Immunopathological basis of lymphocytic choriomeningitis virus-induced chorioretinitis and keratitis

Martin S. Zinkernagel¹,², Beatrice Bolinger², Philippe Krebs², Lucas Onder², Simone Miller², and Burkhard Ludewig²*

¹Clinic of Ophthalmology, ²Research Department, Kantonal Hospital of St. Gallen, 9007 St. Gallen, Switzerland;

Running title: LCMV-induced chorioretinitis

Abbreviations: LCMV, lymphocytic choriomeningitis virus; CTL, cytotoxic T cell; Arm, LCMV strain Armstrong; DC, dendritic cell

Correspondent footnote: Burkhard Ludewig, Research Department, Kantonal Hospital St. Gallen, Rorschacherstrasse 95, 9007 St. Gallen, Switzerland; phone +41 71 4941090; fax +41 71 4946321, email: burkhard.ludewig@kssg.ch

Abstract word count: 196
Text word count: 3845
Abstract
Infection of humans with the rodent-borne lymphocytic choriomeningitis virus (LCMV) can lead to central nervous system (CNS) disease in adults or severe neurological disease with hydrocephalus and chorioretinitis following congenital infection. Although LCMV-induced meningitis and encephalitis have been studied extensively, the immunopathological mechanisms underlying LCMV infection-associated ocular disease remain elusive. We report here that intraocular administration of the neurotropic LCMV strain Armstrong (Arm) elicited pronounced chorioretinitis and keratitis, whereas infection with the more viscerotropic strains WE and Docile precipitated less severe immunopathological ocular disease. Time course analyses revealed that LCMV Arm infection of the uvea and neuroretina led to a monophasic chorioretinitis which peaked between days 7 and 12 after infection. Analyses in T cell-deficient mouse strains showed that LCMV-mediated ocular disease was strictly dependent on the presence of virus-specific CD8\(^+\) T cells and that the contribution of CD4\(^+\) T cells was negligible. Whereas topical application of immunosuppressive agents did not prevent development of chorioretinitis, passive immunization with hyperimmune sera partially prevented retinal and corneal damage. Likewise, mice displaying pre-existing LCMV-specific T cell responses were protected against LCMV-induced ocular disease. Thus, antibody- and/or T cell-based vaccination protocols could be employed as preventive strategies against LCMV-mediated chorioretinitis.
Introduction

The eye is highly vulnerable to virus infection which can precipitate blindness following intraocular infection. Viral retinitis in humans is usually caused by herpesviruses, including cytomegalovirus (CMV) and herpes simplex virus. However, the prevalence of chorioretinitis caused by less common viral pathogens such as West Nile Virus has increased over the recent years (19). Likewise, congenital exposure to lymphocytic choriomeningitis virus (LCMV) can lead to severe chorioretinitis (5, 29, 30). Considering the high prevalence of LCMV in the human population with up to 5% of adults being seropositive for LCMV (11, 23, 27, 38), it is important to investigate the virological and immunopathological mechanisms underlying this ocular disease and to evaluate potential prevention and treatment options.

LCMV was initially isolated by Armstrong and Lillie in 1933 from the cerebrospinal fluid of a woman who was suspected to suffer from St. Louis encephalitis (2). Since then, numerous cases of congenital LCMV infection in humans have been reported (4, 5). In recent years, several cases of acquired LCMV infection in adults, including lethal infection of transplant recipients (17) have been described. Commonly, human infection occurs through ingestion or inhalation of infected murine urine, faeces, or saliva. In acquired LCMV infection, approximately one-third of the patients remain asymptomatic or present with only mild symptoms. About half of the remaining patients develop central nervous system (CNS) disease, predominantly aseptic meningitis or meningoencephalitis, and, less frequently, chorioretinitis. However, in view of the fact that LCMV is not one of the infectious agents routinely evaluated when patients present with uveitis, it has been assumed that the role of LCMV as a causative agent of chorioretinitis is underestimated (9). As a consequence, information regarding the phenotype and incidence is limited, and the mechanisms by which LCMV causes chorioretinitis remain elusive.

