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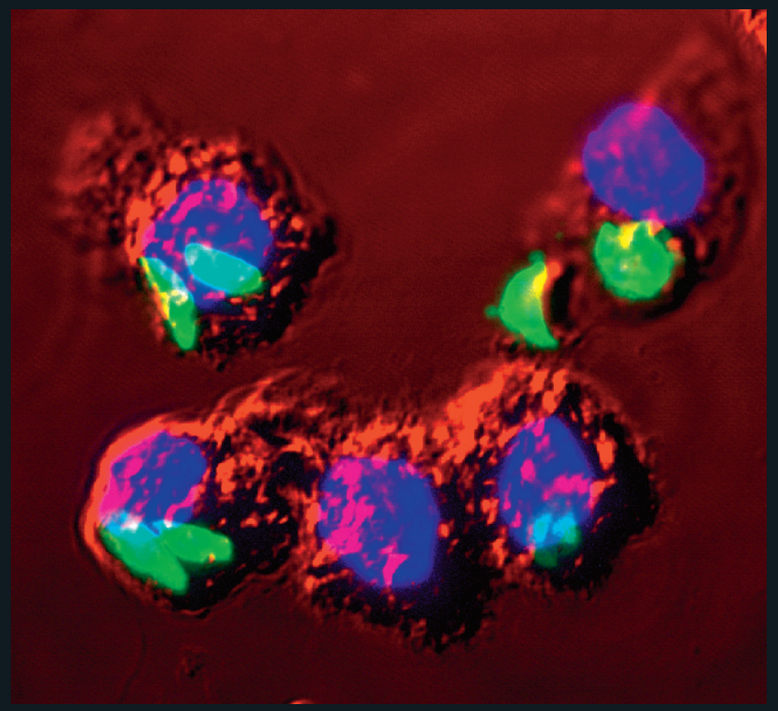
IMMUNE EVASION AND DISSEMINATION OF *TOXOPLASMA GONDII*



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ABSTRACT

Toxoplasma gondii is an obligate intracellular protozoan parasite which propagates in a complex life cycle where infection of Felidae family-members (Cats) results in sexual recombination whereas all other warm-blooded animals are intermediate hosts. Approximately one third of the human population is estimated to be chronically infected by this parasite. Infection is acquired orally by ingestion of undercooked meat harboring tissue cysts or through accidental intake of oocysts in contaminated food-stuffs. Acute disease is generally mild, but may lead to severe neurological and ocular manifestations in the developing fetus. Chronic infection brings subtle pathology, such as memory impairment and latent anxiety, but may upon reactivation in immunocompromised patients cause potentially lethal toxoplasmic encephalitis. The population structure of *Toxoplasma* is dominated by three clonal lineages (type I, II, III) and type II predominates in human infections. Immunity to *Toxoplasma* is biased towards a Th1 type response which mediates long term protection against reactivated disease. Dendritic cells (DCs) and Natural killer (NK) cells are important players in the acute phase of infection and IL-12 and IFN-γ produced by these cells respectively are essential mediators of resistance to the parasite. The overall objective of this work has been to investigate dynamics of parasite dissemination and its impact on the pathogenesis of infection. We have pursued the hypothesis that *Toxoplasma* enables successful dissemination and immune evasion by using leukocytes as Trojan horses during infection. Focus has primarily been on parasite-DC interactions in relation to infection with different parasite strains and on NK cell interactions with infected DCs. In summary we have shown the following:

1. Active invasion of DCs and macrophages by *Toxoplasma* tachyzoites *in vitro* induces a state of hypermotility in these cells which enables transmigration across endothelial monolayers in the absence of auxiliary chemotactic stimuli. This type of hypermotility requires an intracellular parasite and can be blocked by PTX treatment which uncouples the signaling pathway of trimeric G*i*-proteins. Host cell hypermotility is induced by all tree archetypical lineages of *Toxoplasma* but the intensity of this phenotype is clearly higher for type II parasites.
2. Adoptive transfer of *Toxoplasma* infected DCs accelerates dissemination of parasites to the spleen, MLN, brain and testis, and results in exacerbation of infection compared to inoculation of free parasites. PTX-treatment of infected DCs prior to inoculation reversed the observed enhanced dissemination. All tree archetypical lineages exhibits increased dissemination after adoptive transfer of DC-borne parasite but this mode of dissemination clearly favored type II and type III parasites more than type I in syngenic mice.
3. NK cells are the preferentially infected lymphocyte population shortly after parasite inoculation of mice. Infected DCs, but not infected NK cells, exhibit increased sensitivity to NK cell-mediated killing *in vitro*. NK cell killing of infected DCs results in the egress of viable parasites and the subsequent invasion of adjacent NK cells. This mechanism of NK cell infection is perforin-dependent *in vitro*.

The data presented in this thesis shows that parasite strain-dependent manipulation of host cell motility, favors DC-borne parasite dissemination via a Trojan horse type of mechanism, and that NK cell-mediated cellular cytotoxicity may contribute to parasite immune cell sequestration and immune evasion shortly after infection.

LIST OF PUBLICATIONS

This thesis is based on three publications and one manuscript. The individual papers are referred to by Roman numerals.

I. **Lambert H**, Hitziger N, Dellacasa I, Svensson M, Barragan A. Induction of dendritic cell migration upon *Toxoplasma gondii* infection potentiates parasite dissemination. Cell Microbiol. 2006 Oct;8(10):1611-23.

II. Persson CM*, **Lambert H***, Vutova PP, Dellacasa-Lindberg I, Nederby J, Yagita H, Ljunggren HG, Grandien A, Barragan A, Chambers BJ. Transmission of *Toxoplasma gondii* from infected dendritic cells to natural killer cells. Infect Immun. 2009 Mar;77(3):970-6.

* Contributed equally

Erratum. Infect Immun. 2009 Aug;77(8):3516

III. **Lambert H**, Vutova PP, Adams WC, Loré K, Barragan A. The *Toxoplasma gondii*-shuttling function of dendritic cells is linked to the parasite genotype. Infect Immun. 2009 Apr;77(4):1679-88.

IV. **Lambert H**, Dellacasa-Lindberg I, Barragan A. Impact of Infected Leukocytes on *Toxoplasma* Dissemination. Manuscript.

RELATED PUBLICATION

Persson EK, Agnarson AM, **Lambert H**, Hitziger N, Yagita H, Chambers BJ, Barragan A, Grandien A. Death receptor ligation or exposure to perforin trigger rapid egress of the intracellular parasite *Toxoplasma gondii*. J Immunol. 2007 Dec;179(12):8357-65.

CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
B6	B57BL/6 mice
BBB	Blood brain barrier
BLI	Bioluminescence imaging
CD	Cluster of differentiation
CLR	C-type lectin receptor
CpG	Cytosine and guanine separated by a phosphate
CTLA-4	Cytotoxic T lymphocyte antigen 4
CTL	Cytotoxic T lymphocyte
CTX	Cholera toxin
DAP12	DNAX-activating protein of 12 kDa
DC	Dendritic cell
DC-SIGN	DC specific intercellular adhesion molecule-grabbing non-integrin
ECM	Extracellular matrix
FADD	Fas-associated death domain
Fas	Apoptosis stimulating fragment
FasL	Apoptosis stimulating fragment ligand
FSC	Forward Scatter
GFP	Green fluorescence protein
GM-CSF	Granulocyte macrophages- colony stimulating factor
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
HEV	High endothelial venules
HFF	Human foreskin fibroblasts
HIV	Human immunodeficiency virus
IFN-γ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ip	Intraperitoneal
IRF	IFN regulatory factor
ITIM	Immunoreceptor tyrosine-based inhibitory motif
iv	Intravenous
JE	Japanese encephalitis
KIR	Killer cell Immunoglobulin-like Receptors
KLRB1	Killer cell lectin-like receptor B1
KLRG1	Killer cell lectin-like receptor G1
LAIR-1	Leukocyte-associated Ig-like receptor-1
LDM	Long distance migration
LCF	Leishmania chemotactic factor
Mac	Macrophage
MHC	Major histocompatibility complex

MLN	Mesenteric lymph node
Mono	Monocyte
NCRs	Natural cytotoxicity receptors
NF-κB	Nuclear factor of kappa B
NK cell	Natural killer cell
NKp30	NK-cell protein 30
NLR	Nucleotide binding oligomerization domain (NOD)-like receptors
PMN	Polymorphonuclear neutrophil granulocytes
PRR	Pathogen recognizing receptors
PTX	Pertussis toxin
PV	Parasitophorous vacuole
RFP	Red fluorescence protein
RLR	Retinoid acid-inducible gene I (RIG)-like receptor
ROI	Reactive oxygen intermediates
ROP	Rhoptries
SSC	Side Scatter
STAT	Signal transducer and activator of transcription
TBE	Tick-borne encephalitis
Th1	T helper 1
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
WNV	West Nile virus
wt	Wild type

1 INTRODUCTION

The definition of a parasite is an organism that benefits on the host in an unequal manner. A parasite could be a virus, a bacterium, a plant or an uninvited guest. In contrast to many pathogenic bacteria, parasites often establish chronic infections which we carry with us throughout large parts of our lives. Parasites often rely on complex life cycles which may involve several intermediate hosts serving. During six ears of research I have come to realize that parasites are extraordinary creations of evolution. Especially intriguing is their unique ability to manipulate host behavior and turn host conduct to their own advantage. This thesis is mainly focused on the human and veterinary parasite *Toxoplasma gondii*, but other pathogens will also be discussed

1.1 *TOXOPLASMA GONDII*

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects humans and animals worldwide. In addition to a sexual cycle which is limited to members of the *Felidae* family (Cats), the parasite propagates by the use of an asexual cycle in mammals and birds. Transmission to humans occurs through ingestion of undercooked meet from infected animals or by accidental intake of foodstuff contaminated by cat feces. Infection seldom results in acute disease, but latent infection is common and up to one third of the human population is estimated to be infected by this parasite [1]. Research on *Toxoplasma* is spurred for essentially three reasons. First, *Toxoplasma* can cause life-threatening disease, e.g. encephalitis, retinitis, myocarditis and pneumonia [2]. Second, *Toxoplasma* is used as a model-system of apicomplexan parasites which include important disease causing pathogens such as *Plasmodium* (the causative agent of malaria), *Eimeria* and *Cryptosporidium* [3]. Finally, *Toxoplasma* is an important veterinary pathogen with high estimated costs owing to disease, abortion or vaccination in animal farming [4].

1.1.1 Discovery and history of *Toxoplasma*

In 1908 Charles Nicolle and Louis Manceaux, doing Leishmania research at the Pasteur Institute in Tunis, described a blood-borne unicellular parasite from a small rodent called *Ctenodactylus gundi* [5]. In parallel, Alfonso Splendor identified the same protozoan in rabbits in Brazil [6]. Charles Nicolle and Louis Manceaux later named the parasite based on its morphology (toxo = bow, plasma = life) and the animal (gundi ~ gondii) where it was first discovered [7].

In 1937, Albers Sabin and Peter Olitsky described the obligate intracellular nature of the parasite, established the first *in vitro* cultivation system, and suggested transmission by means of ingesting *Toxoplasma*-contaminated tissue [8]. But it was not until 1939, when Abner Wolf and colleagues reported the first case of toxoplasmosis in a three-day old infant, that *Toxoplasma* was recognized as a human pathogen and cause of congenital disease [9]. Two years later Albert Sabin described acquired toxoplasmosis in a 6-y-old old boy with the initials RH [10]. The boy was admitted to hospital due to headache after been hit by a baseball bat. He later

developed lymphadenopathy, enlarged spleen and neurological signs and died on the 30th day of illness. *Toxoplasma* was isolated from mice inoculated with homogenate from the boys' brain and spinal cord. The isolate were given the designation RH in memory of the boy. By 1947, reports on tissue cysts found in autopsy slides indicated prevalent chronic infection among humans [11], but it was not until 1948 when Albert Sabin and Harry Feldman developed the Dye-test that the prevalence of chronically infected individuals could be estimated [12]. It had been known since 1942 that Sulfonamides were effective against *Toxoplasma* [13], but until the Sabin and Feldman dye-test, the only way to diagnose had been by clinical signs or biopsy. The new methods of diagnosing *Toxoplasma* made further clinical investigations possible and the pathology during toxoplasmosis was subsequently recognised and reviewed [14, 15]. The addition of new treatments [16, 17] and new tools for diagnostics [18, 19] during the 1950s and 1960s, were important medical advances that indicated a bright future for *Toxoplasma* related medicine. Further insight into the great prevalence of this parasite and the discovery of the *Toxoplasma* life cycle during the 1970s should have depicted *Toxoplasma* as a relatively harmless micro-organism, or model pathogen, during this time. However, the rise of the HIV epidemic during the 1980s, and the increasing number of reports of pathology as a consequence of chronic toxoplasmosis during the 1990s and beginning of this millennium, has successively swayed both researches and clinicians to re-evaluate the clinical significant and economic impact of *Toxoplasma*.

