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DISSERTATION

Establishment of a murine model of ocular toxoplasmosis

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Abbreviations

AIDS	Acquired immune deficiency syndrome
DAB	Diaminobenzidine
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FTR	Photoreceptor layer
GCL	Ganglion cell layer
HAART	Highly active antiretroviral therapy
H&E	Haematoxylin and eosin
HCl	Hydrochloric acid
HRP	Streptavidin-peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
INL	Inner nuclear layer
IFN	Interferon
i.p.	Intraperitoneal
IPL	Inner plexiform layer
J774A.1	Mouse macrophage-like cell line
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
NF	Nerve fiber layer
NO	Nitric oxide
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PAP	Peroxidase-antiperoxidase
PBS	Phosphate-buffered saline
p.o.	Peroral
RPE	Retinal pigmented epithelium

RPMI	Roswell Park Memorial Institute
s.c.	Subcutaneous
T. gondii	*Toxoplasma gondii*
TNF	Tumor necrosis factor
TGF	Transforming growth factor
TLA	Toxoplasma Lysate Antigen
Tween 80	Polysorbate 80

1 INTRODUCTION

1.1 *Toxoplasma gondii*

1.1.1 The organism and its impact on human health

Toxoplasma gondii is a cosmopolitan protozoan parasite that infects almost any warm-blooded animal. It infects approximately 30% of the human population (2). In immunocompetent adults infection is usually asymptomatic, but may result in toxoplasmosis, which most commonly presents as fever, lymphadenopathy, and headache (3, 4). One of the most prevalent symptomatic outcomes of infection, acquired either congenitally or postnatally, is ocular disease that presents as retinochoroiditis. Furthermore, in immunocompromised individuals acquired infection or a reactivation of a latent infection may lead to severe toxoplasmic retinochoroiditis and life-threatening toxoplasmic encephalitis.

The vast majority of *T. gondii* strains fall into one of the three clonal types: type I, II and III. However, a considerable number of atypical strains have been described so far. These are either recombinant strains of the three basic clonal lineages, or 'exotic' strains not related to any of the three canonical ones. Type I strains are highly virulent to mice and associated with severe disease outcomes in humans as well (5, 6). Type II and type III parasites are considered mouse non-virulent. However, the impact of parasite strains on infection outcome in humans has not yet been fully understood (7).

The life cycle of *T. gondii* consists of two subcycles. The sexual cycle takes place only in the intestinal epithelium of members of the cat family and results in generation of oocysts. Oocysts are shed with the cat's feces and remain in the environment for prolonged periods of time (7). They are ingested by intermediate hosts, including humans, in whom the asexual cycle occurs.
Ingested oocysts containing sporozoites give rise to rapidly dividing tachyzoites that actively enter any nucleated cells and replicate. Subsequently, the infected cells break up, releasing infective tachyzoites and the neighboring cells become infected. Tachyzoites evoke an inflammatory process

that can become clinically apparent (8). However, under pressure of the immune response, tachyzoites will transform into bradyzoites that encyst in the brain, retina, heart, and skeletal muscle. Cysts remain in these tissues for the life of a host (latent infection), unless the immune system of a host becomes compromised. Thus, tissue cysts and the contamination of the environment are important factors in the epidemiology of the infection.

1.1.2 Epidemiology

1.1.2.1 Transmission

Human infection is acquired through ingestion of raw or undercooked meat, most commonly pork and lamb containing tissue cysts; infection by ingestion of unwashed vegetables and consumption of water or soil contaminated with cat feces has also been reported (9). Acquired infection in pregnancy can result in miscarriage or significant neurological sequelae later in life of the newborn. Ocular disease can be acquired pre- or postnatally (10). Both of these infections may result in recurrent disease. In rare cases, organ transplantation or blood transfusion from a seropositive donor to a seronegative recipient may also result in infection.

The life cycle of *T. gondii*, routes of its transmission to humans and clinical manifestations of toxoplasmosis are summarized in Figure 1.

1.1.2.2 Seroprevalence

Seroprevalence varies between populations and geographic regions reflecting hygiene and eating habits of the population. In Europe, seroprevalence ranges between 9.8% in Iceland and other northern countries (10), 26 to 54% among women of child-bearing age in Germany (11), and is as high as 71% among pregnant women in Paris (10). The prevalence of anti-*T. gondii* antibodies steadily decreases in developed countries (1).

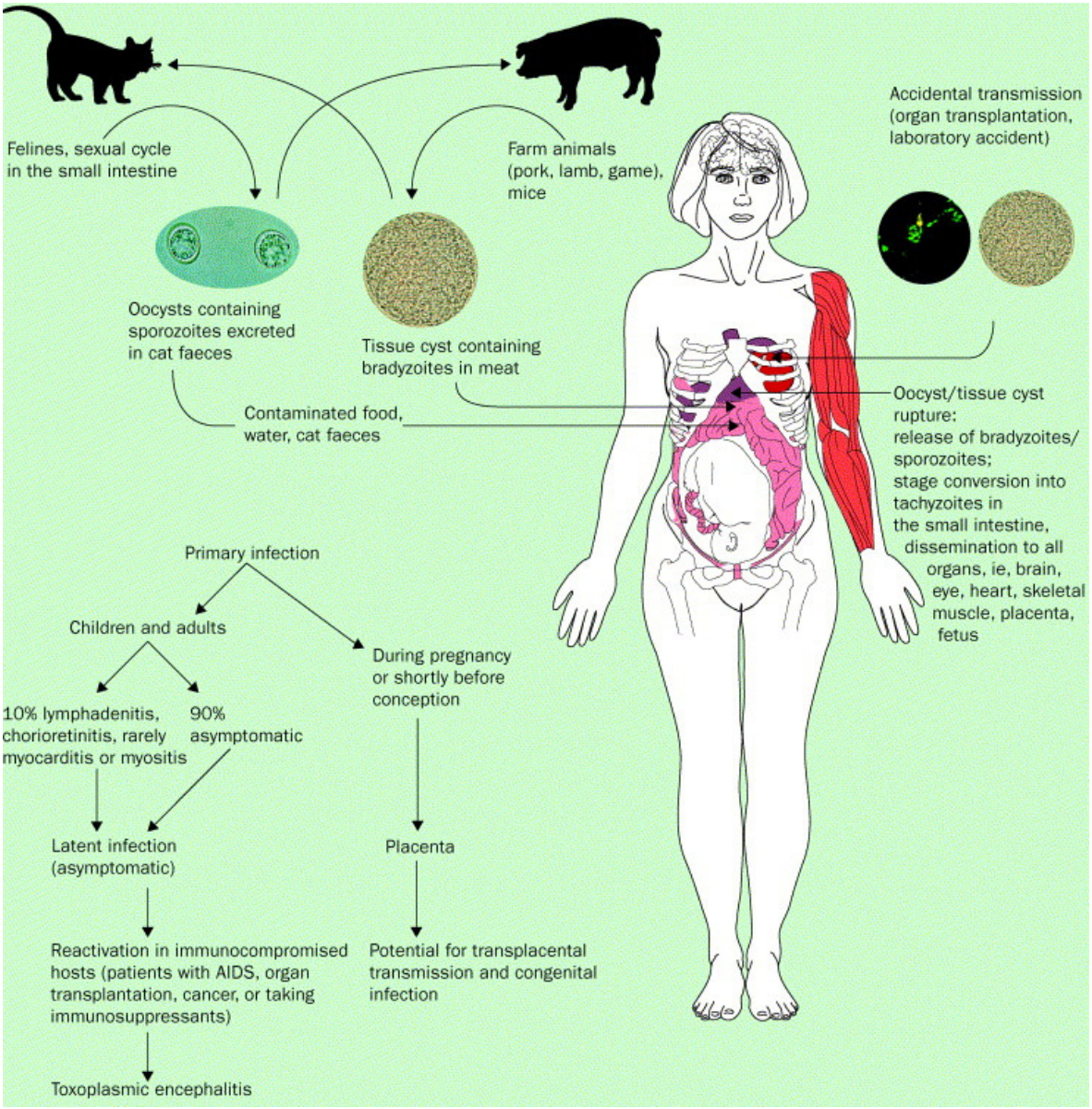

Fig.1: Life cycle of *T. gondii* and clinical manifestations of toxoplasmosis (from (1))

1.2 Ocular toxoplasmosis

Ocular toxoplasmosis is a potentially blinding disease affecting up to 80% of congenitally and between 2 and 20% of postnatally infected individuals (15, 16). Host defense mechanisms and parasite characteristics appear to influence the severity of ocular involvement.

Toxoplasmic retinochoroiditis can result from congenitally and postnatally acquired infection. In traditional teaching, ocular involvement was thought to result mainly form mother to fetus transmission (12). However, this belief has been challenged in recent studies. Gilbert and Stanford suggested that at least 2/3 of toxoplasmic retinochoroiditis is a result of postnatally acquired disease (13). In a study in southern Brazil, 17.7% of the population was affected by ocular toxoplasmosis; the prevalence of toxoplasmic retinochoroiditis increased with age, supporting the notion that ocular involvement more frequently results from postnatally rather than congenitally acquired infection (14).

1.2.1 Clinical presentation

The hallmarks of toxoplasmic retinochoroiditis are unilateral or less frequently bilateral foci of inflammation affecting peripheral or central parts of the retina and choroid. A predilection for macular inflammation has been observed (15). These lesions may occur in the vicinity of a retinochoroidal scar if the episode represents recurrence. However, there is a broad spectrum of clinical manifestations in ocular toxoplasmosis (16). Retinal vasculitis without concomitant foci of retinochoroiditis may be the sign of a recently acquired infection (21). Optic nerve involvement (17) and anterior uveitis (8) have also been described. Toxoplasmic retinochoroiditis in the elderly and immunodeficient patients (i.e., AIDS or immunosuppressive therapy) can present as severe ocular involvement. In immunocompromized hosts, diffuse toxoplasmic retinochoroiditis, multifocal, bilateral lesions, involvement of iris or vitreous tissue, occlusive retinal vasculitis, and other atypical presentations can occur (18). Lesions may develop adjacent to or far from old scars in cases of local reactivation. The latter presentation may suggest acquired infection or dissemination from other affected organs (19, 20).

Patients with active disease experience decreased or blurred vision, metamorphopsia, photophobia, and, less frequently, redness and pain (8, 15). Severe visual impairment and vision loss, the most serious complications, usually are attributed to an involvement of the macula (8).
Toxoplasmic retinochoroiditis is a recurrent disease. Episodes of recurrence are not random, but occur in clusters followed by prolonged disease-free intervals (21). The risk of reactivation is highest right after an episode and decreases with the duration of an interval. Recurrences develop within 2 years in 57% of patients with a previous active retinochoroiditis episode (22). Usually new lesions can be found next to the recent lesions, probably due to proliferation of parasites released from cysts. However, the pathogenesis and risk factors of recurrences are not yet known. Pregnancy (23), the patient's age (21), or lesions to the retina including cataract surgery (24) increase the risk of recurrence. The route of infection (congenital versus postnatal), or antiparasitic therapy at the time of diagnosis of the first episode did not appear to impact the frequency of recurrences (21).

1.2.2 Diagnosis and therapy

Clinical symptoms are considered the main factor in the diagnosis of ocular infection with *T. gondii* (8). In ophthalmoscopy, single or less frequently multifocal white-grayish active lesions and/or pigmented old scars can be observed. Lesions sometimes can be accompanied by vasculitis or papillitis. Serologic screening may assist in the diagnosis. During primary infection IgG antibody titers are usually high and IgM antibodies are detectable, whereas recurrent episodes are characterized by lower IgG concentrations and negative IgM test results. The absence of antibodies against *T. gondii* rules out the diagnosis of ocular toxoplasmosis (8). Detection of *T. gondii*–specific DNA in vitreous fluid by polymerase chain reaction (PCR) can also be helpful in the diagnostic process if the presentation of the ocular involvement is atypical (25).

Often, toxoplasmic retinochoroiditis resolves without treatment within 6 to 8 weeks (26). Thus, ophthalmologists may choose not to treat lesions in immunocompetent patients that are peripheral and not vision-threatening (1). However, in national surveys 40% of German and 15% of uveitis specialists in the USA advised to treat all patients with acute ocular toxoplasmosis regardless of clinical manifestation of the disease (27, 28). Sight-threatening disease and severe inflammatory responses (27) are considered a clear indication for medication. Pyrimethamine, clindamycin,

sulfadiazine, trimethoprim/sulfamethoxazole, azithromycin and atovaquone are used for the treatment of ocular toxoplasmosis. There are no established recommendations for management of the disease. However, among uveitis specialists in Germany and USA, the combination of pyrimethamine plus sulfadiazine, and folinic acid to reduce haematopoietic toxicity, and the monotherapy with clindamycin, administered orally over 4 weeks, are the most commonly used regimens (27, 28). There is no consensus as regards the inclusion of corticosteroids in treatment regimens (28, 29). In immunocompromised patients or patients with a high frequency of recurrences, maintenance treatment should be considered (8). Effective secondary prophylaxis has recently been shown using sulfamethoxazole and trimethoprim over 20 months (30).

1.2.3 Pathogenesis

The eye is an immunoprivileged site. The blood-retina barrier that is composed of retinal pigmented epithelium (RPE) and endothelial cells (31) protects the eye against the parasite and migration of cells and molecules with potentially harmful effects. Due to the blood-retina barrier, the immune response to eye infections is compartmentalized and marked differences can be observed in regard to immune responses in serum and aqueous humor (32). Within the eye, the immune balance is shifted towards suppression of inflammation. MHC class I, which plays a role in activation of cytotoxic CD8+ T cells, is constitutively expressed by cells in the eye, but at low levels (33). TGF-β, an anti-inflammatory cytokine, can also be detected in the eyes of naïve mice (32). Whereas mechanisms suppressing (auto-)immune-mediated pathology protect ocular tissue from damage, the same response may weaken the control of pathogens (33). Therefore, once the blood-retina barrier is disrupted, for example due to *T. gondii* infection, the eye loses its immune privilege and strong inflammation with increased cell infiltration may occur (34, 35).

The inflammatory foci in ocular toxoplasmosis contain mostly macrophages and T lymphocytes, of which CD8+ cells dominate over CD4+ cells (36). T cell depletion in chronically infected mice results in a marked increase of parasite cyst load in the brain and ocular tissue (36) and a greater proliferation of *T. gondii* tachyzoites (37). CD4+ lymphocytes are pivotal in the inflammatory process in the eye due to the activation of macrophages and CD8+ T-cells by production of IFN-γ. Th1-responses, during which the cytokines IL-12, IFN-γ, IL-6 and TNF-α predominate (1, 8), initiate the immune response of the host to *T. gondii* (2). In animal studies, it has been shown that

IFN-γ plays a crucial role in host-defense mechanisms against *T. gondii* infection by upregulating the expression of MHC class I in ocular cells (33, 38). However, an overproduction of IFN-γ may also be harmful through an escalation of damage done to the visual organ (36). IL-10 that possesses anti-inflammatory functions could be found in 50% of patients with ocular toxoplasmosis (8). This cytokine downregulates the levels of IFN-γ and IL-12 and thereby prevents (hyper-)inflammatory changes in the eyes (39). Recently, it was suggested that the "Th17" pathway may contribute to pathogenesis of ocular toxoplasmosis (40).

1.2.4 Animal models of ocular toxoplasmosis

Ocular toxoplasmosis was first described by Jankû in 1923 and it was not before 1939 that ocular involvement resulting from congenital infection became widely reported (15, 41). Scientists soon appreciated the necessity of establishing an animal model that would enable a more in-depth look into pathophysiology, histopathology and treatment possibilities of the disease. The first animal model of ocular toxoplasmosis was described by Hogan in 1951, who infected rabbits with tachyzoites via an intracarotid injection. Frenkel followed his efforts by observing the ocular involvement after intraperitoneal infection in hamsters (42). However, the quest for a satisfactory in vivo model that has lasted over 60 years has not yet finished.

