was evident in the TLR2^{-/-} corneas as compared to WT (Fig. 7B). Interestingly, although there was no difference in the percentage of COX-2 expressing cells in TLR4^{-/-} animals as compared to WT, such animals showed higher proportion of COX-2⁺Gr1⁺ cells as

compared to WT animals (Fig. 7A and B). These results may mean that lower induction of prostaglandins as well as chemokines could be a possible explanation of the resistant and susceptible phenotype in TLR2^{-/-} and TLR4^{-/-} mice respectively.

Discussion

Ocular infection with herpes simplex virus sets off an array of events that succeed in clearing virus from the cornea but leaves the tissue with a CD4⁺ T cell orchestrated chronic inflammatory lesion that impairs vision. We demonstrate that TLR signaling forms a part of the recognition system that sets off a syndrome that eventually culminates in immunopathology. Accordingly, in a comparison of the outcome of the infection in WT mice and those lacking TLR function it was apparent that the absence of TLR2, and to a lesser extent TLR9, resulted in significantly diminished lesions. Similarly, mice lacking the adapter molecule MyD88 were resistant to lesion development but such animals were also unable to control infection and most succumbed to lethal encephalitis. Animals lacking TLR4 developed lesions more rapidly and these became more severe than in WT animals. It could be that modulating TLRs in an appropriate way could represent an approach to control the severity of SK, an important cause of vision impairment.

Past studies had demonstrated that HSV expresses at least 2 TLR ligand activities [16]. These were for TLR2, perhaps a property of one of the envelope major glycoproteins [25], and the TLR9 ligand activity of its DNA [15]. In this report, we also show that HSV infection can cause cells to endogenously express at least two molecules that act as TLR ligands. These were BD3, a TLR4 ligand

[26], and inducible Hsp70 ligand for TLR4 and perhaps also for TLR2 [11]. Our investigations were designed to determine if the absence of TLR2, 4 and 9 impacted on the outcome of an immunopathological reaction in the eye that results from HSV infection.

The most dramatic effects were observed when TLR2^{-/-} mice were infected with a low dose of a virulent strain of HSV. Compared to WT animals, TLR2^{-/-} mice showed considerable resistance to disease expression. Fewer eyes showed clinical lesions, and those that did had reduced levels of neovascularization and stromal inflammatory reactions at both the early and peak times of the response. Reduced inflammatory reactions were also evident in the trigeminal ganglion, a site where virus locates soon after ocular infection and where virus persists when acute infection resolves [1]. Curiously, however, TLR2^{-/-} animals usually expressed notable periocular disease even when the eye itself exhibited minimal or no lesions. We interpret these data to mean that the TLR2 ligand activity of HSV may be a major mechanism by which the virus, usually confined to the epithelium of the infected cornea [1], induces early events necessary for the development of the inflammatory disease. As shown in previous studies, these include cytokines such as IL-6 and IL-1 β and TNF- α [1], the chemokine MIP-1 α , MIP-2 [1], enzymes such as COX-2 associated with the production of inflammatory molecules [1] and molecules responsible for neovascularization of the usually avascular eye such as VEGF and MMP9 [1]. In line with this, when levels of some of these molecules were measured early after infection, all were reduced in amount in TLR2^{-/-} compared to WT eyes. Evidence that cells from TLR2 negative cells make reduced inflammatory cytokine

responses to HSV was previously reported by others [16, 39]. Moreover, such reduced inflammatory reactions in the brain to HSV were advocated to explain why TLR2^{-/-} mice were less susceptible to HSV encephalitis than were WT controls [16]. Similarly in SK, the resistance phenotype of TLR2^{-/-} animals is likely explained by the reduced induction of key inflammatory mediators.

The observation that periocular disease was more severe in TLR2^{-/-} mice was less easy to explain especially since viral clearance kinetics in the eye itself between TLR2^{-/-} and WT showed no significant differences. Similarly, in the brain, Kurt-Jones et al, observed no major differences in viral clearance between control and resistant TLR2^{-/-} animals [16]. Since the periocular lesions appear to be caused by the virus itself, rather than being an immunopathological reaction, conceivably the diminished stimulation of local antiviral molecules such as interferons and perhaps others involved in the acute inflammatory response may explain the outcome in TLR2^{-/-} mice. We are further evaluating this issue.

Since HSV DNA is well known to act as a TLR9 ligand [13, 15], we anticipated that TLR9^{-/-} mice would also react differently to the ocular infection than WT animals. Such TLR9^{-/-} mice were in fact more refractory to lesion expression than WT, but this was only a modest difference. This may be explained by the possibility that the TLR9 ligand activity is inadequately produced under *in vivo* infection conditions to drive the neovascular and inflammatory response, or that any effect of TLR9 is largely overridden by the proinflammatory effect of the TLR2 ligand activity of the virus. We hope to obtain double knockout mice to help resolve these issues. Alternately, there could be reduced Th1 immune response in the absence of TLR9 which could help reduce the lesion severity. We are currently

investigating the generation of HSV specific immune responses in the absence of specific TLRs.

Previous studies on herpetic encephalitis had not revealed a response pattern phenotype for TLR4^{-/-} animals. However, following ocular infection, such animals were significantly, although not dramatically, more susceptible both to neovascularization and SK lesion development. Lesions also occurred earlier than in WT animals and a higher percentage of infected eyes developed lesions. Periocular disease, however, was unusual as it is in WT animals. Although currently we lack a definitive mechanistic explanation for these observations, several ideas are being pursued. These include the possibility that the TLR4 ligand activities might serve to either induce the production of anti-inflammatory cytokines [40], or the induction of some type of regulatory T cell response [41], or that the enhanced reactions occurred because of a heightened anti-HSV immune response in TLR4^{-/-} animals. Currently, we have preliminary support for at least two of these ideas. Thus IL-10 levels in corneal extracts of TLR4^{-/-} mice were less than those present in WT control extracts (Fig. 6). Additionally, the magnitude of T cell responses in TLR4^{-/-} animal was marginally elevated over that of WT animals (data not shown). We have yet to evaluate any differential induction of regulatory T cell responses between TLR4^{-/-} and control animals but such studies are underway.

Our studies also included minimal investigations on the outcome of infection in animals lacking both TLR2 and TLR4. Such animals also showed the resistance phenotype but the magnitude was less than observed in TLR2^{-/-} and MyD88^{-/-} animals. This could mean that the TLR2 ligand effect of the HSV is the

dominant activity but this is partially blunted by the apparent anti-inflammatory effect of TLR4 ligand stimulation. Perhaps the use of TLR4 ligands have some place in the future therapy of SK.

Finally, our studies evaluated HSV induced ocular responses in MyD88^{-/-} mice. Such animals showed approximately the same phenotype as did the TLR2^{-/-} mice which may mean that TLR2 ligand mediated effects are the most significant proinflammatory agonists with HSV infections. MyD88^{-/-} mice, however, showed one major response difference compared to TLR2^{-/-} and other TLR^{-/-} animals. This was their marked susceptibility to herpetic encephalitis with animals usually dying by 15 days p.i. Others have also observed that HSV may prove lethal to MyD88^{-/-} animals infected intranasally [42]. The explanation for this heightened susceptibility to encephalitis remains unclear. Accordingly, although virus was cleared normally in MyD88^{-/-} mice from the eye and TG, this was not true for the brain where it persisted and was still present in dying animals. We anticipate that the susceptibility of MyD88^{-/-} animals might be explained by a deficiency in some critical innate and/or adaptive immune responses that functions in the CNS. In some other infection systems too MyD88^{-/-} animals show reduced ability to generate an effective adaptive immune response [43]. In our preliminary studies we have noted that the antigen presenting cell function in MyD88^{-/-} mice are impaired which might have an effect on the generation of an effective immune response.

In conclusion, our results indicate that the TLR ligand activity of HSV, primarily TLR2, plays a role in the induction of an immunopathological response in the cornea and TG. Other TLR ligands, such as TLR4, may serve to counteract

the severity of the inflammatory response. It is conceivable that manipulation of the TLR ligand response could provide a means to modulate SK lesions.

LIST OF REFERENCES

- [1] P.S. Biswas, B.T. Rouse, Early events in HSV keratitis--setting the stage for a blinding disease, *Microbes Infect* 7 (2005) 799-810.
- [2] S. Deshpande, K. Banerjee, P.S. Biswas, B.T. Rouse, Herpetic eye disease: immunopathogenesis and therapeutic measures, *Expert Rev Mol Med* 2004 (2004) 1-14.
- [3] B. Kim, S. Lee, S. Suvas, B.T. Rouse, Application of plasmid DNA encoding IL-18 diminishes development of herpetic stromal keratitis by antiangiogenic effects, *J Immunol* 175 (2005) 509-516.
- [4] P.S. Biswas, K. Banerjee, M. Zheng, B.T. Rouse, Counteracting corneal immunoinflammatory lesion with interleukin-1 receptor antagonist protein, *J Leukoc Biol* 76 (2004) 868-875.
- [5] B. Kim, Q. Tang, P.S. Biswas, J. Xu, R.M. Schiffelers, F.Y. Xie, A.M. Ansari, P.V. Scaria, M.C. Woodle, P. Lu, B.T. Rouse, Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor pathway genes: therapeutic strategy for herpetic stromal keratitis, *Am J Pathol* 165 (2004) 2177-2185.
- [6] J. McGill, G.M. Scott, Viral keratitis, *Br Med Bull* 41 (1985) 351-356.
- [7] T.H. Mogensen, S.R. Paludan, Reading the viral signature by Toll-like receptors and other pattern recognition receptors, *J Mol Med* 83 (2005) 180-192.
- [8] J. Melchjorsen, L.N. Sorensen, S.R. Paludan, Expression and function of chemokines during viral infections: from molecular mechanisms to in vivo function, *J Leukoc Biol* 74 (2003) 331-343.
- [9] K.W. Boehme, T. Compton, Innate sensing of viruses by toll-like receptors, *J Virol* 78 (2004) 7867-7873.

- [10] E.A. Kurt-Jones, L. Popova, L. Kwinn, L.M. Haynes, L.P. Jones, R.A. Tripp, E.E. Walsh, M.W. Freeman, D.T. Golenbock, L.J. Anderson, R.W. Finberg, Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus, *Nat Immunol* 1 (2000) 398-401.
- [11] A.A. Beg, Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses, *Trends Immunol* 23 (2002) 509-512.
- [12] A.D. Burch, S.K. Weller, Nuclear sequestration of cellular chaperone and proteasomal machinery during herpes simplex virus type 1 infection, *J Virol* 78 (2004) 7175-7185.
- [13] A. Krug, G.D. Luker, W. Barchet, D.A. Leib, S. Akira, M. Colonna, Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9, *Blood* 103 (2004) 1433-1437.
- [14] M. Zheng, D.M. Klinman, M. Gierynska, B.T. Rouse, DNA containing CpG motifs induces angiogenesis, *Proc Natl Acad Sci U S A* 99 (2002) 8944-8949.
- [15] J. Lund, A. Sato, S. Akira, R. Medzhitov, A. Iwasaki, Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells, *J Exp Med* 198 (2003) 513-520.
- [16] E.A. Kurt-Jones, M. Chan, S. Zhou, J. Wang, G. Reed, R. Bronson, M.M. Arnold, D.M. Knipe, R.W. Finberg, Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis, *Proc Natl Acad Sci U S A* 101 (2004) 1315-1320.

- [17] P.G. Kennedy, A. Chaudhuri, Herpes simplex encephalitis, *J Neurol Neurosurg Psychiatry* 73 (2002) 237-238.
- [18] B.M. Mitchell, J.G. Stevens, Neuroinvasive properties of herpes simplex virus type 1 glycoprotein variants are controlled by the immune response, *J Immunol* 156 (1996) 246-255.
- [19] H. Minagawa, S. Sakuma, S. Mohri, R. Mori, T. Watanabe, Herpes simplex virus type 1 infection in mice with severe combined immunodeficiency (SCID), *Arch Virol* 103 (1988) 73-82.
- [20] K. Banerjee, P.S. Biswas, B. Kim, S. Lee, B.T. Rouse, CXCR2^{-/-} mice show enhanced susceptibility to herpetic stromal keratitis: a role for IL-6-induced neovascularization, *J Immunol* 172 (2004) 1237-1245.
- [21] K. Banerjee, S. Deshpande, M. Zheng, U. Kumaraguru, S.P. Schoenberger, B.T. Rouse, Herpetic stromal keratitis in the absence of viral antigen recognition, *Cell Immunol* 219 (2002) 108-118.
- [22] M.R. Dana, S.N. Zhu, J. Yamada, Topical modulation of interleukin-1 activity in corneal neovascularization, *Cornea* 17 (1998) 403-409.
- [23] S. Deshpande, M. Zheng, S. Lee, K. Banerjee, S. Gangappa, U. Kumaraguru, B.T. Rouse, Bystander activation involving T lymphocytes in herpetic stromal keratitis, *J Immunol* 167 (2001) 2902-2910.
- [24] P.G. Spear, B. Roizman, Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion, *J Virol* 9 (1972) 143-159.
- [25] R.W. Finberg, D.M. Knipe, E.A. Kurt-Jones, Herpes simplex virus and toll-like receptors, *Viral Immunol* 18 (2005) 457-465.

- [26] A. Biragyn, P.A. Ruffini, C.A. Leifer, E. Klyushnenkova, A. Shakhov, O. Chertov, A.K. Shirakawa, J.M. Farber, D.M. Segal, J.J. Oppenheim, L.W. Kwak, Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2, *Science* 298 (2002) 1025-1029.
- [27] L.A. O'Neill, Immunology. After the toll rush, *Science* 303 (2004) 1481-1482.
- [28] T. Kaisho, K. Hoshino, T. Iwabe, O. Takeuchi, T. Yasui, S. Akira, Endotoxin can induce MyD88-deficient dendritic cells to support T(h)2 cell differentiation, *Int Immunol* 14 (2002) 695-700.
- [29] J. Thomas, S. Gangappa, S. Kanangat, B.T. Rouse, On the essential involvement of neutrophils in the immunopathologic disease: herpetic stromal keratitis, *J Immunol* 158 (1997) 1383-1391.
- [30] B. Kim, P.P. Sarangi, Y. Lee, S.D. Kaistha, S. Lee, B.T. Rouse, Depletion of MCP-1 increases development of herpetic stromal keratitis by innate immune modulation, *J Leukoc Biol* (2006).
- [31] M. Zheng, S. Deshpande, S. Lee, N. Ferrara, B.T. Rouse, Contribution of vascular endothelial growth factor in the neovascularization process during the pathogenesis of herpetic stromal keratitis, *J Virol* 75 (2001) 9828-9835.
- [32] T. Liu, Q. Tang, R.L. Hendricks, Inflammatory infiltration of the trigeminal ganglion after herpes simplex virus type 1 corneal infection, *J Virol* 70 (1996) 264-271.
- [33] C.C. Bowman, K.L. Bost, Cyclooxygenase-2-mediated prostaglandin E2 production in mesenteric lymph nodes and in cultured macrophages and dendritic cells after infection with *Salmonella*, *J Immunol* 172 (2004) 2469-2475.

- [34] A. Arslan, H.H. Zingg, Regulation of COX-2 gene expression in rat uterus in vivo and in vitro, *Prostaglandins* 52 (1996) 463-481.
- [35] P.S. Biswas, K. Banerjee, B. Kim, P.R. Kinchington, B.T. Rouse, Role of inflammatory cytokine-induced cyclooxygenase 2 in the ocular immunopathologic disease herpetic stromal keratitis, *J Virol* 79 (2005) 10589-10600.
- [36] M. Mitsunari, S. Yoshida, T. Shoji, S. Tsukihara, T. Iwabe, T. Harada, N. Terakawa, Macrophage-activating lipopeptide-2 induces cyclooxygenase-2 and prostaglandin E(2) via toll-like receptor 2 in human placental trophoblast cells, *J Reprod Immunol* 72 (2006) 46-59.
- [37] E. Villamon, P. Roig, M.L. Gil, D. Gozalbo, Toll-like receptor 2 mediates prostaglandin E(2) production in murine peritoneal macrophages and splenocytes in response to *Candida albicans*, *Res Microbiol* 156 (2005) 115-118.
- [38] M.L. Gil, D. Gozalbo, TLR2, but not TLR4, triggers cytokine production by murine cells in response to *Candida albicans* yeasts and hyphae, *Microbes Infect* 8 (2006) 2299-2304.
- [39] R.N. Aravalli, S. Hu, T.N. Rowen, J.M. Palmquist, J.R. Lokensgard, Cutting edge: TLR2-mediated proinflammatory cytokine and chemokine production by microglial cells in response to herpes simplex virus, *J Immunol* 175 (2005) 4189-4193.
- [40] W.H. Kwan, C. Boix, N. Gougelet, W.H. Fridman, C.G. Mueller, LPS induces rapid IL-10 release by M-CSF-conditioned tolerogenic dendritic cell precursors, *J Leukoc Biol* 82 (2007) 133-141.

- [41] W. van Eden, G. Wick, S. Albani, I. Cohen, Stress, Heat Shock Proteins, and Autoimmunity: How immune responses to heat shock proteins are to be used for the control of chronic inflammatory diseases, *Ann N Y Acad Sci* (2007).
- [42] D.S. Mansur, E.G. Kroon, M.L. Nogueira, R.M. Arantes, S.C. Rodrigues, S. Akira, R.T. Gazzinelli, M.A. Campos, Lethal encephalitis in myeloid differentiation factor 88-deficient mice infected with herpes simplex virus 1, *Am J Pathol* 166 (2005) 1419-1426.
- [43] S. Zhou, E.A. Kurt-Jones, L. Mandell, A. Cerny, M. Chan, D.T. Golenbock, R.W. Finberg, MyD88 is critical for the development of innate and adaptive immunity during acute lymphocytic choriomeningitis virus infection, *Eur J Immunol* 35 (2005) 822-830.

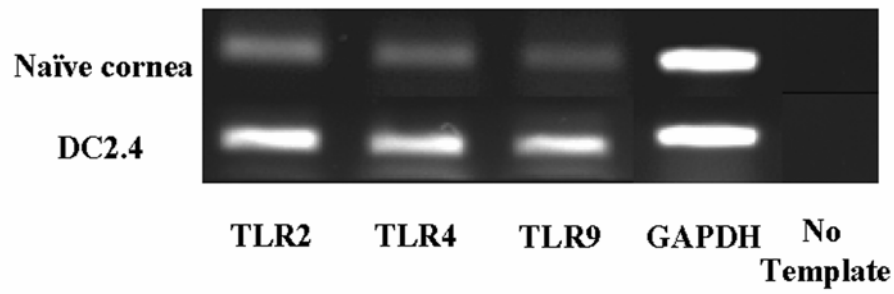
APPENDIX

Figure 1. Induced TLR ligands in the HSV infected corneas

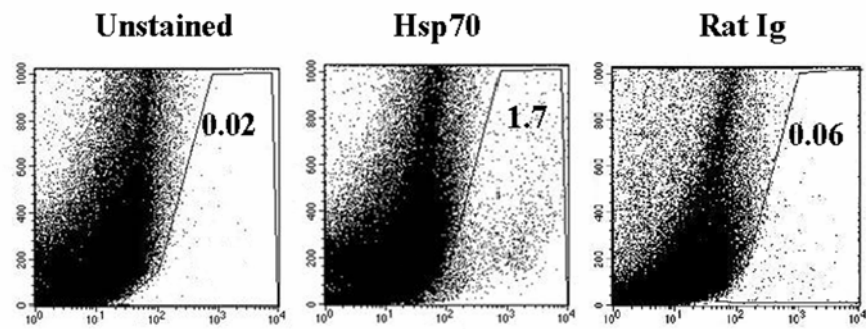
- A. Corneal buttons from uninfected mice were harvested and isolated total mRNA was tested by RT-PCR for TLR2 and TLR4 and TLR9. All three could be demonstrated in the naïve cornea. The DC2.4 cell line was used as positive control.
- B. Single-cell suspension of corneal cells was prepared from four corneas at 24 h p.i. Intracellular staining was done with FITC-labeled anti Hsp70 antibody as mentioned in the material and methods. The number of Hsp70 expressing cells was determined by FACS. Rat IgG was used as the isotype control. The number on the upper-right corner represents the percentage of Hsp70 cells/ 1×10^5 corneal cells at 24 h p.i.
- C. C57BL/6 mice were infected with 2×10^4 pfu of HSV-1RE. At the indicated time points, four corneas from infected mice were collected and total mRNA was extracted. Real time PCR was performed to compare the expression of mouse β -defensin-2 (mBD2) and mouse β -defensin-3 (mBD-3) mRNA in the infected corneas at different time point p.i. as described in material and methods. The bar diagram demonstrate the relative fold increase in the expression of mBD2 and mBD3 as compared to naïve cornea.