1.1.2 Life cycle and parasite stages

Before 1970, the tachyzoite and the bradyzoite were the only stages of *Toxoplasma* known to man. These two stages constitute the asexual life cycle of the parasite present in all intermediate warm-blooded hosts including land and water living mammals, birds, and humans [20]. In the middle of the 1960s, William McPhee Hutchison started to investigate *Toxoplasma* transmission by cat feces. His investigations lay the way for the discovery of the oocyst by several independent groups at the end of the 1960s and the subsequent elucidation of the sexual life cycle of the parasite [20]. A summary of the *Toxoplasma* life cycle is illustrated in figure 1.

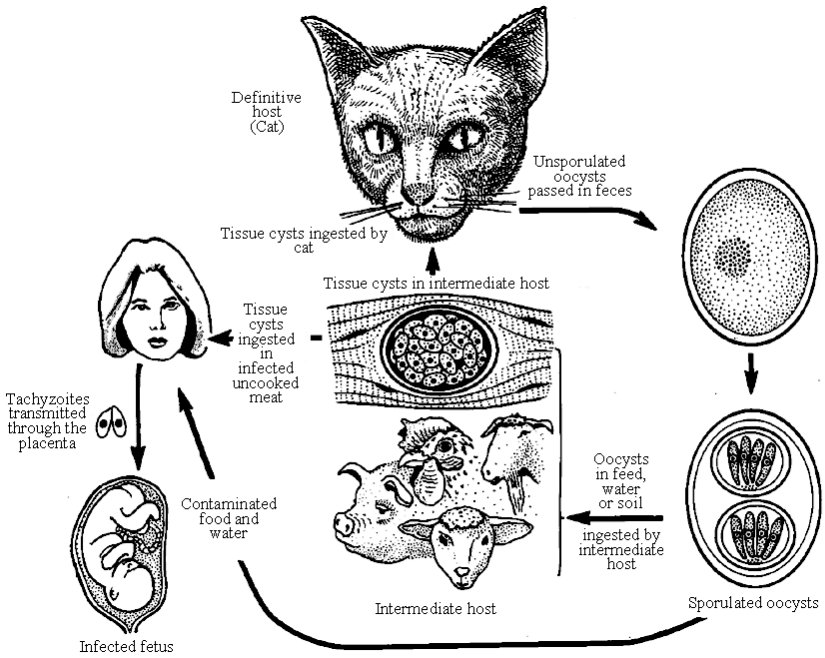

Fig. 1. Life cycle of Toxoplasma.
Adapted from [21]

The asexual cycle

The asexual life cycle of *Toxoplasma* starts by the ingestion of meat containing tissue cysts by a mammal, a bird or any warm-blooded animal. The cyst wall is subsequently degraded by the gastric juices, and bradyzoites are released in the intestine [22, 23]. Bradyzoites then convert into tachyzoites in the small intestine lamina propria and spread to the circulation within 15 h. Tachyzoites disseminate to peripheral organs and muscles, and reconvert back to bradyzoites approximately 5 days after ingestion [22, 24]. The formed tissue cysts may lay dormant for years until the host is eaten by a predator and a new cycle starts.

The tachyzoite was the first *Toxoplasma* form to be isolated and described [7, 25]. Although tachyzoites may infect cats orally, transmission to humans via this stage essentially requires direct blood or tissue transfer from a recently infected individual to a previously unexposed recipient. This is the form which disseminates during acute infection or reactivation of chronic disease and which cause clinical symptoms [24]. It is obligate intracellular and rapidly (Gr. tachy = rapid or accelerated, zoite = life) replicates by a specialized process called endodyogeny [26]. Replication eventually results in egress of fresh tachyzoites and infection of neighbouring cells. As the infection progresses the immune response builds up, stress is applied to the disseminating tachyzoites and conversion into the bradyzoite stage occurs [27].

Although *Toxoplasma* tissue cysts had already been observed in 1928 [28], it was not until 1960 that the bradyzoite stage was properly characterized [23]. This is the chronic stage of the parasite. It forms tissue cysts preferentially in the muscles and brain, and may persist for decades in the host [24]. Bradyzoites are quite resistant to chemical stress but freezing, e.g. of meat containing tissue cysts, eliminates its oral infectivity [23, 24]. The bradyzoite replicates slowly (Gr. brady = slow), exhibits low immunogenicity, and partly protects the host from parasite-induced cell rupture and immunopathology [27, 29, 30].

The sexual cycle

The sexual cycle of *Toxoplasma* begins when a domestic cat or any other member of the *Felidae* family ingests any of the infectious stages (tachyzoites, bradyzoites, and sporozoites) of *Toxoplasma*, e.g. after feeding on infected prey. The parasite then infects the epithelial cells of the ileum and initiates asexual development in a series of distinct morphological schizont stages (designated stage A to E) that exhibit particular division characteristics. Gamete formation is likely initiated by merozoites released from stage D schizonts approximately 2 days post infection of the cat. The female macrogamete contains numerous organelles while the male microgamont harbors up to 21 microgametes. The male microgametes possess a top end perforatorium organelle and flagella, which they use to swim, penetrate and fertilize mature female macrogametes to form zygotes. Several layers of cyst wall are then formed around the parasite, infected epithelial cells rupture and oocysts are released into the intestinal lumen. Oocysts are excreted following defecation and sporulation subsequently takes place in nature. Within 1 to 5 days post excretion sporulated oocysts, containing 2 sporocysts harboring 4 sporozoites each, will be ready to start a new cycle [21].

The oocyst is the infectious stage following sexual recombination of the parasites [22, 31]. This stage is very resistant to all sorts of disinfectants [31], extremely infective and more pathogenic in mice compared to bradyzoites [24]. Oocysts may persist for years in the soil [32] as well as in water [33], and perhaps also inside other free living microorganisms [34]. Therefore, oocysts are probably wide spread in nature where domestic and wild cats roam [32].

1.1.3 Taxonomy and related organisms

Toxoplasma gondii is so far the only known species of the *Toxoplasma* genus. *Toxoplasma* is a unicellular eukaryotic organism of the phylum Apicomplexa. This phylum harbours several other unicellular spore-forming pathogens of human and veterinary importance, e.g. *Plasmodium* spp., *Eimeria* spp. and *Cryptosporidium* spp. Common to the apicomplexan parasites is an apical complex of secretary organelles important for host cell invasion. In addition, many parasites in this phylum, including *Toxoplasma*, harbour an interesting organelle called the apicoplast. The primary functions of this organelle is uncertain, but regular protein traffic across the apicoplast membranes and various metabolic possesses specific to this organelle make it relevant for drug target research [35]. In contrast to several of the other members of the apicomplexan family, different stages of *Toxoplasma* can be cultured, propagated and quantified *in vitro*. In addition, manipulation of the *Toxoplasma* genome is well

established as well as the mouse model for studying host/parasite interactions during infection. Because of these advantages, *Toxoplasma* has emerged as a mode pathogen for this group of parasites [3]. The apicomplexan phylum is further subdivided into classes, e.g. *Toxoplasma*, *Cryptosporodium* and *Eimeria* cluster in the Coccidian group. This group of parasites shares an obligate intracellular nature and infects their host through the gastrointestinal tract. Although coccidian parasites may infect a wide range of animals, including humans, birds, and livestock, they are usually rather species-specific. *Toxoplasma*, with its vast range of potential intermediate hosts and very high prevalence across species, is an exception and stands out in this group as the most promiscuous free-loader [36]. A summary of the scientific classification of some important apicomplexan parasites are depicted in figure 2.

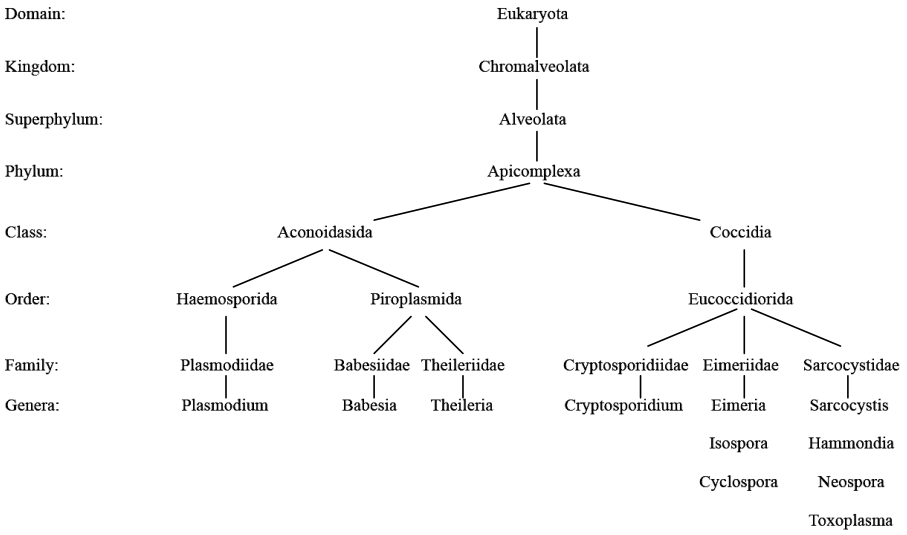

Fig. 2. Scientific classification of a selection of Apicomplexan parasites (Adapted from 'The Taxonomy & Systema Naturae' database).

1.1.4 Population structure and genotype differences

Three clonal lineages

Within the *Toxoplasma* species there are discrete strains of parasites abundantly distributed in nature. Although recent findings indicate that *Toxoplasma* is more genetically diverse than previously realized [37], the majority of strains isolated in Europe, North America and possibly Africa can be classified into one of just three distinct genotypes, entitled type I, II and III [38-40]. Genetically, these strains differ by less than 1 % and exhibit very little within-type diversity [41, 42]. In addition, the polymorphism within these strains is limited to only two allelic classes so that one allele is nearly always shared between two genotypes [41]. Therefore, these three genotypes are likely the result from a recombination event between an "Adam" and an

"Eve" strain in relatively recent evolutionary history [41, 43]. The most successful progeny of this recombination has subsequently evolved to dominate over all other strains in many parts of the world. These events illustrate several important aspects of *Toxoplasma* population biology. First, the sexual cycle of *Toxoplasma* is limited to members of the Felidae family and the infectious stages of the parasite are haploid. Therefore, recombination requires a simultaneous infection of a cat with two distinct *Toxoplasma* lines, a presumably rare event in the wild [41-44]. Second, the strong dominance of the three archetypical strains demonstrates the independence and importance of the asexual cycle for *Toxoplasma* propagation [43]. Finally, natural and experimental sexual recombination of distinct parasite lines is clearly a powerful force driving natural selection and evolution of traits beneficial to parasite propagation and survival [41, 45, 46]. Thus, the success of type I, II and III is likely a result of acquired biological advantages over other recombinants and ancient genotypes.

Virulence

While type I, II and III strongly dominate the *Toxoplasma* population structure in large parts of the world, type II strains are clearly most commonly isolated from patients [38, 47-50]. Therefore, there must be crucial differences between the genotypes important for parasite propagation and survival. For instance, virulence in the mouse model is strictly associated with the parasite genotype. Infection by type I strains are fatal (LD_{50} = 1) whereas infections with type II ($LD_{50} \sim 10^4$) or type III ($LD_{50} \sim 10^6$) strains generally result in controlled infections that persist for life [39]. A major contributor to *Toxoplasma* pathogenicity in mice is the ability to reach high parasite tissue burdens during infection. While avirulent strains may attain this effect following a high inoculation dose, infection with type I strains readily leads to immense parasite loads with massive overproduction of T helper cell type I (Th1) cytokines, ultimately resulting in lethal tissue pathology [51-54]. This difference between avirulent and virulent strains is likely a combination of variations in growth rate, invasion rate, immune stimulation and predisposition for bradyzoite conversion [55]. Virulence of *Toxoplasma* is mainly controlled by quantitative trait loci on chromosome VIIa. Genetic mapping and subsequent cloning of this region has revealed a highly polymorphic rhoptrie-derived serine-threonine kinase (ROP18) secreted into the host cell upon parasite invasion [46]. In parallel, a similar approach identified another functionally related rhoptrie kinase (ROP16) as a key molecule for parasite virulence [45]. The kinase activity of these enzymes is highly linked to pathogenicity and cross transfection with ROP18 into normally avirulent strains increases growth rate and enhance mortality in mice by 4-5 logs [45, 46]. In addition, virulent strains exhibit superior motility which likely facilitates crossing of immunological barriers, a trait significantly less prominent in type II and type III strains [46, 56]. This phenotype probably contributes to the propagation of the parasite and may be especially important for placental transversal as maternal cells do not normally traffic to the fetus [57, 58]. This raises the question of virulence in humans. A few studies have identified a dominance of type I strains in individuals suffering from ocular [59, 60], and congenital toxoplasmosis [48, 50]. However, thus far there is little evidence for a greater virulence of type I parasites in humans [43, 61].