The primary host and reservoir of LCMV is the common mouse, mus musculus. Neonatal mice which lack a fully developed immune system, remain asymptomatic because the
An overwhelming LCMV infection leads to immunological tolerance. Immunocompetent adult mice usually clear the infection with LCMV strains that exhibit slow or intermediate replication kinetics such as Armstrong (Arm) or WE, via a potent cytotoxic T cell (CTL) response (7). Particular LCMV strains, such as the rapidly replicating strain Docile, have been shown to exhaust the CD8⁺ T cell response and therefore establish persistence in multiple tissues (18, 31). Despite varying replication kinetics, the different LCMV strains exhibit distinct patterns of tissue tropism; e.g. Arm causes mainly CNS disease (1), whereas WE elicits severe liver pathology (45).

LCMV-induced pathology of the CNS in mice has been well-studied and represents a reliable model of experimental immunopathology (28). However, only very few studies including those by Silverstein and colleagues more than 40 years ago on LCMV infection of the eye in its natural host (39, 40), and a recent study on LCMV-mediated experimental ocular disease in rats (8), have addressed LCMV-mediated neuroretinal immunopathology. The importance of this disease in humans motivated us to re-address the issue and to thoroughly investigate the immunopathological mechanisms underlying LCMV-induced chorioretinitis. Our study revealed pronounced differences between different LCMV strains with the neurotropic strain Arm eliciting most severe chorioretinitis and keratitis. Virus-specific effector CTL, but not CD4⁺ T cells were mandatory for the development of immunopathological eye disease. Topical immunosuppressive treatment of ongoing intraocular LCMV infection could only improve keratitis but not the potentially more vision-damaging inflammation of the retina. Whereas pre-treatment with hyperimmune sera mitigated severity of the disease, LCMV-induced ocular pathology was abrogated in LCMV immune mice. Hence, our findings suggest that appropriate vaccines could provide protection against this virus-mediated immunopathological disease of the eye.
Materials and Methods

Mice and viruses. C57BL/6 mice were obtained from Charles River (Sulzfeld, Germany). β2-microglobulin-deficient (β2m−/−) (24), CD4+ T cell-deficient MHC class II-knock out (IAb−/−) (12), type II interferon receptor-deficient (Ifngr−/−) mice, type I and II interferon receptor-deficient (Ifna/gr−/−) mice (41), and P14 TCR transgenic mice expressing a TCR recognizing the LCMV-gp33 epitope in the context of H2-D^b (35), were obtained from the Institut für Labortierkunde (University of Zurich, Switzerland). P14 TCR transgenic mice were further crossed with C57BL/6 mice expressing the congenic marker Thy 1.1. Mice were kept in individually ventilated cages under conventional conditions at the Research Department of the Kantonal Hospital St. Gallen. LCMV Armstrong (Arm), WE, and Docile strains, obtained from Rolf M. Zinkernagel (University of Zürich, Switzerland), were propagated on L929 cells at a low multiplicity of infection (moi) and quantified as previously described (26). All animals were treated according to the Statement for the Use of Animals in Ophthalmic and Vision Research promulgated by the Association for Research in Vision and Ophthalmology and to the Federal Swiss Regulations on Animal Welfare.

Virus infection. Intraocular (i.o.) injection was performed with the aid of a surgical microscope. A 35 Gauge needle was introduced through the temporal corner of the limbus and 1.0 µl virus suspension containing $10^3$ pfu of the indicated LCMV strain in MEM medium/2% FCS were applied intravitreally. In order to control for potential injection-associated local inflammatory responses, 1.0 µl PBS was injected i.o. into intravenously (i.v.) LCMV Arm (10^3 pfu) infected mice.

Immunization and topical treatment. For passive immunization, polyclonal immune sera was generated in C57BL/6 mice by repeated i.v. infection with $10^6$ pfu of LCMV Arm. Mice
were treated i.p. with 200 μg ml of the depleting rat anti CD8 mAb (YTS169.4.2) on days 3 and 1 day before LCMV infection. Pooled sera from days 40, 50, and 60 post infection exhibited significant neutralizing titers as determined by focus reduction assay (6). Pooled sera from naive C57BL/6 mice served as control. Active, T cell-based immunity against LCMV was induced by i.v. infection with 200 pfu LCMV WE.

Cyclosporine A 1% eyedrops (obtained from the Pharmacy of the Kantonal Hospital St. Gallen, Switzerland) and prednisolone acetate 1% eyedrops (PredForte®, Allergan, Irvine, California) were applied twice daily with one drop placed on the cornea of mice i.o. infected with LVM Arm.