1.2.4.1 Animal model of ocular toxoplasmosis

Several animal models of ocular toxoplasmosis have been established so far. Non-human primates, cats, rabbits, hamsters, *Calomys callosus*, guinea pigs and mice have been used. All of these animal species have certain advantages and disadvantages for studies on ocular toxoplasmosis.

The visual organ of non-human primates closely resembles the human eye in its anatomy and physiology and so does the immune response to the parasite (43). However, monkeys are much more prone to *T. gondii* infection than humans, due to scarcity of definitive hosts in their natural environment (15). They are also costly as animal models and most of the laboratories cannot provide proper housing conditions for them.

Unlike in humans, choroiditis predominates over retinitis in ocular involvement following infection with *T. gondii* in cats (15). Hamster models of ocular toxoplasmosis are characterized by quick development of pathology post infection, reproducibility, and consistent production of cysts. In addition to that, intraperitoneal infection of hamsters is easily feasible (44). However, unlike in all other above mentioned species, retinal lesions do not resemble those in humans (43). Firstly, hamsters tend to develop bilateral and multifocal lesions and secondly, vitreitis and vasculitis are less pronounced in the course of the disease in these animals. In all of the mentioned species, indirect ophthalmoscopy can be performed in order to control changes in the eye.

Examples of animal models of acquired ocular toxoplasmosis are presented in Table 1.

Table 1: Animal models of ocular toxoplasmosis

Animal species	Parasite dose, strain, and stage	Inoculation route	Dissection days (post-infection)	Observations	Ref.
Calomys callosus	20 ME49 cysts	p.o.	21 and 47 days post infection	Pregnant and non-pregnant females as well as males were infected. 75% of females and 50% of males showed *T. gondii* cysts in the ocular tissue; 40% of fetuses presented ocular lesions.	(45)
Cat	5000 ME49 bradyzoites	Intracarotid	70 days post infection	Cats developed bilateral, multifocal retinitis and choroiditis.	(46)
Monkey (*Macaca fascicularis)*	1×10^5 or 2.5×10^5 of living or heat-killed RH tachyzoites	Intraretinal and s.c.	Not given	Iridocyclitis, vitreitis and retinal edema were observed up to 2 weeks post infection. Retinal vasculitis appeared 6 days post infection and resolved until 3 weeks post infection. No necrotizing retinochoroiditis was observed.	(47)
Rabbit	5000 BK tachyzoites	Primary infection: s.c. Transvitreal challenge of infected rabbits vs. primary infection of naïve rabbits.	Animals were not dissected. Fundoscopic examination was performed to evaluate ocular changes.	All naïve and 91% of primed animals developed retinochoroiditis and vitreal infiltration post transvitreal challenge. Ocular disease in naïve rabbits was more severe compared to challenged animals.	(48)
Syrian Golden Hamster	100 ME49 cysts	i.p.	6 months post infection	Bilateral, white lesions, varying in number and size after 2 -3 weeks post infection; severe retinochoroiditis at 4-5 weeks post infection with vasculitis and, in some animals, vitreitis.	(49)
Syrian Golden Hamster	100 ME49 cysts	p.o.	16 weeks post infection	Small inflammatory foci, varying in size, predominantly at the posterior pole 4 weeks post infection.	(44)

1.2.4.2 Murine models of ocular toxoplasmosis

Murine eyes differ from the human visual organ in anatomy and physiology. Mice have no macula, their lens is proportionally bigger and the volume of vitreous humor is smaller (15). In spite of these facts they have been commonly used to study various aspects of ocular toxoplasmosis. Mice are easily accessible, immunological reagents and a wide range of knock-out mice are available (2). It should also be noted that different strains of the same species may show differences in their susceptibility to infection with *T. gondii*. C57BL/6 mice more easily develop severe ocular lesions and a higher burden of parasites after intraocular (50) and peroral (35) infection compared to BALB/c mice.

1.2.4.3 Parasite inoculation routes to evoke ocular toxoplasmosis in animals

It is known that the route of parasite inoculation can influence the outcome of infection (51). Routes of infection that have been used to induce ocular pathology include intracarotid and intraocular injection, ocular instillation, intraperitoneal and peroral infection. An intracarotid injection of tachyzoites was chosen by Hogan in rabbits (48, 52). Intracarotid injection has the advantage of introducing parasite anatomically close to the eye, which enhances the possibility of development of ocular lesions (46). All cats infected this way developed bilateral, multifocal retinochoroiditis (46). However, this route does not resemble the natural route of infection.

Local infection by intraocular injection of the parasite has been used in monkeys (53), rabbits (48, 54), guinea pigs (55), and mice (50, 56, 57). Direct inoculation leads to intraocular pathology in 100% of infected animals. The intraocular injection can be performed transvitreally through the pars plana (parasites are injected periretinally) (48) or into the anterior chamber of the eye (intracameral route) (37). In experiments involving monkeys as animal models, parasites were injected into the retina (47, 53). Intravitreal injection of tachyzoites breaks the integrity of the vitreous cavity (53), poses a risk of needle damage to the ocular structures and disruption of the blood-retina barrier. In addition, trauma caused by inoculation itself may result in inflammatory changes (35). Tedesco *et al.* proposed an alternative way to introduce parasites into the eye by instillation of the parasite on the surface of an eye in mice; toxoplasmic retinochoroiditis developed and the integrity of ocular tissues was maintained (57).

Ocular toxoplasmosis can result from congenital infection. To study aspects of vertical transmission of *T. gondii* and its impact on the visual organ, mice were infected during gestation and the eyes of their progeny were screened for toxoplasmic retinochoroiditis (58-62). Features characteristic of ocular toxoplasmosis could be found in 82% to 100% of the newborns' eyes (59, 60, 62). However, mice do not develop macular disease and they often present opaque cataract formation (58, 61) which is rare in humans. In addition, the majority of orally inoculated pregnant females does not survive to the last gestational stage (62).

Acquired ocular toxoplasmosis following parasite dissemination can be induced by intraperitoneal or peroral inoculation of adult animals. Intraperitoneal injection has been performed in mice (33, 36, 63, 64) and hamsters (42, 49) and appears to more consistently produce chorioretinal lesions than oral infection.

Peroral infection most accurately resembles the natural infection in humans. Hamsters infected orally with 100 cysts consistently developed ocular toxoplasmosis within 4 to 8 weeks post infection (44), whereas wildtype mice that ingested 5 cysts did not show clinical changes within a month post infection (35, 65). However, in the latter model the presence of the parasite in the ocular tissue was noted (65).

Taken together, the natural oral route of infection has been widely neglected in the quest for an appropriate animal model of ocular toxoplasmosis.

1.2.5 Outcome of infection in murine models of toxoplasmic retinochoroiditis

1.2.5.1 Histopathologic changes in the eyes of mice infected congenitally

Parasite cysts were observed in the eyes of 23% of progeny of Albino mice infected subcutaneously with 10 Beverly *T. gondii* cysts 12 days after mating, mostly in the inner retinal layers. No inflammatory cells or minimal surrounding inflammatory response could be found around the parasites (59, 60), whereas in the eyes of the progeny of C57BL/6 females infected perorally with 5 ME49 *T. gondii* cysts 10.5 days after mating, no parasites but *T. gondii* DNA were observed (62). Mild to diffuse retinochoroiditis (59, 61), alterations in the architecture of retinal layers such as lacunae formation between outer nuclear layer and inner nuclear layer, and inflammatory cells

around the retinal vessels, in the retinal layers, and in the vitreous humor were observed in 82% to 100% of the pups' eyes (62). Primary cataract formation was found in one study (59). Ocular changes appeared in utero after primary infection of a mother during gestation (62).

1.2.5.2 Histopathologic changes in the eyes of wildtype mice with acquired ocular toxoplasmosis

Characteristics of acquired toxoplasmosis in wildtype mice are described in Table 3. Focal retinochoroiditis, cellular inflammatory infiltrate in the retina, vitreous humor, around the blood vessels, and in the choroid are consistently described in animal models of the disease (32, 33, 35, 36, 57, 64). Parasites are predominantly found only in a small portion of the histologic material (36). Usually, *T. gondii* cysts are not associated with inflammatory infiltrates, but free parasites in the blood vessels, if present, are often accompanied by perivasculitis (64). Retinal pigmented epithelium (RPE) migration, another common feature of ocular toxoplasmosis, is consistently described in the eyes of perorally (65), intraperitoneally (32, 36, 64), and intravitreally (57) infected mice. RPE migration and parasite presence contribute to alteration in the architecture of retinal layers (32, 64). Cone formation by the photoreceptor layer (FTR) and an increase in the interstitial spaces between the retinal cells can be noted (64). Focal photoreceptor damage characterized by narrowing of the FTR layer was also observed (36, 65).

Table 2: Characteristics of acquired ocular toxoplasmosis in wildtype mice

Mouse strain	Parasite strain, stage and dose	Inoculation route	Dissection days (post-infection)	Observations	Ref.
C57BL/6 and MRL/MpJ	Infection: 10 PLK cysts Challenge: 50, 500, 5000 or 50,000 PLK tachyzoites	Infection: peroral Challenge: intracameral	Days 6 and 8 post infection	Infection outcome was dose-dependent in naïve and challenged mice (less severe ocular pathology in challenged mice in comparison with primarily infected naïve mice).	(56)
C57BL/6	5×10^3 ME49 bradyzoites	Instillation Intravitreal inoculation	12, 24, 48 h post infection Days 5 and 7 post infection	Both routes of infection resulted in inflammatory changes, edema and formation of lacunae by day 7 after infection. Intravitreal inoculation, unlike instillation, caused mechanical lesions to the eye.	(57)
C57BL/6 BALB/c CBA/J	Immunization: 1×10^5 ts-4 tachyzoites Challenge: 100 tachyzoites of RH, RH-GFP, PLK, or SAG1 deficient mutant RH strain	Immunization: i.p. Challenge: intracameral inoculation	Day 5, 8, 11, 26, 56 or 85 post infection or post challenge	Mild (infection with PLK and SAG1$^{-/-}$strains) or severe (RH strain) inflammation on day 5 post infection in C57BL/6 mice. Similar lesions regardless of parasite strain on day 11. Increased resistance in BALB/c and CBA/J mice compared to C57BL/6 mice (RH infection).	(50)
C57BL/6	50 ME49 cysts	i.p.	60 days post infection	RPE migration in the eyes of all mice.	(64)
C57BL/6	30 ME49 cysts	i.p.	30 days post infection	Parasites within retinal vessels, vasculitis, inflammatory infiltrate in the retinal layers, and lacunae formation by the FTR were observed. Higher IFN-γ and TGF-β and lower IL-10 levels in aqueous humor of infected mice compared to naïve mice.	(32)

1.2.5.3 Histopathologic changes in the eyes of immunocompromised animals with acquired ocular toxoplasmosis

Ocular disease in immunocompromised mice manifests itself in a manner similar to that of the disease in immunocompromised patients (2). Immunodeficiency is established in gene deficient mice or can be induced by depletion of cell subsets or neutralization of cytokines. In animals deprived of CD8+ and B cells, more severe ocular involvement and a greater parasite load in the ocular tissue can be observed following *T. gondii* infection (36, 37). However, CD4+ deficient mice developed less severe inflammatory changes and necrosis than naïve animals but higher parasite loads thereby underlining the dual role (protective and detrimental) of these cells in the immune system (37, 66). Mice lacking IFN-γ (35, 36, 67), TNF-α (36), or IL-6 (33) develop more severe ocular disease. In addition, mice deprived of anti-inflammatory mediators such as IL-10 and NO present exacerbated ocular inflammation compared to control mice (68, 69).

1.3 Aims of the study

Ocular toxoplasmosis continues to be a major health threat in humans. A number of unanswered questions remain, e.g. the role of parasite strains, host genetics, immunopathology, and treatment. Development of an appropriate animal model that mimics human infection would allow an experimental investigation of these questions.

Whereas past studies have investigated the immunopathology of ocular toxoplasmosis using direct injection of the parasite into the eye and intraperitoneal infection of laboratory animals, these inoculation routes do not reflect the natural route of infection which includes oral infection, dissemination in the bloodstream, and passage of the blood-retina barrier.

Therefore, the aim of the present study was to establish a murine model of ocular toxoplasmosis to allow investigation of new strategies for the treatment of toxoplasmic retinochoroiditis. We chose mice due to their easy accessibility and sustainability in laboratory conditions, the wide range of available immunologic reagents, and the similarity between the histopathological characteristics of ocular toxoplasmosis in mice and retinochoroiditis in humans. As it has been suggested that the outcome of ocular toxoplasmosis depends on the mouse strain, infection route, stage, and dose of the parasite, various combinations of these features were investigated. Histological changes and parasite load in the eyes of infected mice were used as a read-out. The following questions were addressed in detail:

1. Does the genetic background of mice impact the development of ocular pathology after systemic infection with *T. gondii*?
2. Do the inoculation route and the infectious inoculum of the parasite impact the development of ocular pathology?
3. Do mice infected with *T. gondii* show characteristic cytokine patterns for Th1 and/or Th17 responses?

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Animals and parasites

2.1.1.1 Animals

Female C57BL/6, NMRI, BALB/c and MMP2$^{-/-}$ (on the C57BL/6-background) mice were maintained in the animal facility of the Charité Campus Benjamin Franklin (Forschungseinrichtung für Experimentelle Medizin). Animals were kept in a specific pathogen-free environment and were provided with water and sterile food *ad libitum*.

2.1.1.2 Parasites

Cysts of the ME49 strain of *T. gondii* were obtained from brains of NMRI mice that had been infected intraperitoneally with 10 cysts at least 4 months before. Mice were sacrificed by asphyxiation with CO_2 and their brains were removed and triturated in PBS. An aliquot of the brain suspension was used to determine the numbers of cysts in the preparation by microscopy.
Tachyzoites of the RH strain transfected with green-fluorescent protein were kindly provided by Dominique Soldati, University of Geneva. Tachyzoites were grown in J774A.1 macrophages. Macrophages were cultured in RPMI 1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin. Medium was changed every 2 to 3 days. After formation of a confluent monolayer, cells were detached with a scraper and split at a 1:5 ratio. After 2 days, monolayers were infected with tachyzoites. Parasites became extracellular within 3 to 5 days. 1 ml of the extracellular parasite suspension in medium was used to infect subsequent flasks with J774A1 macrophages. Parasites were counted in a Neubauer chamber and diluted in RPMI medium.