Fig. 1

A



B



C

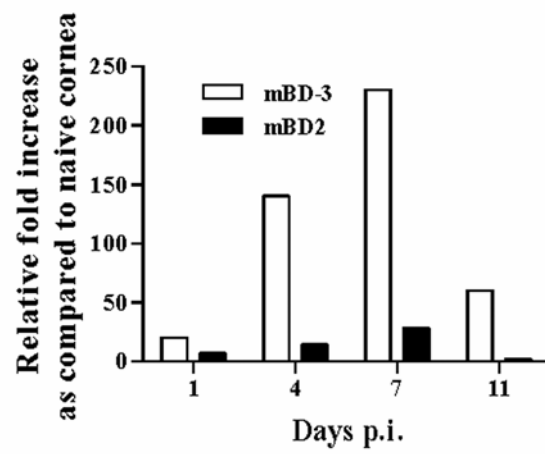


Figure 2. Reduced SK lesions and neovascularization in TLR2^{-/-}, TLR2/4d^{-/-}, and MyD88^{-/-} mice as compared to TLR9^{-/-}, TLR4^{-/-} and WT mice

- A. Bar diagram demonstrates the percentage severity of each group of mice (N=16) infected with 5×10^3 pfu HSV-1 RE at day 15 p.i. TLR2^{-/-}, TLR2/4d^{-/-}, MyD88^{-/-} mice show reduced incidence (HSK score ≥ 3) of disease as compared to TLR9^{-/-}, TLR4^{-/-}, WT animals.
- B. Mean HSK lesion scores at day 8 and day 15 p.i of WT, TLR4^{-/-}, TLR9^{-/-}, TLR2/4d^{-/-}, TLR2^{-/-} and MyD88^{-/-} mice infected with 5×10^3 pfu infection dose. **, ($P \leq 0.001$) and *, ($P \leq 0.05$), statistically significant differences as compared to WT control.
- C. Mean Angiogenesis lesion scores at day 8 and day 15 p.i of WT, TLR4^{-/-}, TLR9^{-/-}, TLR2/4d^{-/-}, TLR2^{-/-} and MyD88^{-/-} mice infected with 5×10^3 pfu infection dose. **, ($P \leq 0.001$) and *, ($P \leq 0.05$), statistically significant differences as compared to WT control animals.

Fig. 2

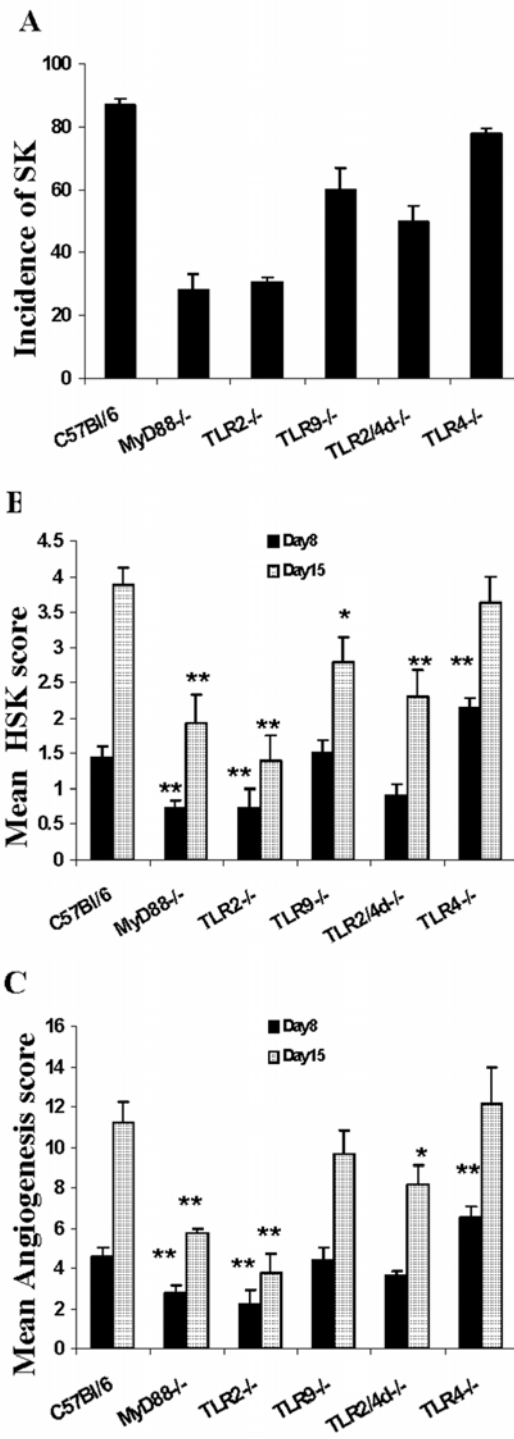


Figure 3. Compromised inflammatory cell influx into the cornea of TLR2^{-/-} , TLR9^{-/-}, TLR2/4d^{-/-} , and MyD88^{-/-} mice as compared to TLR4^{-/-} and WT mice at 48 h p.i.

Mice were infected with 5×10^3 pfu HSV-1 RE. Mice were terminated at 48 h p.i., and eyes were processed for cryo-sections. Hematoxylin and eosin staining was carried out on 6- μ m sections. Dot plots associated with the sections represent the Gr1⁺CD11b⁺ cells in the WT and TLR4^{-/-} corneas (Gr1⁺ on X-axis and CD11b⁺ on Y-axis). The numbers in the dot plots denote the percentage of inflammatory cells expressing both Gr1⁺ and CD11b⁺ markers.

Fig. 3 **Infiltration inflammatory cells into cornea**
at 48 hr p.i.

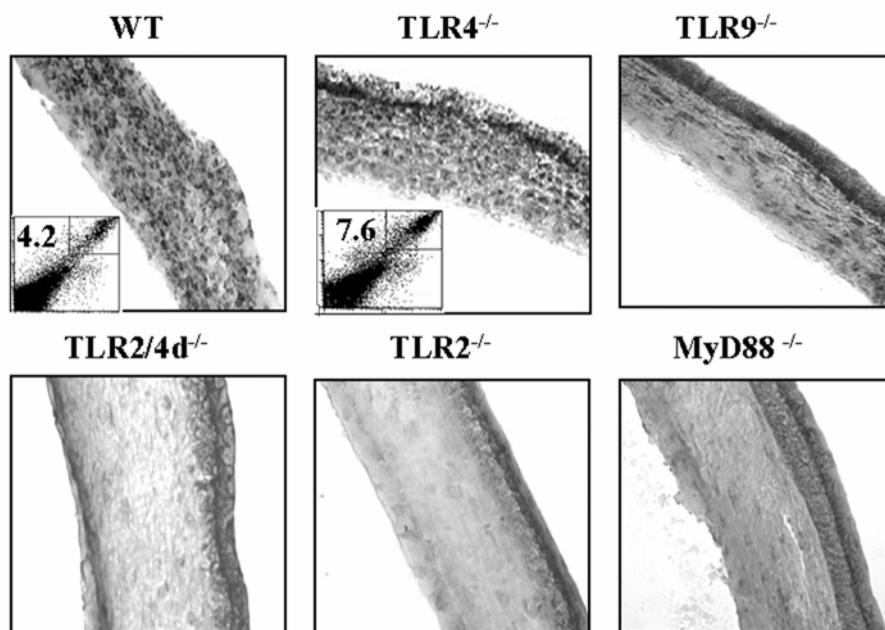


Figure 4. Compromised cell infiltration into the TG of TLR2^{-/-} and MyD88^{-/-} mice as compared to TLR4^{-/-} and WT mice

Single-cell suspensions of TG cells were prepared from four TGs at 48 h and 96 h p.i. for each group of mice. The cells were stained with FITC-labeled anti Gr-1, CD11c, F4/80 and PE labeled CD11b antibody. The number of positive cells was determined by FACS. The bars represent the number of cells/ 4×10^5 cells from the TG at 48 h and 96 h p.i. *, ($P \leq 0.05$) and **, ($P \leq 0.01$), statistically significant difference as compared to WT animals.

Fig. 4

Infiltration of innate immune cells into the Trigeminal ganglia

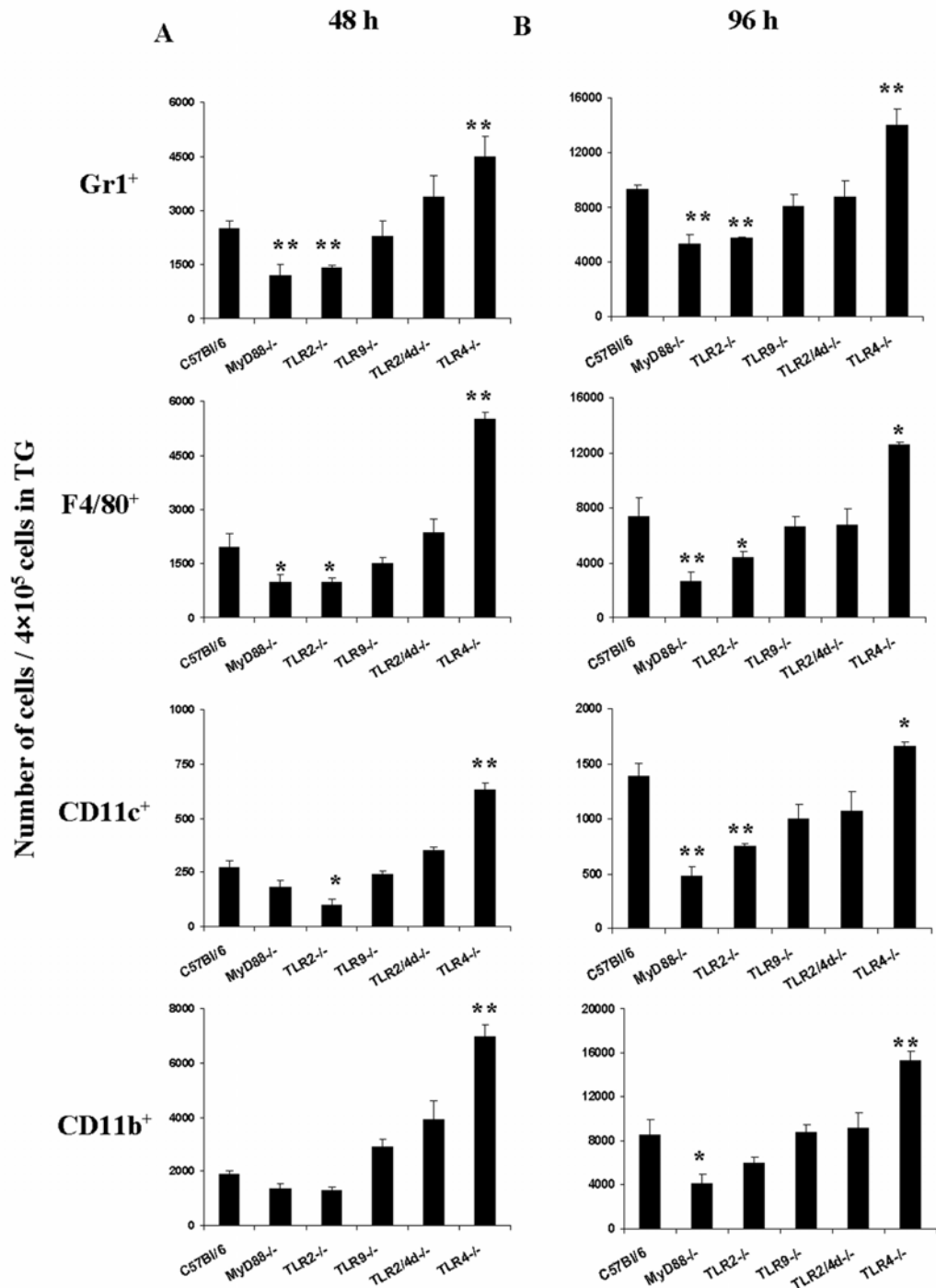


Figure 5. Reduced levels of cytokines and angiogenic factor in the corneas of TLR2-/- and MyD88-/- mice as compared to TLR4-/- and WT mice.

At the indicated time points, six corneas/group were processed to measure the IL-6, IL-1 β , MIP-2 and VEGF levels. Levels of IL-6, IL-1 β , MIP-2 and VEGF were estimated from the supernatants of corneal lysates of WT, TLR2-/-, TLR2/4d-/-, TLR4-/-, TLR9-/- and MyD88-/- animals infected with 5×10^3 pfu HSV-1 RE by an antibody capture ELISA as outlined in materials and methods. *, ($P \leq 0.05$) and **, ($P \leq 0.01$), statistically significant difference as compared to WT animals.

Fig. 5

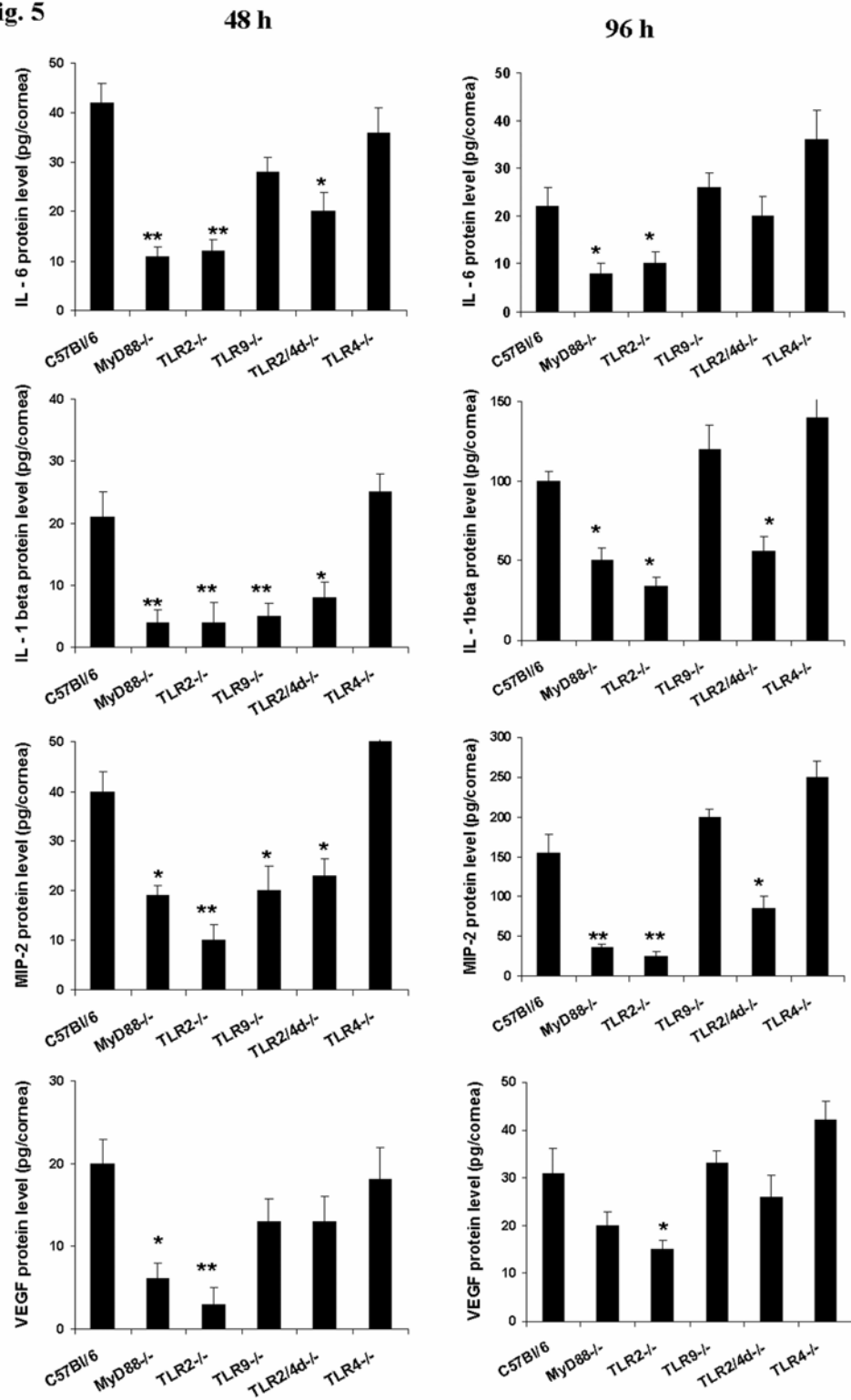


Figure 6. Increased expression of proinflammatory cytokines and reduced levels of IL-10 in TLR4-/- animals as compared to WT

At the indicated time points, six corneas/group and cervical DLNs from individual mice (N=4) were processed to measure the IL-10, IL-6, IFN- γ levels of WT and TLR4-/- animals infected with 5×10^3 pfu HSV-1 RE and cytokines measured by antibody capture ELISA as outlined in materials and methods. *, ($P \leq 0.05$), statistically significant differences as compared to WT animals.

Fig. 6

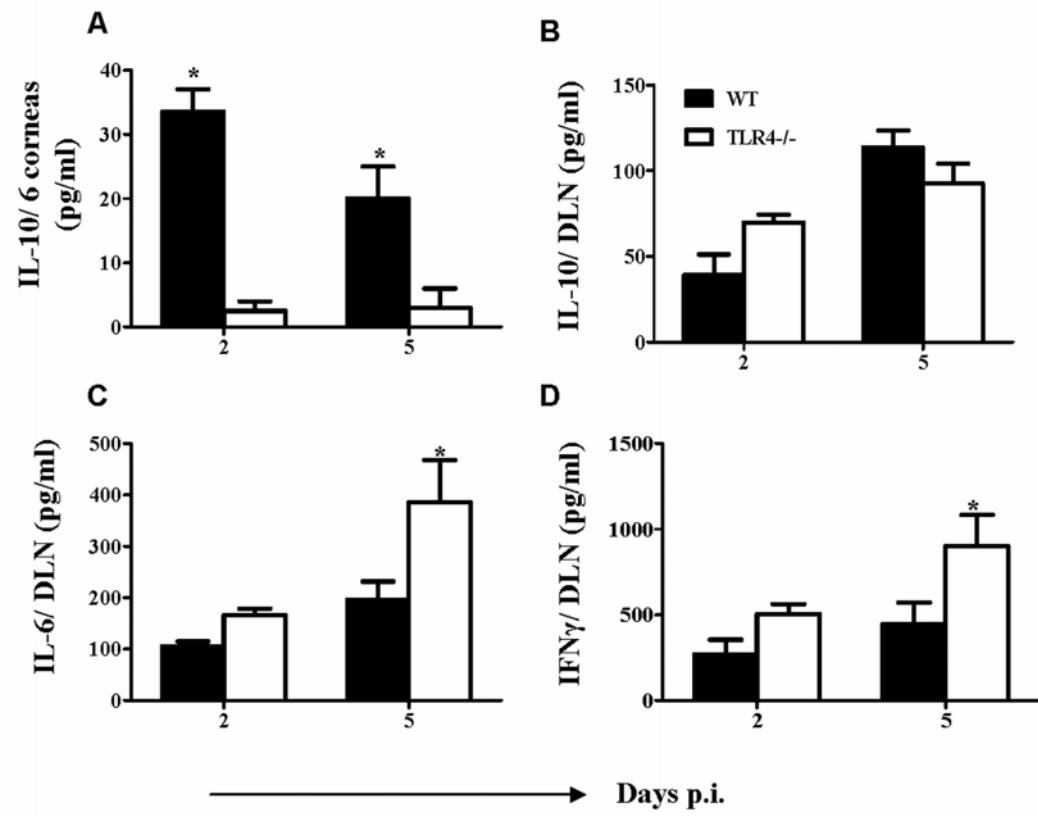


Fig. 7

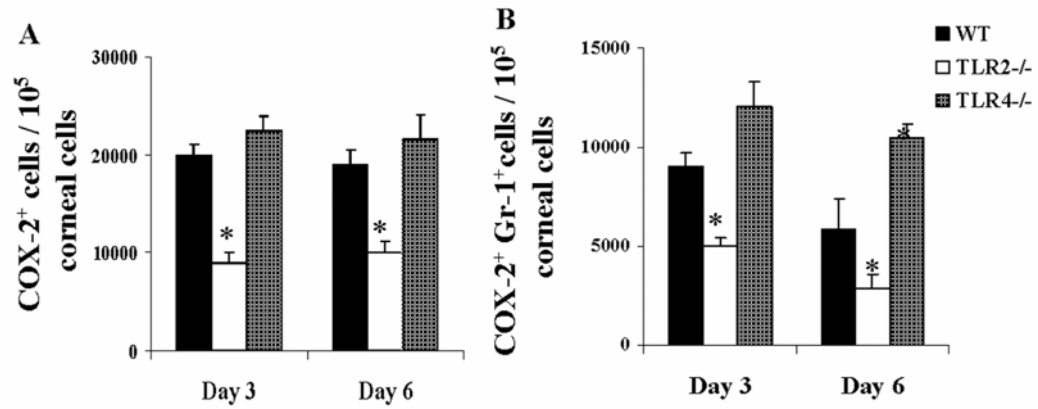


Figure 7. Reduced expression of COX-2 in TLR2^{-/-} animals as compared to WT

Single-cell suspensions of corneal cells were prepared from two corneas (Number of mice = 8) at day 3 and day 6 p.i. Intracellular COX-2 staining was carried out as described in materials and methods. In Fig. 7A and B, bar diagrams demonstrate the total number of COX-2⁺ cells and total number of COX-2⁺Gr1⁺ cells /1×10⁵ corneal cell respectively. *, ($P \leq 0.05$), statistically significant differences as compared to WT animals.

Table 1

Percentage of mortality in various groups by day 15 p.i.