**Adoptive transfer and immunohistology.** For visualization of LCMV-specific CD8⁺ T cells, single cell suspensions from spleens of P14 mice were subjected to hypotonic red blood cell lysis and CD8⁺ T cells were sorted using magnetic separation according to the protocols of the manufacturer (Miltenyi Biotec, Bergisch-Gladbach, Germany). Recipient C57BL/6 mice were injected i.v. with 10⁶ P14-Thy 1.1⁺ splenocytes in 500 μl BSS 12 h prior to LCMV infection. At different time points after LCMV infection, mice were sacrificed and eyes were enucleated and snap-frozen in liquid nitrogen and stored at -80°C. Frozen tissue sections were cut in a cryostat and fixed in acetone for 10 minutes. Sections were incubated with antibodies against CD8 (clone YTS169.4.2), CD4 (YTS191.1.2), LCMV-NP (VL4), Thy1.1 (eBioscience, clone HIS51), or F4/80 (Biomedicals AG, clone BM8) followed by goat anti-rat Ig (Caltag Labs) and alkaline phosphatase-labeled donkey anti-goat Ig (Jackson ImmunoResearch Labs). Alkaline phophatase was visualized by using AS-BI phosphate/New Fuchsin, and sections were counterstained with hemalum. For the quantitative evaluation of swelling of the cornea, 3 to 5 serial cross-sections through the eye were measured by using a Leica DM R microscope, Leica DC300 FX camera and Leica IM1000 (version 1.20) computer-aided morphometry software. For semiquantitative assessment of inflammatory
alterations in retinal lesions, sections were evaluated by two observers in a blinded fashion using the following criteria: grade 0, no infiltration; grade 1, confined minor infiltration (foci of <10 cells) in any retinal layer; grade 2, confined major infiltration (foci of >10 cells) within the retina; grade 3, multiple clusters of inflammatory cells involving at least 10% of the retina; grade 4: multiple clusters of inflammatory cells covering more than 30% of the retina.

**Intracellular cytokine staining.** Spleens were removed at the indicated times after infection. Single cell suspensions of $1 \times 10^6$ splenocytes were incubated for 5 h at 37°C in 96-well round-bottom plates in 200 ml of culture medium containing 25 U/ml IL-2 and 5 mg/ml brefeldin A (Sigma-Aldrich). The cells were stimulated with phorbol myristate acetate (50 ng/ml) and ionomycin (500 ng/ml) as positive control or left untreated as negative control. Peptide-specific responses were analyzed after cells were stimulated with $10^{-6}$ M gp33 (KAVYNFATC) or np396 (FQPGNGQFI) peptides (both from Neosystems, France) and surface stained, as described elsewhere (22). The percentage of CD$^8^+$ T cells producing IFN-γ was determined using a FACScalibur flow cytometer and CellQuest software (BD Biosciences).

**Construction of tetrameric class I-peptide complexes and flow cytometry.** MHC class I (H-2D$^b$) monomers complexed with LCMV-gp33-41 were produced as described (22) and tetramerized by addition of streptavidin-PE (Molecular Probes). At the indicated times after immunization, single-cell suspensions were prepared from spleens or ocular tissue. For isolation of retina-infiltrating cells, dissected ocular tissue was incubated with Collagenase A (Sigma, Buchs, Switzerland) for 60 min at 37°C followed filtration through a 70 μm cell strainer. Aliquots of retina-infiltrating cells, splenocytes, or peripheral blood mononuclear cells (from 300 μl of blood) were stained using 50 μl of a solution containing tetrameric class
I-peptide complexes at 37°C for 10 min followed by staining with anti-CD8-FITC (BD Pharmingen) at 4°C for 20 min. Absolute cell counts were determined by counting leukocytes in an improved Neubauer chamber.

**Statistical analysis.** To determine statistically significant differences, the unpaired two-tailed Student’s t test was used. P values smaller than 0.05 were considered statistically significant. Statistical data analysis was performed using GraphPad Prism version 5 for Windows (GraphPad Software Inc.).