2.1.2 Equipment and instruments

Equipment	Manufacturer	Location
Adjustable volume pipettes (various sizes)	Eppendorf	Hamburg, Germany
Axiostar microscope	Zeiss	Göttingen, Germany
Cell scraper (23 cm)	Nunc	Wiesbaden, Germany
Centrifuge	Heraeus	Hanau, Germany
Cooling plate	Microm	Walldorf, Germany
Dissection tools	Aesculap/Braun	Melsungen, Germany
Drying cabinet	Memmert	Heilbronn,Germany
Incubator	Heraeus	Hanau, Germany
Microscope camera	Inteq	Berlin, Germany
Microtome Microm HM 355	Microm	Walldorf, Germany
Mortar	VWR	Darmstadt, Germany
Multichannel pipette	Eppendorf	Hamburg, Germany
Neubauer chamber	Brand	Wertheim, Germany
Pipetboy	Hirschmann Laborgeräte	Eberstadt, Germany
Pestle	VWR	Darmstadt, Germany
Rotary shaker	Braun	Melsungen, Germany
Rotor Stator, ART Miccra D-1	Roth	Karlsruhe, Germany
Spectrophotometer	Tecan	Crailsheim, Germany
Sterile workbench, Lamina Air Flow Class 100	Gelman	Michigan, USA
Tabletop centrifuge	Eppendorf	Hamburg, Germany
Upright freezer –20°C	Liebherr	Rostock, Germany
Upright freezer –70°C	Sanyo-Fisher Sales	München, Germany
Water bath	GFL	Wunsdorf, Germany

2.1.3 Plastics and glassware

Article	Manufacturer	Location
Cannulae 26G, 22G	Becton Dickinson GmbH	Heidelberg, Germany
Cuvette for spectrophotometer	Eppendorf	Hamburg, Germany
Cover slips	Menzel-Gläser	Braunschweig, Germany
Histology cassettes	Simport, Bernard-Pilon	Beloeil, QC, Canada
Glass jars	VWR	Darmstadt, Germany
Glass slide staining racks	VWR	Darmstadt, Germany
Glass staining dishes	VWR	Darmstadt, Germany
Microscope slide boxes	neoLab	Heidelberg, Germany
Microscope slides	R. Langenbrick GmbH	Emmendingen, Germany
Microtiterplates, coated for ELISA	BD Biosciences	Heidelberg, Germany
Parafilm	Pecheney Plastic Packaging	Chicago, IL, USA
Plastic tubes 15/50 ml	Sarstedt	Sarstedt, Germany
Plastic test tubes 0.5/1.5/2 ml	Eppendorf	Hamburg, Germany
Pipettes 5/10/25 ml	Falcon, BD Biosciences	Heidelberg, Germany
Pipette tips (various sizes)	Eppendorf	Hamburg, Germany
Syringes 1/5/10 ml	Braun	Melsungen, Germany
Syringe 0.45 µm pore filter	Sarstedt	Nümbrecht, Germany
Tissue culture 50 ml flasks	Nunc	Wiesbaden, Germany

2.1.4 Chemicals and reagents

Substance	Manufacturer/Supplier	Location
Ammonium chloride	Roth	Karlsruhe, Germany
Copper(II) sulfate ($CuSO_4$)	Merck	Darmstadt, Germany
Distilled water	Institute of Microbiology and Hygiene, Charité	Berlin, Germany
DAB	Sigma-Aldrich	Steinheim, Germany
Dinatriumhydrogencarbonate	Merck	Darmstadt, Germany
Dinatriumhydrogenphosphate	Merck	Darmstadt, Germany
Entellan	Merck	Darmstadt, Germany
Eosin	Merck	Darmstadt, Germany
Ethanol	Sigma-Aldrich	Steinheim, Germany
EDTA	Sigma-Aldrich	Steinheim, Germany
Formaldehyde (37%)	Sigma-Aldrich	Steinheim, Germany
Glacial acetic acid	Merck	Darmstadt, Germany
Haematoxylin	Merck	Darmstadt, Germany
Isoflurane (Forene®)	Abbott	Illinois, USA
Isopropanol	Sigma-Aldrich	Steinheim, Germany
Potassium alum ($KAI(SO_4)2x12H_2O$)	Merck	Darmstadt, Germany
Sodium dodecyl sulfate	Biorad	München, Germany
Sodium iodide	Riedel de Haen AG	Seelze, Germany
Sulphuric acid (H_2SO_4)	Merck	Darmstadt, Germany
TMB (3,3',5,5'-Tetramethylbenzidine)	Sigma-Aldrich	Steinheim, Germany
Xylol	J.T. Baker	Phillipsburg, NJ, USA
Zinc sulfate ($ZnSO_4$)	Sigma-Aldrich	Steinheim, Germany

2.1.5 Buffers and solutions

Buffer/Solution	Composition
ELISA assay diluent	10% FCS in PBS
ELISA coating buffer	pH 9.5: 0.1 M $NaHCO_3$, Na_2CO_3
ELISA stop solution	2 N H_2SO_4
ELISA substrate solution	0.2 M Na_2HPO_4, 0.1 M Citric acid
ELISA wash buffer	0.05% Tween 20/PBS
Eosin working solution	Eosin, glacial acetic acid, distilled water
Haematoxylin solution	Haematoxylin, sodium iodide, potassium alum
Phosphate buffered saline	Biochrom AG, Berlin

2.1.6 Enzymes and antibodies

Enzyme/antibody	Manufacturer	Location
Streptavidin-peroxidase (HRP)	BD Biosciences	San Diego, CA, USA
Rabbit peroxidase-antiperoxidase (PAP-complex)	DAKO	Hamburg
Anti-mouse IFN-γ	BD Biosciences	San Diego, CA, USA
TLA (Toxoplasma Lysate Antigen)	Institute of Microbiology and Hygiene, Charité (70)	Berlin, Germany
Biotinylated anti-mouse IFN-γ	BD Biosciences	San Diego, CA, USA
Mouse IL-17 conjugate concentrate	R&D Systems GmbH	Wiesbaden, Germany
Mouse/Rat IL-22 conjugate	R&D Systems GmbH	Wiesbaden, Germany
Rabbit anti-*T. gondii* hyperimmune serum	Institute of Microbiology and Hygiene, Charité (70)	Berlin, Germany
Swine anti-rabbit immunoglobulin	DAKO	Hamburg, Germany
Goat anti-mouse IgG:HRP (rat adsorbed)	AbD Serotec	Düsseldorf, Germany

2.1.7 ELISA "Kits"

ELISA	Manufacturer	Location
BD OptEIA Mouse IFN-γ ELISA Set	BD Biosciences	Heidelberg, Germany
Quantikine Immunoassay mouse IL-17	R&D Systems GmbH	Wiesbaden, Germany
Quantikine Immunoassay mouse/rat IL-22	R&D Systems GmbH	Wiesbaden, Germany

2.1.8 Culture media and sera

Medium/serum	Manufacturer	Location
RPMI 1640	GIBCO/Invitrogen	Karlsruhe, Germany
Swine serum	DAKO	Hamburg, Germany
Fetal bovine serum	Biochrom AG	Berlin, Germany

2.1.9 Antibiotics and other materials

Article	Manufacturer	Location
Penicillin/Streptomycin (10,000 U/ml)	Biochrom AG	Berlin, Germany
Fluid nitrogen	Messer Griesheim	Sulzbach, Germany

2.2 Methods

2.2.1 Experimental groups

Animals were divided into experimental groups according to mouse strain, parasite strain, parasite dose, and inoculation route, as shown in Table 3. Six C57BL/6 and two NMRI mice served as an uninfected control group.

In two control BALB/c mice, 25 µl of PBS was instilled onto the right eye, but not onto the left eye to determine any harmful effects of the procedure to the eye. In all BALB/c mice infected via ocular instillation, the left eye remained uninfected to serve as a control for the right, infected eye.

Table 3: Allocation of mice to groups of infection

Group	N° of mice infected	Mouse type	Parasite dose, strain and stage	Inoculation	Dissection days (post-infection)
1	10	C57BL/6	5 ME49 cysts	i.p.	13
2	12	C57BL/6	10 ME49 cysts	i.p.	13
3	16	C57BL/6	5 ME49 cysts	p.o.	14, 21
4	32	C57BL/6	10 ME49 cysts	p.o.	14, 21, 25, 41, and 59
5	10	C57BL/6	20 ME49 cysts	p.o.	10
6	5	NMRI	20 ME49 cysts	p.o.	14, 21
7	5	NMRI	100 ME49 cysts	p.o.	14, 21
8	8	C57BL/6 MMP2$^{-/-}$	10 ME49 cysts	p.o.	25, 41, and 59
9	6	BALB/c	5×10^3 RH tachyzoites	ocular instillation	3, 7

2.2.2 Infection of mice

Mice were infected perorally or intraperitoneally with indicated numbers of ME49 *T. gondii* cysts or via instillation with 25 µl of indicated numbers of tachyzoites onto the right eye. Mice infected by the latter route were anesthetized prior to infection with isofluran and rested for 30 seconds with the parasite solution on their eyes.

2.2.3 Dissection of animals, organ embedding and sectioning

Mice were sacrificed either by CO_2 asphyxiation or by isofluran inhalation. Blood was obtained by cardiac puncture. Eyes were excised, immersed in 5% formalin, and stored overnight. Subsequently, visual organs were embedded in paraffin at the Institute of Pathology, Charité Campus Benjamin Franklin. The paraffin-embedded eyes were hardened on the cooling plate in histology cassettes. Eyes were cut with a microtome into 2 µm sections, mounted on glass slides and dried overnight in the drying cabinet at 56°C.

2.2.4 Histologic staining

2.2.4.1 Haematoxylin and eosin staining

Slides were placed in staining racks. Sections were deparaffinized twice in xylene for 5 minutes each. Sections were then rehydrated in decreasing concentrations of alcohol (100% isopropanol, 96%, 80% and 70% ethanol, for 1 minute each). After washing in distilled water, slides were stained in haematoxylin solution for 5 minutes, washed in distilled water, and counterstained in eosin solution for 30 seconds to 1 minute. Eosin was differentiated by a short dip in 70% ethanol solution and subsequently sections were dehydrated through 80% and 96% ethanol solutions for 1 minute each. Sections were then treated twice with xylene, 5 minutes each. Slides were coverslipped using entellan and dried before microscopic examination. As a result nuclei were stained blue, cytoplasm pink to red, and muscles red.

2.2.4.2 Immunoperoxidase staining for *T. gondii*

Slides were deparaffinized as described above and washed in distilled water. Endogenous peroxidase was blocked by immersing sections in 3% H_2O_2 for 20 minutes. Afterwards, slides were washed in distilled water and twice in PBS. Each of the sections was covered with 100 µl of swine serum (1:10 dilution with PBS) and incubated for 30 minutes at room temperature. The incubation serum was then discarded. Sections were covered with 100 µl of primary rabbit anti-*T. gondii* antibodies (1:2000 dilution with PBS) and incubated overnight in a humid chamber at room temperature. Slides were washed twice for 5 minutes in PBS and covered with secondary antibodies (1:100 dilution with PBS), kept in dark and incubated for 30 minutes at room temperature. Slides were again washed twice for 5 minutes in PBS. Sections were covered with 100 µl of PAP-complex (1:100 dilution with PBS) and incubated for 30 minutes at room temperature. DAB tablets were diluted in 5 ml distilled water, and kept in the dark. Before use, 5 µl of 30% H_2O_2 were added to the solution. Slides were immersed twice for 5 minutes in PBS and washed with distilled water. Sections were then covered with 4 drops of the DAB solution (0.7 mg/ml). After a maximum of 5 minutes (staining was controlled microscopically), slides were washed twice with distilled water. Slides were immersed in Cu-sulphate-solution in 0.9% NaCl for 5-7 minutes to darken the DAB reaction, and washed twice with distilled water. Sections were dipped in haematoxylin for 5 seconds and washed twice in distilled water. Subsequently, sections were immersed in 1% HCl alcohol solution for 15 seconds and washed twice with distilled water. Sections were dehydrated in 70%, 80%, and 96% ethanol solutions and 100% isopropanol for 1 minute each. Afterwards, sections were cleared twice in xylene, each time for 5 minutes. Slides were coverslipped with entellan.

2.2.5 Evaluation of the slides

Retinal layers and choroid were examined for histologic signs of toxoplasmic retinochoroiditis. *T. gondii* tissue cysts within retinal layers were evaluated in haematoxylin and eosin, and PAP-stained sections. Eye sections were evaluated by the following investigators: Agata Katarzyna Dukaczewska, Oliver Liesenfeld (Institute of Microbiology and Hygiene, Charité) and Uwe Pleyer (Department of Ophthalmology, Charité, Campus Virchow Klinikum); to confirm findings, all slides were evaluated by Roberto Carlos Tedesco (Departamento de Ultra-estrutura

e Biologia Celular, Laboratório de Biologia Estrutural do Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil).

2.2.6 Photographic documentation

Images were taken by a digital camera at 10, 40 or 100 magnification. The corresponding magnification is marked below each picture.

Additional images were also taken by Roberto Carlos Tedesco.

2.2.7 Serum extraction and storage

Blood samples obtained by cardiac puncture were allowed to clot for 1-2 hours at room temperature and centrifuged for 10 minutes at 5100 revolutions per minute. Serum was removed and stored at -80°C.

2.2.8 Enzyme-linked immunosorbent assays to detect anti-*T. gondii* antibodies and cytokines

Anti-*T. gondii* IgG antibodies

Murine serum was examined for the presence of anti-*Toxoplasma gondii* IgG antibodies by ELISA according to the following protocol:

ELISA plates were covered with 100 µl of TLA (2 µg/ml) in coating buffer. The plate was covered with an adhesive strip and incubated overnight at 4ºC. Wells were aspirated and washed 5 times by filling each well with about 300 µl of washing buffer using a squirt bottle. After the last wash, wash buffer was removed and the plates were blocked with 200 µl of assay diluent per well. The plate was covered with an adhesive strip and incubated at room temperature for 1 hour. Each well was aspirated and washed as described above. 100 µl of a 1:100 solution of serum sample or control sample in assay diluent was added to each well and incubated for 2 hours at room temperature. Wells were then aspirated and washed. 100 µl of a 1:500 solution of HRP-conjugated anti-mouse IgG antibodies were added to each well and incubated for 1 hour. Each well was aspirated and washed 7 times with 300 µl washing buffer. 100 µl of substrate solution was added to each well and incubated until a change to blue colour was observed in the positive

control. 50 µl of stop solution was added to each well. The optical density of each well was determined using a microplate reader set to 450 nm.

Cytokines

In sera of 10 C57BL/6 mice infected perorally with 5 ME49 *T. gondii* cysts, and sectioned 21 days post infection, IFN-γ, IL-6, IL-17, and IL-22 concentrations were determined by ELISA according to the manufacturer's instructions.

3 RESULTS

3.1 Normal morphology of the retina in control C57BL/6 and NMRI mice

Eye sections of naïve C57BL/6 and NMRI mice were examined to provide a background for comparison with eye sections obtained from infected mice. Eye sections of C57BL/6 and NMRI naïve mice showed normal morphology under light microscopy (Fig. 2A, B). The retinal pigmented epithelium (RPE), photoreceptor layer (FTR), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), nerve fiber layer (NF), and outer limiting membrane were discernible; the inner limiting membrane, located between the nerve fiber layer and the vitreous humor, is not visible under light microscopy (71, 72). It is noteworthy that in NMRI albino mice, retinal pigmented epithelium and choroid do not contain pigment (melanin).

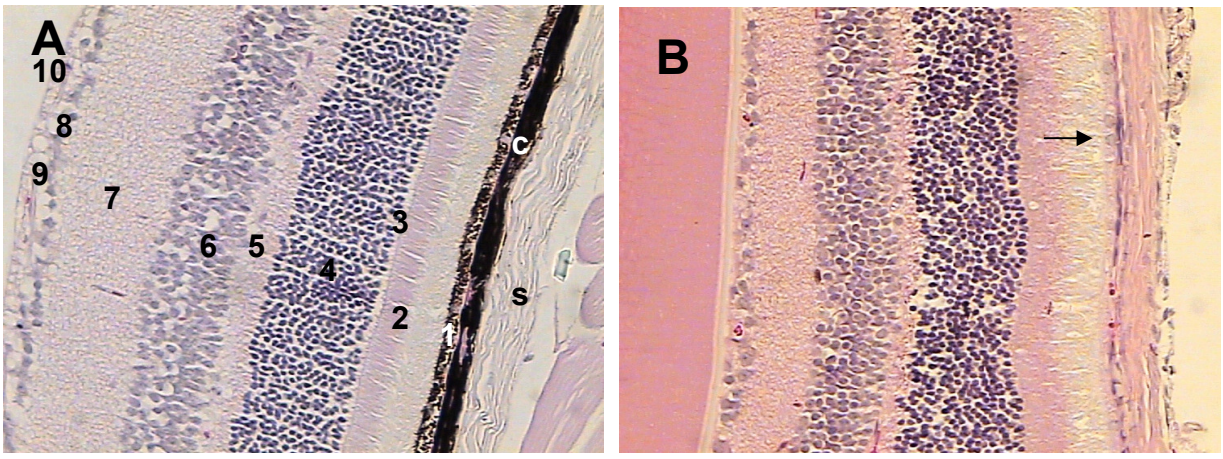

Fig. 2: Eye sections of naïve C57BL/6 (A) and NMRI (B) mice (H&E stain, magnification ×20): Retinal pigmented epithelium (1), photoreceptor layer (2), outer nuclear layer (4), outer plexiform layer (5), inner nuclear layer (6), inner plexiform layer (7), ganglion cell layer (8) and nerve fiber layer (9) are unaffected. Position of outer limiting membrane (3) and inner limiting membrane (10) is marked. Choroid (C) and sclera (S) are also visible. Nuclei of unpigmented cells comprising retinal epithelium (arrow).