Il-12 response

Furthermore, the immune response to *Toxoplasma* is dependent on IL-12 [62, 63] and cells infected with type II strains produce superior amounts of this cytokine compared to type I or III [64-66]. This difference may in part be due to manipulation of different signaling pathways [66, 67] but mainly depend on a type II-specific rhoptrie kinase (ROP16) that inhibits STAT 3 signaling [65]. However, most of the cells producing IL-12 during toxoplasmosis are not infected, and differences in IL-12 production are substantially lower if a mix of infected and uninfected cells is assessed [68]. Therefore, the significance of differential IL-12 production by infected cells is not clear, but may reflect an effort by the intracellular parasite to control the local environment.

Transmission

Finally, propagation ability may influence the prevalence of certain strains. Following the genetic cross giving rise to the three clonal lineages a concurrent acquisition of direct oral infectivity appeared which likely promoted extensive transmission benefits. It is not yet clear if this ability prevails within a certain genotype but type II strains are likely candidates [42]. In addition, although data indicate that both virulent and avirulent strains disseminate rapidly and widely after infection [52, 55, 69], distinct dissemination strategies exist between genotypes (Paper III and IV) [56, 57]. This issue is further discussed elsewhere in this thesis.

In summary, despite the limited genetic diversity between the three archetypical genotypes, each type harbors distinct characteristics decisive for their interaction with the host, and for their propagation. Virulence, immune response and ability to breach biological barriers differ between these strains [55]. Thus, a combination of traits likely explains the high predominance of type II strains in humans.

Atypical strains

In contrast to the parasite population in Europe, North America and Africa, *Toxoplasma* in Latin America seems to harbor a completely different structure. Notably, most of the population data obtained from this continent is based on veterinary studies performed in Brazil [70, 71], and patient isolates are limited [72]. However, the three archetypical genotypes do not appear to dominate this continent, and type II strains are less common [44, 73]. Instead, most isolated strains are distinct and the diversity is vast, indicating that recombination events and/or oocysts transmission are more common [37, 44, 73]. This suggests that Latin American and Eurasian *Toxoplasma* populations may have evolved in parallel until recently when increased trade and shipping initiated mixing of the clonal lineages and "recombinant" or "atypical" strains. Notably, toxoplasmosis seems to be a relatively extensive clinical problem on the South American continent [59, 74-76]. This raises the question of the pathogenesis of non clonal lineages versus type I, II and III. Current available studies on this issue are limited and additional investigations are needed. However, recombination may lead to acquisition of pathogenic mechanisms in new strains, and expansion of such strains in the population could lead to emergent disease [77]. In addition, the growing understanding of *Toxoplasma* diversity and the increasing

number of identified atypical strains imply that a new nomenclature for the *Toxoplasma* population structure may be needed.

1.1.5 Pathogenesis and clinical aspects

Pathogenesis in general may be viewed as a lack of adaptation or an unfortunate consequence of accidental mix of two co-existing organisms. Naturally, all life forms have developed defense mechanism that will protect them in case of attack. However, parasites relying on a particular host for their survival and transmission are compelled to exhibit immune modulator mechanisms that consequently may lead to pathology. *Toxoplasma* is a master of infection, chronically infecting humans and animals worldwide, with estimates of over 50 % infected humans in several countries. For instance, seroprevalence in Southern Europe is over 50 % while sequentially decreasing to around 10 % in Northern Europe (Norway and Sweden) [78, 79]. In the US between 10 to 25 % are seropositive [80, 81], and in Brazil the figures range from 50 to 80 % [82]. In addition, reports from various countries in Asia and Africa present seroprevalence figures between 25 and 50 % [83]. Considering the immense distribution of this parasite, pathology is very rare [1]. Yet, *Toxoplasma* may cause sever disease leading to mental retardation, blindness and death [83].

Risk factors

There are several risk factors for infection with *Toxoplasma*. Eating undercooked meat, especially pork and lamb, seem to be the most common infection route, indicating that most people get infected by tissue cysts [84]. Eating unwashed raw vegetables, poor kitchen hygiene, cleaning the cat litter box, and soil contact are other risk factors [1, 84]. Notably, waterborne outbreaks of *Toxoplasma* infections have been reported on several occasions and accidental intake of oocysts-contaminated drinking water may therefore be an important risk factor where access to clean water is limited [32].

Symptoms

Lymphadenopathy was early recognized as the most frequent symptom of toxoplasmosis [85, 86]. Other symptoms in immune competent individuals include fever, weakness and myalgia. Infiltration of lungs, heart and eyes may further lead to pneumonia, myocarditis and retinitis respectively [2]. In addition, primary infection during pregnancy may transmit to the foetus leading to abortion, permanent neurological damage, hydrocephalus, and/or chorioretinitis with subsequent visual impairment and blindness. The child further faces the risk of recurrent chorioretinitis later in life [83]. Immune compromised individuals are at risk of developing serious illness either by direct primary infection or reactivation of chronic disease. Symptoms include apathy, dementia, motor seizures, ataxia, hemiparesis and coma, ultimately leading to death if not treated [87].

Diagnostics, pathology and treatment

Serology forms the base of *Toxoplasma* diagnostics even if PCR is the preferred method to use for suspected *in utero* infections. Although low levels of *Toxoplasma*-specific IgM antibodies may persist for several years after a primary infection, repeated

measurements monitoring the development of *Toxoplasma*-specific IgG antibodies is a reliable method to conclude the nature of the infection [88, 89]. Once diagnosed, there is generally no need for treatment unless the patient is pregnant, suffers from disseminated infection, ocular toxoplasmosis or immune suppression [83, 90]. The most common treatment includes a combination therapy with pyrimethamine and sulphonamides, with the addition of steroids to patients suffering from ocular toxoplasmosis. Pregnant women should be treated with spiramycin within three weeks of infection and congenitally infected fetuses should be given pyrimethamine and sulphonamides until 12 months of age. Thus far, there are no reports of drug resistance [83, 90]. These drugs all target the tachyzoite stage and are essential to counteract acute pathology following infection or reactivation. Most of the pathology during toxoplasmosis is caused by direct tissue damage by rapidly replicating tachyzoite and inflammation following activation of the immune system [51-54]. In addition, breaching biological barriers such as the blood brain barrier (BBB), blood retina barrier and the placenta, are key aspects of *Toxoplasma* pathogenesis because these events are associated with the most severe pathology [57]. However, once latency has been established in these organs there are no means to eradicate the parasite. The biological benefit of accessing and disrupting vital host organs include impaired functions eventually converting the host into an easier prey. The increasing knowledge of parasite-induced behavior changes indicates that access to the CNS may be an important part of the *Toxoplasma* life cycle [91]. While pathology following acute infection leads to rapid deprivation of the host, behavior manipulation during chronic infection endures a much longer time-frame. Thus, while short-term pathology is attributed to the tachyzoite stage, long-term pathology may be actively induced by bradyzoites to increase the chance of transmission. Behavior changes and pathology associated with *Toxoplasma* infection in humans include schizophrenia, epilepsy, intellectual disability and anxiety [92]. In rodents, examples of behavior changes are unresponsiveness to danger signals and acquisition of predation-encouraging manners [93, 94]. *Toxoplasma* drug research is currently focused on finding a suitable drug to target tissue cysts. The low metabolism of bradyzoites and their location in the CNS are complicating factors in this struggle. However, atovaquone and azithromycin are promising drugs currently under investigation [83, 95, 96]. A cyst-eliminating treatment would be a tremendous clinical advance in the struggle against toxoplasmosis.

Vaccine

Another important clinical aspect is the development of a vaccine against *Toxoplasma*. There are basically three different approaches to this issue. First, as ingestion of undercooked meat, i.e. tissue cysts, are the main source of human infection vaccination of livestock would be a strategy to limit spread to humans. A veterinary vaccine, Toxovax®, is currently available. However, this vaccine is primarily aimed at minimizing the cost of animal fall off by preventing abortion in pregnant ewes [97, 98]. Its protection lasts approximately 18 months and it is not functional to prevent transmission to humans [2, 98]. Second, an effective way of drastically decrease *Toxoplasma* transmission ability would be to develop a vaccine for domestic cats. Such an approach is possible and has shown potential [99, 100], but has not been pursued presumably due to costly production and maintenance of the vaccine. A third type of

vaccine would be designed for use in humans. Currently, no standardized human vaccine exists for any protozoal disease and the commercial interest for a *Toxoplasma* vaccine is probably mediocre at best. In addition, an attenuated vaccine would be risky considering potential effects on the CNS and immunological vaccine-strategies are complicated [98]. Thus, it is not likely that a human *Toxoplasma* vaccine will be developed in the near future.

Screening program

While some nations (e.g. France) routinely screen for *Toxoplasma* in pregnant women, the vast majority of European countries do not recommend screening [101]. The question of whether Sweden should have a screening program for *Toxoplasma* is complicated and exceeds the scope of this thesis. However, considering cost-benefit return, effectiveness of treatment and incidence are important if such a program is to be launched. This requires updated, extensive and stringently performed clinical studies on prevalence and incidence of *Toxoplasma* infection in Sweden, something which is currently lacking.

1.1.6 Immune responses to *Toxoplasma*

The study of the immune response to *Toxoplasma* (reviewed in [102]) began in 1948 with Sabin and Feldman discovering complement-fixing antibodies reacting against extracellular parasites [12]. At the end of the 1960s, IFNγ was highlighted as an important cytokine in the resistance to *Toxoplasma* [103, 104] and ten years later cell mediated immunity to the parasite was described [105]. The development of new efficient immunosuppressive drugs for the treatment of cancer during the 1970s, and the mounting number of HIV infected patients during the 1980s, resulted in rising numbers of immunodeficient individuals exhibiting clinical toxoplasmosis [106, 107]. In 1983 Nathan *et al*. showed that T cell-mediated IFN-γ-dependent activation of infected macrophages lead to elimination of intracellular tachyzoites [108]. The numerous clinical observations in T cell-suppressed AIDS patients subsequently lead to the development of experimental animal models to study toxoplasmosis in immunocompromised individuals. As a result, the essential role of T cells in the control of, and resistance to, *Toxoplasma* was revealed using a mouse model [109]. Concurrently Suzuki *et al*. showed the essential role of IFN-γ during infection by antibody-mediated depletion of this cytokine [110]. Following the discovery of IL-12 at the end of the 1980s [111, 112], this cytokine has emerged as the critical signaling molecule for the activation of the immune response to *Toxoplasma* infection.

Pathogen recognition

The co-ordination of an effective immune response requires the recognition of pathogens. Macrophages and DCs are sentinels of the immune system with a vital role in pathogen recognition. During *Toxoplasma* invasion, the parasite breaches the intestinal epithelium and burrows down to the lamina propria where it encounters these cells [113]. Conserved *Toxoplasma* antigens are recognized by TLR11 [114], TLR2 [115, 116] and TLR4 [116]. These receptors signal through the adaptor protein MyD88 which is essential for resistance to *Toxoplasma* [115]. However, there are strain-

differences in MyD88-dependence [66] and alternative signaling pathways exist [66, 115, 117]. A *Toxoplasma* secreted cyclophilin may activate immune cells, such as DCs, via an MyD88-independent pathway signaling trough the CCR5 receptor [118, 119]. An essential effect of *Toxoplasma* recognition is increased production of IL-12 [120], preferentially by DCs [121]. While interaction with alternative signaling pathways and different cell types contributes to the overall IL-12 production during *Toxoplasma* infection, MyD88 is truly critical for this cytokine response [115, 120]. Clearly, TLR11 is important for MyD88-mediated IL-12 responses in mice, but TLR11$^{-/-}$ still exhibit low levels of IL-12 and survive acute infection [114], indicating redundant cytokine production possibly via CCR5 [117]. By contrast, TLR2$^{-/-}$ display normal IL-12 levels [115], but succumb to high infection doses by normally avirulent parasites [122]. Similarly, TLR4 is not involved in IL-12 production [115], but may be important as a co-activator of cytokine signaling [116]. In addition, TLR4 and TLR9 are believed to function as adjuvant receptors during *Toxoplasma* ileitis in mice [113, 120, 123, 124].

Cytokines

Similar to IL-12, IFN-γ is crucial for resistance to *Toxoplasma* [125]. Although NK cells have been shown to contribute to the early production of IFN-γ during *Toxoplasma* infection, the main source of this cytokine is believed to be CD4^{+} and CD8^{+} T cells [62, 126, 127]. The production of IFN-γ by these cells is dependent on IL-12 produced by innate immune cells activated by the parasite [62]. While macrophages [128] and neutrophils [129, 130] are important cytokine producers, DCs are essential for the production of IL-12 [121, 131]. In addition to IL-12, TNF produced by myeloid cells stimulate supplementary IFN-γ production by lymphocytes [132-134] and work as a co-factor in the activation of microbicidal mechanisms in macrophages [135-137]. Similar functions have been attributed to IL-2 [138-140] which is also important in driving lymphocyte activation and proliferation [139, 141]. These cytokines are typically associated with a Th1 type of immune response. Therefore, interdependent cytokine signaling, between adaptive and innate immune cells, is crucial for the development of an effective immune response to *Toxoplasma* infection.