**Results**

**LCMV-induced chorioretinopathy**

Different LCMV strains exhibit distinct replication patterns (7) and varying target organ tropism (1, 45). In order to assess how strain differences affect LCMV-mediated ocular disease, adult C57BL/6 mice were infected i.o. with 10^3 pfu of LCMV Arm, WE or Docile. In situ analysis on day 12 post infection revealed that the outcome of ocular infection with the different strains varied to a high degree (Fig. 1). I.o. infection with LCMV Arm and WE elicited a severe chorioretinitis with infiltration of CD8^+ and CD4^+ T cells in the retina and pronounced folding of the photoreceptor layer, whereas infection with Docile precipitated only very mild inflammatory reactions (Fig. 1A). Thickening of the cornea and infiltration with CD8^+ T cells was only noted following i.o. infection with LCMV Arm (Fig. 1B). Semi-quantitative analysis of retinal lesions (Fig. 1C) and morphometric determination of cornea swelling (Fig. 1D) confirmed that i.o. infection with LCMV Arm elicited significant ocular disease.
Since LCMV-mediated immunopathology is almost exclusively mediated by virus-specific CD8+ T cells (44), we next determined CTL responses induced by the three different strains following i.o. infection. Tetramer analysis on day 12 post infection revealed that LCMV WE had induced the most pronounced expansion of gp33-specific CD8+ T cells (Fig. 2A and B). However, assessment of the functionality of both gp33- and np396-specific CD8+ T cells showed that only Arm infection had induced fully functional, i.e. IFN-γ producing CD8+ T cells (Fig. 2C). The lack of efficient CD8+ T cell responses in Docile infection is most likely due to the rapid spread of this virus throughout secondary lymphoid organs leading to exhaustion of LCMV-specific CTL responses (7, 31). Indeed, i.o. infection with the three different LCMV strains led to rapid spread to spleens and cervical lymph nodes with high viral titers on day 4 post infection. Whereas Arm and WE titers declined until day 8 post infection, replication of Docile – as in the i.v. infection – could not be halted by the immune system (data not shown). Thus, the different strains used in this study can spread systemically following i.o. inoculation and establish productive infection of secondary lymphoid organs.

Next, we assessed whether LCMV-specific CD8+ can home to ocular tissues following initial replication of the virus within this organ. Tetramer analysis on day 12 post i.o. infection with Arm revealed that high numbers of LCMV-gp33-specific CD8+ T cells accumulated in the retina (Fig. 3A). To visualize LCMV-specific CTL in situ, Thy1.1+ P14 TCR transgenic CD8+ T cells were adoptively transferred into Thy1.2+ C56BL/6 recipients prior to i.o. infection with LCMV Arm. Following LCMV infection, P14 TCR transgenic CD8+ expand vigorously (43) and home, as shown here in Fig. 3B, to virus-infected tissues. It is noteworthy that also under conditions of elevated CTL precursor frequencies, LCMV-specific CTL did not home of ocular tissues if the initial site of replication had not been the eye (Fig. 3B, bottom row). Overall, these initial experiments revealed that i.o. infection with Arm is best-suited to study LCMV-mediated immunopathological ocular disease and that LCMV infection of ocular tissue is a crucial prerequisite for the observed ocular disease.
**Immunopathological mechanisms of LCMV-induced chorioretinitis and keratitis**

Viral infections can precipitate genuine autoimmune reactions which could contribute to the maintenance of the disease process through activation of self-reactive lymphocytes (“epitope spreading”) and/or a cytokine-driven amplification of self-reactive T and B cells (i.e. “bystander activation”) (42). In coxsackievirus B3 infection, for example, a self-limiting virus-mediated inflammation is followed by a prolonged autoimmune phase with immune reactivity against cardiac antigens (16, 25). Likewise, infection of the eye with a neurotropic coronavirus precipitates autoimmune reactivity against retinal antigen (21). Since LCMV-induced inflammation leads to damage of the uvea, various antigens that can induce uveitis may be released (e.g. S-antigen, rhodopsin, or phosducin). To assess whether a self-maintaining chronic autoimmune uveitis develops following acute LCMV infection in the eye, we performed a detailed time course analysis of LCMV-induced chorioretinitis and keratitis following i.o. inoculation of LCMV Arm. The anterior chamber of LCMV Arm infected eyes showed progressive inflammatory reactions starting from day 5 on with decreasing depth of the anterior chamber, production of fibrinous exudates and formation of anterior synechiae (not shown). Immunohistochemical analysis revealed that the infiltration with CD8+ T cells started around the ciliary body by day 5 with loss of integrity of the iris and the ciliary body by day 12. Infiltration was dominated by CD8+ and CD4+ cells. In addition, CD11c+ dendritic cells and F4/80+ macrophages were found in clusters within the inflammatory lesions (not shown). The evaluation of the retinal alterations revealed that the disease activity was highest between days 7 and 12 after infection and that a slow healing process resulted in a complete loss of inflammatory foci in the retina after 2-3 months post infection (Fig. 4A and B).