3.2 Outcome of infection following intraperitoneal infection in C57BL/6 mice

3.2.1 Mortality of C57BL/6 mice following intraperitoneal infection

Ocular pathology following *T. gondii* infection coupled with no mortality is an important feature of a good animal model of ocular toxoplasmosis in humans. Therefore, susceptibility of mice to parasite infection was evaluated. The majority of C57BL/6 mice infected intraperitoneally did not survive infection. Four (40%) of ten mice inoculated intraperitoneally with 5 cysts and three (25%) of twelve mice inoculated intraperitoneally with 10 cysts survived until day 13 post infection; these mice presented symptoms of severe systemic disease (drowsiness, difficulty to walk).

3.2.2 Histological changes in eyes of mice infected intraperitoneally with ME49 *T. gondii* cysts

Two of four C57BL/6 mice infected intraperitoneally with 5 *T. gondii* cysts presented unilateral and two mice presented bilateral ocular involvement 13 days after infection. Unilateral inflammatory infiltrates in the vitreous humor were the most consistent sign of ocular toxoplasmosis (Fig. 3A). Unilateral infiltrates in the vitreous humor and/or GCL were found in all mice infected with 5 cysts. Bilateral RPE migration was observed in two mice and unilateral glial migration in one mouse (Fig. 3B). Whereas all C57BL/6 mice infected intraperitoneally with 10 *T. gondii* cysts developed severe systemic disease, only one of three mice showed unilateral retinitis characterized by RPE migration. The other two animals showed no ocular involvement (Fig. 3C). We did not detect parasites in the eyes of mice infected intraperitoneally.

Taken together, intraperitoneal infection of C57BL/6 mice resulted in high mortality. In addition, no consistent ocular pathology could be achieved in mice infected with 10 cysts.

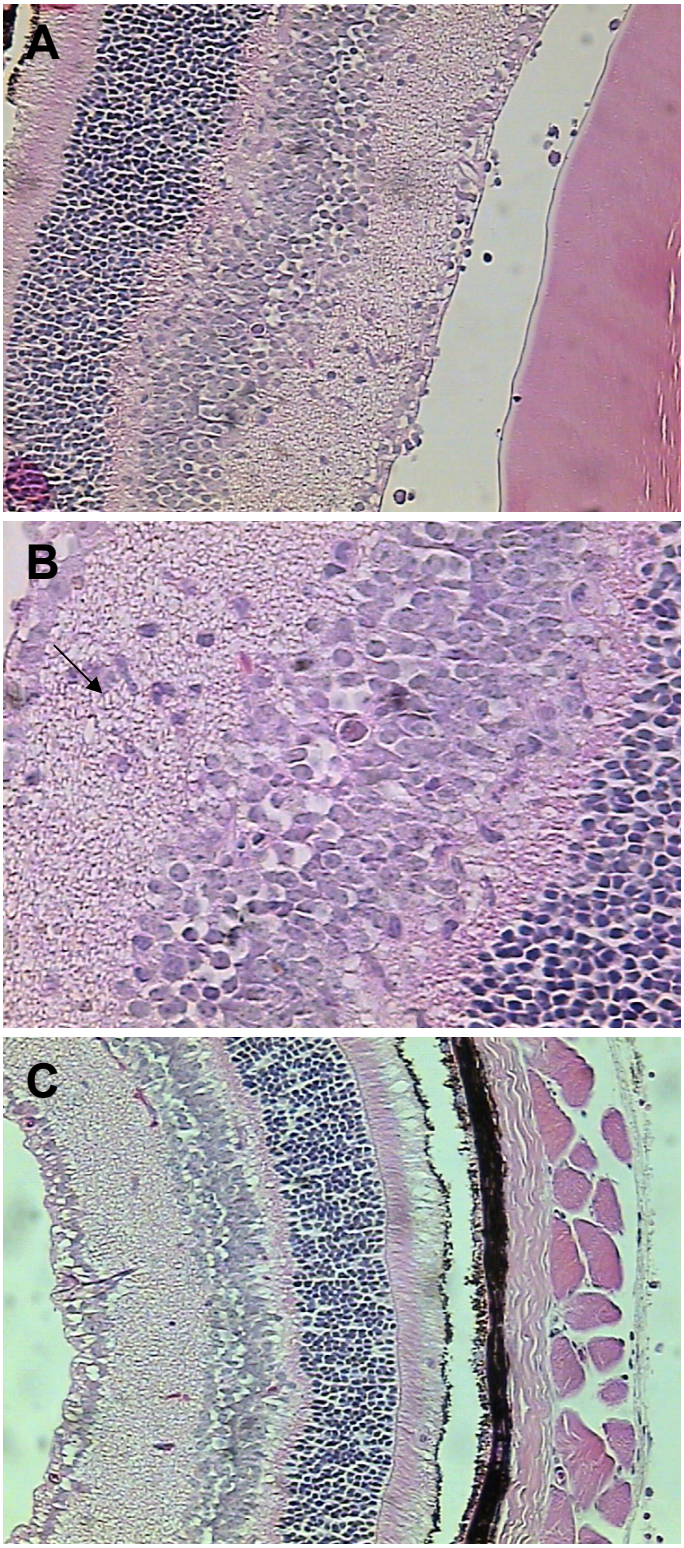

Fig. 3: Ocular histopathology in the eyes of C57BL/6 mice 13 days after intraperitoneal infection (H&E stain): (A) Mild inflammatory infiltrate in the vitreous humor (magnification ×20) and (B) glial migration in the IPL (arrow) in the eye of a mouse infected with 5 cysts (ME49) (magnification ×40). (C) Normal morphology of the retina of a mouse infected with 10 cysts. Separation of the neural retina from the retinal pigmented epithelium is the result of histological processing (magnification ×20).

3.3. Outcome of infection in wildtype C57BL/6, MMP2$^{-/-}$ and wildtype NMRI mice infected perorally with ME49 *T. gondii* cysts

3.3.1 Mortality of mice following peroral infection

Susceptibility of mice to peroral infection with ME49 *T. gondii* cysts varied according to murine genetic background and parasite inoculum. All perorally infected NMRI mice survived the infection with 20 and 100 cysts showing no signs of systemic disease. Survival of perorally infected C57BL/6 mice varied according to parasite inoculum. As shown in Fig. 4, three (19%) of 16 C57BL/6 mice infected with 5 cysts died on day 12 after infection. Thirteen (41%) of 32 C57BL/6 mice infected with 10 cysts died between day 9 and 21 following infection and eight (80%) of 10 C57BL/6 mice infected with 20 *T. gondii* cysts died by day 10 post infection. Two remaining mice from the latter group were severely ill 10 days after infection and were euthanized on day 10 post infection. Two (25%) of 8 MMP2$^{-/-}$ mice infected perorally with 10 cysts succumbed to infection (days 12 and 25 post infection).

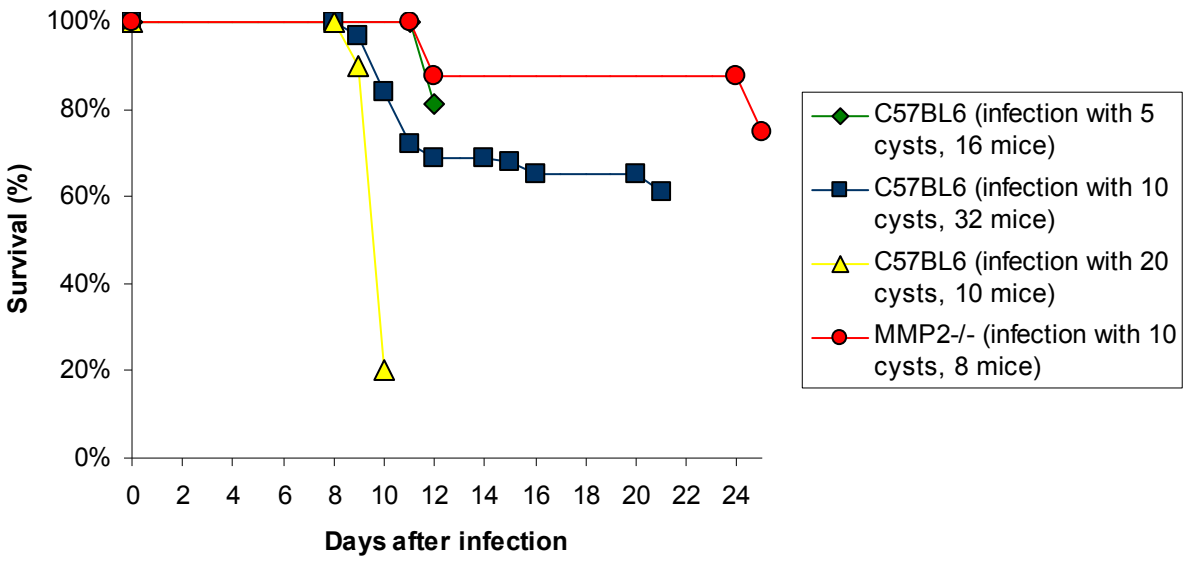

Fig. 4: Mortality of mice infected perorally with 5, 10, and 20 cysts (ME49) of *T. gondii*.

3.3.2 Histological changes in eyes of mice infected perorally with ME49 *T. gondii* cysts

In the eyes of perorally infected C57BL/6 mice, ocular involvement varied in severity ranging from unaffected ocular tissue to multiple changes characteristic of retinochoroiditis (Fig. 5, 6). Histological evaluation disclosed that unilateral or bilateral inflammatory infiltrates in the vitreous, GCL and around the retinal vessels, as well as RPE migration were the most common changes. Cone formation, alteration of disposition of retinal layers and glial migration were also observed. In C57BL/6 mice, peroral infection with 5 cysts resulted in unilateral retinitis in one (33%) mouse 14 days post infection whereas bilateral retinochoroiditis was observed in eight (80%) animals, and unilateral ocular involvement in two (20%) animals 21 days post infection. 21 to 25 days following infection with 10 ME49 *T. gondii* cysts, seven (100%) mice showed bilateral retinochoroiditis. 41 days post infection, two (50%) mice infected with 10 cysts showed bilateral ocular toxoplasmosis, one (25%) mouse unilateral retinochoroiditis, and one (25%) mouse showed bilateral ocular hypoplasia (outcome of infection could not be evaluated due to this (most likely genetic) defect). 59 days post infection, one (50%) C57BL/6 mouse presented bilateral retinochoroiditis and one (50%) mouse did not show any ocular involvement. Following infection with 20 cysts, one (50%) mouse showed unilateral retinitis 10 days post infection and one (50%) mouse presented no ocular pathology. Among C57BL/6 mice infected with 5 cysts, one cyst without inflammatory changes in the surroundings was observed in one (33%) mouse 14 days post infection. 21 days post infection, unilateral cysts were observed in five (50%) mice; in one mouse two cysts were present in one eye. Following infection with 10 cysts, cysts were found in the eyes of four (80%) C57BL/6 mice 21 days post infection; parasites were observed in both eyes (one cyst in each eye) of one of these mice (Fig. 7).

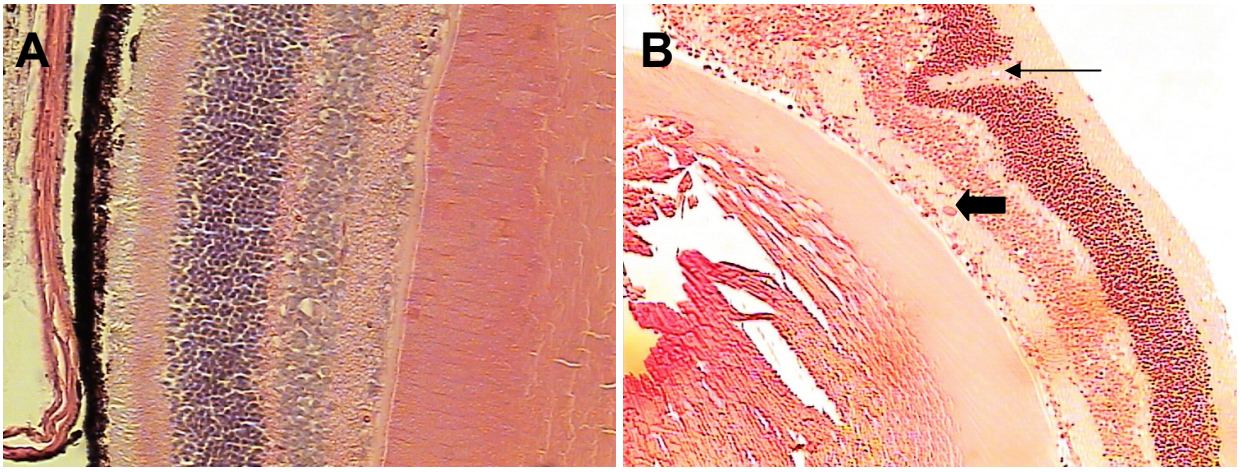

Fig. 5: Ocular tissue of C57BL/6 mice infected perorally with 5 ME49 cysts 21 days post infection (H&E stain, magnification ×10): (A) Unaffected retina; (B) cone formation (arrow) and a *T. gondii* cyst in the IPL (block arrow).