	2×10^4 pfu/eye	5×10^3 pfu/eye
WT	30 %	< 20 %
TLR4 ^{-/-}	45 %	< 20 %
TLR9 ^{-/-}	<15 %	-
TLR2 ^{-/-}	<15 %	-
MyD88 ^{-/-}	100 %	70 %

Table 2

^aVirus titer in log₁₀ pfu/ml in TG and brain at different time points p.i.

	Day4 p.i.		Day7 p.i.	
	TG	Brain	TG	Brain
WT	3.2 ± 0.04	1.7 ± 0.29	^b UD	UD
TLR9 ^{-/-}	3.7 ± 0.08	2.3 ± 0.2	UD	UD
TLR2 ^{-/-}	3.2 ± 0.03	2.3 ± 0.07	UD	UD
MyD88 ^{-/-}	3.7 ± 0.06	2.2 ± 0.20	UD	^c 3.8 ± 0.30

^a Mice were infected with 3 x 10⁵ pfu of HSV-1 RE and killed at day4 or day7 to measure viral titers using a standard plaque assay from brain and TG homogenates (*n* = 3).

^b UD, Undetected, i.e., below the sensitivity of the assay (<10 pfu/ml)

^c Virus was also detected in the brain tissues collected from dying MyD88^{-/-} mice (day-10-15).

Table 3**^aVirus titer in log₁₀ pfu/ml in the cornea at different time points p.i**

	Day3 p.i.	Day7 p.i.
WT	3.2 ± 0.18	^b UD
TLR4 ^{-/-}	3.7 ± 0.21	UD
TLR9 ^{-/-}	3.3 ± 0.17	UD
TLR2 ^{-/-}	3.1 ± 0.08	UD
MyD88 ^{-/-}	3.2 ± 0.10	UD

^a Mice were infected with 3 x 10⁵ pfu of HSV-1 RE. Virus titer was estimated by standard plaque assay from swabs taken from infected corneas of mice (*n* = 4) at the indicated time points.

^b UD, Undetected, i.e., below the sensitivity of the assay (<10 pfu/ml).

Part-III

**Enhanced viral immunoinflammatory lesions in
mice lacking IL-23 responses.**

Research described in this chapter is a slightly modified version of an article that in accepted for publication in *Microbes and Infection* by Pranita P Sarangi, Bumseok Kim, Ahmet Kursat Azkur, Shilpa Deshpande Kaistha, and Barry T. Rouse

*Kim B, *Sarangi PP, Ahmet Kursat Azkur, Deshpande Kaistha S, Rouse BT. Enhanced viral immunoinflammatory lesions in mice lacking IL-23 responses. - *Microbes and infections*. 2008 (article in Press) * Equal contribution of authors.

Copyright © 2008 Published by Elsevier Masson SAS

In this chapter “our” and “we” refers to me and co-authors. My contribution in the paper includes (1) Selection of the topic (2) Compiling and interpretation of the literature (3) Designing experiments (4) understanding the literature and interpretation of the results (5) providing comprehensible structure to the paper (6) Preparation of graphs, figures and tables (7) Writing and editing

Abstract

Herpes simplex virus (HSV) infection of the cornea culminates in an immunopathological lesion (stromal keratitis – SK) that impairs vision. This report shows that HSV infection results in IL-23 up-regulation, but if this response fails to occur, as was noted in p19^{-/-} mice, the severity of lesions, their incidence and the level of viral induced angiogenesis were significantly increased compared to wild-type (WT) animals ($p < 0.05$). The higher disease severity in p19^{-/-} mice appeared to be the consequence of an increased IL-12 response that in turn led to the induction of higher numbers of IFN- γ producing CD4⁺ T cells, the principal orchestrators of SK. Our results indicate that the severity of HSV induced

immunopathological lesions may be mainly the consequence of IL-12 driven Th1 T cell reactions rather than the action of IL-17 producing cells controlled by IL-23.

Introduction

Ocular infection with herpes simplex virus (HSV) culminates in immunoinflammatory lesions in the stroma (stromal keratitis – SK) that is a common cause of human blindness [1]. Understanding the pathogenesis of SK is expected to result in more effective control measures by targeting critical events. Studies in a mouse model of SK have revealed that many key events are set off by HSV infection. Among the early events are the production of several cytokines such as IL-6 and IL-12 [2, 3], chemokines such as MCP-1 and MIP-2 [4, 5] as well as several molecules that induce neovascularization of the normally avascular cornea [6]. Past studies by us and others have shown that IL-12 is upregulated by HSV infection [2, 7] and may be involved in generating IFN- γ producing CD4⁺ T cells that are thought to be the main orchestrators of SK lesions [8]. In support of this, animals unable to generate normal IL-12 responses develop diminished lesions [2]. Currently, the role of the structurally similar IL-23 molecule in the pathogenesis of SK is not understood. This topic is of interest since several autoinflammatory and some anti-microbial inflammatory reactions that were formerly thought to be mainly IL-12/IFN- γ mediated immunoinflammatory events were subsequently shown to be more the consequence of responses influenced by IL-23 that promotes the expansion and survival of pathogenic IL-17 producing T cells [9-11]. Although T cells that produce IL-17 have been demonstrated in human SK lesions [12] the respective role of Th1 and Th17 pathogenic T cells as orchestrators of SK is poorly understood. The present report evaluates the role of

the IL-23/IL-17 axis in SK by comparing the outcome of infection in wild-type (WT) to those in mice unable to generate IL-23 because of p19 knockout. Our results show that HSV infection causes the upregulation of IL-23 but these results in only minimally detectable CD4/IL-17 producing responses in the eye. Furthermore lesions were significantly more severe in animals unable to produce IL-23 making it doubtful if the IL-23/IL-17 axis is mainly responsible for the ocular inflammatory lesions. In fact, in the absence of p19 more abundant IL-12 and Th1 responses were evident. We presume it is the IFN- γ producing Th1, rather than Th17 T cells, that are mainly responsible for mediating SK.

Materials and Methods

Mice

Female C57BL/6 mice, 6 to 7 weeks old, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). P19 deficient mice were kindly provided by Dr. Nico Ghilardi (Genentech, Inc., CA). All investigations followed guidelines of the Committee on the Care of Laboratory Animals Resources, Commission of Life Sciences, National Research Council. The animal facilities of the University of Tennessee (Knoxville) are fully accredited by the American Association of Laboratory Animal Care.

Virus

HSV-1 strain RE (kindly provided by Dr. Robert Lausch, University of Alabama, Mobile, AL) was used in all procedures. Virus was grown in Vero cell monolayers (American Type Culture Collection, Manassas, VA; Cat. no. CCL81), titrated, and stored in aliquots at -80°C until used.

Corneal HSV infection

Corneal infections of all mouse groups were conducted under Avertin deep anesthesia. The mice were scarified lightly on their corneas with a 30-gauge needle, and a 2 µl drop containing different plaque-forming units (PFU) of HSV-1 RE was applied to the eye and gently massaged with the eyelids.

Real-time quantitative PCR

The total cellular RNA was isolated from corneas and draining lymph nodes (DLNs) by using an RNeasy protect mini kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. All samples were treated with RNase-free DNase (Qiagen). The extracted RNA was reverse transcribed using oligo (dt) primers and reverse transcriptase enzyme (Promega, Madison, WI) according to standard protocol. The cDNA obtained was used as a template for real-time quantitative PCR and real-time PCR was performed using a QuantiTect SYBR Green PCR kit (Qiagen). The primers used were murine p19 forward (TGCTGGATTGCAGAGCAGTAA) and reverse (GCATGCAGAGATTCCGAGAGA). Initially, mRNA levels were normalized to the GAPDH mRNA level. Briefly, a standard curve was generated using the PCR product of interested gene cloned into a pCR-XL-TOPO vector (Invitrogen, San Diego, CA). After spectrophotometric determination of the plasmid DNA concentration, the copy number was calculated using the following formula: $(X \text{ g/}\mu\text{l DNA} / (\text{plasmid length in bp} \times 660) \times 6.022 \times 10^{23} = Y \text{ molecules/}\mu\text{l})$. Test samples were run in triplicate with a dilution series and the mean value of a particular dilution was used. Data are expressed as copy number per sample.

Clinical observations

The eyes were examined on different days after infection for the development of clinical lesions by slit-lamp biomicroscopy (Kawa Co, Nagoya, Japan), and the clinical severity of keratitis of individually scored mice was recorded. 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 stromal keratitis. The severity of angiogenesis was recorded as described previously [6]. In reference to the angiogenic scoring system, the method relied on quantifying the degree of neovessel formation based on three primary parameters: 1) the circumferential extent of neovessels (as the angiogenic response is not uniformly circumferential in all cases); 2) the centripetal growth of the longest vessels in each quadrant of the circle; and 3) the longest neovessel in each quadrant was identified and graded between 0 (no neovessel) and 4 (neovessel in the corneal center) in increments of ~0.4 mm (radius of the cornea is ~1.5 mm). 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 to 16) for each eye at a given time point.

Cytokine assay

For cytokine (IFN- γ , IL-12p35, IL-12p40) assay, splenocytes from mice were suspended in 10% RPMI-1640, and 10^6 cells in 1 ml were stimulated *in vitro* with irradiated syngeneic-enriched naïve splenocytes pulsed with UV-inactivated HSV (MOI, 1.5 before UV inactivation). Similar number of cells was Con A-stimulated

(5 $\mu\text{g}/10^6$ cells/ml) in 96-well plates. Plates were incubated at 37°C for 72 h. The supernatant fluid was collected and stored at -80°C until use. These supernatants were screened for the presence of individual cytokines by enzyme-linked immunosorbent assay (ELISA).

Flow cytometric analysis

Single-cell suspensions were prepared from the SK corneas or lymphoid organs (spleen and DLNs). For the preparation of corneal samples, four corneas per group were dissected, pooled together, and digested with 60 U/ml Liberase (Roche Diagnostics, Alamenda, CA) for 60 min at 37°C in a humidified atmosphere of 5% CO₂. After incubation, the corneas were disrupted by grinding with a syringe plunger on a mesh and a single-cell suspension was made in complete RPMI 1640 medium. Next, the single-cell suspensions obtained from corneal or lymphoid samples were stained for FACS. Briefly, single cells were first blocked with an unconjugated anti-CD32/CD16 mAb (BD Biosciences, San Jose, CA) for 15 min at 4°C in FACS buffer and stained by anti-CD4-APC, anti-CD8-PerCp, CD11c-PerCp, or CD11b-APC antibody (BD Biosciences) for 30 min. Cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) solution for approximately 30 minutes, followed by washing with perm washing solution. Intracellular staining was done with anti-IFN- γ -PE, anti-IL-12-PE, or anti-IL-17-PE antibody (BD Biosciences) for 30 minutes on ice and washed twice with perm wash. Finally, the cells were washed with FACS buffer and samples were acquired on a FACScan (BD Biosciences). The data were analyzed using the CellQuest 3.1 software (BD Biosciences).

Protein quantification of corneal or lymphoid organs lysates by ELISA

The lysates from HSV infected cornea or lymphoid organs (spleen and DLNs) were used for the measurement of IFN- γ , IL-12, IL-23, or TGF- β by a standard sandwich ELISA protocol. For preparation of sample lysates, four corneas or lymphoid organs/time point (n = 4) were collected and minced with liquid nitrogen. Minced pieces were collected in 1 ml of DMEM without FCS and homogenized using an ultra sonicator (Heat systems-Ultrasonics, NY). The lysates were then clarified by centrifugation at 12,000 rpm for 5 min at 4°C. The supernatant was collected and stored at -80°C till further use. The ELISA plate was coated with anti-mouse capture antibody (100 μ l/well of the capture antibody at various concentrations, BD Biosciences) and incubated at 4°C overnight. The plate was washed with 0.05% tween20/PBS and blocked with 3% BSA for 2 h at 37°C. After washing, serially diluted lysates were added to the plate and incubated at 4°C overnight. The plate was washed and then incubated with biotinylated detection antibody (BD Biosciences) for 2 h. Finally, peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratory, PA) was added. The color reaction was developed using ABTS (Sigma-Aldrich, St. Louis, MO) and measured with an ELISA reader (Spectramax 340; Molecular Devices, Sunnyvale, CA) at 405 nm. Quantification was performed with Spectramax ELISA reader software version 1.2.

Virus recovery and titration

Swabs of the corneal surface were collected at various time points post infection (p.i.). The swabs were put into sterile tubes containing 600 μ l serum free DMEM with 10 international units (IU) penicillin/ml and 100 μ g streptomycin (Life

Technologies, Grand Island, NY)/ml and were stored at -80°C. For detection and quantification of virus, the samples were thawed and vortexed. Individual subsamples (200 µl each sample) were diluted further, and viral titers were determined by a plaque assay performed on Vero cells as described elsewhere [13].

Statistical analysis

Significant differences between groups were evaluated by using the Student's t test. $P < 0.05$ was regarded as a significant difference between two groups. Results are expressed as mean $\pm$ standard error.

Results

Expression of IL-23 after HSV-1 infection

WT C57BL/6 mice were infected with HSV-1 (5×10^5 PFU) and at various times p.i. were killed to determine the levels of p19 mRNA by quantitative real-time PCR in both corneal and DLNs RNA samples. As is shown in Fig.1A, minimal or undetectable mRNA levels of p19 were present in naïve corneas or lymph nodes, but by day 2 p.i. abundant levels of IL-23 mRNA were detected. In other experiments, IL-23 protein levels were quantified in corneal extracts as well as extracts of lymphoid organs by ELISA in animals infected with different doses of virus (5×10^3 , 5×10^4 , 5×10^5 PFU). Peak levels were found with the highest dose used for infection (Fig. 1B). These results indicate that IL-23 is up-regulated by HSV infection and could perhaps participate in the immunopathogenesis of herpetic SK.

The absence of IL-23 production results in more severe disease

The expression of disease was compared in WT and p19^{-/-} mice at various times p.i. upon infection with different doses of virus (5×10^3 , 5×10^4 , 5×10^5 PFU). At high doses of virus used for infection (5×10^4 and 5×10^5 PFU) disease severity in both WT and p19^{-/-} animals was of similar magnitude (Fig. 2A). However, at a low dose of infection (5×10^3 PFU) highly significant differences were observed in the susceptibility of WT and p19^{-/-} animals. In the latter, more eyes developed lesions and the mean severity scores of both angiogenesis and SK lesions were significantly more severe than in WT animals ($P < 0.05$, Fig. 2B). Lesions also occurred slightly earlier in p19^{-/-} animals. Overall an average result of three separate experiments reveal that 80% of p19^{-/-} developed SK of 3 or greater where as only 33% of WT animals developed such severity (Fig. 2C). Thus the failure to generate an IL-23 response results in more severe lesions at least at minimal doses of infection.

As the presence of replicating virus may contribute to lesion severity, we compared the levels of virus in the eye swabs collected from both groups of animals at different time points. As is evident in Fig. 2D, virus clearance occurred earlier in the corneas of p19^{-/-} as compared to WT animals ($p < 0.05$). Thus although lesions were more severe in p19^{-/-} animals virus was cleared from the epithelium earlier than in WT.

P19^{-/-} animals develop more severe Th1 immune response

In an attempt to explain why p19^{-/-} mice showed more severe responses than WT animals, we infected both groups with the low dose of virus (5×10^3 PFU) and lymphoid samples were collected at day 20 post infection to compare the

magnitude of the HSV specific T cell responses. This was done by stimulating T cells with antigen and comparing the induced cytokine response by ELISA. As shown in Fig. 3A, splenocytes from p19^{-/-} mice produced significantly higher ($p < 0.05$) levels of IFN- γ as compared to WT controls. Similarly, although no difference was observed in the production of p40 subunit, the IL-12p35 level was significantly higher in p19^{-/-} as compared to WT ($p < 0.05$). These results may mean that in the absence of p19 the Th1 type of immune response is increased.

To determine which cell populations contribute to the Th1 type immune response against HSV, isolated splenocytes from both groups having SK lesions were stimulated with anti-CD3 and anti-CD28 to analyze cytokine production. As assessed by intracellular cytokine staining assay, the percentage and absolute number of IFN- γ expressing CD4⁺ T cells as well as IFN- γ expressing CD8⁺ T cells was significantly higher ($p < 0.05$) in p19^{-/-} mice as compared to WT control animals (Fig. 3B). Similar results were also obtained when cells were stimulated with syngeneic naive splenocytes pulsed with HSV (Fig. 3C). In addition, we also compared the numbers of IL-12 producing cells in the infected DLNs and spleen in the two groups of animals. As shown in Fig. 4A, higher numbers of IL-12 expressing cells were noted in the p19^{-/-} animals as compared to WT. The majority of cells producing IL-12 were found to be CD11c⁺ CD11b⁻ (Fig. 4B). Total cell numbers in the DLNs and spleen of p19^{-/-} were marginally increased as compared to WT animals (not shown) indicating that relative population of IFN- γ or IL-12 expressing cell types were greater in lymphoid organs of p19^{-/-} animals. Thus these data indicate that p19^{-/-} mice generated a greater Th1 type of immune response than did WT animals.

The nature of the response in p19^{-/-} and WT corneas was also measured using corneas isolated from the infected animals of both the groups. As shown in the Fig. 5A, the protein levels of IFN- γ and IL-12 were significantly higher in the lysates of p19^{-/-} corneas as compared to WT at day 20 p.i. ($p < 0.01$). We next determined the number of IFN- γ expressing cells in the collagenase digested corneas of each group. As shown in Fig 5B, there was approximately two and four fold increase in the total number of IFN- γ producing cells in the p19^{-/-} corneas after anti-CD3 and CD28 stimulation at day 10 and day 20 respectively as compared to WT. Such IFN- γ producing cell types in SK corneas were mainly CD4⁺ T cells confirming that pathogenesis of herpetic SK is contributed by Th1 type CD4⁺ T cells (Fig. 5C). Similarly, the numbers of IL-12 producing cells were also higher in p19^{-/-} corneas as compared to WT controls (Fig. 5B). Total cell numbers in the SK cornea of p19^{-/-} mice were also greater than those of WT mice at day 10 and 20 p.i. (Fig. 5D) suggesting both absolute numbers and frequencies of such cells in the corneas of p19^{-/-} animals were higher as compared to WT animals. These data indicate that the p19^{-/-} mice generated a more abundant Th1 type of immune response in the corneas than WT.

P19^{-/-} mice develop less Th17 immune response

Previous studies showed that IL-23 expands a pathogenic CD4⁺ T cell population that produces IL-17 and contributes to inflammation [9]. To record such an effect, isolated splenocytes, DLN cells, and corneal cells from both groups having SK lesions were stimulated with anti-CD3 and anti-CD28 to analyze IL-17 production. As assessed by intracellular cytokine staining assay, comparable and little levels of IL-17-producing CD4⁺ T cells were observed in the spleen, DLNs, and corneas of

both p19^{-/-} and WT mice at day 10 post infection (data not shown). At day 20 post infection, however, higher levels of IL-17-producing CD4⁺ T cells were observed in the SK cornea, spleen, and DLNs of WT compared to p19^{-/-} mice (Fig. 6A). Since TGF- β is critical for the induction of Th17 cells [14], we measured the protein levels of TGF- β in the corneas and lymphoid tissues of p19^{-/-} and WT animals. We could not find significant differences in the TGF- β levels in the naïve and infected p19^{-/-} animals corneas as compared to WT. Interestingly, significantly higher levels of TGF- β was found in the cervical DLNs but not the spleens of p19^{-/-} animals at 5 days p.i. (Fig. 6B). These data indicate that IL-23 is needed for the maintenance of the Th17 cells post infection.

Discussion

In many immunoinflammatory lesion events thought formerly to represent the consequences of IL-12 controlled IFN- γ producing Th1 type T effector cells were subsequently shown to be more the consequence of pathologic CD4⁺ T cells that produce IL-17 [11]. The differentiation and expansion of the latter was influenced by IL-23 rather than IL-12. We, and others, had shown the relevance and importance of IL-12 and IFN- γ producing CD4⁺ T cells in the orchestration of HSV induced inflammatory reactions in the cornea [1, 2, 8, 15, 16], but any role for Th17 mediated events had not been studied. The present investigation shows that ocular infection does result in IL-23 induction but the extent of the ocular Th17 response in the cornea was very limited. Furthermore, we observed that the severity of HSV induced SK lesions were in fact more severe than in WT animals if mice were unable to generate IL-23 because of p19 knockout. Thus in this viral-induced immunopathological reaction, SK lesions would appear to be mainly

orchestrated by Th1 cells as advocated by several groups prior to our knowledge about the existence of the IL-23/IL-17 axis of inflammatory disease mediation.