Parasite clearance

To launch an effective immune response against *Toxoplasma*, restrict parasite growth and initiate microbicidal activity, infected cells require IFN-γ-dependent assistance. Macrophages, monocytes and neutrophils are likely the main leukocyte cell populations responsible for parasite clearance, but nonhemopoietic cells are also required [125]. IFN-γ signals mainly through STAT1 to activate several antimicrobial effector mechanisms [142]. For instance, iNOS activity is upregulated leading to increased production of microbicidal ROIs. However, in contrast to IFN-γ$^{-/-}$ or IL-12$^{-/-}$ mice, iNOS$^{-/-}$ mice survive the acute phase of infection and do not succumb until the chronic phase, indicating the presence of other functional IFN-γ dependent mechanisms [143]. Consequently, IFN-γ activates a family of 48-kDa GTP-binding proteins with specialized roles in microbial resistance [144]. Certain members of this p47 GTPase family exhibit specific anti-*Toxoplasma* activity through degradation of parasitophorous vacuole (PV) proteins and suppression of *Toxoplasma* growth [145, 146]. These mechanism are essential to parasite resistance and specific p47 GTPase knockout animals rapidly succumb during the acute phase of infection [145, 147].

Cellular immunity

Several leukocyte cell types are essential to *Toxoplasma* infection. DCs mediate critical IL-12 production and presentation of antigen [121, 148, 149], T cells producing IFN-γ provide long term protection [109, 125], and Gr-1^+ cells mediate early resistance to the parasite [150, 151]. However, several cell populations with important functions for *Toxoplasma* infection, including monocytes, neutrophils and pDCs, express Gr-1 [151]. During acute infection, CXCR2 is important for recruiting neutrophils to the inflammatory site. Although they survive infection, $CXCR2^{-/-}$ mice suffer from impaired infiltration, decreased levels of IFN-γ and increased cyst numbers in the brain [152]. In addition, neutrophils produce chemokines and cytokines that attract and activate DCs, and may play a role in instructing the T cell response by influencing DC activity [151]. While neutrophil infiltration from the blood begins within hours of infection, CCR2-mediated recruitment of Gr-1^+ inflammatory monocytes occurs from the blood and bone marrow within days [68, 152, 153]. $CCR2^{-/-}$ mice suffers from uncontrolled replication of parasites at the site of infection and extensive immunopathology, presumably caused by neutrophils [153-155], ultimately resulting in death [153, 156]. These studies show that essential regulatory and effector mechanisms executed by neutrophils and inflammatory monocytes are likely complimentary in function and time. Furthermore, CD8 and CD4 T cells mediate long term protection against reactivated disease [157]. IFN-γ produced by these cells are important to activate bystander cells to kill parasites [125, 158]. Therefore, immunocompetent individuals are likely ignorant to reactivation of *Toxoplasma*. By contrast, in patients suffering from AIDS or treatment with immune modulating drugs, where T cells are often suppressed, reactivation may lead to toxoplasmic encephalitis, resulting in uncontrolled replication of parasite in the brain and death if not acutely treated [107]. The role of DCs and NK cells during *Toxoplasma* infection is further described elsewhere in this thesis.

Humoral immunity

Antibodies mediate resistance to secondary *Toxoplasma* infection [159]. In addition, they assist the clearing of parasites during acute infection. IgM initially acts on egressing tachyzoites by blocking cell invasion, mediating agglutination and enabling complement activation [160]. Following class switch, IgG provides additional opsonisation, a possibility of ADCC, and blocks re-invasion [159]. Once immunity has been established, IgG protects the fetus during pregnancy [161]. Interestingly, the antibody response seems to play a role in the development of intra-cerebral cysts, indicating that antibodies may influence stage conversion [159]. In addition, B cell deficient mice succumb to infection due to uncontrolled tachyzoite-mediated tissue pathology in the brain despite normal levels of IFN-γ. Administration of *Toxoplasma*-specific antibodies to these mice prevents pathology [162]. These studies indicate that antibodies are the body's first line of defense against *Toxoplasma* and that effective humoral immune responses against extracellular parasites are crucial.

1.2 DENDRITIC CELLS

In 1867 the German medical student Paul Langerhans, at the Berlin Pathological Institute, investigated the innervations of the skin using a gold chloride staining technique. He observed non-pigmentary cells with a dendrite-like morphology which he assumed to be nerve cells and later came to bear his name [163, 164]. Research on this new cell type progressed slowly until 1961 when Michael Birbeck identified cytoplasmic Langerhans' cells-specific organelles which he termed Birbeck granules [165]. Although the exact function of the Birbeck granules remained elusive [166], Langerhans cells (LCs) were subsequently identified in the lymph and lymph nodes [167], and their ability to present antigen [168] and activate T cells [169] were acknowledged. However, it was not until 1973, when Ralph Steinman and Zanvil Cohn characterized a small population of "large stellate" cells in the peripheral lymphoid organs of mice and coined the term "dendritic cell" (DC), that modern DC research was initiated [170]. In the beginning of the 1980s the haematopoietic origin of DCs and LCs started to emerge [171-173].

1.2.1 Dendritic cell development

DCs are a heterogeneous family of widely distributed haematopoietic APCs performing several important functions including bridging the innate and adaptive immune system during infection and maintaining tolerance to self [174]. While the majority of DCs subsets predominately arise from myeloid precursor cells, lymphoid progenitors have the potential of giving rise to some DC subsets [175, 176].

In vitro, DCs can be derived from bone marrow (BM) precursor cells by the addition of granulocyte-macrophage colony-stimulating factor (GM-CSF) to cell cultures [177, 178]. Similarly, human $CD34^+$ BM precursors differentiate into DCs in the presence of GM-CSF and TNF [179-182]. DCs can also be generated from human monocytes in the presence of GM-CSF and IL-4 and are then usually referred to as monocyte-derived DCs (MDDCs) [183-185]. These techniques have been revolutionary to the DC field, and presented new opportunities to study DC biology [186]. Thus, a lot of the current knowledge of DC function comes from studies with *in vitro* generated DCs. Different developmental pathways have also been identified *in vivo*. The myeloid origin of DCs has been shown *in vivo* by the restoration of DC populations in the spleen and thymus after transplantation of mouse BM common myeloid progenitors (CMPs) into irradiated recipients [187-189]. Interestingly, similar experiments using BM common lymphoid progenitors (CLPs) demonstrated the ability of these cells to differentiate into splenic and thymic DCs *in vivo* [187, 189]. Further studies identified the receptor kinase fms-like tyrosine kinase 3 (Flt3L) to be expressed by all BM DC precursors regardless of their lymphoid or myeloid lineage orientation [176, 190]. Therefore, although the majority of DCs *in vivo* appear to be of myeloid origin [175], functional differences among DC subsets are likely to reflect maturation status or compartment adjustment rather than ontogeny [191].

1.2.2 Dendritic cell subsets and distribution

The fact that myeloid and lymphoid DC precursors exhibit redundancy in the generation of functional DCs, suggests that other factors control the phenotype and development of DC subpopulations. The appearance of different DC subsets in nearly all tissues indicates that residence is important for the development and function of individual DC populations [192]. The DC population found under steady-state condition in mice and humans is traditionally split into conventional/resident and migratory DCs, and plasmacytoid DCs (pDCs) [193-195].

Cell precursors in the circulation continuously enter tissues and peripheral lymphoid organs to maintain DC turnover. In the mouse blood, two distinct DC precursors with the ability to differentiate into mature DCs have been described [196]. The $CD45RA^{neg}CD11c^{int}CD11b^{pos}$ population represents conventional DCs which mature under the influence of TNF, acquire the ability to stimulate T cells, and produce IL-12 in response to microbial stimuli. The $CD45RA^{pos}CD11c^{low}CD11b^{neg}$ population represents the pDCs which mature upon stimulation with e.g. viruses or CpG, and primarily produce large quantities of type I interferons [197-199]. In the human blood, similar DC populations have been described [200]. In addition, human and mouse monocyte subsets are important to maintain the DC pool [201]. Monocytes have been shown to differentiate after trans-endothelial migration into subendothelial matrix *in vitro*. Cells which remained in the tissue differentiated into macrophages while cells migrating back in an ablumenal-to-lumenal direction exhibited DC characteristics [202].

The epithelial tissues of the body are constantly surveyed by DCs operating as sentinels of the immune system [194]. Important entry points for pathogens are the skin, lungs and intestinal mucosa. Migratory DC subsets, expressing myeloid lineage markers such as CD11c and CD11b, predominate in these tissues. The epidermis is populated by LCs while the dermis contains mainly interstitial DCs [203-205]. In the intestine and lung, there are similar DC populations resident in the mucosal and submucosal tissue respectively [206, 207]. As a first line of defense, LCs and mucosal DCs are situated in the basolateral space of the mucosa. These cells perform immune surveillance of the luminal surface by extending processes between epithelial cells, sample the lumen for microbial components, and retract [206, 208]. In addition, the interstitial DCs are located under the basal membrane to survey this barrier for an eventual breach.

Secondary lymphoid tissues such as spleen and lymph nodes primarily harbor blood-derived resident cDCs that can be further divided into three main subpopulations depending on surface expression, function and localization [209]. First, the three main DC subsets are generally defined as $CD8^{pos}CD4^{neg}$, $CD8^{neg}CD4^{pos}$ or double negative (DN). The $CD8^{pos}$ DCs are also $CD205^{pos}$ while the $CD8^{neg}$ DCs are $CD11b^{pos}$ [210]. Second, $CD8^{pos}$ DCs are located in the T cell areas of the spleen and lymph nodes, while $CD4^{pos}$ and DN DCs reside in the marginal zones. Thirdly, $CD8^{pos}$ DCs may capture antigens from migrating DCs [211], cross-prime cytotoxic T cells [212], and induce a Th1-biased cytokine response in CD4 T cells [213]. By contrast, $CD4^{pos}$ DCs appear to be predominantly cytokine producers [214] and induce a Th2-biased response [213].

1.2.3 Recognition of pathogens and maturation

DCs express pathogen recognizing receptors (PRRs) that bind conserved pathogen associated molecular patterns (PAMPs) on microbial surfaces. Activation of DCs via these receptors initiates a chain of events ultimately leading to antigen presentation and T cell expansion. There are different families of PRRs. First, nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) are cytosolic receptors that bind molecules produced during the synthesis or degradation of peptidoglycan, an essential component of the bacterial cell-wall. NLRs detect intracellular bacteria such as *Shigella flexneri* and *Listeria monocytogenes* [215]. Second, C-type lectin receptors (CLRs) recognize sugar molecules on pathogens and self cells. This receptor family binds several viral pathogens including HIV, and is important for anti-fungal defense [216]. Thirdly, TLRs are an important group of PRRs that recognize a diverse range of different microbial molecules. For example, TLR4 and TLR5 recognize extracellular bacterial LPS and flagellin respectively. By contrast, TLR3, 7/8 and 9 are expressed intracellularly and detect nucleic acids from bacterial and viral pathogens [217]. DC subsets, express distinct patterns of TLRs and may therefore be suited to tackle different pathogens and dedicate immune responses accordingly [200]. Binding of these receptors initiates a signaling cascade, mediated by the adaptor protein MyD88, which ultimately leads to activation and translocation of important transcription factors, such as NF-κB and IRF [217, 218].

DC-interaction with microbial antigens initiates a series of events referred to as DC maturation (reviewed in [174]). Immature and mature DCs differ with respect to expression of cell surface markers, function and cytokine production. Essentially, immature DCs exhibit a high endocytic capacity but express low or intermediate levels of molecules involved in antigen presentation and lymphocyte priming. Upon maturation, the endocytic capacity is reduced and co-stimulatory molecules (e.g. CD80, CD86, CD40 and CD54), cytokine receptors (e.g. CD25) and MHC class I and II, are up-regulated. This maturation event further separates the two DC entities functionally. First, immature DCs which present self antigens on MHC molecules are important for the induction of peripheral tolerance under steady state. Maturation and up-regulation of certain co-stimulatory molecules enables activation of T cells and directed immunity [219, 220]. Second, the immune surveillance function of immature DCs is discontinued upon maturation and the cell instead transforms into a professional APC and initiator of the immune response [220]. Finally, since T cell activation requires relocation to secondary lymphoid organs, immature and mature DCs express different chemokine receptors and therefore partly exist in different compartments in the body.

1.2.4 Migration and antigen presentation

Immature DCs express chemokine receptors (e.g. CCR1, CCR2, CCR5 and CCR6) that respond to ligands (e.g. MIP-1α, MCP-1, MIP-1β, and MIP-3α) produced at the inflammatory site [95, 221]. Therefore, inflammatory DCs/monocytes, DC precursors in the blood, and resident DCs are recruited to this area during infection. Encounter with a pathogen induces down modulation of these chemokine receptors, DC maturation and the subsequent expression of CCR7 [174]. The ligands for CCR7 (CCL19 and CCL21) are expressed by stromal cells in secondary lymphoid organs and

this receptor therefore guides the activated DCs to the naïve T cells during the initial phase of the immune response [222]. DCs that reside in the periphery, sample antigens, and migrate to draining lymph nodes for presentation to T cells, are called migratory DCs. This group includes mainly Langerhans/mucosal and dermal/interstitial DCs. Upon microbial invasion or inflammation, additional DCs appear which are not present during steady-state. These cells are termed inflammatory DCs and derive primarily from monocytes. They too migrate, present antigen, express co-stimulatory molecules and produce cytokines. *In vitro* GM-CSF-generated DCs, which are frequently used in many studies, are believed to be similar to these cells [195]. The expression and regulation of chemokine receptors are essential to direct migrating and inflammatory DCs to lymphoid organs.