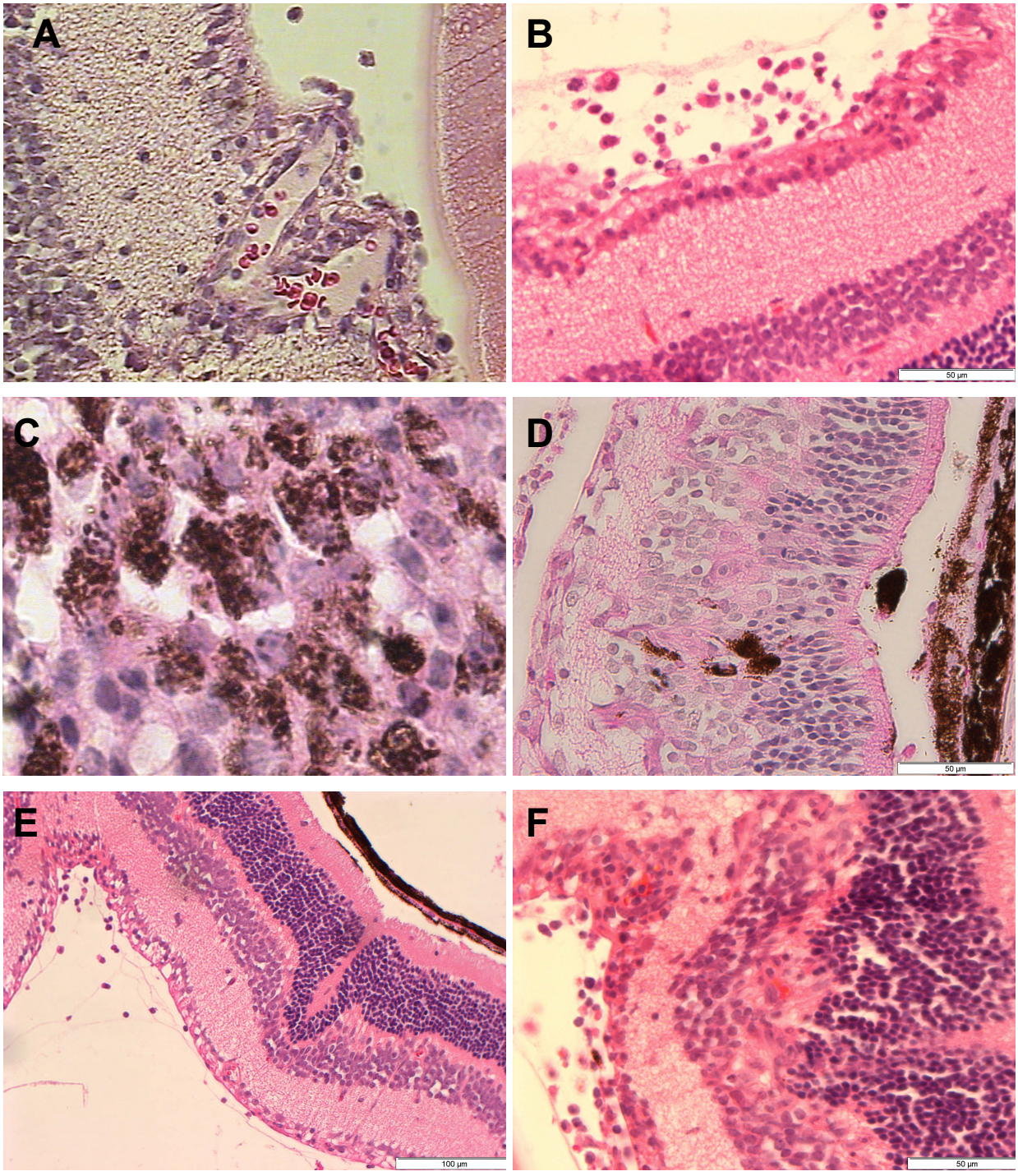

Fig. 6: Retinochoroiditis in the eyes of C57BL/6 mice perorally infected with ME49 *T. gondii* cysts (H&E stain): (A) Perivascular and vitreal inflammatory infiltrate, and glial migration in a mouse infected with 10 cysts 21 days post infection (magnification ×40); (B) extensive inflammatory infiltrate in the vitreous and GCL in a mouse infected with 5 cysts 21 days post infection; (C) RPE migration to the INL in a mouse infected with 10 cysts 21 days post infection (magnification ×100); (D) RPE migration to the ONL, OPL and INL in the eye of a mouse infected with 10 cysts 41 days post infection; (E) cone formation and inflammatory infiltrate in

the vitreous humor and GCL in a mouse infected with 10 cysts 21 days post infection; (F) cone formation and alteration in the disposition of INL, OPL and ONL; inflammatory infiltrate in the vitreous humor, GCL and around a retinal vessel in a mouse infected with 10 cysts 21 days post infection.

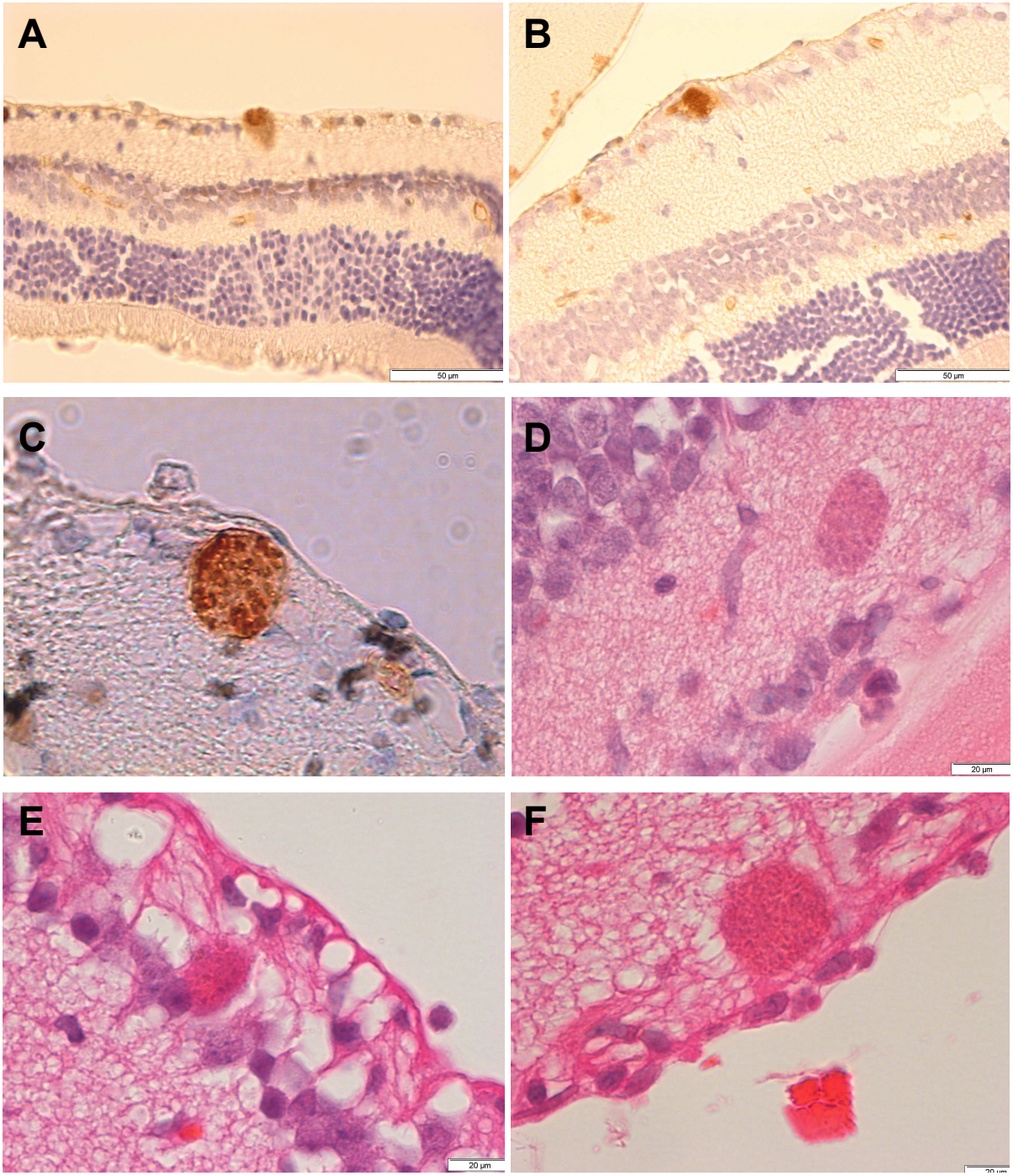

Fig. 7: *T. gondii* cysts in the eyes of C57BL/6 mice infected perorally with ME49 *T. gondii* (A-C: PAP stain; D-F: H&E stain): (A) and (B) Two cysts in the GCL in one eye of a mouse infected with 5 cysts 21 days post infection; (C) eye tissue of a mouse infected with 10 cysts 21 days post infection. *T. gondii* cyst in the GCL (magnification ×100); (D) parasite cyst in the IPL in an eye of a mouse infected with 5 cysts 21 days post infection; (E) cyst in the GCL in the left eye of a mouse infected with 10 cysts 21 days post infection, and (F) in the right eye of the same mouse.

All MMP2$^{-/-}$ mice infected perorally with 10 ME49 *T. gondii* cysts presented bilateral ocular toxoplasmosis characterized by vitreal and perivascular inflammatory infiltrates, RPE migration and/or architectural changes of the retina at 25, 41 and 59 days post infection (Fig. 8). Bilateral RPE migration was present in all MMP2$^{-/-}$ mice 41 (two mice) and 59 (two mice) days post infection, and in one of two mice 25 days post infection. One MMP2$^{-/-}$ mouse presented unilateral RPE migration 25 days post infection. Unilateral nflammatory infiltrates in the vitreous humor, GCL and/or around the retinal vessels were observed in two mice 25 days post infection, in one mouse 41 days post infection, and in one mouse 59 days post infection. Bilateral architectural changes, such as lacunae formation, alteration in the disposition of retinal layers and augmentation of interstitial spaces within FTR layer were found in one mouse 25 days post infection and in one mouse 41 days post infection. Unilateral architectural changes of the retina were observed in one mouse 25 days post infection, in one mouse 41 days post infection and in one mouse 59 days post infection. No parasite cysts were found in the eyes of MMP 2$^{-/-}$ mice. Severity of ocular lesions in C57BL/6 and MMP2$^{-/-}$ mice 25, 41 and 59 days after peroral infection with 10 cysts was similar, but MMP2$^{-/-}$ mice appeared to have a stronger predilection for development of bilateral disease than wildtype mice.

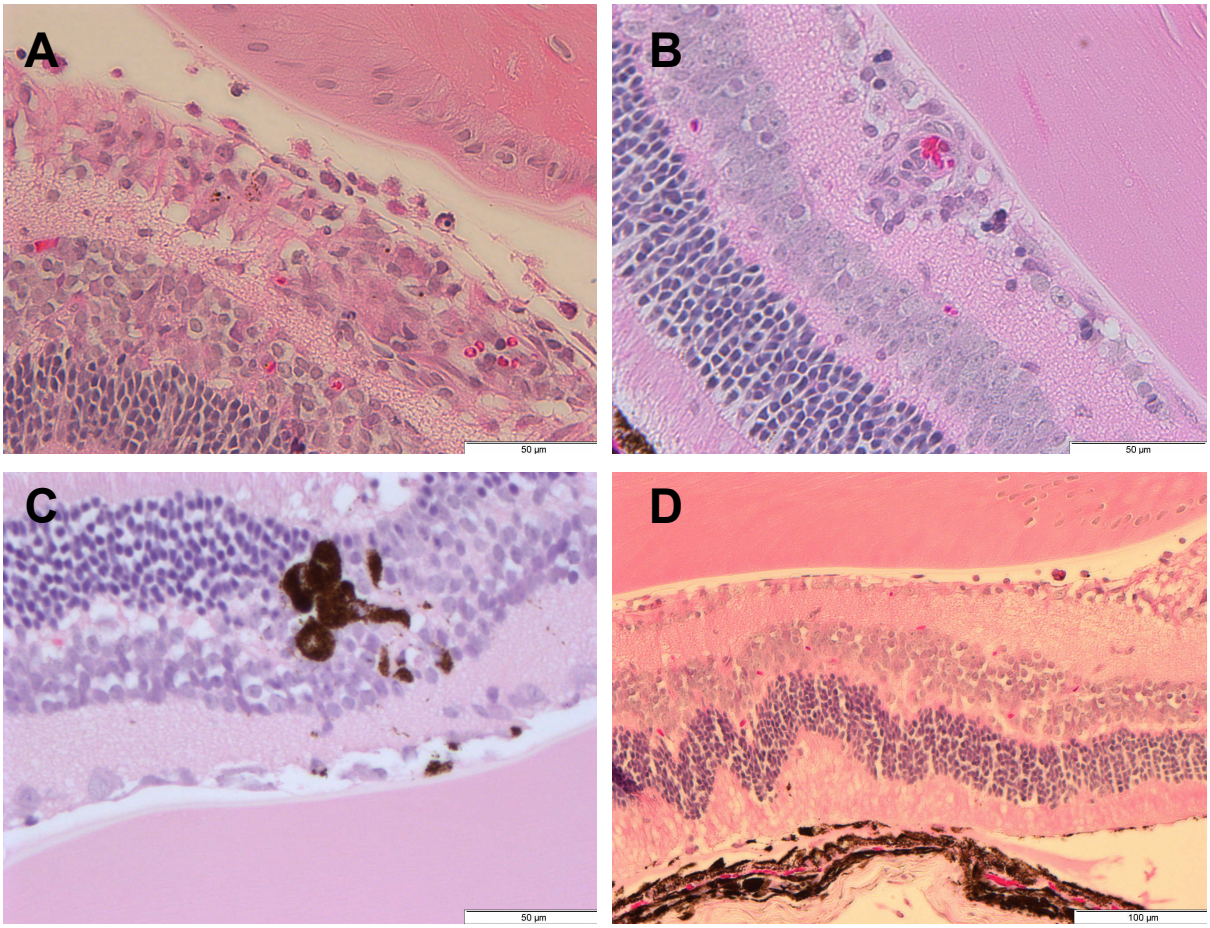

Fig. 8: Ocular histopathology in the eyes of MMP2$^{-/-}$ mice infected perorally with 10 ME49 *T. gondii* cysts (H&E stain): (A) Extensive vitreal inflammatory infiltrate in a mouse 25 days post infection; (B) perivascular inflammatory infiltrate 41 days post infection; (C) RPE migration reaching ONL, OPL, INL, IPL and GCL 59 days post infection; (D) cone formation and augmentation of the interstitial spaces within FTR layer 41 days post infection.

Figure 9 shows changes characteristic of retinochoroiditis found in *T. gondii* infected NMRI mice. Among five NMRI mice infected perorally with 20 cysts, three (60%) presented unilateral and two (40%) presented bilateral retinochoroiditis 14 days post infection. Retinochoroiditis was characterized by mild to extensive vitreal infiltrates, as well as infiltrates in the GCL. In one mouse unilateral glial migration was observed. Unilateral RPE migration was present in two mice. Cone formation and alteration in the disposition of retinal layers were also observed. 21 days post infection, five (100%) NMRI mice infected with 20 cysts showed bilateral retinochoroiditis. Three mice presented bilateral and two unilateral inflammatory infiltrates in the vitreous humor, GCL, and around the retinal vessels. Unilateral and bilateral RPE migration was found in two and one mice, respectively. Bilateral cone formation was observed in four mice. All NMRI mice infected with 100 cysts sectioned at 14 (n=5) and 21 (n=5) days post infection showed bilateral retinochoroiditis. Bilateral inflammatory infiltrates in the vitreous humor and GCL were observed in four (80%) mice and unilateral ones in one (20%) mouse at both time points. 14 days post infection, one (20%) mouse presented unilateral RPE migration; two mice showed bilateral cone formation and one mouse unilateral cone formation. 21 days post infection, one (20%) mouse presented bilateral RPE migration, three (60%) mice unilateral RPE migration and one (20%) mouse no RPE migration. In four mice bilateral and in one mouse unilateral cone formation was observed. One cyst was found at the boarder of INL and OPL in a mouse infected with 20 cysts 14 days post infection. Among mice infected with 100 cysts, in one (20%) mouse three cysts were found unilaterally in the OPL 14 days post infection, and in two (40%) mice unilateral individual cysts were found in the GCL 21 days post infection (Fig. 10).