The evidence that the IL-12/IFN- γ and IL-23/IL-17 represent separate mechanisms of cell mediated tissue damage appears now to be well accepted [9, 11, 17, 18]. In fact, based on the use of knockout animals, as well as approaches using *in vivo* cytokine neutralizing antibodies, the trend has usually been to show that lesions assumed to represent the consequence of IL-12/ IFN- γ mediated events in fact primarily involved IL-23/IL-17 mediated mechanisms. This has been shown true for several autoimmunities and some inflammatory reactions against microbes [10, 11, 17]. Reasons why one type of inflammatory reaction prevails over another are usually not available. However, with some microbes and some purified microbial products more effective induction of IL-23 compared to IL-12 has been shown to occur. This happens with *Bordetella pertussis* [19] as well as with stimulation by peptidoglycan from *Staphylococcus aureus* [20]. In addition, various toll-like receptor (TLR) ligands were shown to differentially stimulate IL-12 and IL-23 production from dendritic cells (DCs) [21, 22]. HSV has been shown to possess both TLR2 and 9 ligand activity [23] and in addition causes the release of endogenous TLR ligands such as hsp70 from infected cells [24, 25]. One or more of these TLR ligands could stimulate IL-12 and IL-23 production differentially in SK corneas. We are currently attempting to compare the relative IL-12/IL-23 inducing capacity of HSV, infected cell extracts and known TLR ligands in various cell types derived from the eye. We anticipate that HSV may be a better inducer of IL-12 than IL-23 and that the resultant IFN- γ response from innate cells, that includes neutrophils [26], may serve to suppress any concomitant

Th17 responses. Such suppression of Th17 responses by IFN- γ has been shown to occur *in vitro* [27]. Moreover, HSV may also be a weak inducer of TGF- β which appears to act as a cofactor for Th17 development [28]. In our current study, there was no significant increase in the TGF- β levels in the WT corneas post infection. Additionally, we did not find any significant difference in the levels of TGF- β in the corneas of p19^{-/-} animals as compared to WT animals. Taken together these events may explain the minimal Th17 response in the eye during HSV induced immunopathology, and such ideas are being further investigated.

Arguing forcefully against a major role for IL-23/Th17 induced tissue damage in the SK system was the observation that without the ability to produce IL-23 because of p19^{-/-}, lesions were more severe than in WT. In preliminary results, we have also confirmed the previous observation of the Ghiasi group [2] that corneal scarring becomes less severe in animals unable to make IL-12 because of p35^{-/-}. Moreover, animals unable to respond to IL-12 because of STAT4 knockout develop only mild lesions of SK [29]. The reasons for the enhanced pathology in p19^{-/-} animals appeared to be associated with the increased IL-12/IFN- γ response that occurred in such animals. These enhanced responses were noted both in the spleen as well as in ocular inflammatory sites. The better IFN- γ -producing CD4⁺ T cell response likely accounted for the more severe SK lesions in p19^{-/-} animals. Presumably in the absence of p19 more of the available p40 subunit may be able to dimerize with p35 subunit leading to more IL-12 production. Such a finding was also suggested to the observation of Becker et al [22] in an experimental colitis model wherein p19^{-/-} animals were shown to develop more severe colitis than the intact animals. They also observed elevated

levels of IL-12 production and the reversal of the phenotype by administration of blocking antibody to the p40 subunit.

In our model, p19^{-/-} animals also cleared the infecting virus more promptly presumably because of the enhanced production of IFN- γ in p19^{-/-} compared to WT animals. Furthermore, greater numbers of CD11c⁺CD11b⁺ cells as well as CD11c⁺CD11b⁻ cells were also observed in the ocular tissues of p19^{-/-} animals compared to WT (data not shown). Such cells may be myeloid and plasmacytoid DCs respectively, an abundant source of IFN- α which also might explain the more rapid clearance of virus from the eyes of p19^{-/-} animals [30].

In summary, our results make the case that SK remains a disease whose mechanism of tissue damage appears to be mainly the consequence of an IL-12 influenced Th1 IFN- γ producing type of T cell rather than by a pathogenic Th17 producing mechanism. The latter likely plays only a minor role but this might synergize with the Th1 pathogenic response as has been suggested to occur in EAE model [31]. We are currently attempting to confirm our observations using adoptive T cell transfer approaches with *in vitro* generated Th1 and Th17 specific cells along with evaluating the effects of inhibition studies with specific anti-sera. Understanding the mechanisms is considered important as it could impact on measures used to control this important tissue damaging disease process.

LIST OF REFERENCES

- [1] S.P. Deshpande, M. Zheng, S. Lee and B.T. Rouse, Mechanisms of pathogenesis in herpetic immunoinflammatory ocular lesions, *Vet. Microbiol.* 86 (2002), pp. 17-26.
- [2] Y. Osorio, S.L. Wechsler, A.B. Nesburn and H. Ghiasi, Reduced severity of HSV-1-induced corneal scarring in IL-12-deficient mice, *Virus Res.* 90 (2002), pp. 317-326.
- [3] R.R. Fenton, S. Molesworth-Kenyon, J.E. Oakes and R.N. Lausch, Linkage of IL-6 with neutrophil chemoattractant expression in virus-induced ocular inflammation, *Invest. Ophthalmol. Vis. Sci.* 43 (2002), pp. 737-743.
- [4] J. Thomas, S. Kanangat and B.T. Rouse, Herpes simplex virus replication-induced expression of chemokines and proinflammatory cytokines in the eye: implications in herpetic stromal keratitis, *J. Interferon Cytokine Res.* 18 (1998), pp. 681-690.
- [5] W.J. Cook, M.F. Kramer, R.M. Walker, T.J. Burwell, H.A. Holman, D.M. Coen and D.M. Knipe, Persistent expression of chemokine and chemokine receptor RNAs at primary and latent sites of herpes simplex virus 1 infection, *Virol. J.* 1 (2004), pp. 5.
- [6] M. Zheng, S. Deshpande, S. Lee, N. Ferrara and B.T. Rouse, Contribution of vascular endothelial growth factor in the neovascularization process during the pathogenesis of herpetic stromal keratitis, *J. Virol.* 75 (2001), pp. 9828-9835.
- [7] U. Kumaraguru and B.T. Rouse, The IL-12 response to herpes simplex virus is mainly a paracrine response of reactive inflammatory cells, *J. Leukoc. Biol.* 72 (2002), pp. 564-570.

- [8] S. Gangappa, S.P. Deshpande and B.T. Rouse, Bystander activation of CD4(+) T cells can represent an exclusive means of immunopathology in a virus infection, *Eur. J. Immunol.* 29 (1999), pp. 3674-3682.
- [9] S. Aggarwal, N. Ghilardi, M.H. Xie, F.J. de Sauvage and A.L. Gurney, Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17, *J. Biol. Chem.* 278 (2003), pp. 1910-1914.
- [10] D.J. Cua, J. Sherlock, Y. Chen, C.A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wiekowski, S.A. Lira, D. Gorman, R.A. Kastelein and J.D. Sedgwick, Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain, *Nature* 421 (2003), pp. 744-748.
- [11] C.L. Langrish, Y. Chen, W.M. Blumenschein, J. Mattson, B. Basham, J.D. Sedgwick, T. McClanahan, R.A. Kastelein and D.J. Cua, IL-23 drives a pathogenic T cell population that induces autoimmune inflammation, *J. Exp. Med.* 201 (2005), pp. 233-240.
- [12] J. Maertzdorf, A.D. Osterhaus and G.M. Verjans, IL-17 expression in human herpetic stromal keratitis: modulatory effects on chemokine production by corneal fibroblasts, *J. Immunol.* 169 (2002), pp. 5897-5903.
- [13] J.S. Babu, J. Thomas, S. Kanangat, L.A. Morrison, D.M. Knipe and B.T. Rouse, Viral replication is required for induction of ocular immunopathology by herpes simplex virus, *J. Virol.* 70 (1996), pp. 101-107.
- [14] P.R. Mangan, L.E. Harrington, D.B. O'Quinn, W.S. Helms, D.C. Bullard, C.O. Elson, R.D. Hatton, S.M. Wahl, T.R. Schoeb and C.T. Weaver, Transforming

growth factor-beta induces development of the T(H)17 lineage, *Nature* 441 (2006), pp. 231-234.

[15] J. He, H. Ichimura, T. Iida, M. Minami, K. Kobayashi, M. Kita, C. Sotozono, Y.I. Tagawa, Y. Iwakura and J. Imanishi, Kinetics of cytokine production in the cornea and trigeminal ganglion of C57BL/6 mice after corneal HSV-1 infection, *J. Interferon Cytokine Res.* 19 (1999), pp. 609-615.

[16] A. Heiligenhaus, D. Bauer, M. Zheng, S. Mrzyk and K.P. Steuhl, CD4+ T-cell type 1 and type 2 cytokines in the HSV-1 infected cornea, *Graefes Arch. Clin. Exp. Ophthalmol.* 237 (1999), pp. 399-406.

[17] K.I. Happel, P.J. Dubin, M. Zheng, N. Ghilardi, C. Lockhart, L.J. Quinton, A.R. Odden, J.E. Shellito, G.J. Bagby, S. Nelson and J.K. Kolls, Divergent roles of IL-23 and IL-12 in host defense against *Klebsiella pneumoniae*, *J. Exp. Med.* 202 (2005), pp. 761-769.

[18] C.A. Murphy, C.L. Langrish, Y. Chen, W. Blumenschein, T. McClanahan, R.A. Kastelein, J.D. Sedgwick and D.J. Cua, Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation, *J. Exp. Med.* 198 (2003), pp. 1951-1957.

[19] G. Fedele, P. Stefanelli, F. Spensieri, C. Fazio, P. Mastrantonio and C.M. Ausiello, Bordetella pertussis-infected human monocyte-derived dendritic cells undergo maturation and induce Th1 polarization and interleukin-23 expression, *Infect. Immun.* 73 (2005), pp. 1590-1597.

[20] X.T. Ma, X.J. Zhang, B. Zhang, Y.Q. Geng, Y.M. Lin, G. Li and K.F. Wu, Expression and regulation of interleukin-23 subunits in human peripheral blood

mononuclear cells and hematopoietic cell lines in response to various inducers, *Cell Biol. Int.* 28 (2004), pp. 689-697.

[21] S. Vanden Eijnden, S. Goriely, D. De Wit, M. Goldman and F. Willems, Preferential production of the IL-12(p40)/IL-23(p19) heterodimer by dendritic cells from human newborns, *Eur. J. Immunol.* 36 (2006), pp. 21-26.

[22] C. Becker, H. Dornhoff, C. Neufert, M.C. Fantini, S. Wirtz, S. Huebner, A. Nikolaev, H.A. Lehr, A.J. Murphy, D.M. Valenzuela, G.D. Yancopoulos, P.R. Galle, M. Karow and M.F. Neurath, Cutting edge: IL-23 cross-regulates IL-12 production in T cell-dependent experimental colitis, *J. Immunol.* 177 (2006), pp. 2760-2764.

[23] A. Sato, M.M. Linehan and A. Iwasaki, Dual recognition of herpes simplex viruses by TLR2 and TLR9 in dendritic cells, *Proc. Natl. Acad. Sci. U.S.A.* 103 (2006), pp. 17343-17348.

[24] K. Kobayashi, E. Ohgitani, Y. Tanaka, M. Kita and J. Imanishi, Herpes simplex virus-induced expression of 70 kDa heat shock protein (HSP70) requires early protein synthesis but not viral DNA replication, *Microbiol. Immunol.* 38 (1994), pp. 321-325.

[25] R. Wang, T. Town, V. Gokarn, R.A. Flavell and R.Y. Chandawarkar, HSP70 enhances macrophage phagocytosis by interaction with lipid raft-associated TLR-7 and upregulating p38 MAPK and PI3K pathways, *J. Surg. Res.* 136 (2006), pp. 58-69.

[26] L. Li, L. Huang, S.S. Sung, P.I. Lobo, M.G. Brown, R.K. Gregg, V.H. Engelhard and M.D. Okusa, NKT cell activation mediates neutrophil IFN-gamma

production and renal ischemia-reperfusion injury, *J. Immunol.* 178 (2007), pp. 5899-5911.

[27] Y. Iwakura and H. Ishigame, The IL-23/IL-17 axis in inflammation, *J. Clin. Invest.* 116 (2006), pp. 1218-1222.

[28] S.M. Wahl, Transforming growth factor-beta: innately bipolar, *Curr. Opin. Immunol.* 19 (2007), pp. 55-62.

[29] K. Banerjee, P.S. Biswas and B.T. Rouse, Role of Stat4-mediated signal transduction events in the generation of aggressor CD4⁺ T cells in herpetic stromal keratitis pathogenesis, *J. Interferon Cytokine Res.* 27 (2007), pp. 65-75.

[30] D.A. Leib, T.E. Harrison, K.M. Laslo, M.A. Machalek, N.J. Moorman and H.W. Virgin, Interferons regulate the phenotype of wild-type and mutant herpes simplex viruses in vivo, *J. Exp. Med.* 189 (1999), pp. 663-672.

[31] L. Shriver, M. Mann and B.N. Dittel, Th17 cells alone are not sufficient to induce CNS autoimmunity, but can synergize with Th1 cells to induce EAE, *J. Immunol.* 178 (2007), 129.24.

APPENDIX

Figure 1. Expression of IL-23p19 mRNA and protein in the corneas and lymphoid organs after HSV-1 ocular infection

(A) WT mice were infected with 5×10^5 PFU of HSV-1 on their scarified corneas. At different time points, mRNA was extracted from the corneas and DLNs, and reverse transcribed into cDNA. Kinetic expression of IL-23p19 mRNA was measured by real-time PCR. Test samples were run in triplicate with a dilution series and the mean value of a particular dilution was used. Data are expressed as copy number per sample. (B) WT mice (n = 3) were ocularly infected with different doses of virus (5×10^5 , 5×10^4 , 5×10^5 PFU) and levels of IL-23 protein were estimated from lysate supernatants of corneas, DLNs, or spleen of mice by an antibody capture ELISA as described by materials and methods.

Fig. 1

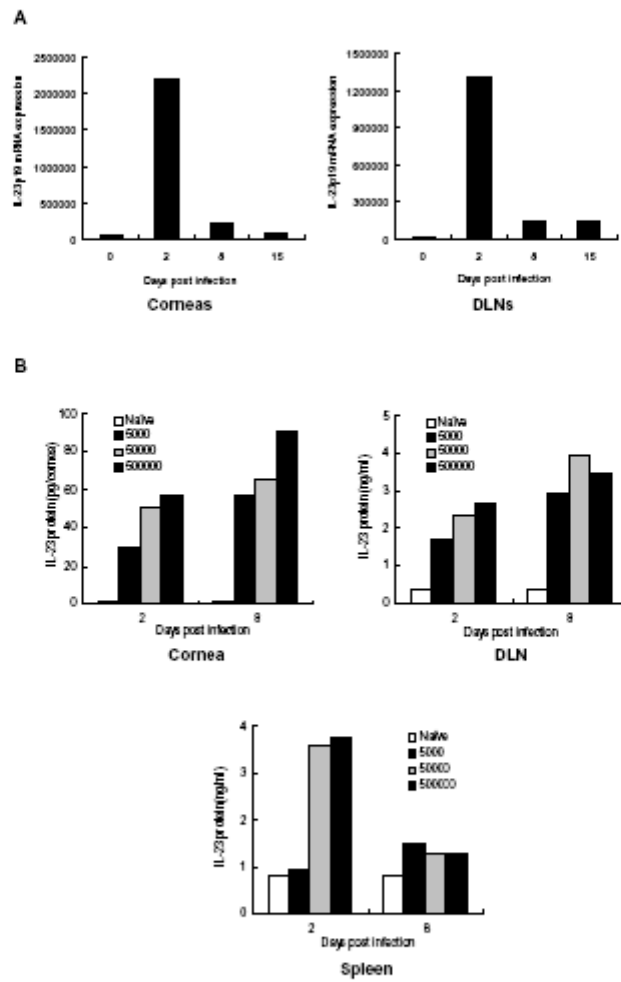


Figure 2. IL-23p19^{-/-} mice have higher SK lesion severity, incidence, ocular angiogenesis, and enhanced viral clearance

Corneas of IL-23p19^{-/-} and WT mice (n = 4) were infected with various doses of HSV-1 (5×10^3 , 5×10^4 , 5×10^5 PFU), and the levels of SK severity and neovascularization were measured in the corneas by biomicroscopy in groups of p19^{-/-} (●) and WT (○) mice. (A) SK lesion severity and angiogenesis scoring was conducted on day 18 p.i.. Results are representative of one of three independent experiments. Statistically significant differences in SK and angiogenic score were observed between the groups (** $p < 0.01$). (B) Stromal lesion severity and angiogenesis in IL-23p19^{-/-} and WT mice (n = 4) infected with low dose of HSV-1 (5×10^3 PFU) were measured at different time intervals. Results are representative of three independent experiments. Statistically significant differences in SK and angiogenic score were observed between the groups (* $p < 0.05$, ** $p < 0.01$). Images were taken by stereomicroscopic imaging system at day 19 after virus infection (original magnification, $\times 40$). (C) The incidence of clinical SK lesions was considered as over score 2 at day 14 p.i.. (D) Infectious virus titers were compared in the different mice groups (n = 4) following virus infection with 5×10^3 PFU. At days 4 and 6 p.i., swabs of the corneal surface were collected and were put into serum free DMEM with antibiotics. Individual samples were diluted further, and viral titers were determined by a plaque assay performed on Vero cells as described materials and methods. Statistically significant differences in number of virus on the cornea (* $p < 0.05$) were observed between the groups.

Fig. 2

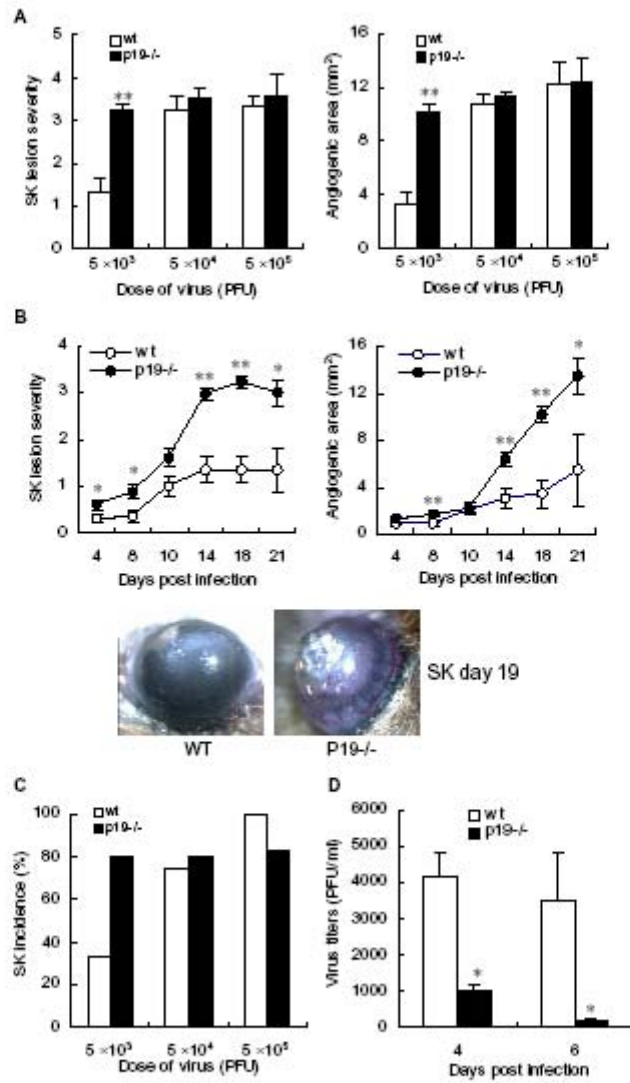


Figure 3. Increased Th1 type immune responses in the spleen of IL-23p19-/- mice after HSV-1 infection

(A) At day 20 p.i. (5×10^3 PFU/eye), spleen cells of the different mice groups were used in antigen specific ELISA. For cytokine ELISA assay, splenocytes from mice were sampled, suspended, and stimulated with irradiated syngeneic-enriched naïve splenocytes pulsed with UV-inactivated HSV (MOI, 1.5) at 37°C for 72 h. Levels of individual cytokines were estimated from supernatant fluid by an antibody capture ELISA as outlined in materials and methods. Statistically significant differences in levels of individual cytokines (* $p < 0.05$, ** $p < 0.01$) were observed between the groups. At day 10 and 20 p.i., splenocytes of the different mice groups ($n = 4$) were used in intracellular cytokine staining assay. For intracellular cytokine staining assay, isolated spleen cells from both groups having SK lesions were stimulated with anti-CD3 and anti-CD28 (B) or stimulated with syngeneic naïve splenocytes pulsed with HSV (C) for 16 h. Single cells were extracellularly stained with anti-mouse CD4 (APC) and CD8 (PerCp) antibody and then intracellularly stained with anti-mouse IFN- γ PE antibody. Values shown in each graph reflect the percentage or number of spleen cells expressing IFN- γ . Statistically significant differences were observed between the groups (* $p < 0.05$, ** $p < 0.01$). Results are representative of two independent experiments.