Immediately after antigen encounter macropinocytosis of DCs is increased. This increase is transient but facilitates enhanced antigen presentation [223]. As the DCs mature receptor-mediated endocytosis, phagocytosis and macropinocytosis is successively reduced with sustained high expression of MHC class I and II molecules [174]. The intracellular pathways for antigen processing and loading onto MHC molecules have been well characterized and thoroughly reviewed [224, 225]. In brief, endogenous antigens are degraded by the proteasome, transferred to the endoplasmic reticulum (ER) and loaded onto MHC class I molecules. Antigen loading provide stability and the complex is subsequently transported to the cell surface for presentation to CD8 T cells. Exogenous antigens are acquired through phagocytosis or macropinocytosis and confined to endocytic vesicles. Here, they are loaded onto MHC class II molecules, transferred to the cell surface, and presented to CD4 T cells. In addition, exogenous antigens may be presented on MHC class I molecules by a mechanism termed cross-presentation [226].

DCs are professional APCs with a superior ability to induce T cell proliferation compared to B cells and macrophages [227]. Following antigen encounter, DCs mature and migrate to the T cell areas of the draining lymph node. The DCs cluster with the T cells and the DC antigen-MHC complex connects with the T cell receptor. CD8 or CD4 molecules stabilize the interaction by binding MHC class I or II respectively. Co-stimulatory molecules on the DC, e.g. CD80/86 and CD40, interact with CD28/CTLA-4 and CD40L on the T cell. Such interactions condition the DC to produce cytokines which, in a paracrine fashion, stimulates the T cell. Collectively, these events provide the essential signals for T cell activation and proliferation [228]. The T cell response is further polarized by cytokines in the microenvironment produced by innate immune cells, such as DCs. These cytokines activate distinct transcription pathways in the T cells leading to directed immune responses. Consequently, IL-12 and IL-18 induce Th1 type immunity, characterized by a CD4 T helper cell-assisted CTL response and production of IL-2, IFN-γ and TNF, which are important to mount cellular immunity against intracellular microorganisms. In contrast, Th2 cells produce IL-4, IL-5, and IL-13, and are required for humoral immunity to control helminths and other extracellular pathogens. Moreover, TGF-β and IL-6 induce Th17 cells which produce IL-17 and play an important role in the clearance of extracellular bacteria and fungi [229]. In addition, TGF-β together with IL-2 induce IL-10 producing regulatory T cells important to maintain peripheral tolerance, prevent autoimmunity and limit immune-mediated tissue damage during infection [230].

Finally, DCs can affect NK cell-function by cytokine secretion or direct cell-cell contact. IL-12 and IL-18 promotes IFN-γ production by NK cells, and DCs may induce NK cell proliferation. NK cells may further mediate DC activation and maturation, or eliminate DCs as a consequence of NKp30 or TRAIL signaling [231].

1.2.5 Dendritic cells during *Toxoplasma* infection

During *Toxoplasma* infection, production of IL-12 is an essential part of the immunological cascade leading to the production of protective IFN-γ and elimination of virulent tachyzoites [62, 63, 117, 128]. Using a diphtheria toxin-based system that allowed ablation of DCs to be induced *in vivo* [232], Liu and colleagues showed that DCs constitute an essential source of IL-12 during *Toxoplasma* infection in mice. Deprivation of DCs lead to host mortality which could be reversed by the replenishment with wt DCs producing IL-12 [121]. Notably, adoptive transfer of MyD88- or IL-12p35 deficient DCs did not restore resistance to infection [121]. Production of IL-12 by DCs can occur via several mechanisms. Yarovinsky *et al*. showed that *Toxoplasma* activates IL-12 production from DCs via the secretion of a profiline-like molecule signaling through TLR11 and MyD88 [114, 120, 233, 234]. In addition, an MyD88-independent signaling pathway, involving a parasite derivative called Cyklofilin-18 (C-18) and the chemokine receptor CCR5, has been described [118, 119]. However, this pathway does not explain the MyD88-dependence of the IL-12 response observed by Liu and colleagues [121]. Also, $TLR11^{-/-}$ mice have low but detectable IL-12 levels, display increased number of brain cysts, but survive past the acute infection [114]. In contrast, $IL\text{-}12^{-/-}$ and $MyD88^{-/-}$ mice rapidly dies from infection [115]. Thus, a proper immune response to *Toxoplasma* requires DCs producing IL-12 via an MyD88 dependent pathway. This pathway exhibits receptor redundancy and is supported by alternative MyD88-independent pathways. Importantly, humans do not express a functional TLR11 and there is according to my knowledge no evidence for CCR5-mediated IL-12 production by human APCs [235]. Therefore, alternative pathways to IL-12 production, such as CD40 ligation or signaling via different TLRs, are likely to be important in clinical toxoplasmosis [117, 236].

The fact that *Toxoplasma*-infected severe combined immunodeficiency disease (SCID) animals clearly demonstrate increased survival compared to IFN-γ deficient mice, shows that the innate immune system is independently and potently activated by this parasite [128]. However, DC biology implicates sampling of exogenous antigens in peripheral tissue with subsequent maturation and migration to lymph nodes for priming of naïve T cells. Before the crowning of DCs as the supreme APCs, macrophages were believed to perform this function [237]. Interestingly, DCs appear to remain in an immature state upon infection with *Toxoplasma* [238], and the initial activation of CD8 T cells during toxoplasmosis may not require professional APCs as several infected cell types appear to present antigen on MHC class I [239]. Yet, DCs can stimulate class I- [148, 240], and class II-restricted [241] responses during *Toxoplasma* infection. Work by Reis e Sousa and colleagues have shown that when live tachyzoites or soluble tachyzoite antigen are injected into mice, $CD11c^{+}$ IL-12 producing cells rapidly redistribute to the T cell areas of the spleen [131]. Recently,

activated but uninfected DCs were shown to be the major APCs for priming CD8 T cells in secondary lymphoid organs, suggesting that cross-presentation by uninfected DCs plays a role in the activation of T cells during toxoplasmosis [148]. In addition, Pepper and colleagues demonstrated that pDCs express MHC class II during *Toxoplasma* infection and preferentially upregulate the co-stimulatory molecule CD83 compared to other DC subsets. Activated pDCs exhibit TLR11 dependent IL-12 production *in vitro*, induce proliferation of naïve T cells *ex vivo* and presented antigens *in vivo* [149]. These results indicate that while infected DCs may be rendered immature during *Toxoplasma* infection, bystander DC subsets can alert the adaptive immune system and activate T cells.

Although DCs appear to be preferentially parasitized early during *Toxoplasma* infection, their precise role may differ as a consequence of infection with different parasite strains or as a result of differential host susceptibility [240, 242, 243]. Recently, we and others have generated data suggesting that DCs function as transporters of tachyzoites early during infection (Paper I and III) [242, 243]. These findings are discussed further below.

1.3 NATURAL KILLER (NK) CELLS

The NK cell was identified in 1975 by Swedish and American groups working in parallel [244-247]. The name derives from the ability of this cell type to kill certain tumour cells without prior target exposure [244]. At present, NK cells are recognized for multiple functions including tumour suppression, anti-viral defence and immune cell regulation [248]. NK cells represent an innate lymphocyte population that confines its function to a limited range of germ-line encoded receptors [249]. Apart from the functional characterization of this cell type, a phenotypic classification has been developed. According to this classification, NK cells in humans and mice are defined as $CD3^-$ $CD56^+$ and $CD3^-$ $NK1.1^+$, respectively [250]. However, an alternative definition based on NKp46 has been suggested [251].

1.3.1 NK cell distribution and trafficking

NK cell development is initiated and takes place primarily in the bone marrow, but differentiation may also occur in the spleen, lymph nodes, liver and thymus. Development is dependent on several factors but IL-15 plays a determinant role [252]. NK cells distribute to distinct compartments and are preferentially found inside vessels or sinuses of lymphoid and non-lymphoid organs. NK cells constitute between 2 to 10 % of the lymphocyte population in the mouse spleen, blood, liver and lung, while mouse lymph nodes and thymus are sparsely populated [253]. However, during inflammation the number of NK cells in the lymph nodes increase dramatically [254]. Human NK cells distribute similarly in non-lymphoid organs, but the predominance of NK cells in human lymph nodes and blood appears to be higher compared to the mouse [253, 255]. In addition, NK cells may be found in the skin [256] and massive numbers infiltrate the uterus during pregnancy [257].

NK cell trafficking starts by the recruitment of NK cells from the bone marrow into the blood. This recruitment is dependent on sphingosine 1-phosphate receptor 5 ($S1P_5$) [258] which responds to high sphingosine 1-phosphate concentrations in extracellular fluids like the blood and lymph [259]. Once in the circulation, NK cells express CD62L which, by binding to glycosylated L-selectins, mediate extravasation through HEVs [260]. In addition, NK cells may exit via the marginal sinuses to enter the red pulp of the spleen and subsequently redistribute to non-lymphoid organs [253].

During inflammatory conditions, NK cells respond predominantly to chemokines binding to CCR2, CCR5, CXCR3 or CX_3CR1. Chemokine signaling is not dependent on tissue, but instead specific to the inflammatory condition, i.e. different pathogens stimulate NK cell recruitment via specific receptors [253]. Upon activation by chemokines, NK cells adhere to the vascular endothelium, mainly via leukocyte function-associated antigen-1 (LFA-1), and extravasate into the inflamed tissue [261].

1.3.2 Target cell recognition and cytotoxicity

To escape immunity and increase survival, microbes infect cells and modify their appearance to their own benefit. Similarly, tumour cells accumulate mutations improving their ability to withstand immune attack [262]. Since $CD8^+$ T cells are activated to kill cells presenting foreign or altered peptides on MHC class I molecules, intracellular microbes and tumour cells may down modulate these molecules to avoid recognition. The "missing-self" hypothesis states that NK cells identify target cells based on the absence of self MHC class I molecules [263]. However, while NK cells may kill in the absence of MHC, a correctly assembled peptide expressed on a self MHC class I molecule will send an inhibitory signal to the NK cell. This inhibition is potent and derives from high affinity interactions which normally overrides potentially activating signals from stress induced, low affinity ligands on the target cell. Interestingly, certain intracellular pathogens induce the expression of MHC class I-like molecules on the cell surface, supposedly to avoid elimination by NK cells [264]. However, vigorous target cell activation leading to extensive signaling via activating receptors may result in NK cell activation despite sustained MHC class I expression, so-called "induced-self" activation. Thus, NK cells distinguish self/healthy from non-self/sick by a balance between activating and inhibitory receptors [265]. NK cell receptors are divided into either the immunoglobulin superfamily or the C-type lectin superfamily. Both these families contain inhibitory as well as activating receptors (reviewed in [249, 265]). In addition, the receptors are structurally very diverse, highly polymorphic and stochastically expressed leading to a diverse receptor repertoire in the adult NK cell populations within every individual.

Inhibitory receptors

Inhibitory receptors signal through intracellular immunoreceptor inhibitory tyrosine-based motifs (ITIMs). There are both MHC class I-binding and non-MHC class I-binding inhibitory receptors. Receptors binding to non-MHC class I-ligands, such as KLRG1, KLRB1 and LAIR-1, have mainly been suggested to function in immune

tumour surveillance and peripheral tolerance [266]. Traditionally, receptors to inhibitory MHC class I-ligands are divided into three groups. First, the Ly49 receptors are C-type lectins expressed as homodimers on mice NK cells. The majority of isoforms in the Ly49 group are inhibitory, with Ly49C exhibiting the broadest specificity, followed by Ly49A [267]. Second, the CD94/NKG2A heterodimer binds the non-classical MHC class I molecule HLA-E in humans and Qa-1 in mice. Thirdly, monomeric killer cell immunoglobulin-like receptors (KIRs) belong to the immunoglobulin superfamily, are expressed on human NK cells and recognize human MHC class I molecules.

Activating receptors

Many of the activating receptors signal through adaptor proteins linked to intracellular immunoreceptor activating tyrosine-based motifs (ITAMs). There are several groups of activating receptors. First, NKG2D is a C-type lectin-like protein widely expressed on human and mouse NK cells. While its ligands (e.g. Rae1 in mice and MICA/B in humans) are normally absent from resting or unaffected cells, they are usually extensively expressed in stressed or infected targets [267]. Second, CD94/NKG2C, Ly49D, Ly49H, and several KIR molecules bind classical- and non-classical MHC class I molecules as well as several pathogen associated ligands. These receptors associate with the adaptor protein DAP12 containing an ITAM domain. Thirdly, natural cytotoxicity receptors (NCRs), such as NKp30, NKp44 and NKp46 are mostly expressed on human NK cells and may activate upon binding various ligands on tumours or virus-infected cells [268]. Next, CD16 is an Fcγ receptor which, upon binding IgG antibodies attached to target cells, mediates ADCC. In addition, the 2B4 receptor is functionally expressed on both human and mouse NK cells and carries a dual function. While high expression and cross-linking of this receptor appears to promote inhibitory signals, low expression functions to activate the NK cells [269]. Finally, several other receptors, including integrins, CD2, DNAM-1 and CD28 are certainly important for NK cell activation [249, 265, 270].