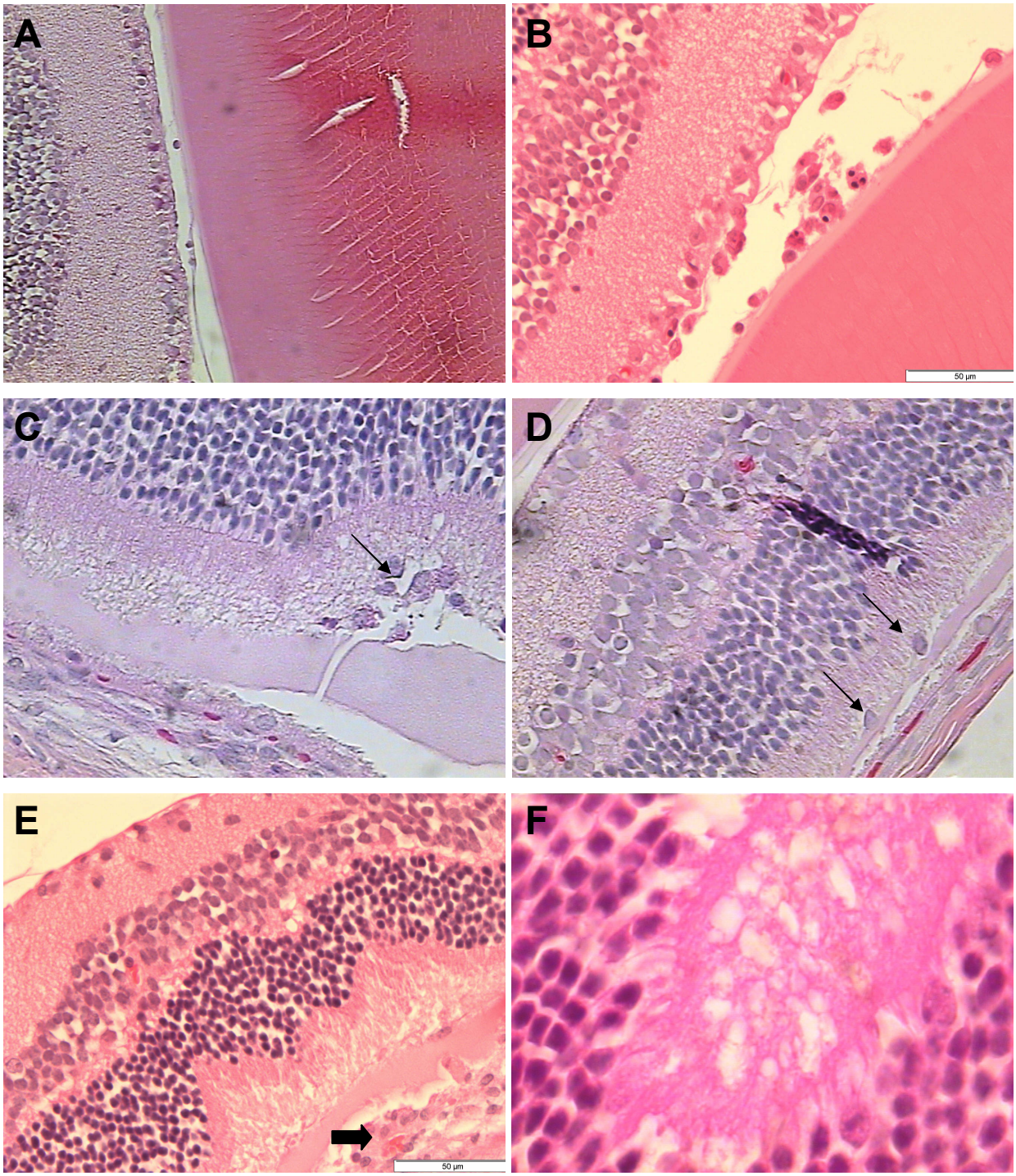

Fig. 9: Inflammatory changes in the eyes of NMRI mice infected perorally with ME49 *T. gondii* cysts (H&E stain): (A) Mild inflammatory infiltrate in the vitreous humor of a mouse infected with 20 cysts 21 days post infection (magnification ×20); (B) vitreal inflammatory infiltrate in a mouse infected with 100 cysts 14 days post infection; (C) RPE migration to the FTR layer (arrow) in a mouse infected with 20 cysts 21 days post infection (magnification ×20); (D) RPE migration to the FTR layer (arrows) in an NMRI mouse infected perorally with 100 cysts 21 days post infection (magnification ×20); (E) choroiditis (block arrow), cone formation, and glial migration in a mouse infected with 20 cysts 14 days post infection; (F) cone formation in the eye of a mouse infected with 100 cysts, sectioned 21 days post infection (magnification ×100).

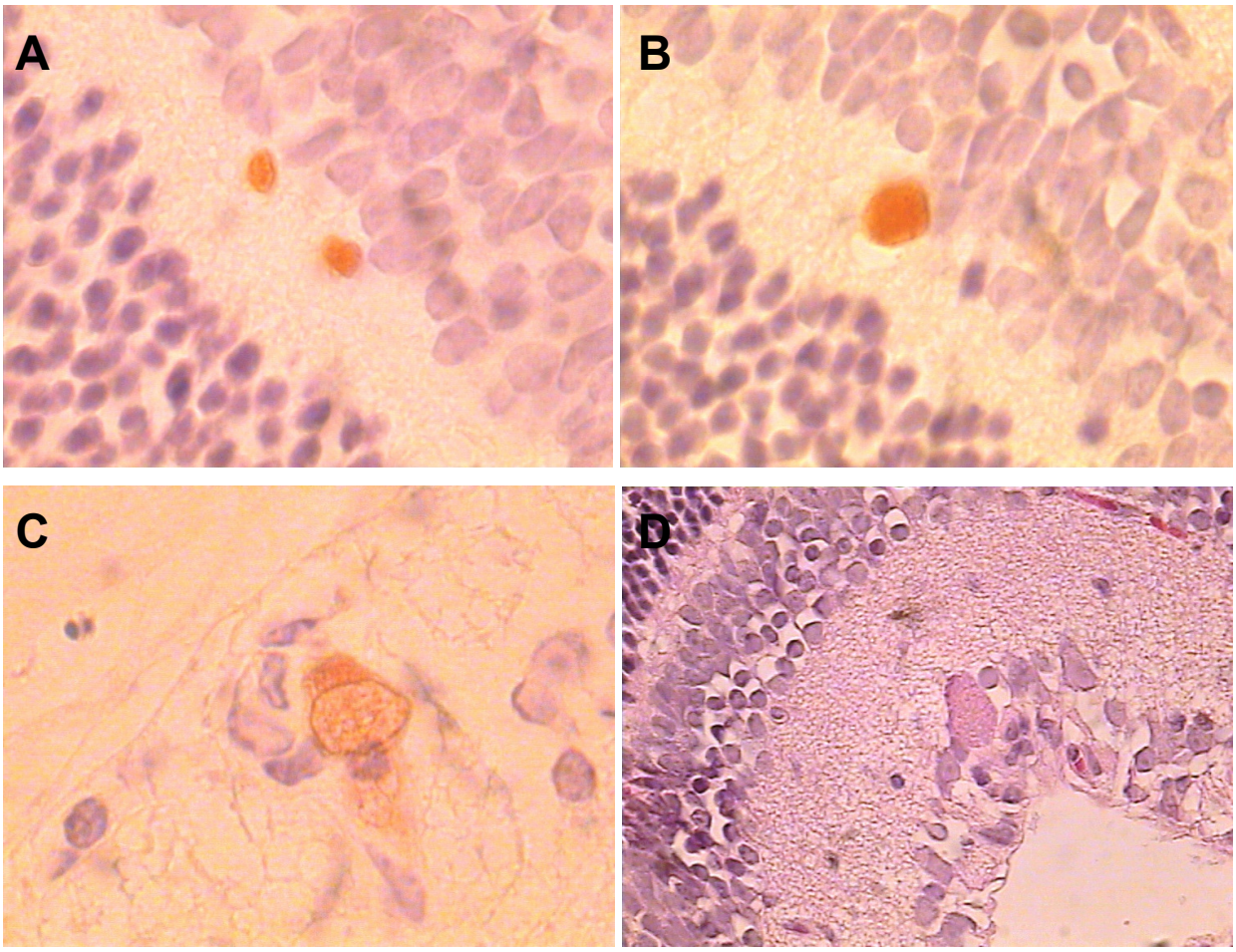

Fig. 10: *T. gondii* cysts in the eyes of NMRI mice infected with 100 cysts 14 (A and B) and 21 (C and D) days post infection (A-C: PAP stain, magnification ×100; D: H&E stain, magnification ×40). (A) and (B) Unilateral cysts in the OPL; (C) cyst in the vicinity of a blood vessel in the GCL; (D) cyst at the boarder of GCL and IPL.

Taken together, we observed that all C57BL/6 mice infected perorally with 10 cysts presented bilateral retinochoroiditis 21 to 25 days following infection. Similarly, all MMP2$^{-/-}$ mice infected with 10 cysts developed bilateral retinochoroiditis 25, 41, and 59 days post infection. Histological evaluation of the eyes of NMRI mice disclosed bilateral retinochoroiditis in all mice infected perorally with 20 cysts at 21 days post infection; at a higher dose of 100 cysts all mice developed bilateral retinochoroiditis between 14 and 21 days post infection. Moreover, NMRI mice are more resistant to peroral infection with *T. gondii* than C57BL/6 and MMP2$^{-/-}$ mice as they do not present symptoms or signs of systemic disease after infection.

3.4 Outcome of infection in BALB/c mice infected via ocular instillation of 5×10^3 RH tachyzoites

3.4.1 Susceptibility of BALB/c mice to ocular instillation of *T. gondii*

All BALB/c mice infected via ocular instillation of tachyzoites survived until the day of dissection showing no signs or symptoms of systemic disease.

3.4.2 Histological changes in eyes of mice infected via ocular instillation of 5×10^3 RH tachyzoites

In control BALB/c mice 25 µl of PBS was instilled onto the right eye only to determine if the process of instillation itself was harmful to the eye. No differences in retinal architecture were observed between the right and left eye in control mice, indicating that the deposition procedure did not cause architectural or inflammatory changes in the retina (Fig. 13A and B). BALB/c mice were infected via instillation of 5×10^3 RH tachyzoites in 25 µl of PBS onto the right eye. The left eye of each mouse remained uninfected to serve as a control. Mice were sectioned 3 and 7 days post infection. Mice did not present with systemic signs or symptoms of disease and we did not observe histological changes characteristic of retinochoroiditis in the infected eyes (Fig. 11). Mice that received PBS as a control also did not show either inflammatory or architectural changes of the retina (Fig. 12). In BALB/c mice no parasites were observed in histological sections of the eyes.

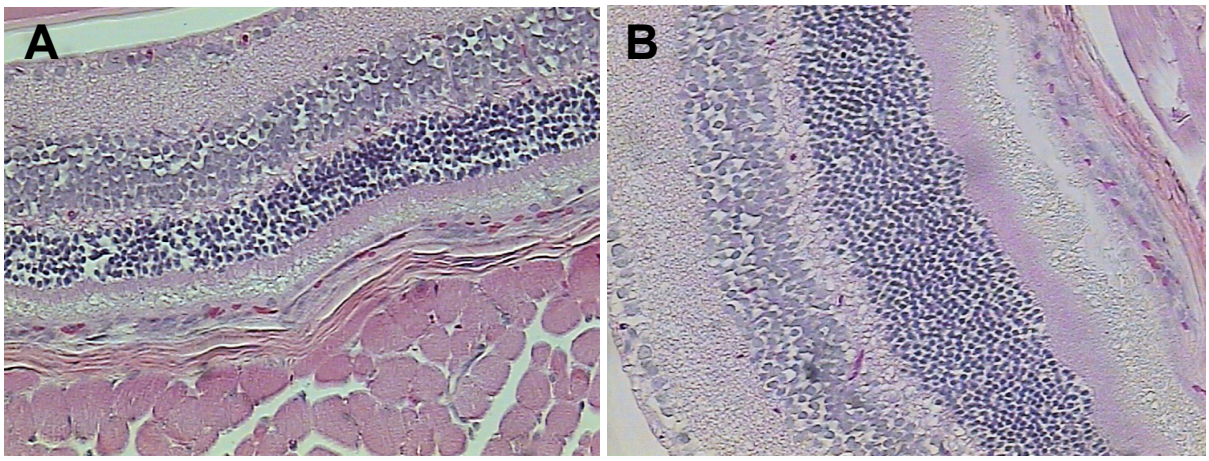

Fig. 11: Histological evaluation of eyes of control BALB/c mice (H&E stain, magnification ×20): (A) Normal retinal morphology in the right eye 7 days after instillation of 25 µl PBS; (B) untreated control left eye.

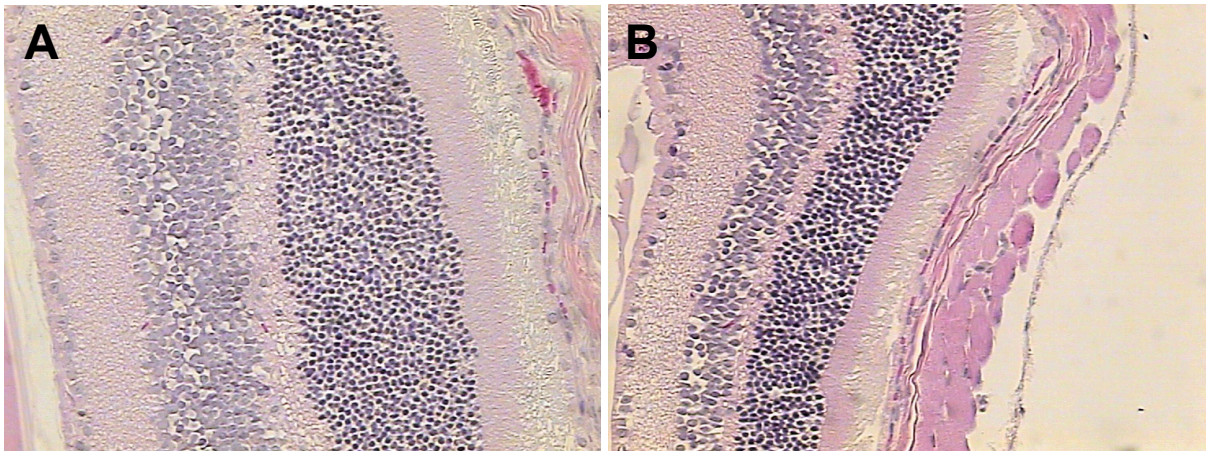

Fig. 12: Histological sections of eyes of BALB/c mouse infected via ocular instillation of 5×10^3 RH tachyzoites onto the right eye, euthanized 7 days post infection (H&E stain, magnification ×20): (A) Normal retinal morphology of the right eye; (B) section of the left untreated eye of the same mouse.

3.5 Anti-*Toxoplasma* IgG antibodies in sera of *T. gondii* infected mice

Sera of all *T. gondii* infected mice were screened for the presence of anti-*T. gondii* IgG antibodies to determine if mice had been infected successfully. In sera of all perorally and intraperitoneally infected mice anti-*T. gondii* IgG antibodies were detectable. In contrast, anti-*T. gondii* IgG antibodies were undetectable in serum samples obtained from mice infected via ocular instillation. These results indicate that oral and intraperitoneal administration of tissue cysts but not ocular instillation of tachyzoites resulted in infection of mice.

3.6 Concentrations of IL-6, IFN-γ, IL-17 and IL-22 in serum samples of C57BL/6 mice infected perorally with 5 *T. gondii* cysts

To investigate whether ocular inflammation correlates with changes in the cytokine profiles in mice following infection with *T. gondii*, sera of mice obtained 21 days post infection were screened for concentrations of the pro-inflammatory cytokines IL-6, IFN-γ, IL-17, and IL-22 by ELISA. IL-6 and IL-17 were undetectable in sera of perorally infected C57BL/6 mice. Only in one naïve mouse was IL-6 detected (113 pg/ml). Whereas IFN-γ was undetectable in sera of naïve mice, it was detected in sera of infected mice. Concentrations varied widely. Table 4 shows IFN-γ and IL-22 concentrations and their correlation with histological changes. The mean concentration of IFN-γ in sera of infected mice was 552.66 ± 597.38 pg/ml. Mean concentrations of IL-22 detected in sera of naïve mice were 389 ± 58.59 pg/ml and did not change significantly compared to infected mice (377.14 ± 128.17 pg/ml). Due to the limited number of sera analyzed we did not find a strong correlation between serum levels of IFN-γ and IL-22 and the severity of ocular changes following peroral infection with *T. gondii*.