Fig. 3

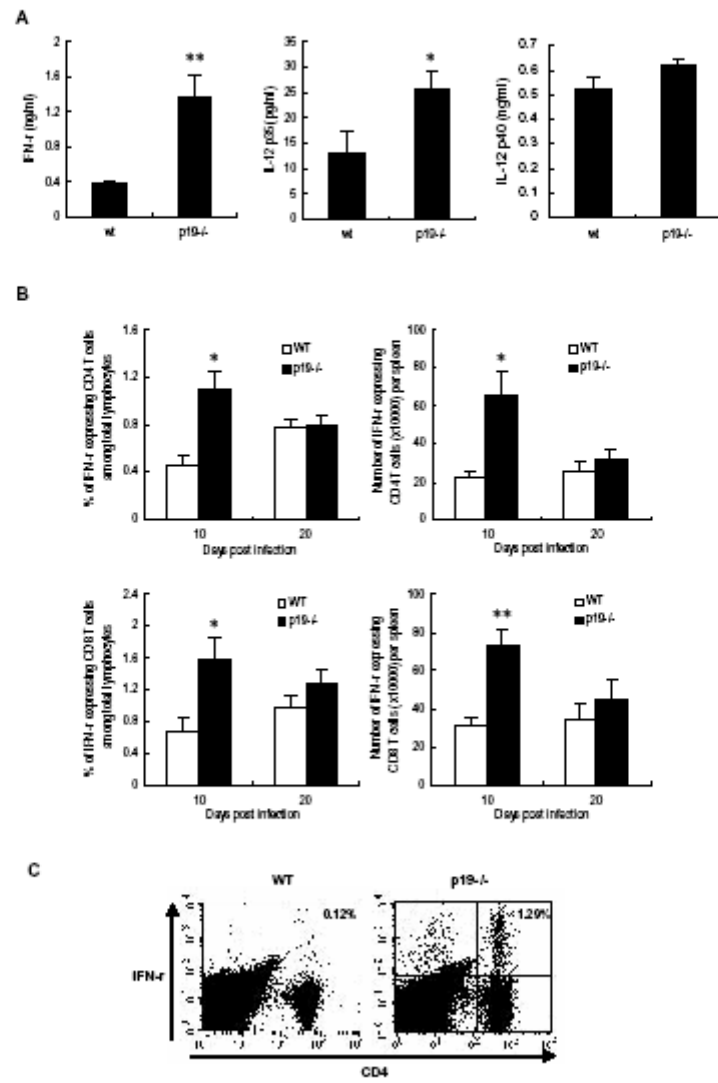
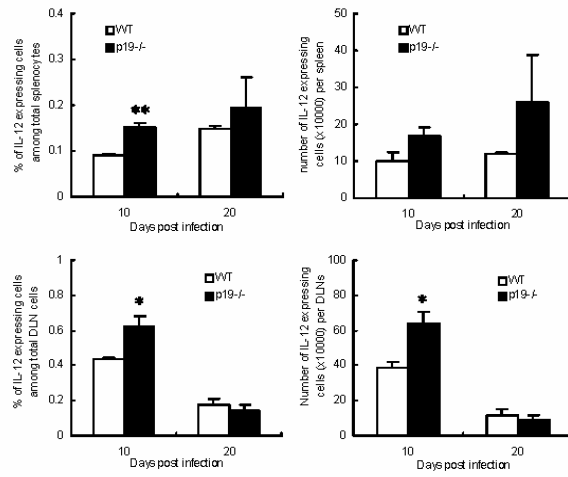


Figure 4. Increased population and number of IL-12 expressing cells in the lymphoid organs of p19^{-/-} mice after HSV-1 infection

At day 10 and 20 p.i., splenocytes or DLNs cells of the different mice groups (n = 4) were used in intracellular cytokine staining assay. Isolated splenocytes or DLNs cells from both groups having SK lesions were stimulated with anti-CD3 and anti-CD28 for 16 h. Single cells were extracellularly stained with anti-mouse CD11b (APC) and CD11c (PerCp) antibody and then intracellularly stained with anti-mouse IL-12 PE antibody (A and B). Values shown in each graph reflect the percentage or absolute number of spleen cells expressing IL-12. At day 10 and 20 p.i., total numbers of splenocytes and DLNs cells of the different mice groups (n = 6) were measured (C).

Fig. 4

A



B

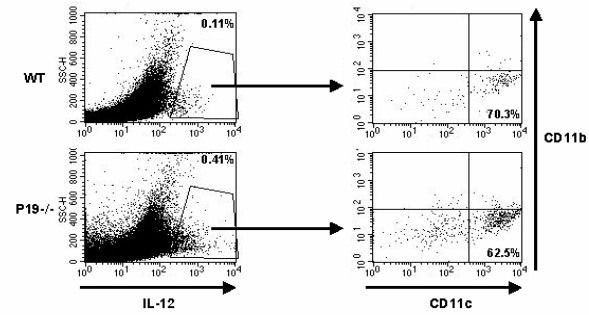


Figure 5. Increased Th1 type immune responses in the SK corneas of p19^{-/-} mice

At day 20 p.i., 6 corneas/group that were infected with 5×10^3 PFU HSV-1 were processed to measure the IFN- γ or IL-12 cytokine levels (A). Levels of individual cytokines were estimated from supernatants of corneal lysates of mice by an antibody capture ELISA as outlined in materials and methods. Statistically significant differences in levels of cytokines (** $p < 0.01$) were observed between the groups. Alternatively, isolated corneal cells from both groups having SK lesions (10 and 20 post infection) were stimulated with anti-CD3 and anti-CD28 for 16 h. Single cells were extracellularly stained with anti-mouse CD4 APC antibody and then intracellularly stained with anti-mouse IFN- γ or IL-12 PE antibody (B and C). Cells were gated on lymphocytes and values shown in each plot represent the percentage of CD4⁺ T cells expressing IFN- γ (C). At day 10 and 20 p.i., total cell numbers of collagenase digested cornea of the different mice groups (n = 6) were measured (D). Statistically significant differences in the absolute cell numbers of SK cornea (* $p < 0.05$, ** $p < 0.01$) were observed between the groups.

Fig. 5

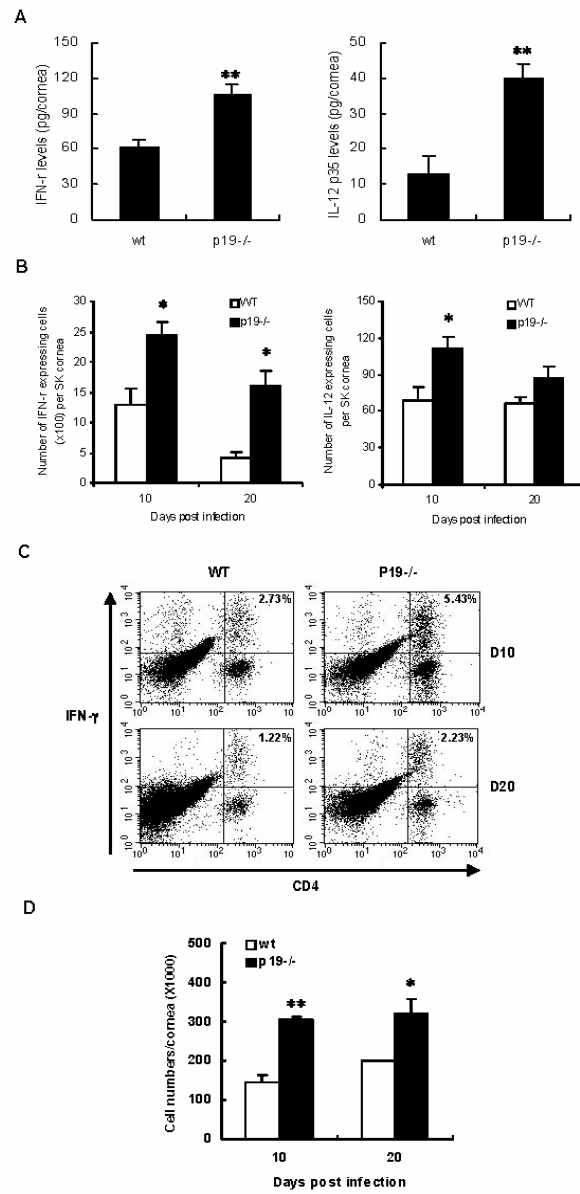
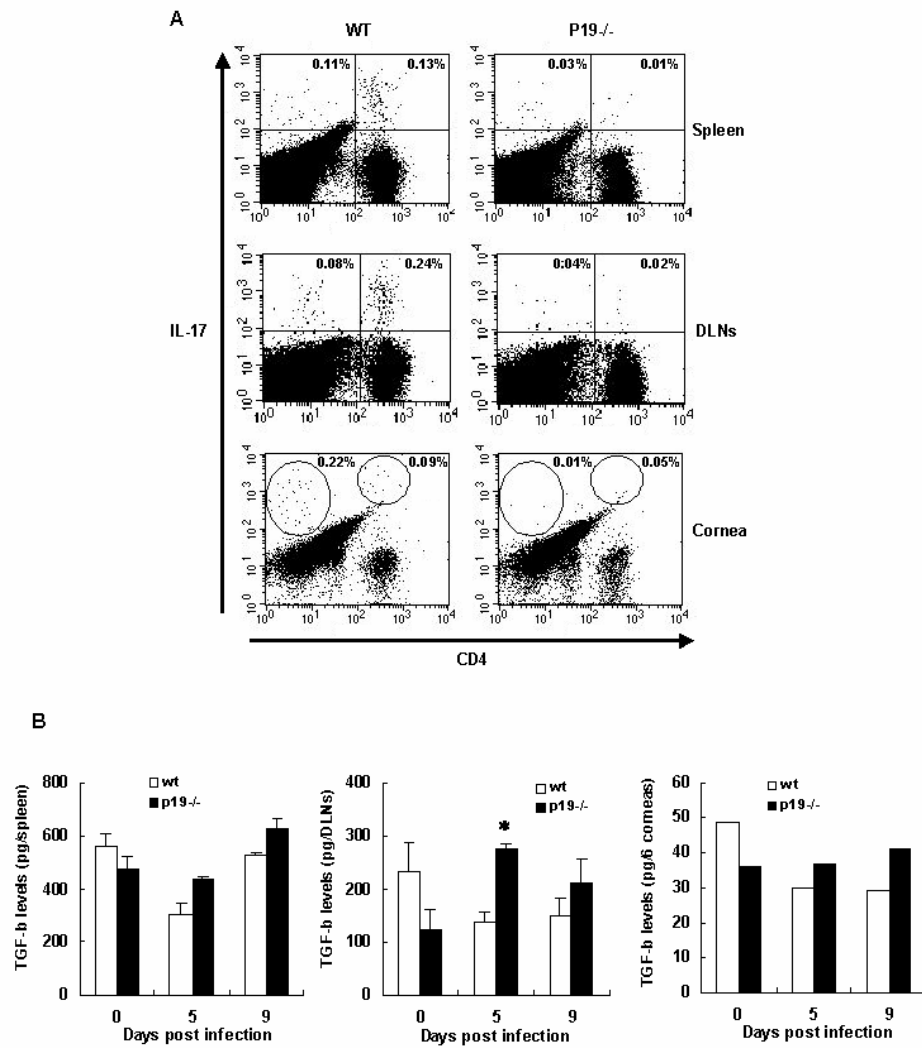


Figure 6. Decreased Th17 immune response in p19^{-/-} mice after HSV-1 infection

At day 20 p.i., splenocytes, DLN cells, and corneal cells from WT and p19^{-/-} mice having SK lesions were stimulated with anti-CD3 and anti-CD28 for 16 h. Single cells were extracellularly stained with anti-mouse CD4 APC antibody and then intracellularly stained with anti-mouse IL-17 PE antibody. Cells were gated on lymphocytes and values shown in each plot represent the percentage of cells expressing IL-17 (A). Data are representative of two similar experiments. At day 5 and 9 p.i., DLNs, spleen, and 6 corneas/group that were infected with 5×10^3 PFU HSV-1 were processed to measure the TGF- β cytokine levels (B). Levels of TGF- β were estimated from supernatants of each tissue's lysates of mice by an antibody capture ELISA as outlined in materials and methods.

Fig. 6



Part-IV

**IL-10 and natural regulatory T cells: Two
independent anti inflammatory mechanisms in
HSV induced ocular immunopathology**

Research described in this chapter is a slightly modified version of an article that in accepted for publication in *Journal of Immunology* by Pranita P Sarangi, Sharvan Sehrawat, Susmit Suvas, and Barry T. Rouse

Sarangi PP, Sehrawat S, Suvas S and Rouse BT. IL-10 and natural regulatory T cells: Two independent anti inflammatory mechanisms in HSV induced ocular immunopathology. (*Journal of Immunology-Accepted*)

In this chapter “our” and “we” refers to me and co-authors. My contribution in the paper includes (1) Selection of the topic (2) Compiling and interpretation of the literature (3) Designing experiments (4) understanding the literature and interpretation of the results (5) providing comprehensible structure to the paper (6) Preparation of graphs, figures and tables (7) Writing and editing

Abstract

Two prominent anti-inflammatory mechanisms involved in controlling HSV-1 induced corneal immunopathology (Stromal Keratitis-SK) are the production of the cytokine IL-10 and the activity of natural regulatory T cells (nTregs). It is not known whether under *in vivo* conditions, IL-10 and nTregs influence the corneal pathology independently or in concert. In the current study using wild-type (WT) and IL-10^{-/-} animals, we have assessed the activity of nTregs in the absence of IL-10 both under *in vitro* and *in vivo* conditions. The IL-10^{-/-} animals depleted of nTregs prior to ocular infection showed more severe SK lesions as compared to the undepleted IL-10^{-/-} animals. In addition, nTregs purified from naïve WT and IL-10^{-/-} animals were equally able to suppress the proliferation and the cytokine production from anti-CD3 stimulated CD4⁺CD25⁻ T cells *in vitro*. Furthermore,

intracellular cytokine staining results indicated that non regulatory cells expressing B220 and CD25 markers were the major IL-10 producing cell types in the lymphoid tissues of HSV infected mice. In contrast, in the infected corneas, cells with the CD11b⁺Gr1⁺ phenotype along with a minor population of Foxp3⁺CD4⁺ and a few F4/80⁺ cells produced IL-10. Our current investigations indicate that at least two independent anti-inflammatory mechanisms are involved in limiting the corneal lesions in SK, both of which may need to be modulated to control SK therapeutically.

Introduction

Ocular infection with herpes simplex virus (HSV) results in a chronic immunoinflammatory reaction in the cornea that can result in blindness. Studies in animal models of stromal keratitis (SK) have revealed that the infection, possibly in part because of its TLR ligand activity sets off a range of critical cellular and molecular events [1], but the actual SK lesions appear to be orchestrated mainly by CD4⁺ T cells that are primarily type 1 cytokine producers [2, 3]. SK lesions may resolve spontaneously and when this occurs in the mouse model, it may be associated with the up regulation of IL-10 production [4]. Additionally, the severity of SK may also be affected by the activity of Foxp3⁺ regulatory T cells [5]. Accordingly, the depletion of such cells may result in more severe lesions [5] and recently we have shown that the adoptive transfer of *in vitro* generated Foxp3⁺ Tregs may modulate the severity of SK lesions [6].

One potential mechanism by which the Tregs carry out their regulatory effects is by the production of inhibitory cytokines such as IL-10 and TGF- β [7]. In fact some types of Tregs may be induced by IL-10 and mediate regulation

principally by producing IL-10 [8]. In some infectious disease models, these inducible Tregs (sometimes referred to as Tr1 cells) are considered to play a pivotal role in viral pathogenesis [9]. That IL-10 is critical for resolution of inflammatory lesions has been shown in many systems including SK [10]. Thus SK lesions may be more severe in IL-10^{-/-} animals [11] but it is unclear if this reflects the failed function of some type of regulatory T cell or be the consequence of failure of IL-10 production by some innate inflammatory cell type. Thus macrophages and some dendritic cells (DCs) subtypes as well as highly activated CD4⁺ T cells may be major sources of IL-10 production [12, 13] and certain pathogens may encode ligands that can stimulate IL-10 production from some cells [14, 15].

In the present study, we have compared the pathogenesis of SK in wild-type (WT) and IL-10^{-/-} mice in an attempt to further define if Treg mediated control of the inflammatory reaction is dependent or independent of IL-10 production. Our results show that compared to WT controls increased SK lesions occur in the absence IL-10 as well as if nTregs are deleted. Interestingly, depletion of nTregs even in IL-10^{-/-} animals resulted in even more enhanced SK lesion severity compared to IL-10^{-/-} mice indicating that the effect of Tregs mainly occurred independently of IL-10 production. In support of this finding, Tregs purified from IL-10^{-/-} and WT appeared equally active at suppression and could inhibit the cytokine production from TCR stimulated CD4⁺CD25⁻ T cells isolated from naive and infected WT animals. Furthermore, in the infected corneas, non regulatory cells with the CD11b⁺Gr1⁺ phenotype along with a minor population of Foxp3⁻CD4⁺ and a few F4/80⁺ cells produced IL-10. Our results show that at least

two independent anti-inflammatory mechanisms namely, IL-10 and nTregs are involved in controlling corneal immunopathology in SK. We discuss the implication of our studies for future novel therapies for SK

Material and methods

Animals

IL-10^{-/-} breeders were obtained from the Jackson laboratory (Bar Harbor, ME) and were maintained in the animal facility of University of Tennessee. C57BL/6 mice were obtained from Harlan Sprague-Dawley, (Indianapolis IN). Foxp3-GFP mice [16] were kind gift from Dr.Mohammed Oukka (Harvard Medical School). To prevent bacterial superinfection, all mice received prophylactic treatment with Sulfamethoxazole/Trimethoprim (Biocraft, Elmond Park, NY) at the rate of 5 ml/200 ml drinking water after virus infection. All experimental procedures followed the guidelines the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. The animal facilities of the University of Tennessee (Knoxville) are fully accredited by the American Association of Laboratory Animal Care.

In vivo depletion of nTregs and HSV-1 infection

IL-10^{-/-} and C57BL/6 animals were given 0.7mg of anti-CD25 monoclonal antibody pc61 mAb (Bioexpress) i/p, 3 days prior to corneal infection. Depletion was tested by staining for CD4⁺CD25⁺ cells in the spleen and lymph nodes (Fig. 1A). More than 80% depletion was achieved by day 3 after depletion. HSV-1 strain RE (obtained from R. Lausch, University of South Alabama College of Medicine) was used in all procedures. Virus was propagated and stored as described previously [17]. For all experiments 6-8 week old females were used

except for comparison of IL-10^{-/-} with Treg depleted IL-10 animals in which 9-10 week old male mice were used. Corneal infections of all mouse groups were conducted under deep anesthesia induced by intra-peritoneal injection of Avertin (Sigma-Aldrich, St. Louis, MO). The mice were scarified on their corneas with a 27 gauge needle and infected with a 3 µl drop containing 5×10^5 plaque-forming units (PFU) of HSV-1 was applied to the eye and gently massaged with the eyelids.

Clinical observation

The eyes were examined on different days post infection (p.i.) for the development of clinical lesions by slit-lamp biomicroscopy (Kawa Co., Nagoya, Japan), and the clinical severity of keratitis lesions and development of neovascularization of individually scored mice was recorded as described previously [18]. In brief, 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 stromal keratitis. Similarly, the angiogenic scoring system relied on quantifying the degree of neovessel formation based on three primary parameters: 1) the circumferential extent of neovessels (as the angiogenic response is not uniformly circumferential in all cases); 2) the centripetal growth of the longest vessels in each quadrant of the circle; and 3) the longest neovessel in each quadrant was identified and graded between 0 (no neovessel) and 4 (neovessel in the corneal center) in increments of ~0.4 mm (radius of the cornea is ~1.5 mm). 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 to 16) for each eye at a given time point.

Histopathology

For histopathologic analysis, eyes from different groups of mice were extirpated at the indicated time point p.i. and snap-frozen in OCT compound (Miles, Elkart, IN). Six micron thick sections were cut, air dried in a desiccation box, and stained with hematoxylin and eosin (Richard Allen Scientific, Kalamazoo, MI).

Flow cytometry

For flow cytometry measurement of the infiltrating cells, four corneas per time point per group were collected at indicated time points by dissecting the corneal buttons above the limbus by a scalpel. Similarly, two trigeminal ganglia (TGs) were collected per time point. Corneas or TGs were digested in Liberase (Roche Diagnostics Co., Indianapolis, IN) for 45 min at 37°C. Cervical draining lymph nodes (DLNs) and spleens were also collected from individual animals. Single cell suspension was prepared as described elsewhere [19]. The Fc receptors were blocked with unconjugated anti-CD16/32 (BD Pharmingen, San Diego, CA) for 30 min. Samples were incubated with fluorescein isothiocyanate (FITC) labeled anti-Gr-1, anti-CD11b, anti-CD11c, anti-NK1.1, anti-B220, anti-CD19, anti-CD25 (7D4), anti-CD8 (BD Pharmingen), anti-F4/80 (eBioscience), and PerCpCy5.5 labeled anti-CD4 (BD Pharmingen), antibody for 30 min. For intracellular cytokine staining PE labeled anti-IL-10, anti-IL-17, anti-IFN- γ and FITC labeled anti-IFN- γ , anti-TNF- α , antibodies were used (BD Pharmingen, San Diego, CA). For Foxp3⁺IL-10⁺ cell staining, mice expressing Foxp3-GFP were used and GFP⁺IL-10⁺ cells were measured in such mice by using intracellular IL-10 staining. Intracellular staining for IL-10, TNF- α and IFN- γ was performed using Fixation/Permeabilization Solution Kit with BD GolgiPlug (BD Bioscience). For

intracellular cytokine staining cells were stimulated with anti-CD3 and anti-CD28 (1 µg/ml) or PMA/Ionomycin or UV inactivated HSV (3MOI). Foxp3 staining was performed using mouse regulatory T cell staining kit (eBiosciences). All samples were collected on a FACScan (BD Biosciences, San Diego, CA), and data were analyzed by using cell Quest 3.1 software (BD Biosciences).