Cytotoxicity

Cell mediated cytotoxicity is a highly organized process. In contrast to the tissue damage following membrane disruption during necrosis, NK cells and cytotoxic T cells induce cell death in a programmed and controlled fashion with sustained membrane integrity resulting in apoptotic bodies that are subsequently removed by surrounding phagocytes [271]. NK cell cytotoxicity is mediated primarily via two pathways.

In the first pathway perforin, granzymes and granulysin are released into the immunological synapse following NK cell degranulation as a response to activation. Perforin rapidly anchors in the target cell membrane and polymerizes in the presence of Ca^{2+} to form cylindrical pores which allows ionic exchange and granzyme entry, subsequently leading to cell death [272]. Mice deficient in perforin suffer from spontaneous lymphomas and are susceptible to viral infections [273]. Similarly, humans lacking perforin exhibit suppressed immune responses to intracellular pathogens [274], indicating the importance of this molecule to cellular cytotoxicity. Cytotoxic granzymes enter the cell trough perforin channels and via Ca^{2+}-dependent or mannose-6-phosphate-dependent pathways [275]. Granzymes and granulysins are

serine-proteases and lysosomal glycoproteins respectively, capable of inducing apoptosis in a caspase-dependent or -independent manner [275, 276]. Once inside the cell, these proteins mediate DNA disintegration, trigger the caspase cascade, activate the cytochrome-C releasing factors Bid, and hydrolyze amino acids leading to mitochondrial membrane destabilization. These events ultimately result in cell death and fragmentation of the target cell into apoptotic bodies [275].

The second pathway involves interactions between FasL and TRAIL on NK cells and death receptors on the target cell. Fas binding to FasL results in receptor trimerization followed by recruitment of FADD proteins which activate caspase 8 or 10 and assemble a death-inducing signaling complex. Subsequent procaspase activation leads to Bid hydrolysis, mitochondrial outer membrane destabilization, cytochrome-C release and DNA degradation [275]. NK cell-mediated FasL induced cell death has been shown to contribute to tumour suppression [277]. Similarly, TRAIL suppresses tumour cells [278] and is involved in the elimination and regulation of DCs *in vivo* [279]. TRAIL has two transducing receptors (DR4 and DR5) which function similarly to Fas [275].

1.3.3 NK cells during *Toxoplasma* infection

Upon microbial invasion, the innate immune response performs an important function of restricting the infection until adaptive immunity kicks in. As central players in this response, NK cells produce IFN-γ and TNF which in turn may activate infected cells to clear intracellular pathogens. NK cells execute important functions during infection with certain viruses, e.g. *herpes* spp. while their contribution to resistance during bacterial infections is unclear [280]. Depletion of NK cells during infection with protozoan parasites like *Trypanosoma*, *Leishmania* and *Toxoplasma*, have further shown that this cell type is important to restrict parasite tissue burdens, limit parasitemia and increase host survival. [281]. Below follows a more detailed description on the role of NK cells during *Toxoplasma* infection.

NK cells are important for the innate immune response to *Toxoplasma*. Functional deprivation or depletion of NK cells results in severe disease and increased death rate [282, 283]. In addition, NK cells may be crucial for the resistance to *Toxoplasma* in immune compromised individuals suffering from diminished T cell numbers [128, 284]. Essentially, NK cell-effector functions include cytotoxicity and cytokine production [248]. During the initial phase of *Toxoplasma* infection, pro-inflammatory cytokines from DCs [131], neutrophils [129, 150], and macrophages [64, 128, 285], stimulate IFN-γ production by NK cells [62, 126, 128] witch mediates early parasite-resistance [62]. While NK cells augment cytotoxicity in mice infected with *Toxoplasma* [286], and target intracellular [140] as well as extracellular parasites [287], this function seems to be of minor importance for the resistance to the parasite since mice selectively deprived of this function have no apparent phenotype [288]. In addition, cytotoxicity does not limit the spread of tachyzoites (Paper II) [133, 289, 290]. Furthermore, the importance of NK cells for the early resistance to *Toxoplasma* has been nicely illustrated by Khan *et al*. who showed that the susceptibility of $CCR5^{-/-}$ mice to infection with *Toxoplasma* resulted from lack of infiltrating NK cells normally

responding to signals via this receptor. Adoptive transfer of $CCR5^+$ NK cells resulted in the restoration of adequate inflammatory responses leading to increased cytokine production and ability of the knockout recipients to survive infection [283]. Thus, cytokine production and inflammation management are essential NK cell-functions during *Toxoplasma* infection. Recently, NK-DC cross-talk has been shown to be important for the regulation of immune responses to certain tumours and intracellular pathogens [231]. During *Toxoplasma* infection, NK cells appear to enhance IL-12 production by DCs via a NKG2D-dependent pathway [291] and augment specific CD8 T cell responses [291, 292]. Collectively, these studies recognize NK cells as important regulators of the early immune response to *Toxoplasma*.

1.4 THE TROJAN HORSE HYPOTHESIS

1.4.1 What is a Trojan horse?

The ancient epics "The Iliad" and "The Odyssey" by the Greek poet Homer, tell the story of the great city of Troy and how it came to fall. Further details of the legend are given in Virgil's Latin epic poem *The Aeneid*.

The relinquished son of the King of Troy, Paris, had unrighteous been designated to adjudicate a beauty contest between the supreme god Zeus wife and two of his daughters. The winner was to receive an apple with the inscription "For the fairest" brought to them by Eris, the goddess of strife. The goddesses each promised Paris a beautiful gift if he would pick her: Hera offered power, Athena offered military glory and wisdom, and Aphrodite offered him the most beautiful women in the world as his wife… Paris gave the apple to Aphrodite…

Helen was a secret daughter of Zeus, notorious for her beauty. As a condition for marriage, her stepfather Tyndareus made all the Greek warrior leaders promises to collect avenge for any insult to her. Helen married Menelaus, the powerful king of Sparta, while her half sister, Klytaimnestra, married Agamemnon, the most powerful leader in the hole of Greece.

During a visit in Sparta, Paris and Helen fell in love, and together they returned to Troy. Tyndareus was furious and appealed to the oath of the Greek warriors. Thus, the powerful armies of Greece landed on the shores of Troy. For ten years the warriors withstood endless battles and diseases without any side prevailing. The city of Troy was besieged but could not be concurred.

Finally, the Greeks devised the strategy of building a great wooden horse, hide soldiers inside it and then fake their concurrence and withdrawal, leaving the wooden horse as a fictitious offering to the god Athena. The Trojans went out of the city, celebrating their victory, and unsuspectingly brought the horse within the city walls. At night, the Greek soldiers went out of the horse and opened the gates for the returning Greek army. The city was destroyed, the people slaughtered, and Helen was brought back to Sparta.

The story of the Trojan horse is intriguing because it describes how a dangerous and cunning plan is executed and accomplished. As a metaphor, the Trojan horse is a smart tool for the infiltration and weakening of a target, and a clever way to hide a transport from one location to another when other means of transfer are not accessible. In a biological setting, a Trojan horse can be a pro-drug delivering cytotoxins to tumour

cells [293], an endocytic pathway ferrying nanoparticles across a cell membrane into a target cell [294], a monoclonal antibody engineered to transport large molecules across the BBB [295], or a cell carrying a pathogen across a biological barrier [296]. The last mentioned example will be the focus of the rest of this chapter.

Intracellular pathogens take advantage of the fact that they are protected from the devastating effects of the humoral arm of the immune system. In addition to the cover and shielding from immune attack that this lifestyle may provide, the Trojan horse mechanism offers relocation and access to sites that are normally protected from pathogens. The persistent struggle of the Greeks to capture Troy eventually resulted in the adventure with the wooden horse. Thus, this instrument was an adaptation to the art of war, grown out of many failed trails to access a heavily guarded area. This was by no means a safe trip. Would the soldiers in the horse have been captured they would certainly have been killed. Similarly, the biological Trojan horse would be the result of pathogens evolving in concert with the immune system, using common pathways to facilitate productive transfer, albeit in a very dangerous manner. As a countermove, the immune system has developed advanced intracellular defense strategies, the cellular arm of the immune system and programmed cell death. A pathogen exploiting a cellular Trojan horse needs to circumvent these systems and control the relocation of the host cell. This requires additional adaptation and the development of immune escape mechanisms.

In summary, the Trojan horse mechanism presents a way for a pathogen to evade the humoral immune system, escape from the inflammatory site, relocate, and access remote parts of the host anatomy, making use of the host's own trafficking pathways for its dissemination. So, is the Trojan horse mechanism of dissemination effective? This will be further discussed below.

1.4.2 Leukocyte trafficking and trails of Trojan horses

In the multi-cellular organism cell populations deriving from different progenitors specialize to form organs and balance homeostasis. Specific effector functions are often anatomically-related depending on neighboring or endocrine interactions. Hepatocytes, nerve cells and epithelia, are local-restricted cells with limited abilities to relocate. Thus, these cell types represent improbable Trojan horses, although they may provide immune shielding for intracellular pathogens [297, 298]. By contrast, leukocytes are haematopoietic cells that originate in the bone marrow and populate the blood. They traffic the tissues, draining to the lymphatic system and back into the circulation [299]. They provide immune surveillance, protection, tissue repair and maintenance, interact with microbes and are essential for pathogen clearance. Thus, the inherent migratory pathways and functions of leukocytes make them potential Trojan horses.

DCs and macrophages

Peripheral entry points for pathogens include the gastro-intestinal and genital mucosa, the urinary tract, the lungs, skin and eyes. In common to all these locations is the epithelial barrier which is firmly sealed by tight junctions [300]. In case of a breach, the sub epithelial tissue is guarded by tissue macrophages and perambulated by DCs [174,

194, 301, 302]. Macrophages and DCs are known targets for many intracellular bacteria including *Listeria monocytogenes* [303, 304], *Mycobacterium Tuberculosis* [305], and *Salmonella* spp. [306, 307]. These pathogens exploit the phagocytosis process for access to the intracellular environment. However, macrophages and DCs are also targeted by other pathogens not relying on phagocytosis for uptake, e.g. *Toxoplasma*. Although DCs and macrophages share many characteristics including distribution, phagocytosis, antigen presentation, and cytokine secretion, macrophages are not primarily professional APCs [174, 301]. Instead, macrophages function to initiate inflammation, clear pathogens and repair tissue [301, 308]. They further exhibit robust microbicidal killing pathways rendering them dangerous to approach. The benefit of using a tissue macrophage as a Trojan horse therefore appears limited. By contrast, DCs are primarily professional APCs, which after pathogen encounter exhibit a retrograde migratory behaviour, disseminating to draining lymph nodes and entering the circulation [309]. DCs may also cross biological barriers and enter normally immune privileged sites [310, 311]. In addition, important functions of DCs, as sentinels of the immune system and initiators of adaptive immunity, may be manipulated by intracellular pathogens to their own benefit. Thus, the inherent biology of DCs makes them ideal Trojan horses.

Neutrophils

In the blood, neutrophils are the main leukocyte cell population [312]. In response to IL-8-like chemokines, circulating neutrophils rapidly enter the inflammatory site and exert antimicrobial functions including phagocytosis. They subsequently undergo apoptosis, preferably killing the ingested pathogens, and are cleared by macrophages [313, 314]. The short half life of neutrophils, their reluctance to return to the circulation once exited, and the potent antimicrobial mechanisms exhibited by this cell type, argues against the neutrophil as a preferred Trojan horse. However, parasites (e.g. *Leishmania*) may still confiscate neutrophils for immune protection and transfer to other cell types [315, 316].

Monocytes

There are two main populations of monocytes in the blood of humans and mice. These subsets are generally referred to as inflammatory monocytes ($CD14^{low}CD16^{pos}$ in humans and $Ly6C^{pos}CCR2^{pos}CX3CR1^{low}$ in mice) and resident monocytes ($CD14^{high}CD16^{neg}$ in humans and $Ly6C^{low}CCR2^{neg}CX3CR1^{pos}$ in mice) respectively [317]. Resident monocytes represent the large majority of monocytes in the blood. They remain in the circulation for long periods of time and continuously migrate into tissues to sustain cell turnover and differentiation [308]. In contrast, inflammatory monocytes are recruited from the bone marrow in response to inflammation, exhibit a short transit time in the blood and rapidly enter the inflammatory site [153, 318]. Subsequent upregulation of CD11c and MHC class II, migration to draining lymph nodes and activation of T cell proliferation, suggest that these cells may differentiate into professional APCs [318]. Therefore, inflammatory monocytes could transport pathogens away from the inflammatory site while resident monocytes are more likely to carry intracellular microbes in the blood.