After i.o. infection with LCMV Arm, the cornea remained clear until day 5. On day 7, clinical signs of ocular disease were externally visible, which started with mild opacity of the cornea.
This was followed by a progressive loss of corneal clarity peaking on day 12 and persisting until the end of the experiment. Infiltrations were detected in the corneal stroma, and were accompanied by loss of endothelial cells and disruption of the lamellar array (Fig. 4C). Corneal thickness measurement showed progressive oedema of the cornea with a maximum between days 7 and 12 post infection (Fig. 4D). Resolution of the corneal tissue damage was comparable to retinal inflammation, i.e. the swelling of the cornea was completely resolved 2-3 months post infection (Fig. 4D). It is noteworthy that i.v. infection with LCMV ARM even following i.o. application of carrier PBS did not result in ocular inflammation (Fig. 4A and C), indicating that replicating virus had to be present within the eye to cause the disease. In summary, the monophasic development and synchronous resolution of both chorioretinitis and keratitis suggest that autoimmune reactions against uveal proteins may not play a dominant role in prolongation of the ocular disease following LCMV infection.

Next, we determined the relative contribution of CD8\(^+\) versus CD4\(^+\) T cells to the disease process using i.o. infection of MHC II-deficient (IA\(^{b/-}\)) and β2-microglobulin-deficient (β2m\(^{-/-}\)) mice. Lack of CD4\(^+\) T cells in MHC class II-deficient mice (12) did only mildly mitigate chorioretinitis (Fig. 5A and B) and did not affect corneal swelling (Fig. 5C). The lack of LCMV antigen in the retina of IA\(^{b/-}\) mice on day 12 post infection (Fig. 5A) suggests that CD8\(^+\) T cells had completely cleared the viral infection even without support from Th cells. Indeed, the lack of CD8\(^+\) T cells in β2m\(^{-/-}\) mice completely prevented chorioretinitis and keratitis (Fig. 5A-C), but resulted in persistence of LCMV antigen in the retina (Fig. 5A). Overwhelming LCMV infection which leads to widespread distribution of the virus and exhaustion of virus-specific CD8\(^+\) T cells, can be observed in mice lacking the type I interferon (IFN) receptor (32). Since the lack of the type II IFN receptor ameliorates autoimmune diseases (15), we assessed whether the lack of both the type I and II IFN receptor, or the lack of the type II IFN receptor alone impacts on chorioretinitis and keratitis following i.o. LCMV Arm inoculation. As shown in Fig. 5A-C, ifnagr\(^{-/-}\) mice did not develop
chorioretinitis or keratitis. The persistence of viral antigen in the retina of ifnagr\textsuperscript{-/-} mice (Fig. 5A) indicates that the disease developed only in the presence of a functional virus-specific CD8\textsuperscript{T} cell response. In the absence of the type II IFN receptor, immunopathological ocular disease developed comparable to C56BL/6 controls (Fig. 5A-C) indicating that the type II IFN receptor did most likely neither affect the development of the antiviral T cell response nor the disease process.

**Prevention and treatment of LCMV-induced chorioretinitis and keratitis**

Antibodies play only a minor role for the resolution of acute LCMV infection (10). However, pre-existing neutralizing antibodies are able to lower viral titers, and protect against lethal LCMV choriomeningitis (3, 37). In order to assess whether neutralizing antibodies could prevent LCMV-induced chorioretinitis and keratitis, we passively immunized C57BL/6 mice with anti-LCMV hyperimmune serum before i.o. challenge. The presence of neutralizing antibodies efficiently attenuated LCMV-specific CD8\textsuperscript{T} cell responses (Fig. 6A). The reduced vigour of the CTL response was associated with significantly diminished chorioretinitis and keratitis (Fig. 6B and C). However, LCMV Arm-induced ocular disease was nearly completely abolished in LCMV WE immune mice (Fig. 6D and E). It is noteworthy that pre-immunization with WE did not affect clearance of Arm following i.o. infection. Thus, these vaccination experiments indicate that appropriate T cell vaccination and passive immunization with neutralizing antibodies represent potential means for prevention of LCMV-induced ocular disease.