Virus specific CD8⁺IFN-γ⁺ staining

To determine the number of IFN-γ producing CD8⁺ T cells in the infected draining lymph node (DLN) and spleen, intracellular cytokine staining was performed as previously described [20]. Single-cell suspension of infected DLN and spleen was prepared. 10⁶ cells/well were cultured in 96-well U bottom plates. Cells were left untreated or stimulated with SSIEFARL peptide (HSVgB₄₉₈₋₅₀₅ [synthesized at Genemed Synthesis, Inc., CA]) (1 µg/ml) for 5 h at 37°C in 5% CO₂. Brefeldin A (10 µg/ml) was added to the culture for the intracellular cytokine accumulation. Cell surface marker and intracellular cytokine staining for IFN-γ was performed using a Cytofix/Cytosperm kit (BD Pharmingen). All samples were collected with a FACScan and were analyzed with Cell Quest 3.1 software.

Purification of CD4⁺CD25⁺ and CD4⁺D25⁻ T cells

CD4⁺CD25⁺ and CD4⁺D25⁻ Tregs were purified from the lymph nodes of WT and IL-10^{-/-} mice using a Treg isolation kit (Miltenyi Biotec, CA) according to the suggested protocol. The purity of cells ranged from 89–92% (Fig. 8A). Accessory cells were isolated from spleens of WT mice by depleting Thy1.2⁺ cells using Thy1.2 magnetic beads (Miltenyi Biotec, CA). Thy1.2 depleted cells were irradiated before adding to the cultures. CD4⁺D25⁻ T cells from Thy1.1 B6 animals were used for in vitro cultures.

Cytokine inhibition and in vitro suppression Assay

Purified Tregs (5×10^5 /well) from IL-10^{-/-} and WT animals were cultured with CD4⁺CD25⁻ T cells (5×10^5 /well) isolated from naïve and infected WT animals along with irradiated splenocytes (1×10^6 /well). Anti-CD3 antibody was added to the wells (1 µg/ml). Cells were cultured in a 48 well plate and the culture supernatants were collected after 48 h and the levels of IFN-γ and IL-2 measured by sandwich ELISA. For in vitro suppression assays, in one of the experiment CD4⁺CD25⁻ T cells from Thy1.1 mice (1×10^5 /well) were labeled with CFSE (0.5 µM) and were cultured in a 96 well plate with different dilutions of WT or IL10^{-/-} CD4⁺CD25⁺ T cells (1:1, 1:2, 1:4, 1:8, 1:16, 1:32) in the presence of anti-CD3 antibody (1 µg/ml) and antigen presenting cells (APCs) (2×10^5 /well).

Cytokine ELISA

Cervical DLNs from individual mice and six corneas per group were collected at the indicated time points. The DLNs and corneas were sonicated and the level of IL-6, IL-12 and IL-10 were measured in the supernatants. Similarly, culture supernatant from the *in vitro* suppression assay was collected and the level of IL-2 and IFN-γ were determined by a standard sandwich ELISA protocol. Anti IL-6, IL-12, IL-2, IL-10 and IFN-γ capture and detection antibodies were purchased from BD Pharmingen and standard recombinant murine IL-6, IL-12, IL-2, IL-10 and IFN-γ were obtained from R&D Systems (Minneapolis, MN). Biotinylated detection antibodies were purchased from BD Pharmingen. The color reaction was developed using ABTS (Sigma-Aldrich, St. Louis, MO) and measured with an ELISA reader (Spectramax 340; Molecular Devices, Sunnyvale, CA) at 405 nm.

Quantification was performed with Spectramax ELISA reader software version 1.2.

Statistical Analysis

Statistical significance was determined by Student's *t* test. $P < 0.05$ was regarded as a significant difference between the groups.

Results

The absence of IL-10 and depletion of nTreg results in increased SK severity

The outcome of HSV infection was followed and compared in three groups of mice that were age and sex matched and ocularly infected with the same dose of HSV-1 RE. The pattern of disease that developed differed significantly in the three groups. Thus both IL-10^{-/-} and Treg depleted animals showed heightened susceptibility compared to WT animals (Fig.1B and C). Clinical lesions were evident in IL-10^{-/-} animals before those in other groups. At 8 days p.i., the incidence of SK (lesion score ≥ 3) was 0 and 30% in WT and IL-10^{-/-} animals respectively (not shown). The majority of IL-10^{-/-} animals had developed moderately severe lesions (≥ 3) by 16 day p.i. (90% in the experiment shown in Fig. 1B). In contrast, less than 40% of WT animals in the same experiment developed ≥ 3 lesions and almost two fold more than WT in the nTreg depleted group (Fig. 1B). The pattern of more severe responses in IL-10^{-/-} and Treg depleted animals was also evident when the extent of angiogenesis was quantified in the different groups (Fig.1E and F). Eyes showing median responses in each group were selected for histological analysis. As is evident in Fig. 2A, inflammatory reactions were more severe than in WT in both the IL-10^{-/-} and Treg depleted animals. In other experiments, corneal samples were collected from 8

randomly selected eyes taken from animals at 17 days p.i. to prepare single cell suspensions following collagen digestion for phenotypic analysis by flow cytometry. Firstly, total viable cell numbers recovered from IL-10^{-/-} and Treg depleted animals was greater than WT animals (Fig.2C). In addition, the frequency of cells with the neutrophil phenotype was also higher in IL-10^{-/-} and nTreg depleted WT as compared to WT animals (Gr1⁺CD11b⁺ cells, Fig. 2B). With regard to total CD4⁺ T cells, the highest numbers of such cells were present in the eyes of IL-10^{-/-} animals (Fig. 2D). Comparing the ratio of Foxp3⁺CD4⁺ T cells to Foxp3⁻CD4⁺ T cells, the ratio was lower in IL-10^{-/-} animals as compared to WT (Fig. 2E). Of note, before infection, the number of Foxp3⁺ cells in the secondary lymphoid tissues was comparable to WT in IL-10^{-/-} mice (data not shown).

Cellular analyses were also performed in single cell suspensions of pooled trigeminal ganglia from the 3 groups of animals. As with the corneas, inflammatory cell numbers were elevated in the TG of IL-10^{-/-} and nTreg depleted compared to WT animals (Fig 2F). In addition, the frequency of both CD8⁺ and CD4⁺ T cells were both elevated compared to WT with the highest frequencies present in IL-10^{-/-} animals (Fig. 2G).

Along with changes in inflammatory cell influx in the different groups of mice, the numbers of IFN- γ and IL-17 producing CD4⁺ T cells were also measured at day 17 in each group by intracellular cytokine staining. Once again the highest frequencies of cytokine producing cells were present in the corneas of IL-10^{-/-} with Treg depleted showing less and WT the least amount (Fig. 3A-C). By intracellular cytokine staining, we also looked for the cytokine producing CD4⁺ T cells in the cervical lymph node and the spleen of these mice. As shown in Fig. 4,

about three and eight fold increases in the numbers of CD4⁺ T cells producing IFN- γ and TNF- α were found in the DLN and spleens in the absence of nTregs and IL-10 respectively. This increase in proinflammatory mediators in the cornea and secondary lymphoid tissues along with higher Treg numbers in IL-10^{-/-} animals may mean an inability of nTregs to suppress the disease pathology in the proinflammatory milieu [21, 22] or that the pathogenic Th1 type CD4⁺ T cells present at the site are resistant to nTregs mediated suppression [23, 24].

We also determined the magnitude of SSIEFARL specific (immunodominant epitope in C57BL/6 mice) CD8⁺ T cell responses and cytokine production by CD8⁺ T cells in these animals. As shown in the Fig. 5A and B, higher magnitude of immune responses were noted in the absence of either Tregs (three fold) or IL-10 (two fold). Interestingly, in contrast to CD4⁺ T cells, the increase in the total CD8⁺IFN- γ ⁺ was greater in the absence of nTregs as compared to IL-10 which could mean different pathways of immune response generation could be influenced by IL-10 and nTregs.

Treg effects are still evident in IL-10^{-/-} mice

One explanation for the greater susceptibility of IL-10^{-/-} animals could relate to the function of adaptive or natural Tregs. Thus the former cell type is usually dependent on IL-10 so would not be expected to be present in IL-10^{-/-} animals. Studies in some cases have indicated that Foxp3⁺ natural Tregs may also function at least in part by their production of IL-10 [25]. Another explanation could be the dysfunction of nTregs in the presence of high concentration of proinflammatory mediators, such as IL-6 and IL-12, at the site [22, 23]. In this

regard, higher levels of IL-6 and IL-12 were found in the DLN and corneal lysates of IL-10^{-/-} animals as compared to WT (Fig. 6).

Experiments were performed in older IL-10^{-/-} male mice, which are more resistant to HSV induced lesions than the 6-8 weeks old female mice used in previous experiments. To evaluate the role of nTregs in controlling SK lesion development, Treg depleted IL-10^{-/-} (DEP-IL-10^{-/-}) were compared with non depleted IL-10^{-/-} animals. As shown in Fig. 7A-D, depletion of CD25⁺ cells from IL-10^{-/-} animals resulted in increased SK lesions (7A and D) as well as angiogenic responses (7B and D). At day 20 p.i., 55% eyes in depleted IL-10^{-/-} animals had SK scores of ≥ 3 compared with 25% in undepleted IL-10^{-/-} mice (7C). At this time point, the SK scores were 2.3 ± 0.3 ($p \leq 0.05$) in Treg depleted IL-10^{-/-} mice as compared to 1 ± 0.2 in normal IL-10^{-/-} animals. A similar pattern was also evident in the development of neovascularization in the depleted IL-10^{-/-} animals. At day 20 p.i., Treg depleted IL-10^{-/-} animals had neovascularization scores of 10.8 ± 1.1 as compared to 7.4 ± 1 in control IL-10^{-/-} animals. Additionally, there was an earlier onset of SK lesions in the Treg depleted IL-10^{-/-} animals as compared to normal IL-10^{-/-} animals. The incidence of SK at day 12 p.i. was 30 % and 58 % in the IL-10^{-/-} and DEP-IL-10^{-/-} group respectively (Fig. 7C). Increased numbers of IFN- γ ⁺CD4⁺ T cells (Fig 7E) and other inflammatory cell influx were also noted in the corneas and TG of such animals as compared to the controls (not shown). Similarly, as shown in Fig. 7F, the virus specific CTL response, as assessed by the frequency and the absolute number of virus specific CD8⁺IFN- γ ⁺ T cells in the secondary lymphoid tissues, was also significantly increased in the absence of nTregs in the IL-10^{-/-} animals. These results indicate that even in IL-10^{-/-} animals,

nTregs can exert their suppressive activity and that their function could be independent of IL-10 in HSV induced corneal immunopathology.

To support the apparent IL-10 independent *in vivo* suppressive activity of nTregs, we isolated CD4⁺CD25⁺ nTregs from uninfected WT and IL-10^{-/-} animals and those were cultured with CFSE labeled CD4⁺CD25⁻ cells isolated from naïve and 12 day post infected WT animals. Cells were stimulated with anti-CD3 and accessory cells *in vitro* for 48 h. As is shown in Fig. 8B-D, both groups of nTregs could equally suppress the proliferation (Fig. 8B) and cytokine production (Fig. 8D) from WT CD4⁺CD25⁻ T cells.

What is the main cellular source of IL-10 in SK

Several reports have showed the production of IL-10 by Foxp3⁺ Tregs [25-27]. To determine if in SK nTregs themselves produce IL-10 post infection, we measured the IL-10 production using Foxp3-GFP mice at day 5, 11 and 18 p.i. As shown in Fig. 8E and table 1, we could not detect substantial numbers of GFP⁺IL-10⁺ cells in the lymphoid tissues although very few such cells were present in the infected corneas at later time points post infection (Table. 1). Additionally, IL-10⁺IFN- γ ⁺CD4⁺ T cells were not evident in either cornea or lymphoid tissues (not shown). These data suggest that non regulatory cells could be the major cellular source of IL-10 p.i.

To further study the phenotype of the IL-10 producing cells in the infected corneas and secondary lymphoid tissues, intracellular cytokine staining was performed with cells taken from infected WT corneas, spleens and DLNs at day 5, 11, and 20 p.i. The cells were stained with surface markers such as c-kit, Gr-1, CD11c, CD11b, B220, CD19, CD25, CD3, NK1.1, and CD4 along with

intracellular IL-10. As shown in Table. 1, the majority of the IL-10 producing cells in the spleen and DLNs also expressed B220, CD25 and F4/80 markers. More than 50% of the cells also expressed c-kit and Gr1 markers (data not shown). Interestingly, as shown in the Table 1, in the infected corneas non regulatory cells with the CD11b⁺Gr1⁺ phenotype along with a minor population of Foxp3⁺CD4⁺ and a few F4/80⁺ cells produced IL-10.

Influence of nTreg on IL-10 production

Since our results indicated that Treg could have IL-10 independent suppression in SK, and that these cell types did not constitute a significant part of the IL-10 producing cell types, we determined whether the activity of nTregs could influence the production of IL-10 p.i. In many reports, Tregs were shown to suppress immune responses via IL-10 production [8, 28]. Interestingly, as measured by intracellular cytokine staining and cytokine ELISA, an increase in the absolute number and frequencies of IL-10 producing cells as well as IL-10 proteins was noted in the lymphoid tissues in the absence of Tregs in the WT animals as compared to undepleted WT animals (Fig. 9A and B). But the mean fluorescence intensity (MFI) of IL-10⁺ cells remained comparable among Treg depleted and undepleted WT animals and similar was the case of phenotype of IL-10 producing cells (Data not shown). This could be due to the generalized suppressive activity of Tregs on the IL-10 producing cell types such as macrophages or DCs or the activated CD4⁺ T cells. In contrast as is evident in the Fig. 9C, the level of IL-10 in the corneas of Treg depleted WT mice was lower as compared to undepleted WT animals at day 4 and day 9p.i.

Discussion

Many chronic immunoinflammatory diseases are difficult to control therapeutically. One such example is SK caused by HSV infection of the eye. It is anticipated that control measures will improve once mechanisms involved in lesion resolution are all identified and their respective role fully understood. With regard to SK, few studies have been done, but reports have demonstrated that upregulation of certain cytokines such as IL-10 and TGF- β help resolve lesions [29, 30]. Recently, observations in a variety of inflammatory diseases indicate that several types of regulatory cells control and resolve inflammatory lesions and some of these appear to function by producing either or both IL-10 and TGF- β [31]. With SK, natural regulatory T cells were shown previously to influence lesions but the mechanisms by which this is achieved were not identified [5]. In some systems, nTregs appear to function independently of any cytokine production [32], whereas in others IL-10 production is a necessary event [25, 28].

In this report, we show that both nTreg and IL-10 participate in the control of SK lesion severity, but these two mechanisms proceed largely independently of each other. Our results show that the severity of SK lesions become enhanced if animals are unable to produce IL-10, because of gene knock out, or have been depleted of nTreg. Furthermore, depletion of nTreg in IL-10^{-/-} mice led to even higher levels of disease severity than in undepleted IL-10^{-/-} mice. In addition, nTreg purified from WT and IL-10^{-/-} animals appeared to be equally suppressive *in vitro* to the activity of non regulatory cells. Few if any Foxp3⁺CD4⁺ Treg in both the ocular lesions or draining LN were IL-10 producers, In fact, the major source of IL-10 appeared to be non T cells such as granulocytes and mononuclear

inflammatory cells. Accordingly, these HSV induced immunoinflammatory lesions can be modulated independently by both IL-10 and nTreg activity which could mean that future novel therapies will need to manipulate both mechanisms to achieve maximal effects.

The means by which inflammatory lesions are influenced by regulatory T cells remain poorly understood especially in responses caused by pathogens [33]. In some virus induced immunoinflammatory lesions, some advocate a major role for adaptive Tregs induced by antigen in the presence of IL-10 [9, 34]. These so-called Tr1 cells regulate responses by producing an abundance of IL-10 [9, 34]. Their role in SK remains unexplored. In other systems, such as Leishmania infections, cells of the nTreg phenotype control lesions but these appear to act by producing IL-10 [35, 36]. The same may also be true in other parasite mediated lesions as well as in bacterial induced colitis [36, 37]. Our results showing that a significant nTreg effect could be demonstrated in mice genetically unable to produce IL-10 strongly argues against IL-10 being a dominant mechanism by which nTreg act *in vivo*. Similarly such cells isolated from IL-10^{-/-} mice acted equally to those from WT animals *in vitro* indicating no role for IL-10 to mediate their function as others have argued to be the case using different approaches [38].

It is conceivable that several functional subsets of the Foxp3⁺ regulatory phenotype exist. Those thymic derived autoantigen specific cells, that are primarily depleted by pc61 mAb in naïve mice, may function in a different way to those converted *in vivo* from CD4⁺Foxp3⁻ foreign antigen specific precursors. This conversion process was shown recently to occur by several groups [39-41] including ourselves [6]. We suspect that the nTregs preexisting at the time of

infection are signaled in some way by the HSV infection and these control SK in a manner that does not depend on IL-10 production. In contrast, the Foxp3⁺ Tregs that act in more chronic infections may largely represent Foxp3⁺ converts and these may function primarily by producing IL-10. Since we have recently succeeded in generating Foxp3⁺ Treg converts from non regulatory precursors [6], we are now in the position to compare these with normal nTregs for their ability to modulate SK as well as their dependence on IL-10 production. Such studies are in progress.

Although our results could not define the site at which IL-10 and nTregs function to control SK lesions, we suspect they may be quite different. We anticipate that IL-10 acts mainly at the ocular site to control lesions. Moreover, the major source of this cytokine was shown to be non regulatory cells having the CD11b⁺Gr1⁺ and F4/80⁺ phenotype. Some of these cells may be equivalent to the CD11b⁺Gr1⁺ myeloid suppressor cells described in some tumor systems [42]. We also demonstrated that the IL-10 appeared to act by inhibiting the production of proinflammatory cytokines such as IFN- γ , IL-17 and TNF- α . Cells producing such cytokines were more abundant in lesions of IL-10^{-/-} than in WT animals. The results of several other observations also support a similar anti-inflammatory effect of IL-10 [43, 44].

Although our observations as well as former reports show the presence of Tregs at the site of inflammation [5, 24, 45], we can not assess whether or not they exert a suppression effect in the inflamed tissue. In fact whether Tregs function at inflammatory site remains debatable. Thus, one report showed that Tregs taken from an inflammatory site failed to suppress pathogenic T cells isolated from the

lesions when tested *in vitro* [24], perhaps because their activity was inhibited by one or more inflammatory cytokines as has been shown to occur *in vitro* [22, 23]. Interestingly, others have shown that Tregs do proliferate at such sites and that they can suppress IFN- γ , although not IL-17 production, by CD4⁺CD25⁻ T cells isolated from the lesion site [45]. In our current study, WT mice depleted of nTreg had higher levels of proinflammatory cytokines in the lesions as well as in the lymphoid tissues. Conceivably, such cytokines could inhibit their function, but this issue requires further evaluations using Tregs isolated from the inflamed cornea at different time points p.i.

A body of evidence favors the idea that nTregs mainly act to regulate in lymphoid tissues [46]. Our data favors this idea too. Accordingly, in nTreg depleted WT animals the absolute numbers of IL-10 producing cells in the draining LN and spleen were increased accounting at least in part for the inhibitory effects that occurred. In addition, Treg depletion resulted in better HSV specific immune response providing more of the effector cells responsible for mediating SK.

One surprising result of our observations was that an effect of nTreg was easy to demonstrate even when the cells were called upon to function in an environment rich in cytokines assumed to impair their function. Thus we demonstrated marked elevations of cytokines such as IL-6 and IL-12 in the LNs and ocular tissues of infected IL-10^{-/-} compared to similarly infected WT animals. Despite this, an effect of nTreg depletion was readily apparent in the IL-10^{-/-} infected mice. These results may mean either that in the *in vivo* inflammatory environment the inhibitory effects of some cytokines observed *in vitro* is not a

major effect, or that the Tregs function at certain sites such as in LNs where effector cells are being generated is not inhibited by inflammatory cytokines. Some recent observations on EAE also support the concept that effector cell functions in lymphoid sites may be inhibited by Foxp3⁺ Tregs whereas at the same time effectors at lesion site may resist such regulation[24]. These issues are under further investigation in the SK system.