Table 4: Comparison of ocular pathology in C57BL/6 mice infected perorally with 5 ME49 *T. gondii* cysts with serum levels of IFN-γ and IL-22

Mouse N°	Description of histological eye sections		Cytokine levels (pg/ml)	
	Left eye	Right eye	IFN-γ	IL-22
1	Inflammatory infiltrate in the GCL, also around a retinal vessel	RPE migration to the FTR; 2 cysts in the GCL	1363.33	210.86
2	RPE migration to the ONL, OPL and INL	Inflammatory infiltrate in the vitreous humor and in the GCL; RPE migration to the FTR; cone formation by the FTR	746.67	279.43
3	Inflammatory infiltrate in the vitreous humor and around a blood vessel in the GCL and INL; RPE migration to the FTR; a *T. gondii* cyst in the GCL, in the vicinity of a blood vessel	Cone formation by the FTR; changed architecture of ONL, OPL and INL	1680.00	325.14
4	RPE migration to the FTR; cone formation by the FTR	Inflammatory infiltrate in the vitreous humor	undetectable	522.29
5	Inflammatory infiltrate in the vitreous humor and GCL; RPE migration to the FTR and INL; glial migration; cone formation by the FTR; ONL is disrupted by the FTR	Discreet inflammatory infiltrate in the vitreous humor	undetectable	336.57
6	Normal retina	Inflammatory infiltrate in the vitreous humor and GCL; RPE migration to the INL and GCL; cone formation by the FTR; a cyst in the IPL	163.33	273.71
7	Inflammatory infiltrate in the IPL and GCL	Inflammatory infiltrate in the vitreous humor and around the blood vessels in the GCL	undetectable	533.71
8	RPE migration to the ONL; Cone formation by the FTR	1 cyst in the GCL	396.67	588.00
9	Discreet inflammatory infiltrate in the vitreous humor	Inflammatory infiltrate in the vitreous humor and GCL; RPE migration to the ONL; 1 cyst at the boarder of INL and OPL	863.33	305.14
10	RPE migration to the FTR	Normal retina	313.33	396.57

4 DISCUSSION

Ocular toxoplasmosis remains one of the most important clinical presentations of infection with *T. gondii* in humans. With the decline in AIDS patients suffering from toxoplasmic encephalitis due to the introduction of HAART, ocular toxoplasmosis has become the most frequent clinical presentation of infection with *T. gondii*. Unfortunately, while acute episodes associated with the presence of the tachyzoite stage of the parasite in the eye can be treated, none of the treatments tested to date is able to prevent recurring disease due to cyst rupture. Only the combination of trimethoprim and sulfamethoxazole has been shown to reduce the number of recurrences when given in a continuous prophylactic regimen (30). Due to the lack of suitable animal models, animal studies on the treatment of acute ocular toxoplasmosis in humans are scarce (73-75), and evidence of efficacy of the most commonly used regimens (clindamycin and 'bactrim' (27, 28)) is mostly based on clinical observations (16, 76). Additionally, in spite of numerous studies in mice and humans (32, 33, 35-37, 65, 68, 69, 77-79), the pathogenesis of ocular toxoplasmosis remains poorly understood. Therefore, to enable powerful studies of pathogenesis and treatment of the disease, an animal model of ocular toxoplasmosis that mimics important characteristics of disease in humans is essential.

In the past a number of animal models of ocular toxoplasmosis have been investigated (44-49, 53). Non-human primate, cat, rabbit and hamster models have the advantage that ophthalmological investigation of the eye is possible but are costly and only very few species-specific immunological reagents are available for these species to study (immuno)pathogenesis. Furthermore, gene knock-out animals of these species are also missing. Recent studies therefore focused on murine models of ocular toxoplasmosis; however, the majority of these studies were based on the intraocular and intraperitoneal rather than the peroral (natural) route of infection (32, 35, 50, 56, 57, 64). In the present study, we therefore investigated the value of a variety of murine models of ocular toxoplasmosis that would mimic key clinical and other features of ocular toxoplasmosis in humans. The impact of variables including the route of infection, parasite inoculum and genetic background of mice on the development of ocular pathology was determined following infection of mice with cysts of *T. gondii*. Furthermore, we analyzed whether surrogate markers of Th1 and Th17 immune responses that are associated with (immuno)pathology correlate with the presence and/or severity of ocular pathology.

4.1 Influence of the route of infection and parasite inoculum on the development of ocular pathology

Peroral inoculation of *T. gondii* is the natural infection route. The ingested parasite is exposed to the lymphoid tissue in the mucosa of the gastrointestinal tract (44) and antigens of the parasite are presented by cells of the intestinal mucosa (80) evoking a characteristic local followed by a systemic immune response of the host. This initial interaction of the parasite with the host is circumvented when mice are infected by non-peroral routes. In the present study peroral infection with ME49 *T. gondii* cysts evoked uni- or bilateral ocular pathology in all inbred C57BL/6 mice that had ingested 5 or 10 *T. gondii* cysts 21 to 41 days prior. We also observed ocular pathology in outbred NMRI mice that had ingested 20 or 100 cysts. All of the characteristic histological changes of ocular toxoplasmosis that develop after intraperitoneal or intraocular infection of wildtype mice could be found in perorally infected mice: inflammatory infiltrates, predominantly in the inner retinal layers and in the vitreous humor (32, 68, 79), RPE migration (32, 36, 63, 64), disorganization of retinal architecture (57, 64), lacunae formation by the FTR (32, 57, 64, 79) and glial migration (57). Retinitis, vitreitis, as well as RPE disruption were also observed in humans suffering from toxoplasmic retinochoroiditis (8, 81). As previously observed, parasite cysts were found only in a small portion of histologic sections (63), and were noted mostly in the GCL (49, 60). The presence of some cysts was associated with inflammatory infiltrates in the inner retinal layers and vitreous humor (32).

Whether or not the route of infection (intraperitoneal vs. oral) impacts the development of ocular pathology has not yet been determined in a murine model. Results of the present study indicate that mice perorally and intraperitoneally infected with *T. gondii* develop similar ocular changes, where inflammatory infiltrates in retina and vitreous humor, as well as RPE migration predominate as signs of toxoplasmic retinochoroiditis. *T. gondii* cysts could not be observed in mice infected intraperitoneally, though.

Whereas unilateral or bilateral ocular toxoplasmosis developed in all animals as early as 13 days post intraperitoneal infection with 5 cysts, we observed a consistent pattern of ocular pathology in perorally infected mice only 21 days post infection. Thus, ocular pathology in C57BL/6 mice infected intraperitoneally appears to develop faster than in mice infected perorally; this finding is not unexpected based on the circumvention of the intestinal mucosa during intraperitoneal infection. On the other hand, systemic effects of oral infection appeared to be less severe than those of the intraperitoneal infection. 81% of mice infected orally with 5 cysts survived in good

health until day 21, whereas the majority of mice infected intraperitoneally with the same inoculum succumbed to infection by day 13 post infection; mice showed symptoms of severe systemic disease characterized by ruffled fur, marked weight loss, drowsiness, and difficulty to walk. In conclusion, both infection routes lead to development of ocular pathology in C57BL/6 mice, but the peroral route may be the superior infection model to the intraperitoneal infection route due to a better survival.

Thus, the natural, peroral inoculation of parasites should be the infection route of choice in investigations of ocular toxoplasmosis. However, only few animal models based on peroral administration of the parasite have been investigated so far (44, 65). Norose *et al.* used the oral inoculation route in C57BL/6 mice, and observed mild focal retinitis, mild inflammatory infiltrates, RPE migration, and disorganization of the retinal architecture 28 days post infection with 5 Fukaya *T. gondii* cysts (65). In the present study toxoplasmic retinochoroiditis appeared to develop faster, but the use of 5 Fukaya cysts by Norose enabled C57BL/6 mice to survive for at least 30 days post peroral infection (65). However, individual differences in the virulence of specific strains of *T. gondii* are known (82) and may have contributed to the decrease in time to pathology and length of murine survival.

As alternative routes of infection, direct intraocular inoculation of the parasite (47, 48, 50, 56, 57, 68, 79, 83) and intracarotid infection have been used to investigate ocular toxoplasmosis (15, 46, 52). Intraocular administration of parasites does evoke toxoplasmic retinochoroiditis in C57BL/6 mice with characteristic signs found in humans. As a disadvantage, intraocular infection causes mechanical lesions that may blur the ocular pathology induced by *T. gondii* (57). Tedesco *et al.* showed that toxoplasmic retinochoroiditis can be induced in C57BL/6 mice by instillation of ME49 parasites on the eye surface to avoid injection-related lesions of the retina and choroid (57). He observed parasites in the retinal vessels, RPE and glial migration, edema formation in the inner nuclear and ganglion layers, and focal retinochoroiditis in the eyes of C57BL/6 mice infected by instillation onto the eye of 5×10^3 ME49 bradyzoites in PBS. In the present study, infection via instillation of 5×10^3 tachyzoites on the surface of BALB/c mice did not evoke any ocular pathology, although the mouse virulent RH-strain tachyzoites were used. As anti-*T. gondii* IgG antibodies were undetectable in sera of BALB/c mice, the parasite instillation did not evoke infection. This may be due to the fact that BALB/c mice are less prone to infection with *T. gondii* than C57BL/6 mice (35, 50, 82). To conclude, ocular instillation of *T.gondii* is not a reliable route to evoke ocular pathology in BALB/c mice.

It was previously reported that ocular pathology resulting from *T. gondii* infection depends on the parasite inoculum administered (56). Results of the present study do not support this hypothesis, as all outbred NMRI mice infected perorally – regardless of parasite inoculum – developed unilateral or bilateral ocular pathology. 21 to 41 days post peroral infection all inbred C57BL/6 mice infected with 5 and 10 *T. gondii* cysts developed ocular pathology as well. Opposite results were obtained by Hu *et al.* in a murine model of ocular toxoplasmosis based on intraocular infection (56). Naïve C57BL/6 mice inoculated intracamerally with 500 PLK (type II strain) tachyzoites presented moderate inflammatory changes in the eyes, whereas mice that had received 50 tachyzoites showed no ocular inflammation by day 8 post infection (56). On the other hand, increased susceptibility to infection with greater parasite inocula was coupled with higher mortality among C57BL/6 mice in the present study. These results are consistent with those obtained by Hu *et al.* where mice infected with 50,000 PLK tachyzoites died of a systemic infection (56).

In conclusion, the inoculum does not appear to impact the development of ocular pathology in perorally infected mice, but it influences survival post infection in perorally and intracamerally infected C57BL/6 mice.

4.2 Impact of the genetic background of mice on the development of ocular pathology after peroral infection with *T. gondii*

Results of the present study show that there are no vast differences in ocular pathology between different strains of mice following peroral infection with *T. gondii*. Histological changes were similar in outbred NMRI and inbred C57BL/6 mice known to be susceptible to toxoplasmic encephalitis (82). However, RPE migration was more difficult to observe in albino NMRI than in C57BL/6 mice due to the lack of pigment in the eyes of NMRI mice. A comparison of ocular outcomes in C57BL/6 and NMRI mice following *T. gondii* infection had not been reported before. However, predilection for retinal lesions was compared between C57BL/6 and BALB/c mice by Norose *et al.* (35). Following peroral infection of C57BL/6 and BALB/c mice with 5 Fukaya cysts, inflammatory changes could not be detected at 12 days post infection. On the other hand, the *T. gondii* load measured by PCR was significantly higher in retinas of C57BL/6 mice than in BALB/c mice at the same time point (35). These results and results of other studies (84) point towards a higher susceptibility to parasite replication of C57BL/6 compared to other mouse

strains whereas histological changes do not differ in the eyes of C57BL/6, BALB/c and NMRI mice following peroral inoculation.

In the present study we also confirmed that C57BL/6 mice are more susceptible to early death than NMRI mice. All NMRI mice infected with 20 or 100 ME49 cysts survived until the end of the experiment (day 14 and 21) in good health, whereas, depending on parasite inoculum, 19% to 80% of C57BL/6 mice died between day 9 and 21 following infection as previously reported (82). As outbred NMRI mice do not succumb to peroral infection with *T. gondii* and are easier to breed than inbred C57BL/6 mice, the cost of *in vivo* studies of ocular toxoplasmosis using NMRI mice is markedly lower. Taken together, the NMRI murine model represents an attractive alternative to the C57BL/6 murine model of acquired ocular toxoplasmosis. However, whereas murine immunological tools can be applied to any mouse strain, the lack of gene knock-out NMRI mice is a limitation.

Matrix metalloproteinases (MMPs) remodel the extracellular matrix and thereby may be engaged in the healing of injured nervous and ocular tissue (85-87). At the same time MMPs are involved in the immunopathology of inflammatory bowel disease (88), and play a role in disruption of the blood-brain barrier (89-91). MMP-2 has been shown to contribute to choroidal neovascularisation (85, 92) and proliferative diabetic retinopathy (93). MMP-9, but not MMP-2 increased inflammatory responses in a murine model of keratitis following *P. aeruginosa* infection (94). Since the role of MMPs has been investigated in parasitic neurologic (cerebral malaria (95) and neurocysticercosis (96)) but not in ocular diseases, we studied the role of MMP2 in ocular toxoplasmosis. All $MMP2^{-/-}$ mice developed bilateral ocular pathology 25 to 59 days post peroral infection, whereas the clinical presentation ranged from no ocular involvement to unilateral or bilateral disease in wildtype mice at the same time point. Thus, MMP2 does not contribute to ocular pathology but may play a rather protective role in the development of ocular pathology following *T. gondii* infection. Interestingly, in the present studies survival rates of wildtype mice following peroral infection with 10 ME49 *T. gondii* cysts were lower than those of $MMP2^{-/-}$ mice; similarly, $MMP2^{-/-}$ mice were also reported to be protected against early death and intestinal pathology following peroral infection with 100 ME49 cysts (97).

4.3 Correlation between serum cytokine levels and ocular pathology following infection with *T. gondii*

Inflammatory immune responses driven by T cells can be subdivided into Th1, Th2, and Th17 responses (98). Whereas IFN-γ is a key cytokine of Th1 responses that control the replication of intracellular parasites (8, 33, 36), IL-17 and IL-22 have been associated with Th17 responses (48) that drive immunopathology in uveitis (40). We therefore investigated the association between concentrations of Th1 and Th17 cytokines in the serum and severity of ocular pathology in C57BL/6 mice infected perorally with 5 ME49 *T. gondii* cysts.

IFN-γ serum levels were below the limit of detection in naïve mice, but were elevated in 70% of infected mice. The finding of elevated IFN-γ serum levels confirms the results of previous studies in mice infected intraperitoneally with 30 ME49 *T. gondii* cysts (32). The IFN-γ levels, however, did not correlate with severity of ocular involvement in our studies: IFN-γ was detected in sera of mice presenting unilateral ocular involvement, but remained undetected in individual mice presenting bilateral disease. In patients with ocular toxoplasmosis concentration of IFN-γ in the aqueous humor also did not correspond with the degree of uveal inflammation (78). Therefore, it appears that concentrations of IFN-γ, both in serum as well as in aqueous humor, do not determine the severity of ocular involvement in toxoplasmosis but may rather contribute to the control of parasites numbers (35). The fact that IFN-γ was only measured in the serum but not in the eyes in the present study may also have contributed to the lack of association between IFN-γ concentrations and severity of disease.