Inflammatory processes of unclear origin in the uvea (so-called autoimmune uveitis) are commonly treated with immunosuppressive regimen including steroids or cyclosporine. We thus evaluated the effectiveness of such topical treatment options in LCMV-induced immunopathology of the cornea and retina. Daily topical application of prednisolone eyedrops led to a significant reduction of corneal swelling, but did not influence retinitis severity (Fig.
Likewise, local application of cyclosporine which inhibits T cell activation, led to a small improvement in corneal immunopathology, but exerted no significant effect on the inflammation in the retina (Fig. 7A-C). In summary, these results indicate that appropriate vaccination against LCMV represents the most promising approach to avoid this potentially debilitating ocular disease.

Discussion

LCMV infection in humans can lead to severe and potentially lethal disease. It is mainly the CNS that is affected in humans with aseptic meningitis in immunocompetent adults, or chorioretinitis and structural brain anomalies following congenital infection. Studies on LCMV-induced choriomeningitis in mice during the last decades have revealed great insight into the delicate virus-host balance and the particular immunopathological mechanisms underlying the disease (28). However, a major drawback in studying LCMV-induced CNS disease following intracranial injection is the uniformly lethal course of the disease. Once LCMV has established an infection in the meninges and effector CTL have gained access to the CNS, the ensuing immunopathological damage within the CNS does not permit studies beyond the acute phase of the viral infection. Thus, detailed studies on later stages of the disease such as the healing process, or potential autoimmune complications, are impractical in the intracranial infection model. The present study established that the retina, as a readily accessible part of the CNS, is well-suited to investigate the long-term effects of LCMV-mediated immunopathological disease in this organ system.

Two read-out systems were found to be robust and reliable in the assessment of LCMV-induced ocular disease. Assessment of the accumulation of mononuclear inflammatory cells in the retinal layers (predominantly CD8+ T cells) provided a direct measure for the damage in this particular part of the CNS. During infection with LCMV, and primarily with the Arm
strain, it appears that cells in the cornea become infected with the virus, leading to CTL infiltration and a pronounced corneal thickening. Morphometric determination of the cornea swelling reaction provided an exact second measure for the vigour of the immunopathological reaction. Using the i.o. infection with LCMV Arm and the carefully validated read-out systems, the study revealed that LCMV-mediated ocular disease depends critically on the delicate balance between functional antiviral CTL and the presence of viral antigen in the eye. In the absence of CD8+ T cells, or under conditions of impaired CTL function (i.e. exhaustive conditions during LCMV Doc infection or in the absence of the type I IFN receptor), viral antigen persisted in the eye, but disease did not develop. Correspondingly, rapid reduction of the viral load through administration of neutralizing antibodies or generation of efficient antiviral T cells effectively reduced or even prevented chorioretinitis and keratitis.

Activation of CD8+ T cells during viral infection critically depends on professional APCs (mainly dendritic cells and macrophages) that present antigenic peptides in the context of host MHC class I molecules to naïve T cells within the secondary lymphoid organs (20, 34, 36). However, the details of how and where viral antigen from the intraocular space is presented to CTLs, remains unclear. A recent study examined the route by which antigen from the anterior segment of the eye migrate to draining lymph nodes (14). Attempts to trace the route of APC migration from the anterior uveal tissues showed that dendritic cells or macrophages did not migrate to draining lymph nodes. Therefore, immune responses in the draining lymph nodes were assumed to be initiated by antigens escaping from the eye extracellularly via the canal of Schlemm into the blood (14). This view is compatible with our results which suggest that i.o. injection of LCMV leads to a generalized LCMV infection with a systemic specific T cell response similar to that of i.v. infected animals.

Immunopathological destruction of the retina might also be caused by viruses which are considered as being mainly cytolytic. Indeed, CMV which represents an important ocular pathogen in immune-suppressed patients causes destruction of retinal cells mainly through
direct cytopathicity. This finding has been well-documented in AIDS patients before the era of highly active antiretroviral therapy (HAART) (13). However, with the introduction of HAART, a new form of CMV retinitis has evolved, termed immune recovery uveitis which is associated with an improvement in T cell numbers and enhanced CD8$^+$ T cell reactivity against CMV (33). Although the exact nature of this syndrome in humans is currently unknown, the findings presented in this study suggest that immunopathological damage by activated T cells recognizing persisting viral antigen in the eye might be the underlying pathological mechanism.