LIST OF REFERENCES

- [1] P.P. Sarangi, B. Kim, E. Kurt-Jones, B.T. Rouse, Innate recognition network driving herpes simplex virus-induced corneal immunopathology: role of the toll pathway in early inflammatory events in stromal keratitis, *J Virol* 81 (2007) 11128-11138.
- [2] S. Deshpande, K. Banerjee, P.S. Biswas, B.T. Rouse, Herpetic eye disease: immunopathogenesis and therapeutic measures, *Expert Rev Mol Med* 6 (2004) 1-14.
- [3] M.G. Niemialtowski, B.T. Rouse, Predominance of Th1 cells in ocular tissues during herpetic stromal keratitis, *J Immunol* 149 (1992) 3035-3039.
- [4] J.S. Babu, S. Kanangat, B.T. Rouse, T cell cytokine mRNA expression during the course of the immunopathologic ocular disease herpetic stromal keratitis, *J Immunol* 154 (1995) 4822-4829.
- [5] S. Suvas, A.K. Azkur, B.S. Kim, U. Kumaraguru, B.T. Rouse, CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions, *J Immunol* 172 (2004) 4123-4132.
- [6] S. Sehrawat, S. Suvas, P.P. Sarangi, B.T. Rouse, In vitro generated antigen specific CD4+CD25+Foxp3+ regulatory T cells control the severity of virus induced immunoinflammatory lesion, *Immunology 2007 meeting abstract* 178 (2007).
- [7] M. Miyara, S. Sakaguchi, Natural regulatory T cells: mechanisms of suppression, *Trends Mol Med* 13 (2007) 108-116.
- [8] A. O'Garra, P.L. Vieira, P. Vieira, A.E. Goldfeld, IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage, *J Clin Invest* 114 (2004) 1372-1378.

- [9] A.J. MacDonald, M. Duffy, M.T. Brady, S. McKiernan, W. Hall, J. Hegarty, M. Curry, K.H. Mills, CD4 T helper type 1 and regulatory T cells induced against the same epitopes on the core protein in hepatitis C virus-infected persons, *J Infect Dis* 185 (2002) 720-727.
- [10] T.M. Tumpey, V.M. Elner, S.H. Chen, J.E. Oakes, R.N. Lausch, Interleukin-10 treatment can suppress stromal keratitis induced by herpes simplex virus type 1, *J Immunol* 153 (1994) 2258-2265.
- [11] T.M. Tumpey, H. Cheng, X.T. Yan, J.E. Oakes, R.N. Lausch, Chemokine synthesis in the HSV-1-infected cornea and its suppression by interleukin-10, *J Leukoc Biol* 63 (1998) 486-492.
- [12] K.W. Moore, R. de Waal Malefyt, R.L. Coffman, A. O'Garra, Interleukin-10 and the interleukin-10 receptor, *Annu Rev Immunol* 19 (2001) 683-765.
- [13] C.F. Anderson, M. Oukka, V.J. Kuchroo, D. Sacks, CD4(+)CD25(-)Foxp3(-) Th1 cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis, *J Exp Med* 204 (2007) 285-297.
- [14] Y. Yanagawa, K. Onoe, Enhanced IL-10 production by TLR4- and TLR2-primed dendritic cells upon TLR restimulation, *J Immunol* 178 (2007) 6173-6180.
- [15] S.C. Higgins, E.C. Lavelle, C. McCann, B. Keogh, E. McNeela, P. Byrne, B. O'Gorman, A. Jarnicki, P. McGuirk, K.H. Mills, Toll-like receptor 4-mediated innate IL-10 activates antigen-specific regulatory T cells and confers resistance to *Bordetella pertussis* by inhibiting inflammatory pathology, *J Immunol* 171 (2003) 3119-3127.

- [16] E. Bettelli, Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, V.K. Kuchroo, Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells, *Nature* 441 (2006) 235-238.
- [17] P.G. Spear, B. Roizman, Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion, *J Virol* 9 (1972) 143-159.
- [18] B. Kim, P.P. Sarangi, Y. Lee, S. Deshpande Kaistha, S. Lee, B.T. Rouse, Depletion of MCP-1 increases development of herpetic stromal keratitis by innate immune modulation, *J Leukoc Biol* 80 (2006) 1405-1415.
- [19] S. Deshpande, M. Zheng, S. Lee, K. Banerjee, S. Gangappa, U. Kumaraguru, B.T. Rouse, Bystander activation involving T lymphocytes in herpetic stromal keratitis, *J Immunol* 167 (2001) 2902-2910.
- [20] U. Kumaraguru, B.T. Rouse, Application of the intracellular gamma interferon assay to recalculate the potency of CD8(+) T-cell responses to herpes simplex virus, *J Virol* 74 (2000) 5709-5711.
- [21] S. Dominitzki, M.C. Fantini, C. Neufert, A. Nikolaev, P.R. Galle, J. Scheller, G. Monteleone, S. Rose-John, M.F. Neurath, C. Becker, Cutting edge: trans-signaling via the soluble IL-6R abrogates the induction of FoxP3 in naive CD4+CD25 T cells, *J Immunol* 179 (2007) 2041-2045.
- [22] C. Pasare, R. Medzhitov, Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells, *Science* 299 (2003) 1033-1036.
- [23] I.L. King, B.M. Segal, Cutting edge: IL-12 induces CD4+CD25- T cell activation in the presence of T regulatory cells, *J Immunol* 175 (2005) 641-645.

- [24] T. Korn, J. Reddy, W. Gao, E. Bettelli, A. Awasthi, T.R. Petersen, B.T. Backstrom, R.A. Sobel, K.W. Wucherpfennig, T.B. Strom, M. Oukka, V.K. Kuchroo, Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation, *Nat Med* 13 (2007) 423-431.
- [25] S. Sakaguchi, F. Powrie, Emerging challenges in regulatory T cell function and biology, *Science* 317 (2007) 627-629.
- [26] C.L. Maynard, L.E. Harrington, K.M. Janowski, J.R. Oliver, C.L. Zindl, A.Y. Rudensky, C.T. Weaver, Regulatory T cells expressing interleukin 10 develop from Foxp3(+) and Foxp3(-) precursor cells in the absence of interleukin 10, *Nat Immunol* 8 (2007) 931-941.
- [27] C.M. Freeman, B.C. Chiu, V.R. Stolberg, J. Hu, K. Zeibecoglou, N.W. Lukacs, S.A. Lira, S.L. Kunkel, S.W. Chensue, CCR8 is expressed by antigen-elicited, IL-10-producing CD4⁺CD25⁺ T cells, which regulate Th2-mediated granuloma formation in mice, *J Immunol* 174 (2005) 1962-1970.
- [28] K.J. Maloy, L. Salaun, R. Cahill, G. Dougan, N.J. Saunders, F. Powrie, CD4⁺CD25⁺ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms, *J Exp Med* 197 (2003) 111-119.
- [29] N.A. Kuklin, M. Daheshia, S. Chun, B.T. Rouse, Immunomodulation by mucosal gene transfer using TGF-beta DNA, *J Clin Invest* 102 (1998) 438-444.
- [30] M. Daheshia, N. Kuklin, S. Kanangat, E. Manickan, B.T. Rouse, Suppression of ongoing ocular inflammatory disease by topical administration of plasmid DNA encoding IL-10, *J Immunol* 159 (1997) 1945-1952.
- [31] B.T. Rouse, Regulatory T cells in health and disease, *J Intern Med* 262 (2007) 78-95.

- [32] A.M. Thornton, E.M. Shevach, CD4⁺CD25⁺ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production, *J Exp Med* 188 (1998) 287-296.
- [33] B.T. Rouse, S. Suvas, Regulatory T cells and immunity to pathogens, *Expert Opin Biol Ther* 7 (2007) 1301-1309.
- [34] N.A. Marshall, M.A. Vickers, R.N. Barker, Regulatory T cells secreting IL-10 dominate the immune response to EBV latent membrane protein 1, *J Immunol* 170 (2003) 6183-6189.
- [35] J. Ji, J. Masterson, J. Sun, L. Soong, CD4⁺CD25⁺ regulatory T cells restrain pathogenic responses during *Leishmania amazonensis* infection, *J Immunol* 174 (2005) 7147-7153.
- [36] Y. Belkaid, R.B. Blank, I. Suffia, Natural regulatory T cells and parasites: a common quest for host homeostasis, *Immunol Rev* 212 (2006) 287-300.
- [37] A. Izcue, J.L. Coombes, F. Powrie, Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation, *Immunol Rev* 212 (2006) 256-271.
- [38] E.M. Shevach, R.S. McHugh, C.A. Piccirillo, A.M. Thornton, Control of T-cell activation by CD4⁺ CD25⁺ suppressor T cells, *Immunol Rev* 182 (2001) 58-67.
- [39] S. Yamagiwa, J.D. Gray, S. Hashimoto, D.A. Horwitz, A role for TGF- β in the generation and expansion of CD4⁺CD25⁺ regulatory T cells from human peripheral blood, *J Immunol* 166 (2001) 7282-7289.
- [40] W. Chen, W. Jin, N. Hardegen, K.J. Lei, L. Li, N. Marinos, G. McGrady, S.M. Wahl, Conversion of peripheral CD4⁺CD25⁻ naive T cells to CD4⁺CD25⁺

regulatory T cells by TGF-beta induction of transcription factor Foxp3, *J Exp Med* 198 (2003) 1875-1886.

[41] C.M. Sun, J.A. Hall, R.B. Blank, N. Bouladoux, M. Oukka, J.R. Mora, Y. Belkaid, Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid, *J Exp Med* 204 (2007) 1775-1785.

[42] P. Sinha, V.K. Clements, S.K. Bunt, S.M. Albelda, S. Ostrand-Rosenberg, Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response, *J Immunol* 179 (2007) 977-983.

[43] E.H. Wilson, U. Wille-Reece, F. Dzierszynski, C.A. Hunter, A critical role for IL-10 in limiting inflammation during toxoplasmic encephalitis, *J Neuroimmunol* 165 (2005) 63-74.

[44] M.C. Kullberg, A.G. Rothfuchs, D. Jankovic, P. Caspar, T.A. Wynn, P.L. Gorelick, A.W. Cheever, A. Sher, *Helicobacter hepaticus*-induced colitis in interleukin-10-deficient mice: cytokine requirements for the induction and maintenance of intestinal inflammation, *Infect Immun* 69 (2001) 4232-4241.

[45] R.A. O'Connor, K.H. Malpass, S.M. Anderton, The inflamed central nervous system drives the activation and rapid proliferation of Foxp3⁺ regulatory T cells, *J Immunol* 179 (2007) 958-966.

[46] Q. Tang, J.A. Bluestone, Regulatory T-cell physiology and application to treat autoimmunity, *Immunol Rev* 212 (2006) 217-237.

APPENDIX

Figure 1. Increased SK lesions in the absence of IL-10 and nTregs

- A. Percentage of CD4⁺CD25⁺ T cells in the spleens at day 4 of the animals depleted of nTregs by α -CD25 antibody (pc61 mAb, 0.7mg) treatment. Staining for CD25 was performed with FITC labeled anti-CD25 (clone7D4) antibody. Left dot plot shows the spleen of an untreated control animal.
- B. The bar diagram demonstrates the percentage severity of each group of mice infected with 5×10^5 pfu HSV-1 RE at day 16 p.i. The IL-10^{-/-} and Treg depleted mice showed increased incidence (SK score ≥ 3) of disease as compared to WT animals.
- C. SK lesion scores of individual eyes at day 16 p.i of WT, DEP-WT (Treg depleted WT), and IL-10^{-/-}, mice infected with 5×10^5 pfu infection dose.
- D. SK lesion scores in three groups of mice at different time points p.i. *, ($P \leq 0.05$), statistically significant differences as compared to WT control.
- E. Angiogenesis lesion scores of individual eyes at day 16 p.i of WT, DEP-WT, and IL-10^{-/-}, mice infected with 5×10^5 pfu infection dose.
- F. Angiogenesis sores at different time points p.i. in the three groups of mice. *, ($P \leq 0.05$), statistically significant differences as compared to WT control.

A

No depletion α -CD25 depletion

CD25

CD4

20
80

3
97

B

Incidence of SK
Score ≥ 3

WT DEP-WT IL10^{-/-}

38 75 90

C

SK Scores

WT DEP-WT IL-10^{-/-}

D

SK Scores

○ WT
□ DEP-WT
▲ IL10^{-/-}

4 8 12 16

Days p.i.

E

Angiogenesis Scores

WT WT-DEP IL-10^{-/-}

F

Angiogenesis Scores

Days p.i.

Figure 2. Increased inflammation in the corneas of mice deficient in IL-10 and nTregs

A. Mice were infected with 5×10^5 pfu HSV-1 RE. Mice were terminated at Day 17 p.i., and eyes were processed for cryo-section. Hematoxylin and eosin staining was carried out on 6- μ m sections. The figure shows the pictures of the section taken at 10x magnification.

B. Single-cell suspensions of the infected corneas were prepared from six corneas at day 17 p.i. for each group of mice. The cells were labeled for Gr1⁺CD11b⁺ (PMNs) cells. The dot plots represent the Gr1⁺CD11b⁺ cells in the WT, DEP-WT and IL-10^{-/-} corneas. The number on the dot plots denotes the percentage of inflammatory cells expressing both Gr1⁺ and CD11b⁺ markers.

C. The bars represent the total number of viable cells present per cornea of different groups of mice.

D. The cells isolated from infected corneas at day 17 p.i were stained for CD4 marker and the bars represent the total number of CD4⁺ T cells present per cornea of different groups of mice.

E. Intra-nuclear staining for CD4⁺Foxp3⁺ nTregs was performed with the cells isolated from corneas. The bars represent the percentage of CD4⁺Foxp3⁺ cells present in the total CD4⁺ T cells per cornea of different groups of mice.

F. Single-cell suspensions were prepared from four TGs at day 17 p.i. for each group of mice. The cells were labeled for Gr1⁺CD11b⁺ cells. The dot plots represent the Gr1⁺CD11b⁺ cells in the WT, DEP-WT and IL-10^{-/-} corneas

Figure. 2 cont.

The number on the dot plots denotes the percentage of inflammatory cells expressing both Gr1⁺ and CD11b⁺ markers in TG.

G. The cells from TGs were labeled for CD4⁺ and CD8⁺ cells. The dot plots represent the CD4⁺ and CD8⁺ cells in the WT, DEP-WT and IL-10^{-/-} corneas.

The number on the dot plots denotes the percentage of inflammatory cells expressing CD4⁺ and CD8⁺ markers.

Fig 2

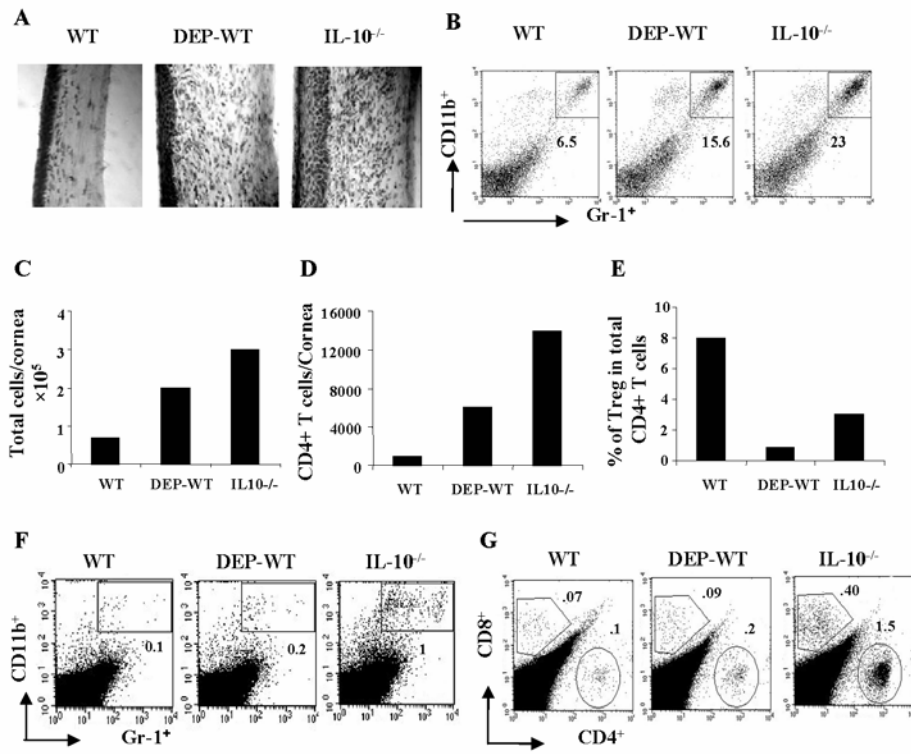


Figure 3. Increased number of CD4⁺IFN- γ ⁺ T cells in the corneas of mice deficient in IL-10 and nTregs

Single-cell suspensions of corneal cells were prepared from six corneas at day 17 p.i. Cells were stimulated with either anti-CD3 and anti-CD28 antibody or UV-HSV. Intracellular IFN- γ staining along with CD4 surface staining was carried out as described in materials and methods. The results in the figure represent the data from one of two similar experiments.

- A. Dot plots represent the frequencies of CD4⁺IFN- γ ⁺ T cells when gated on the lymphocytes.
- B. The bar diagram demonstrates the total number of CD4⁺IFN- γ ⁺ in the cornea in different groups.
- C. The bar diagram demonstrates the total number of CD4⁺IL-17⁺ in the cornea in different groups.

Fig. 3

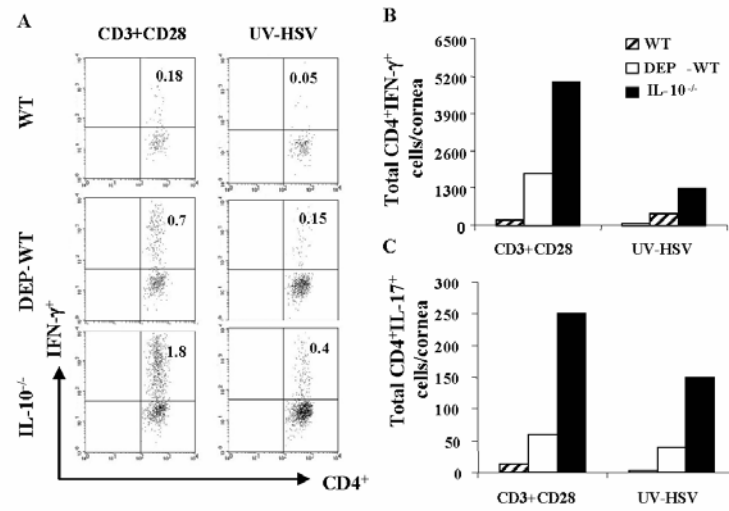


Figure 4. Increased number of IFN- γ ⁺ and TNF- α ⁺ CD4 T cells in the secondary lymphoid tissues of IL-10^{-/-} mice as compared to nTregs depleted and WT mice

Single-cell suspensions of the individual spleen and the cervical draining lymph nodes (DLN) were prepared from four mice of each group at day 17 p.i. Cells were stimulated with either anti-CD3 and anti-CD28 antibody or UV-HSV as described in the materials and methods. Intracellular cytokine staining along with CD4 surface staining was carried out as described in materials and methods. In fig. A and B, dot plots represents the frequencies of CD4⁺IFN- γ ⁺ and CD4⁺TNF- α ⁺ T cells respectively in the lymphoid tissues of different groups of mice when gated on the lymphocytes. Bar diagram demonstrates the total number of CD4⁺IFN- γ ⁺ and CD4⁺TNF- α ⁺ T cells per DLN or Spleen in different groups (N=4 per group). *, ($P \leq 0.05$), statistically significant differences as compared to WT animals. Note: Similar pattern was also noticed in the number of CD4⁺IL-17⁺ T cells.

Fig. 4

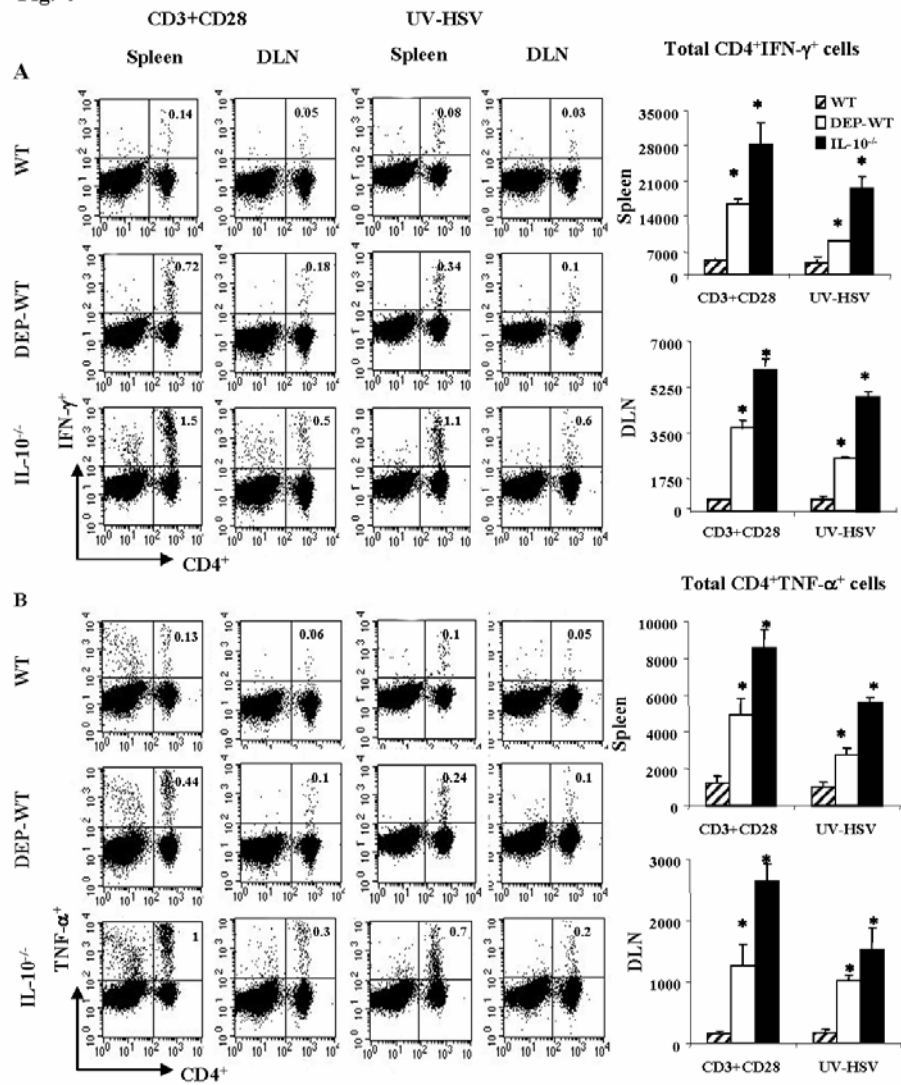


Figure 5. Increased number of SSIEFARL peptide specific CD8⁺IFN- γ ⁺ T cells in the secondary lymphoid tissues of mice deficient in nTregs as compared to IL-10^{-/-} and WT

Single-cell suspensions of the spleen and the cervical lymph nodes were prepared from four mice of each group at day 17 p.i. SSIEFARL specific CD8⁺ IFN- γ ⁺ staining was carried out as described in materials and methods.