IL-6 has been reported to be involved in the generation of Th17 responses (99). We did not detect IL-6 consistently in naïve or infected mice. Calabrese *et al.* reported contrasting results in peritoneally infected mice (32): IL-6 was not detected in sera of naïve mice but slightly elevated in sera of infected mice. In the latter experiment IL-6 titers were significantly elevated in aqueous humor in infected mice. Other investigators reported elevated IL-6 concentrations in murine (33) and human (100) eyes, but not in serum (100) following infection. In a murine model of toxoplasmic retinochoroiditis IL-6 was shown to control the ocular parasite load (33), but concentrations of IL-6 did not correspond with severity of ocular pathology in patients presenting with ocular toxoplasmosis (78).

It has recently been suggested that IL-17 mediates toxoplasmic encephalitis (101) and ocular toxoplasmosis (40, 78). In the present study, IL-17 was undetectable in sera of both naïve and infected mice. As the ELISA assay used in our experiment may not be sensitive enough to detect

IL-17 in murine sera, the use of more sensitive techniques such as polymerase chain reaction may be of help in future studies of the disease. However, in patients presenting with ocular toxoplasmosis IL-17 levels were markedly elevated in the aqueous humor but not in sera and IL-17 titers did not correlate with degree of ocular involvement (78). In conclusion, although IL-17 amplifies the inflammatory process within the eye bulb (40), it does not seem to impact the severity of the disease.

The other characteristic Th17 cytokine, IL-22, possesses both pathogenic and protective functions in various inflammatory diseases (97, 98, 102). It was reported to play a role in disruption of the blood-brain barrier, thereby contributing to the development of central nervous tissue inflammation (103). IL-22 was reported to induce apoptosis in retinal pigmented epithelium cells, which may also facilitate the disruption of the blood-retina barrier and thereby ocular inflammation in autoimmune uveitis (104). The role of IL-22 in ocular infection has not yet been investigated. In the present study IL-22 serum levels in mice infected with *T. gondii* did not differ from those detected in naïve mice and did not correlate with the severity of ocular disease. It would therefore be of interest to determine aqueous humor levels of IL-22 in individuals presenting with ocular toxoplasmosis in order to assess if IL-22 is produced in the eye in the course of toxoplasmic retinochoroiditis.

Levels of most cytokines involved in the development of ocular toxoplasmosis are higher in the aqueous humor than in serum (32). These results suggest that cytokines are preferentially expressed locally within the eye bulb following infection with *T. gondii*. A comparison of severity of ocular pathology and cytokine levels in aqueous humor in individual mice would be difficult to conduct due to the very small volume of aqueous humor in the murine eye. Calabrese *et al.* pooled ocular aspirates obtained from 100 mice into one sample to enable the determination of cytokine levels in murine eyes using ELISA (32). This procedure, however, excludes comparison of cytokine levels and ocular pathology in individual mice. On the other hand, studies using aqueous humor can be conducted in individuals with toxoplasmic retinochoroiditis, as ocular samples may be obtained for different analyses anyway (8).

Several authors described uveitis-specific cytokine patterns (Table 5). Whereas IFN-γ was found to be consistently elevated in all types of uveitis diseases in both mice and humans, IL-17 was elevated in individuals affected with autoimmune uveitis and toxoplasmic retinochoroiditis. Therefore, some authors suggested that immunopathology of ocular toxoplasmosis comprises autoimmune process (105). An increase in IL-10 and IL-4 levels in the human eye and a decrease in the murine eye and serum in the course of ocular toxoplasmosis were observed, which leads to an assumption that there may be species characteristic differences in cytokine patterns. In the

present study we confirmed that IFN-γ is involved in the course of ocular toxoplasmosis. On the other hand IL-22 was proven not to be involved in the course of the disease, as the cytokine levels in naïve mice did not differ from those in infected mice. We failed to prove that cytokine patterns correlate with ocular pathology, which confirmed the results acquired by Lahmar *et al.* in humans (78). Detection of particular cytokines in the aqueous humor or serum of uveitis patients may contribute to the diagnosis of uveitis diseases (78, 106) and may also reveal targets for new treatment strategies (78, 107).

Table 5: Cytokine patterns in various uveitis diseases

Uveitis type	Sample	Host	Cytokine patterns		Ref.
Viral uveitis	Aqueous humor	human	Increase of GM-CSF, G-CSF, IFN-γ, IL-1β, IL-6, MIP-1β	Increase of IL-8, MCP-1, TNF-α, IL-4 and IL-10	(78)
Intermediate uveitis			Increase of IL-17, G-CSF		
Toxoplasmic retinochoroiditis			Increase of IFN-γ, MIP-1β, IL-5, IL-6, IL-12, IL-17		
Idiopathic uveitis	Aqueous humor	human	Elevation of IL-6, IL-8, MCP-1 and IFN-γ, decrease in TGF-β2 and CXCL12		(106)
Fuchs' heterochromic cyclitis			Upregulation of IL-2, IL-12 and IL-13		
Viral uveitis			Elevation of IL-10 in herpes viral uveitis, MCP-1 and IL-8		
Behçet disease			Elevation of MCP-1 and IL-8, downregulation of TGF-β and CXCL12		
Viral uveitis	Supernatants of eye-derived cell cultures	mouse	Upregulation of IL-4, IFN-γ and TNF-α		(105)
Autoimmune uveitis	Peripheral mononuclear blood cells	human	Elevated IL-17 levels		(108)
Autoimmune uveitis (Behçet disease)	Supernatants of peripheral blood mononuclear cells	human	Elevated IL-23, IL-17 and IFN-γ levels		(109)
Toxoplasmic retinochoroiditis	Serum and aqueous humor	mouse	Upregulation of IL-6, Fas and FasL in aqueous humor; downregulation of IL-10 in serum and aqueous humor; upregulation of TGF-β in serum and downregulation in aqueous humor; undetectable levels of IL-4 in infected and naïve mice; similar levels of TNF-α and IL-12 in aqueous humor of naïve and infected mice		(32)
Toxoplasmic retinochoroiditis	Serum	mouse	Upregulation of IFN-γ; similar IL-22 levels in naïve and infected mice; undetected IL-17		present study

4.4 Conclusion and outlook

In the present study, we investigated the impact of a variety of factors on the course of ocular toxoplasmosis. We proved that the infection route (peroral versus intraperitoneal), parasite inoculum and genetic background among wildtype mice do not impact the severity of toxoplasmic retinochoroiditis, but influence survival of mice post parasite administration. We also proved that MMP-2 plays a protective role against ocular pathology and murine mortality in the course of toxoplasmosis.

As peroral administration of parasites is the natural route of infection in humans, in vivo studies of the disease should be preferentially based on peroral infection of animals. NMRI and C57BL/6 mice perorally infected with ME49 *T. gondii* cysts developed ocular pathology characteristic of toxoplasmic retinochoroiditis in the present study. The pathology, i.e. focal inflammatory infiltrates in the retina, vasculitis, vitreitis and presence of the cysts in the retina, resembled that in humans (8). This model should prove useful to study various aspects of ocular toxoplasmosis since mice are relatively inexpensive, can be easily sustained in laboratories, and a whole array of immunologic agents are available. A disadvantage of the murine model is the small size of the animals, which makes it very difficult to conduct studies of the aqueous humor and excludes the use of ophthalmologic tools applicable in humans to observe the eye fundus (16). Taken together, the advantages of the murine model outnumber the disadvantages, and murine studies can be ideally complemented by studies in humans.

The present study has limitations. First, since the number of mice used in this experiment was limited, the results must be taken with caution. Second, the impact of parasite strain on ocular pathology has not been investigated in this study. The majority of human infections is caused by type II *T. gondii* strains (82). However, an increasing number of atypical parasite strains are found in South America (110). As parasites of other clonotypes are characterized by varying virulence (111), it is of interest to investigate the pathogenesis of infection with atypical *T. gondii* strains in an animal model of toxoplasmic retinochoroiditis. Various aspects of postnatally acquired ocular toxoplasmosis still remain obscure, such as immunopathology of *T. gondii* infection, individual susceptibility of an immunocompetent host to the parasite, the influence of genetic variation (112) and age of the host (113) on ocular outcome. All of these should be investigated in murine models of ocular toxoplasmosis. However, in

immunopathology studies, the C57BL/6 murine model based on peroral *T. gondii* infection must be used, as gene-deficient NMRI mice are not available on the NMRI background.

As there is an urgent need to develop new therapeutic approaches to ocular toxoplasmosis future studies must focus on the pharmaceutical aspect of ocular toxoplasmosis (114). The murine model in NMRI mice based on peroral *T. gondii* infection has already served as a springboard for investigation of a new therapy regimen for toxoplasmic encephalitis, where atovaquone (115), especially when given in the form of nanosuspensions to increase its uptake through the host barriers, was shown to be a promising new therapeutic strategy. Two novel 1-hydroxyquinolones were also proven to have an antitoxoplasmic activity in a murine model of toxoplasmic encephalitis (116). Therefore, antiparasitic effects of atovaquone- as well as related compounds should be tested for the treatment of ocular toxoplasmosis in the murine model based on peroral *T. gondii* infection.

5 SUMMARY

Toxoplasma gondii, an obligate intracellular protozoan parasite, infects around 30% of human population worldwide. Toxoplasmic retinochoroiditis, observed in 2%-20% of infected immunocompetent individuals, is the major cause of posterior uveitis and poses a threat of visual impairment if the macula is involved. Due to the lack of suitable animal models of acquired ocular toxoplasmosis, the efficacy of the most commonly used therapeutic regimens of an acute infection and the immunopathogenesis of infection have not been determined. Moreover, animal models established so far have mostly been based on intraocular and intraperitoneal infection that does not resemble the natural infection route in humans.

The aim of this study was to establish a murine model of ocular toxoplasmosis based on natural, peroral infection that allows the investigation of (immuno)pathology and therapy of the disease. To achieve this goal, we compared the influence of a variety of factors (parasite inoculum, infection route and murine genetic background) on ocular pathology. Additionally, we investigated the correlation between Th1 and Th17 cytokine patterns in murine sera and the severity of toxoplasmic retinochoroiditis in perorally infected C57BL/6 mice.

First, histopathological features of toxoplasmic retinochoroiditis did not differ between perorally and intraperitoneally infected C57BL/6 mice, but the signs of ocular toxoplasmosis in intraperitoneally infected mice developed faster. However, the majority of intraperitoneally infected animals succumbed to infection in contrast to perorally infected mice. Instillation of *T. gondii* RH tachyzoites onto the eyes of BALB/c mice failed to evoke infection. Second, the influence of the murine genetic background on ocular pathology could not be confirmed, as C57BL/6 and NMRI mice developed similar histopathological changes including retinal and vitreal inflammatory infiltrates, migration of retinal pigmented epithelium (RPE), disorganization of retinal architecture and presence of *T. gondii* cysts. RPE migration was more difficult to detect in NMRI mice due to lack of pigment in murine eyes. Furthermore, the availability of gene-knock-out C57BL/6 but not NMRI mice represents a major advantage for the investigation of immunopathology of the disease. Third, the parasite inoculum did not influence ocular pathology but impacted murine survival post infection in perorally infected C57BL/6 mice.

The immune response was characterized by elevated concentrations of IFN-γ in sera of the majority of perorally infected C57BL/6 mice. In contrast, IL-22 concentrations were similar in

sera of naïve and infected mice; IL-6 and IL-17 were not detected. Cytokine levels did not correlate with severity of ocular pathology.

In conclusion, peroral infection of NMRI mice with 20 or 100 *T. gondii* cysts as well as infection of C57BL/6 mice with 5 or 10 *T. gondii* cysts provides a reproducible model of ocular toxoplasmosis that can serve as a springboard for investigation of new therapeutic regimens and immunopathology of the disease.

6 ZUSAMMENFASSUNG

Toxoplasma gondii, ein obligat intrazelluläres Protozoon, infiziert ca. 30% der Weltbevölkerung. 2-20% der infizierten immunkompetenten Menschen entwickeln eine Toxoplasma Retinochoroiditis, die die Hauptursache einer posterioren Uveitis darstellt und zu Blindheit führen kann, wenn die Makula betroffen ist.

Infolge des Mangels an Mausmodellen der okulären Toxoplasmose liegen nur unzureichende Informationen zur Immunpathogenese der Infektion und der Wirksamkeit von etablierten und neuen Behandlungsschemata vor. Etablierte Tiermodelle basieren vor allem auf der intraokulären oder intraperitonealen Infektion, die aber nicht den natürlichen oralen Infektionsweg beim Menschen darstellen. Der Ziel der vorliegenden Arbeit war es, ein Mausmodell der okulären Toxoplasmose zu etablieren, das auf dem natürlichen oralen Infektionsweg basiert und die detaillierte Aufklärung der Immunopathologie und Therapieeffizienz erlauben würde. So wurde der Einfluss von verschiedenen Faktoren wie parasitäres Inokulum, Infektionsweg und genetischer Hintergrund der Mäuse auf die okuläre Pathologie untersucht. Darüber hinaus untersuchten wir den Zusammenhang zwischen Konzentrationen von Th1- und Th17-Zytokinen in Serum und dem Schweregrad der Retinochoroiditis.

Histopathologische Merkmale der Retinochoroiditis unterschieden sich nicht zwischen peroral und intraperitoneal infizierten Mäusen. Während sich die Zeichen der okulären Toxoplasmose in intraperitoneal infizierten Mäusen schneller entwickelten, zeigten intraperitoneal infizierte Mäuse auch eine deutlich erhöhte Letalität. Im Gegensatz dazu konnte durch Instillation von *T. gondii* Tachyzoiten auf das Auge keine Infektion hervorgerufen werden.

Auch konnte kein bedeutender Einfluss des genetischen Hintergrunds der Mäuse auf die okuläre Pathologie festgestellt werden, da C57BL/6- und NMRI-Mäuse ähnliche histopathologische Veränderungen wie entzündliche Infiltrate in Retina und Glaskörper, Migration des retinalen Pigmentepithels, Auflösung der Retinastruktur und *T. gondii* Zysten in der Retina aufwiesen. Die Migration des retinalen Pigmentepithels war jedoch in NMRI-Mäusen aufgrund des Fehlens von Pigment in den Augen schwieriger zu erfassen. Darüberhinaus stellt auch die Verfügbarkeit von diversen “Knock-out”-Mäusen auf dem C57BL/6-Hintergrund einen wesentlichen Vorteil für die Untersuchung der Immunopathogenese der okulären Toxoplasmose in C57BL/6-Mäusen dar.

Auch konnte gezeigt warden, dass das Inokulum die okuläre Pathologie nicht, wohl aber das Űberleben der C57BL/6-Mäuse nach oraler Infektion beeinflusst.

Die Immunantwort war durch erhöhte IFN-γ-Konzentrationen im Serum der Mehrheit der oral infizierten C57BL/6-Mäuse charakterisiert. Im Gegensatz dazu unterschieden sich die IL-22-Konzentrationen im Serum von naïven und infizierten Mäusen nicht; IL-6 und IL-17 konnten nicht nachgewiesen werden. Es konnte in der vorliegenden Arbeit keine Korrelation der Zytokin-Konzentrationen mit dem Schweregrad der okulären Pathologie nachgewiesen werden.

Zusammenfassend stellt die orale Infektion von NMRI-Mäusen mit 20 oder 100 *T. gondii* Zysten sowie die orale Infektion von C57BL/6-Mäusen mit 5 oder 10 *T. gondii* Zysten ein reproduzierbares Modell der okulären Toxoplasmose dar. Derartige Mausmodelle könnten wichtige Hinweise für die Evaluierung von neuen Behandlungsschemata liefern und der Klärung der Immunopathologie der okulären Toxoplasmose dienen.

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8 ADDITION

CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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Erklärung

„Ich, Agata Katarzyna Dukaczewska, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: Establishment of a murine model of ocular toxoplasmosis selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

_____ _____

Datum Unterschrift