An enhanced understanding of the factors that trigger ocular immune responses and ocular immunopathology may facilitate therapeutic interventions. The presented results suggest that treatment strategies against immunopathological uveitis should aim at reducing excessive local intraocular immune reactivity without impairing general immune defence mechanisms for elimination of extraocular virus. Lessons learned from the LCMV-induced chorioretinitis model may also shed light on the pathogenesis of other retinal diseases.

Acknowledgements

We thank Rolf Zinkernagel for helpful discussion and careful reading of the manuscript. We thank Rita de Giuli for generating MHC class I tetramers, Harindra Hewage for excellent animal husbandry, and Andre Fitsche for help with immunohistochemistry. This study was supported by a grant from the OPOS Foundation St. Gallen, Switzerland. The authors declare that they do not have competing commercial or financial interests.
References


Figure legends

**Figure 1:** In situ analysis of LCMV-induced chorioretinitis and keratitis. C57BL/6 mice were infected intraocularly (i.o.) with $10^3$ pfu of LCMV strains Armstrong (Arm), WE, or Docile (Doc). (A) Eyes were analyzed on day 12 post-infection by immunohistology for the presence of CD8$^+$ and CD4$^+$ T cells and LCMV nucleoprotein (NP). Representative images of the retina (A) and cornea (B) from three independent experiments are shown. (C) Semi-quantitative analysis of immunopathological retinitis following infection with the indicated LCMV strains. Data represent mean values of retinitis score ± SEM (Arm, n=16; WE, n=5; Docile, n=6). (D) Measurement of corneal thickness using digital morphometry. Values represent mean corneal thickness in micrometers ± SEM (Arm, n=13 mice; WE, n=5 mice; Docile, n=6 mice; naive C57BL/6 mice (Ctrl), n=10). **, P < 0.001; *, P < 0.05; ns, not significant.

**Figure 2:** LCMV-induced CD8$^+$ T cell responses after i.o. infection. C57BL/6 mice were infected i.o. with $10^3$ pfu of LCMV Arm, WE and Docile and CD8$^+$ T cell responses were analyzed on day 12 post infection. (A) Representative dot blots of MHC I tetramer analysis for the indicated viral strain. Values in the upper right quadrant indicate percentage of H2-D$^b$/gp33-binding cells within the CD8$^+$ T cell compartment. (B) Mean values of gp33-tetramer-positive cells in the CD8$^+$ compartment ± SEM (Arm, n=3 mice; WE, n=3 mice; Docile, n=3 mice). (C) CTL reactivity determined by intracellular IFN-γ secretion in response to restimulation with LCMV gp33 and np396 peptide. Values indicate percentage mean ±SEM of IFN-γ secreting cells within the CD8$^+$ T cell compartment (Arm, n=3 mice; WE, n=3 mice; Docile, n=3 mice).
Figure 3: Recruitment of LCMV-specific CD8\(^+\) T cells to the retina after i.o. infection. (A) C57BL/6 mice were infected i.o. with 10\(^3\) pfu of LCMV Arm and CD8\(^+\) T cell responses were analyzed on day 12 post infection by tetramer analysis. Representative dot blots of MHC I tetramer analysis are shown with values in the upper right quadrant indicating mean percentage ± SEM (n=3 mice) of H2-D\(^b\)/gp33-binding cells within the CD8\(^+\) T cell compartment. (B) In situ analysis for the presence of P14 TCR transgenic CD8\(^+\)Thy1.1\(^+\) T cells in the retina. Thy1.2\(^+\) C57BL/6 mice received 10\(^6\) purified P14 TCR transgenic cells 12 h before i.o. (upper and middle row) or i.v. (lower row) infection with LCMV Arm. Representative images of the retina are shown.