T cells and B cells

Naïve T cells travel in the blood, extravasate through the HEVs, survey the lymphatic system and return to the circulation via the thoracic duct [299]. If they are activated by an APC in the lymph node or spleen, they subsequently relocate to execute effector functions [319, 320]. By contrast, B cells are less prone to circulate, but rather move within secondary lymphoid organs until they are activated and transform into an antibody secreting plasma cell or memory cell. They subsequently relocate to other lymphoid organs or the bone marrow [299]. Since naïve T cells and B cells generally reside in the circulation or lymph nodes, they are less likely to be initial targets for pathogens in need of a Trojan horse. However, while B cell function and relocation pathways appear less beneficial to an intracellular pathogen, activated T cells traffic in tissues and may access normally immunoprivileged sites [311]. Thus, T cells may be suitable targets for pathogens looking to exploit a Trojan horse later during infection.

NK cells

NK cells are present in secondary lymphoid organs, liver, lungs and blood. Little data are available on the circulation of NK cells but they appear to redistribute throughout all NK cell-populated organs [253]. NK cells are recruited to inflammatory sites upon infection and human NK cells may express the central homing receptor CCR7, indicating that they have the potential to return to the lymphatic system after pathogen encounter [321]. Interestingly, NK cell trafficking has recently been shown to be dependent on the $S1P_5$ receptor [258], which is highly expressed in the CNS [322]. Indeed, NK cells may traffic to the CNS upon inflammation [323]. In addition, the main functions of NK cells is to kill abnormal cells as well as produce cytokines, and they are not well equipped to handle intracellular pathogens [248]. Thus, NK cell trafficking and functions may be useful for a pathogen looking for a Trojan horse.

1.4.3 Pathogens exploiting cellular Trojan Horses

The basic concept of the Trojan horse is that leukocytes are infected in the periphery and then transport the intracellular microbe to a site where it causes disease. A true Trojan horse mechanism implicates assisted transition into an otherwise restricted area, e.g. across the BBB [296]. By contrast, assisted transfer away from an inflammatory site alone rather implies immune evasion which is discussed elsewhere in this thesis.

Most commensal microbes are not human parasites per se, as they do not normally cause damage to their host but rather live in symbiosis with it. Occasionally, this symbiosis is disrupted which may cause damage to the host. Meningitis or sepsis can be looked upon as illnesses owing to the unfortunate translocation of generally innocuous bacteria into an area where immune control is reduced. As a consequence of co-existence, evolution has supplied such bacteria with mechanisms to protect them from immune attack (e.g. by resisting phagocytosis or phagolysosome fusion) but simultaneously generating potential pathogens. Therefore, the pathogenesis of most commensal microbes does not promote transmission. In contrast, parasites profit on their host in an unequal manner, taking advantage of host cellular functions and

manipulating the immune response to their own benefit of propagation. Thus, the biological intention of different pathogens to breach biological barriers and access immune restricted sites diverge. Yet, several different pathogens, including bacteria, virus and protozoa have been proposed to use a Trojan horse mechanism.

Bacteria

Studies on *Listeria monocytogenes*, a causative agent of meningitis, have shown that, in addition to other means of extracellular transition [324], this bacterium breaches the BBB even if only intracellular bacteria are present in the circulation [325], and invasion of Ly-6C^{high} monocytes into the brain coincide with both bacterial invasion and up-regulation of brain macrophage chemoattractant protein-1, indicating cell-mediated transfer [326]. On the basis of phagocyte- [327] and DC-mediated [328] bacterial relocation, a similar mechanism has been proposed for disseminated *Mycobacterium tuberculosis* infection [324, 329]. In addition, several species of non-motile (*Brucella* spp.) and intracellular bacteria (*Riskettsia* spp.) can be found in the CNS [324], indicating a Trojan horse mechanism mediating their transfer. Interestingly, *Salmonella* appear to access the circulation by manipulating the motility CD18^{+} cells [330, 331]. Whether this mechanism also promotes transfer of this bacterium to local anatomic units where it occasionally causes pathology, e.g. the joints, testicles and kidneys, is not clear. In addition, many bacteria (e.g. *Salmonella*, *Legionella*, *Cholera*, *Mycobacterium* etc) can infect and multiply within free-living protozoa like *Acantamoeba*. Therefore, certain protozoan may themselves be Trojan horses of the microbial world [332].

Viruses

The human immunodeficiency virus (HIV) infects monocytes, macrophages, DCs and CD4 T cells [297]. While DCs have been shown to transfer HIV to T cells in the lymph nodes [333-335], macrophages and monocytes have been suggested as viral carriers to the CNS [336-338], where HIV can establish a reservoir [297] and cause debilitating neurological disease [339]. On the basis of identification of infected macrophages/microglia in the brain, a similar mechanism has been suggested for the hepatitis C virus neuroinvasion [340]. Although it is well known that other members of the *flaviviridae* virus family (e.g. Dengue, TBE, JE and WNV) may cross the BBB and cause disease, very little is known about the translocation processes for this group of pathogens. However, blockade or deletion of cellular factors involved in extravasation of leukocytes, such as ICAM-1 [341, 342], LFA-1 [343], or MIP-1 [344], often reduces viral load and dissemination to the CNS in experimental animal models [345].

Protozoa

Plasmodium, the causative agent of malaria, uses a slightly different Trojan horse mechanism for its propagation. The sporozoite stage of this parasite is believed to rely on active motility and passive transport for its relocation from the midgut of the *Anopheline* mosquito, via the salivary glands, to the mammalian hepatocytes [346]. It transforms into a merozoite and apparently detach from host hepatocytes by a Trojan horse-like mechanism involving budding of parasite-filled vesicles [347]. It

subsequently invades red blood cells and develops into gametocytes [348]. The blood pool of erythrocytes may be viewed as a circulating immunoprivileged organ that in addition to immune shielding, provide an opportunity for assisted transportation into the definitive host, i.e. a Trojan horse mechanism. The infected erythrocytes also directly contribute to immune evasion by adhering to DCs which reduce their ability to stimulate T cells [349]. Advanced manipulation of immune cell function is also exhibited by other apicomplexan parasites. For instance, *Theileria* makes use of lymphocytes as intermediate cellular hosts to proliferate and transform before moving on to infect red blood cells [350]. *Leishmania* and *Toxoplasma* are other protozoans reported to use the Trojan horse mechanism to propagate. These parasites are further discussed below.

To summarize, the Trojan horse mechanism has two parts. The first part consists of the host biology, i.e. the innate migratory pathways of leukocytes. This component should theoretically underlie the conceivably unintentional cellular transfer of commensal bacteria. The second part consists of parasite manipulation of the host cell for their benefit of propagation. The cellular mechanisms underlying this manipulation and their relevance for the pathogenesis of infectious disease awaits further clarification.

1.5 IMMUNE CELL SEQUESTRATION BY INTRACELLULAR PATHOGENS

Many obligate intracellular pathogens escape the immune system by accessing the intracellular environment of a host cell. In addition, parasites relying on chronic infection for their propagation are dependent on manipulation of the immune response for their persistence. Thus, immune cell confiscation and subsequent host cell management is an important way for pathogens to seclude themselves during infection. However, should the pathogen be exposed, it needs to counter the attack by the immune system and again fade away. This may require the transmission to a new host cell, like a criminal changing car to escape the pursuing police. For certain pathogens, such a sequestration event may be part of a cunning ‘plan’ to fool the immune system by taking advantage of normally antimicrobial functions. In this section I discuss this mechanism in the light of different parasitic infections.

Leishmania spp. spread by sandflies but depend on vertebrates as their definitive hosts. Inside the vertebrate the promastigote stage needs to infect macrophages to transform into amastigotes and multiply. Interestingly, after cutaneous *Leishmania* infection, the promastigote actively releases a chemotactic factor (LCF) which attracts neutrophils [351], an event beneficial to the parasite [352, 353]. Parasites are subsequently phagocytosed, but survive by blocking the oxidative burst initiated by the PMN [353, 354]. Following the *Leishmania* infection, the PMNs start producing the chemokine MIP-1β. This promotes accumulation of macrophages to the near surroundings of the parasite infected cell [355]. Although *Leishmania* promotes increased survival of the infected neutrophil [356], the inherent short half-life of this cell type makes apoptosis unavoidable [314]. Consequently, parasite-containing apoptotic bodies are phagocytosed by nearby macrophages subsequently getting infected [353]. Because ingestion of apoptotic cells is a standard chore of macrophages, this seemingly results

in down modulation of antimicrobial functions rather than the opposite [357, 358]. Collectivelly, these studies suggest a model whereby immune cell sequestration, dependent on normally antimicrobial functions, in conjunction with a Trojan horse-like mechanism, facilitates *Leishmania* propagation [315, 316, 357]. Interestingly, a similar mechanism may be important for the survival and virulence of *Chlamydiae pneumoniae* [316].

While macrophages and monocytes may function as Trojan horses during HIV transfer to the CNS [336-338], a Trojan horse-like model for DC-mediated HIV *trans*-infection of T cells have been suggested [335]. Since DCs reside in close proximity to HIV entry points like the genital mucosal surfaces, they are likely primary targets for the virus [359]. Although DCs are relatively resistant to infection [360], the expression of DC specific intercellular adhesion molecule-grabbing non-integrin (DC-SIGN) on their cell surface enable high affinity binding of HIV envelope protein gp120 [334]. After viral encounter in the periphery, DCs migrate to the draining lymph nodes where T cells reside [359]. Upon DC-T cell interaction, intracellular or membrane bound virus in transit, relocate from the DC to their major cellular target, the CD4 T cell [333, 335, 361]. Recent findings suggest that the majority of viruses conveyed in *trans* originate from the plasma membrane of the DC rather than from the intracellular compartment [362]. Nevertheless, HIV uses the migratory pathways of DCs to enable transit to, and the confiscation of, a secondary host cell, i.e. an immune cell sequestration mechanism.

Plasmodium falciparum is commonly associated with sequestration during infection. Essentially, gradual modification of the infected erythrocyte cell surface leads to adherence to the endothelium, erythrocyte agglutination, rosetting, parasite egress and reinfection of new red blood cells. This mechanism is important because it stimulates and preserves a high parasitic load in the blood which is beneficial to transmission. In addition, adherence in the microvasculature protects the parasite from clearance in the spleen and is strongly linked to virulence and pathology [363]. While this type of sequestration promotes parasite propagation and survival in a cyclic manner, other pathogens may be using this mechanism in a more linear fashion. In this thesis I present data indicating that NK cell mediated killing of *Toxoplasma* infected DC leads to rapid egress of viable parasites and subsequent infection of the NK cell (Paper II). This sequestration mechanism is discussed further below.

2 AIMS OF THIS THESIS

The studies in this thesis have explored the hypothesis that *Toxoplasma* uses leukocytes as Trojan horses to evade the immune system and to disseminate in the host. The specific objectives were:

1. To investigate cell-borne parasite dissemination in relation to the ability of *Toxoplasma* tachyzoites to infect DCs and other APCs, and its role in the pathogenesis of infection (Paper I and IV)

2. To investigate the dynamics of cell-borne parasite dissemination in relations to DC-NK cell interactions during infection (Paper II)

3. To investigate differences in dissemination of *Toxoplasma* genotypes in relation to their ability to infect DC, and the role of these differences in the pathogenesis of infection (Paper III).

4. To investigate migration and dissemination of lymphoid and myeloid leukocyte populations after infection with *Toxoplasma* tachyzoites (Paper I, III, IV).

3 METHODS

Detailed descriptions of experimental procedures are published in the original papers on which I base this thesis. In this chapter follows a more general description of advantages and disadvantages of some of the more important techniques used.

Parasite cultivation

The parasite cultivation system I have been using throughout this work has many advantages. This system allows us to culture the parasite *in vitro* using cell-line material, i.e. count, view and transfect the parasite without involving animals. In addition, stage conversion can be induced *in vitro* and quantification of viable parasites can be assessed by plaquing assay. However, keeping the parasite disconnected from its natural environment changes its course of development. Consequently, several *in vitro* adapted strains have lost their ability for sexual replication [105]. In addition, adapted strains tend to replicate faster *in vitro* than clinical isolates. This should be kept in mind when interpreting the results of experiments performed with these strains. The majority of experiments underlying this thesis have been performed with transfected strains. Parasite lines used include: RH-LDM [56] a clone derived from RH-GFP [364], ME49/PTG-GFPS65T [364], PRU [149] and derivatives RH-LDMluc and PTGluc [52]. Additional strains used are indicated in the respective papers.

Bioluminescence imaging (BLI)

Traditionally, infection progression has been assessed by euthanizing individual animals at single time points followed by evaluation of host-pathogen interactions in specific organs, e.g. by plaquing assay. To complement this conventional approach, we have been using Bioluminescence imaging (BLI) together with an In Vivo Imaging System (IVIS 100, Xenogen Corp.). Essentially, BLI detects light produced by the reaction of luciferase enzymes with a defined substrate. Hitziger *et al*. used luciferase enzymes from the firefly (*Photinus pyralis*) to generate archetypical bioluminescent luciferin-dependent reporter parasites [52], which have been used for these studies. Luciferin is a non-immunogenic, non-toxic, low molecular weight molecule, which penetrates intact blood-tissue barriers, e.g. the blood-brain barrier, and rapidly distributes in the body after ip injection. Within 10 min it reaches a plateau which is maintained for approximately 30 min [365, 366]. Thus, substrate availability is not a limitation for *in vivo* imaging with firefly luciferase.