- A. The dot plots in the figure represent the frequencies of CD8⁺IFN- γ ⁺ cells (CD8⁺ on the x-axis and IFN- γ ⁺ on the y-axis). The numbers on the upper right quadrants represent the percentage of CD8⁺IFN- γ ⁺ T cells in the gated population of lymphocytes in the spleens. The plots are representative of four animals analyzed per each group (N=4).
- B. The bar diagram demonstrates the total number of double positive cells per DLN or Spleen in different groups. *, ($P \leq 0.05$), and **, ($P \leq 0.01$) statistically significant differences as compared to WT animals

Fig. 5

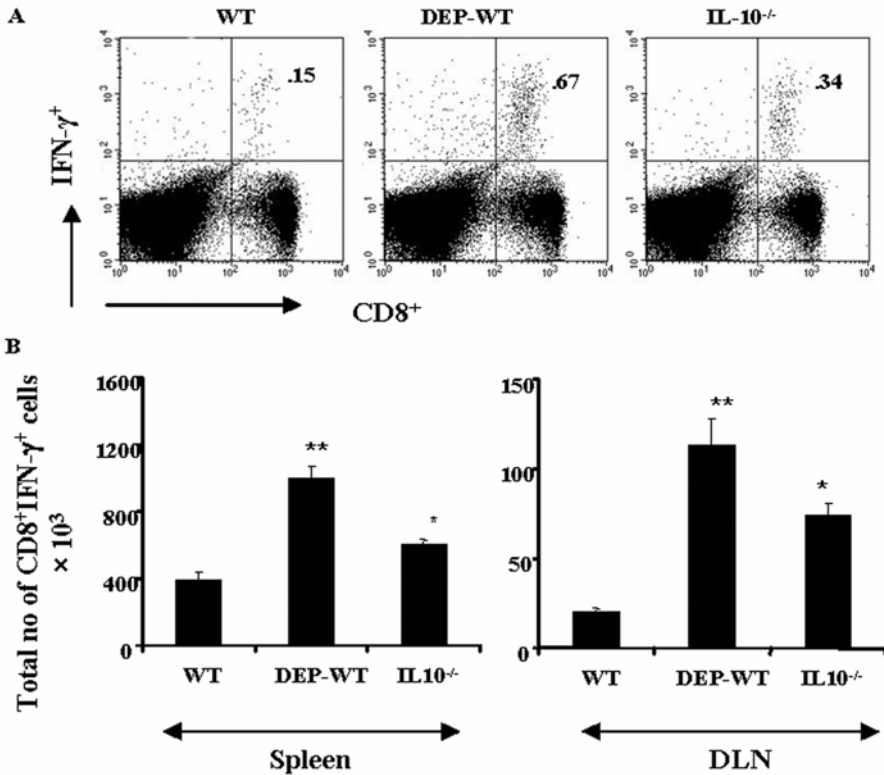
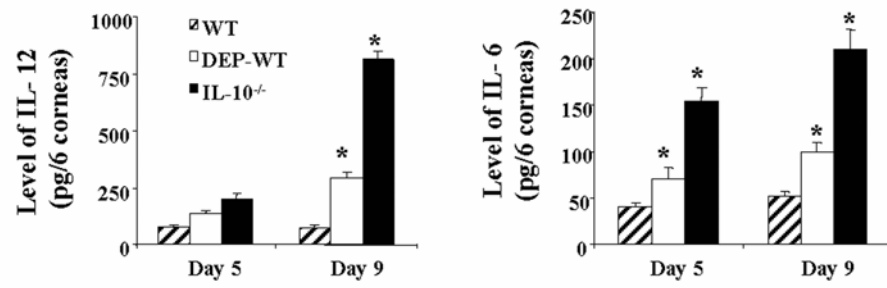


Figure 6. Increased expression of inflammatory mediators in the corneas and lymphoid tissues of mice deficient in IL-10 and nTregs

At the indicated time points, six corneas/group and cervical lymph nodes from individual animals were processed to measure the IL-6, and IL-12 levels. Level of cytokines were measured in the supernatants of tissue lysates of WT, DEP-WT and IL-10^{-/-} animals infected with 5×10^5 pfu HSV-1 RE using sandwich ELISA as outlined in materials and methods. Bar diagrams in Fig. A, and B represent the levels of IL-12 and IL-6 in the corneal and DLN lysates of the three groups of animals. *, ($P \leq 0.05$), statistically significant difference as compared to WT animals.

Fig. 6

A



B

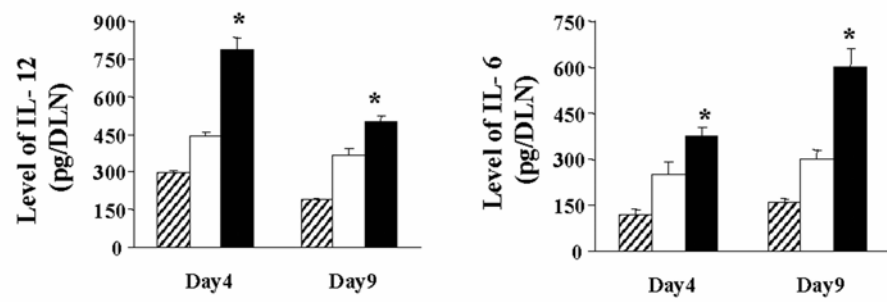


Figure 7. Increased SK severity in the absence of nTregs in the IL-10^{-/-} animals

- A. Mean SK lesion scores at different time point p.i. of Treg depleted IL-10^{-/-} (DEP-IL-10^{-/-}) and undepleted IL-10^{-/-} animals. *, ($P \leq 0.05$), statistically significant differences as compared to undepleted IL-10^{-/-} mice.
- B. Mean angiogenesis scores at different time point p.i. of Treg depleted IL-10^{-/-} (DEP-IL-10^{-/-}) and undepleted IL-10^{-/-} animals. *, ($P \leq 0.05$), statistically significant differences as compared to undepleted IL-10^{-/-} mice.
- C. Bar diagram demonstrate the percentage severity (SK score ≥ 3) of both the groups of mice at day 14 and day 20 p.i.
- D. Eye pictures showing the lesion severity at day 18 p.i. in IL-10^{-/-} and DEP-IL-10^{-/-} animals.
- E. Single-cell suspensions of corneal cells were prepared from six corneas at day 20 p.i. Cells were stimulated with either anti-CD3 and anti-CD28 antibody or UV-HSV. Intracellular IFN- γ staining along with CD4 surface staining was carried out as described in materials and methods. Bar diagram demonstrate the total number of CD4⁺IFN γ ⁺ T cells/ cornea at day 17 p.i.
- F. Single-cell suspensions of the spleen and the cervical lymph nodes were prepared from four mice of each group at day 20 p.i. SSIEFARL specific CD8⁺IFN- γ ⁺ staining was carried out as described in materials and methods. The bar diagram demonstrates the total number of double positive cells per DLN or spleen in different groups. *, ($P \leq 0.05$), statistically significant differences as compared to undepleted IL-10^{-/-} animals.

Fig. 7

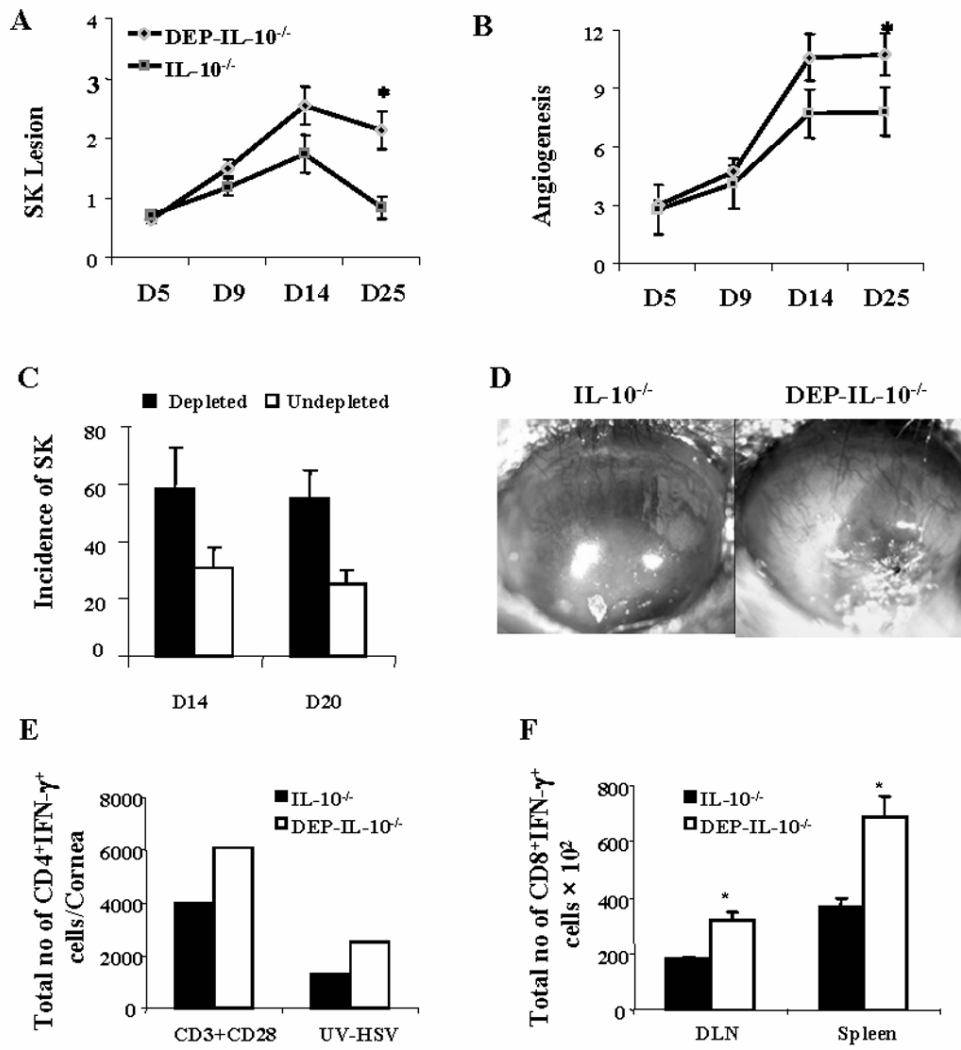


Figure 8. nTregs from both IL-10^{-/-} and WT animals can equally suppress the proliferation and cytokine production from conventional T cell isolated from WT animals

CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were isolated as described in materials and methods and were cultured in the presence of irradiated splenocytes and anti-CD3. Culture supernatants were collected at 48 hr and the level of cytokines was analyzed by sandwich ELISA. For measuring proliferation of non Tregs, CD4⁺CD25⁻ from Thy1.1 mice were labeled with CFSE and the extent of suppression activity was assessed from the intensity of CFSE staining.

- A. Dot plots show the purity of isolated CD4⁺CD25⁺ T cells from WT and IL-10^{-/-} mice.
- B. Histograms demonstrate the suppression of proliferation of conventional T cells by nTregs isolated from IL-10^{-/-} (lower panel) and WT (upper panel) animals. Proliferation of CD4⁺CD24⁻ T cells are shown at 1:1, 1:4 and 1:8 dilutions of CD4⁺ CD25⁺ nTregs. Grey dotted lines denote the division of CD4⁺CD25⁻ T cells in the absence of nTregs and the solid lines show the suppression in the presence of nTregs.
- C. Bar diagrams show the levels of IFN- γ in the 48 hr culture supernatant in the presence and absence of nTregs. As shown in the figure, both the nTregs could equally suppress the cytokine production.
- D. Bar diagrams show the levels of IL-2 in the 48 hr culture supernatant in the presence or absence of nTregs.
- E. GFP-Foxp3 mice were infected with HSV-RE and intracellular staining for IL-10 was performed as outlined in the materials and methods. As shown in the

Figure 8 cont.

dot plots, no IL-10⁺ and GFP⁺ cells were detected in the lymphoid tissues after infection.

Fig. 8

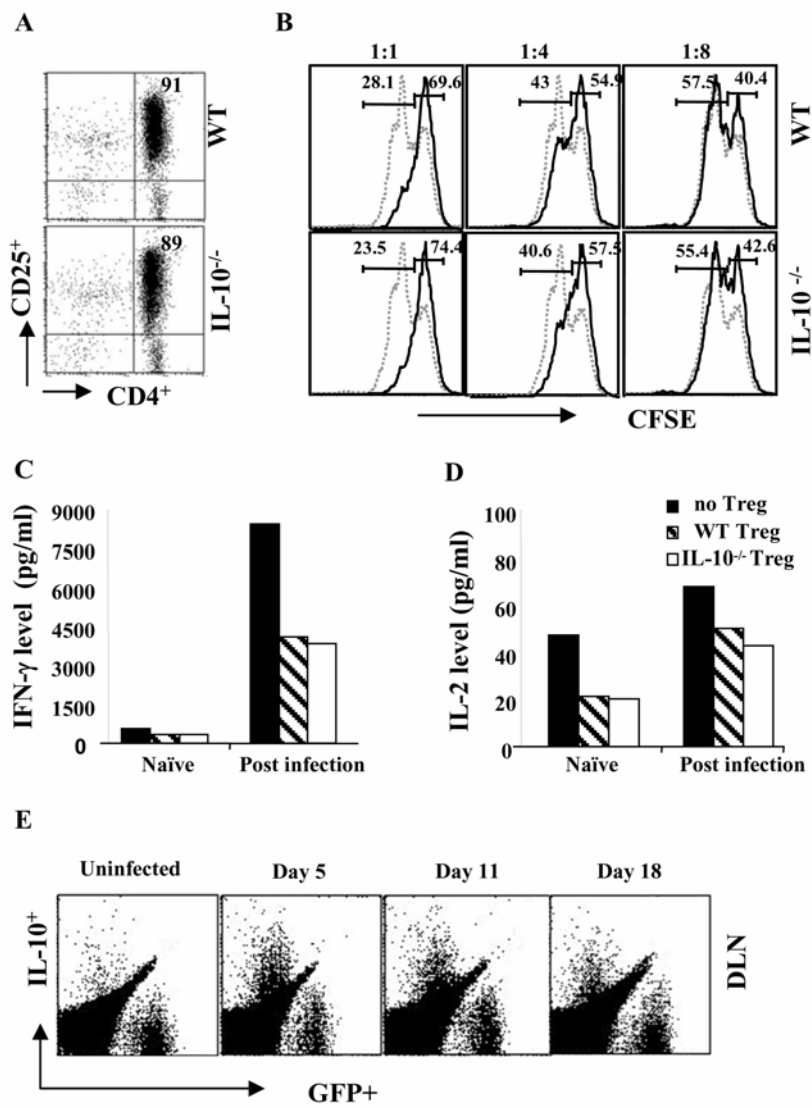
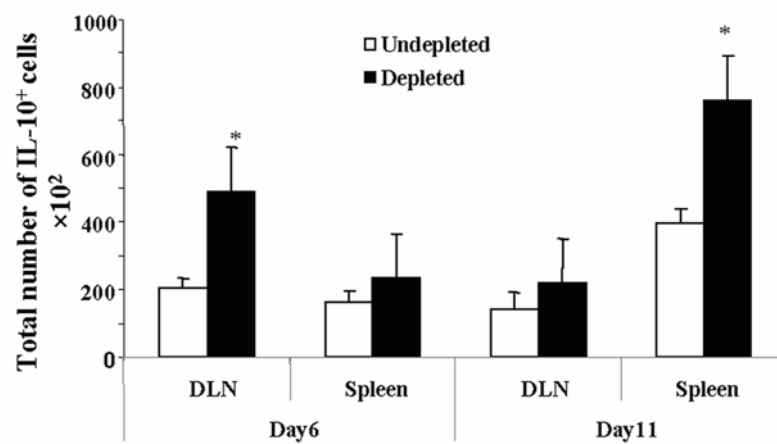


Figure 9. A. Tregs do not produce IL-10 but can influence the IL-10 production from other cell types in the lymphoid tissues

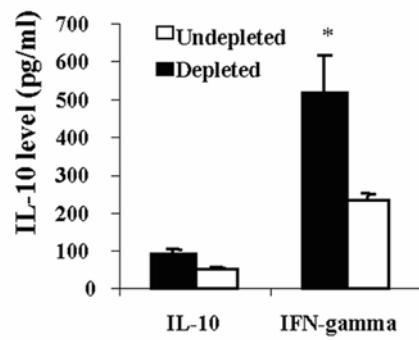
To ascertain the effect of Tregs on IL-10 production, total numbers of IL-10⁺ cells were measured in the Treg depleted and undepleted animals. A. The bar diagram demonstrates an increase in the number of IL-10⁺ cells in the lymphoid tissues of Treg depleted animals. B. The bar diagram demonstrates an increase in the IL-10 and IFN- γ protein levels in the Treg depleted animals as compared to undepleted WT animals at day 6 p.i. C. The bar diagram demonstrates the level IL-10 in the corneal lysates of Treg depleted animals as compared to undepleted WT animals at day 4 and 9 p.i. *, ($P \leq 0.05$), statistically significant differences as compared to undepleted WT animals.

Fig. 9

A



B



C

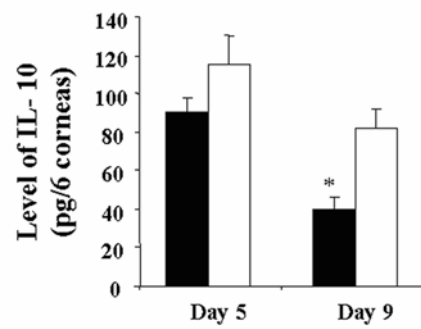


Table 1.**Phenotype of IL-10 producing cells in various tissues**

Days p.i.	Surface markers	% of Total IL-10 ⁺ cells		
		Cornea	Spleen	DLN
5	CD4 ⁺	30	<5	5±2
	Foxp3 ⁺	0	UD	UD
	Gr1 ⁺ CD11b ⁺	50	-	-
	F4/80 ⁺	10	6±2.6	<5
	B220 ⁺	-	62±4.2	65±11
	CD25 ⁺	-	40±4	45±8
11	CD4 ⁺	5-10	7±2.5	5±3.2
	Foxp3 ⁺	2	UD	UD
	Gr1 ⁺ CD11b ⁺	80	-	-
	F4/80 ⁺	5	14±3.2	7.6±3
	B220 ⁺	-	77±4.3	89±2
	CD25 ⁺	-	65±3	73±4.3
20	CD4 ⁺	5-10	UD	UD
	Foxp3 ⁺	2-5	<1	<1
	Gr1 ⁺ CD11b ⁺	70-80	-	-
	F4/80 ⁺	<5	20±5	25±2.5
	B220 ⁺	-	83±3	84±4
	CD25 ⁺	-	55±5	60.5±6

* The number of Foxp3+IL-10+ cell number is determined by using GFP⁺IL10⁺ cell number. After day 20 p.i.an increase in the number of these double positive cells was noticed in the infected corneas but not in the lymphoid tissues.

Vita

Pranita Pragnyadipta Sarangi was born on June 05, 1979, in Bhubaneswar, India. She grew up in Bhubaneswar and attended Sainik School, Bhubaneswar and completed her pre university education in 1996. She was then admitted in College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology in 1996. She received her Bachelor in Veterinary Science and Animal Husbandry (B.V. Sc. & A. H.) in 2002. After graduation, she joined Heifer Project International (HPI)/India as veterinary extension officer till Jan 2003. Then she worked as junior veterinary officer in the Govt. of Orissa, Dept. of animal husbandry and fisheries, Orissa, India till July 2003. She joined the University of Tennessee, Knoxville in August 2003 and received a Doctor of Philosophy (Ph.D) from the graduate program of Comparative and Experimental Medicine, Department of Pathobiology, College of Veterinary Medicine in March 2008. She is going to pursue her post doctoral training under Dr. Minsoo Kim at Department of Microbiology and Immunology, David H. Smith Center for Vaccine Biology and Immunology, University of Rochester Medical Center, University of Rochester, NY.