Figure 4: Time course analysis of immunopathological alteration in retina and cornea following i.o. infection with 10\(^3\) pfu LCMV Arm. Eyes were analyzed at the indicated time points post infection. (A) Immunohistological analysis for the presence of CD8\(^+\) T cells. Representative images of the retina are shown. (B) Semi-quantitative analysis of immunopathological retinitis. Data represent mean values of retinitis score ± SEM (day 3, n=7; day 5, n=4, day 7, n=12; day 12, n=16; day 24, n= 4; day 36, n=4; day 90, n=3). (C) Immunohistological staining for CD8. Representative images of the cornea are shown. (D) Measurement of corneal thickness using digital morphometry. Values represent mean corneal thickness in micrometers ± SEM (day 3, n=5; day 5, n=4, day 7, n=12; day 12, n=13; day 24, n= 3; day 36, n=3; naive C57BL/6 mice (Ctrl), n=10).

Figure 5: Impact of CD8\(^+\) and CD4\(^+\) T cells and of the IFN system on LCMV-induced ocular disease. (A) The indicated mouse strains were infected i.o. with 10\(^3\) pfu of LCMV Arm and eyes were analyzed on day 12 post infection by immunohistology for the presence of CD8\(^+\) T cells and LCMV nucleoprotein (NP). Representative images of the retina are shown. Semi-
quantitative analysis of immunopathological retinitis (B) and measurement of corneal thickness using digital morphometry (C) following infection of the indicated mouse strains. Data represent mean values of retinitis score ± SEM (B) and mean corneal thickness ± SEM (C) (B6, n=16; IA<sup>β<sub>2<sup>−/-<sub>2</sub></sup>+, n=3; β2m<sup>−/-<sub>−</sub></sup>, n=4; ifnagr<sup>−/-<sub>−</sub></sup>; n=6; ifngr<sup>−/-<sub>−</sub></sup>; n=4). **, P < 0.001; *, P < 0.05; ns, not significant.

**Figure 6:** Prevention of LCMV-induced chorioretinitis and keratitis. (A-C) C57BL/6 mice were pre-treated with hyperimmune serum (HIS) or normal mouse serum (NS) before i.o. challenge with 10<sup>3</sup> pfu of LCMV Arm (A) Representative dot blots of MHC I tetramer analysis on day 12 post infection. Values in the upper right quadrant indicate mean percentage ± SEM of H2-D<sup>b</sup>/gp33-binding cells within the splenic CD8<sup>+</sup> T cell compartment. (HIS, n=12 mice; NS, n=7 mice). (B) Semi-quantitative analysis of immunopathological retinitis (upper panel) and measurement of corneal thickness using digital morphometry (lower panel). (HIS, n=12 mice; NS, n=7 mice). (C) In situ analysis of CD8 stained cryosections of retina from C57BL/6 mice treated with hyperimmunserum (HIS) against LCMV or with naive serum (NS) as control. (D and E) Analysis of immunopathological retinitis (D, left panel) and measurement of corneal thickness using digital morphometry (D, right panel) of C57BL/6 mice receiving LCMV WE eight days before i.o. infection with LCMV Arm. Values represent mean retinitis grade ± SEM (right panel) and mean corneal thickness ± SEM on day 12 after LCMV Arm infection (n=6 mice).

**Figure 7:** Analysis of LCMV Arm infected eyes treated with prednisolone acetate 1% eyedrops (PredForte®, PF) or cyclosporine A 1% eyedrops (CSA). (A) In situ analysis (CD8 stain) of retina and cornea of C57BL/6 mice treated topically with PF or CSA (day 12 post i.o. infection). Representative images of the retina and cornea from two independent experiments.
are shown. (B) Semi-quantitative analysis of immunopathological retinitis (upper panel) and measurement of corneal thickness using digital morphometry (lower panel). Values represent mean retinitis grade ± SEM (upper panel) and mean corneal thickness ± SEM on day 12 after LCMV Arm infection (untreated (none), n=16 mice; CSA, n=6 mice; PF, n=6 mice; naive C57BL/6 (Ctrl), n=10 mice). **, P < 0.001; ns, not significant.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A

B

C

CD 8 LCMV NP

Retinilis (grade)

Cornea thickness (μm)

B6 IAb−/− βm−/− Ifnag−/− Ifngr−

ns

ns

**

ns

ns

*
Figure 6
Figure 7

A

Retina

<table>
<thead>
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<th>PF</th>
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Cornea

| CSA | PF |

B

Retinitis (grade)

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<th>PF</th>
<th>Ctrl</th>
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C

Cornea thickness (µm)

| None | CSA | PF | Ctrl |

ns

**