The advantages of the IVIS system over traditional systems are the possibility of evaluating dissemination, pathogen burden and infection progression in the same animal over time. Thus, the IVIS may reveal unanticipated biological variations in pathogen distribution without wasting animal lives [52, 365]. This is so far the only technique which permits this type of assessment.

Flow cytometry

Flow cytometry is a widely used technique for counting, examining and sorting cells via the use of fluorescent labeled antibodies. The technique is based on laser excitation of fluorophore-conjugated antibodies bound to cells hydro-dynamically fixed and aligned in a stream of fluid. Detectors in line with (Forward Scatter/FSC), and

perpendicular to the laser light beam (Side Scatter/SSC and fluorescence detectors), may then be used to depict size (FSC), granularity (SSC) and emission from individual fluorophore marked cells. A combination of filters and mirrors directs the wavelength specific light onto there detectors. The impulses are converted into electrical signals, amplified, and processed for computer analysis. I have used this technique mainly to quantify transmigrated cells and for examination of membrane bound markers of cell maturation and activation.

Adoptive transfers

This is a useful method to experimentally approach immunological questions. It can be combined with different techniques to investigate specific responses, e.g. by the use of OVA-specific transgenic T cells [239, 241, 290], and is often useful to restore immunological functions deleted by knock out techniques [121, 153, 242, 283]. In my studies I have been using this method to investigate the impact of infected cells on the dissemination of *Toxoplasma* tachyzoites (Paper I, III and IV), or to assess cell-cell interactions *in vivo* (Paper II).

When conducting adoptive transfer experiments comparing different cell populations, sometimes infected by different strains, normalizations of cell viability, parasite viability, infection level and replication speed are crucial for an acceptable comparison.

I have also tried to address the relative contribution of *Toxoplasma*-mediated migratory induction on DC-borne parasite dissemination (Paper I). The lack of a suitable control, e.g. a parasite that does not induce cell migration *in vitro*, diminishes the conclusions one can draw from these experiments. However, the use of different cells populations (Paper IV), and the ability to block migration with PTX (Paper I), constitutes relevant controls.

The intraperitoneal infection model

All infections in this thesis were done using the ip inoculation model. This model has been well characterized and extensively used in multiple studies [52, 53, 56, 68, 156, 242]. The benefit of this model is that it allows reproducible quantification of disseminated parasites in association to inoculation dose (Paper I and III). In addition, it permits comparison between strains (Paper III) and may be used to study short term cell to cell interactions *in vivo* (Paper II). By contrast, oral infection provides a more physiological model. However, quantification following oral infection is precluded because of the need for stage conversion and epithelial breaching. Furthermore, it has been shown that infection mode influences the dissemination pattern of *Toxoplasma* [52, 367]. Yet, the models share the requirement for parasites, or parasite infected cells, to actively cross biological barriers such as the basal lamina and endothelial layers, to reach the lymphatic system or blood before systemic dissemination to organs, e.g. the spleen or the brain, can occur.

Cell extraction and culture

I have used several protocols for extraction and *in vitro* culture of DCs. While most of these protocols are widely used and relatively straight foreword, extraction of mouse intestinal DCs was challenging and required optimization. Eventually I succeeded in reproducing the purification of an MHC class $II^{+}CD11c^{+}$ expressing cell population.

While the MHC class II expression on small intestine $CD11c^+$ cells did not change after infection with *Toxoplasma*, MHC class II expression increased on $CD11c^+$ cells from the Peyer's patches (Paper III). In addition, viability for both these cell populations, but for small intestine DCs in particular, rapidly declined once in culture. Such dissimilarities may reflect a variation in maturation stage between these two DC populations, or possibly a difference in the permissiveness to infection. Furthermore, these cells clearly exhibited reduced transmigration frequencies compared to *in vitro* generated DCs. Although viability was kept high through rapid usage of these cells following isolation, it is likely that augmented cell death, as a result of post purification stress and tachyzoite challenge, affected their transmigration frequency.

In vitro migration studies

Migration of infected cells was assessed using two different approaches. The transwell system was used for quantification, control experiments, and studies on the underlying mechanism of induced host cell migration (Paper I, III and IV). To compliment this assay, real-time confocal microscopy was used for qualitative evaluation of motility phenotype (Paper I and III), measurements of migratory speed and distance covered (Paper I and III), and cell-cell interactions (Paper II).

4 RESULT AND DISCUSSION

4.1 *TOXOPLASMA*-INDUCED CELL MIGRATION

When first assessing the means by which *Toxoplasma* tachyzoites disseminate, a well founded supposition was necessary. Several reports indicated that extracellular tachyzoites were sensitive to antibodies and complement, albeit with differences between host species [12, 160, 368, 369]. In addition, the early systemic presence of tachyzoites after infection indicated circulatory spread of the parasite [367, 370, 371]. The Trojan horse hypothesis was intriguing as it would explain how the parasite accessed the circulation while staying shielded from immune attack.

The transwell system had previously been used to study migration of *Toxoplasma* tachyzoites across cellular monolayers, and proven instrumental as a model of parasite migration *in vivo* [56, 372]. Consequently, we addressed the question whether *Toxoplasma* tachyzoites are capable of modulating the migration of leukocytes. Tachyzoites were used to infect different leukocyte populations *in vitro* and migration was assessed in the transwell system. In summary, we found a distinct actin-dependent migratory induction of infected DCs, and infected macrophages, which was not present in other leukocyte cell types (Paper I and IV). This induction was dependent on infection dose, required a live intracellular parasite, and was linked to the parasite genotype (Paper I, III and IV). Further investigations on infected DCs showed that the induced migration could be blocked by pertussis toxin (PTX) but did not depend on CCR5-, CCR7-, MyD88-, or Toll/interleukin-1 receptor signaling (Paper I). Below, I will discuss these results in the context of their contribution to the field of *Toxoplasma* research.

In this thesis, I present data on migration of primary and *in vitro*-derived human and mouse DCs, human and mouse macrophages, primary human monocytes, T-cells, B-cells and NK-cells. In addition, migration of tachyzoite-infected neutrophils [373] and T-cells (Ellen Robey, personal communication) have been compared to uninfected cells *in vivo* (Table 1). In summary, only differentiated cells of monocytic origin, i.e. DCs and macrophages, are induced to migrate upon tachyzoite invasion. Supporting this notion are similar migratory phenotypes in microglia (Dellacasa *et al*., Manuscript) and osteoclasts (Vutova *et al*., Manuscript). Differences in migratory induction of myeloid cells may partly be explained by differentiation (Paper IV). Yet, in contrast to other isolated lymphoid cell types, primary human blood DCs and mouse intestinal DCs exhibited migratory activation upon infection with a type II strain (Paper III). This shows that other determinants are involved in the induction of host cell migration.

TABLE 1. Migration of infected leukocytes.

Cell type	DC[1]		Mac[2]		Mono[1,2]		PMN[3]		T-cells[2,4]		B-cells[2]		NK-cells[2]	
Host	H	M	H	M	H	M	H	M	H	M	H	M	H	M
Transwell	+++	+++	+++	+++	-	ND	ND	ND	-	ND	-	ND	-	+
Microscopy	+++	+++	ND	ND	ND	ND	ND	ND	-	ND	-	ND	ND	ND
In vivo	ND	ND	ND	ND	ND	ND	ND	-	ND	-	ND	ND	ND	ND

H, human cells; M, mouse cells; +, induction; -, no induction

[1] Paper I, and Diana *et al.*, 2004 [374]

[2] Paper IV, and unpublished data

[3] Chtanova *et al.*, 2008 [373]

[4] Ellen Robey, personal communication

Cell motility is an essential element of the immune system. Its importance in immune cell development, differentiation, surveillance and function of leukocytes is indisputable. Features such as extravasation, antigen presentation, phagocytosis and chemotaxis depend on actin polymerisation and cell migration. Regulatory mechanisms are often multi-dependent and redundant, but nevertheless stringently controlled to assure a proper immune response. Interestingly, DCs and macrophages exhibit a substantial migratory activation upon *Toxoplasma* tachyzoite infection. This activation requires a live intracellular parasite and commences very rapidly (< 3 h) after parasite invasion (Paper I, III and IV), indicating a post-transcriptional or tightly regulated control of this phenotype. Leukocytes generally use an amoeboid type of migration, which is characterized by leading edge pseudopod formation, surface receptor substrate interactions, mid-region contraction, and rear cell movement [299]. In line with this, tachyzoite-infected DCs form membrane protrusions, followed by retraction and net forward motion (Paper I), indicating amoeboid-like migration of infected DCs. This type of polarized cell migration is reminiscent of cells responding to a chemokine gradient, but it may also be observed during chemokinesis or migration that lacks directionality [375].

PTX and CTX are exotoxins produced by bacteria. These agents are known to block chemokine signaling and effect cell migration [376, 377]. Although, no added chemokines are required for *Toxoplasma* induced cell migration, PTX treatment blocked transmigration of infected DCs *in vitro* (Paper I), indicating involvement of common chemokine signaling pathways. By contrast, CTX treatment had no effect or slightly increased migration of infected DCs (Paper I). While different G proteins are targeted by PTX (targets G*i*) and CTX (targets G*s*), the net result of there action is increasing levels of cAMP [378, 379]. Thus, cAMP likely plays a minor role in tachyzoite-mediated migratory activation of DCs. Instead, alternative signaling through the G*iα*-subunit, or blocking of an unidentified receptor, is a more likely mechanism. Further, chemokine signals are received, decoded and transmitted by G protein-coupled receptors (GPCRs). The functions of these receptors are not mutually dependent, i.e. transmission by an GPCR does not require a chemokine [320]. Thus, activation of host cell motility by *Toxoplasma* may involve a chemokine receptor pathway, albeit activated from inside the cell.

In addition to migratory activation, chemokine signalling pathways control leukocyte navigation routes for immune surveillance, homing, and extravasation [320]. These events are highly inter-reliant and strictly dependent on cell migration. *Toxoplasma* infection of DCs leads to a number of physiological and phenotypic changes that could alter their cellular trafficking. Fore example, infected DCs exhibit reduced maturation characteristics [238], does not respond to CCL19 (Paper I), and exhibit increased migratory activity (Paper I). Such data implicates that the common migratory pathways of DCs, following pathogen invasion, are disrupted in favor of routes depending on alternative chemokine signaling. Thus, infected DCs may bypass the lymphatic system and choose a direct haematogenous course. In support of this notion, infected DCs are rare in the lymph nodes after infection [148] and adoptive transfer of DC-borne tachyzoites resulted in a substantial increase in parasite dissemination to the spleen but only a slight increase to the MLN (Paper I). However, several studies have shown that lymphoid organs are parasitized early and extensively during infection [53, 367, 371], and T cell priming [148] as well as IL-12 production [121] characterize uninfected DCs. Thus, while uninfected DCs are essential in protecting the host from toxoplasmosis, infected DC may serve primarily to aid parasite immune evasion and dissemination.

Just as chemokine signaling is important for cell trafficking, integrins play a determinant role in leukocyte migration events during homeostasis and inflammation. Adhesion molecules are used by leukocytes to "crawl" along 2-D surfaces *in vitro* (e.g. transwell) and *in vivo* (e.g. endothelial surface), and to arrest before diapedesis [380]. These processes require polarized cell adhesion and integrin-mediated attachment, preferably through LFA-1 [381]. By contrast, 3-D migration *in vitro* (e.g. Matrigel^{TM}) and *in vivo* (ECM environments) is integrin independent, instead relying on ECM scaffolding structures, shape change and squeezing [382]. In addition, the conformational state, turnover and distribution of integrins is highly related to their function [380, 383, 384]. The modulated expression of cell surface adhesion molecules observed on infected DCs (Paper I) and macrophages [385], could therefore have variable *in vivo* implications. First, the proficient translocation of infected DCs across endothelial bilayers (Paper I) shows that *Toxoplasma* infection stimulates diapedesis and indicates that down modulation of integrins does not necessarily compromise extravasation. Second, migration through tissue should not be affected by the observed down modulation of several integrins (Paper I) [382, 385]. Thirdly, down modulation of ICAM-1 on infected DCs and macrophages (Paper I) [385] in conjunction with the immature phenotype of these cells [238], could result in reduced relocation [383], but more likely suggests an impact on the antigen presenting ability of the infected cell [386]. In addition, ICAM-1 expression tend to vary as the cell matures [383] and a proper kinetic study on the expression of this marker is necessary for further conclusions.

While the rapid onset of host cell migration upon tachyzoite invasion probably derives from mechanisms other than changes in underlying host gene-expression, the trait exhibited by the parasite is indicative of an underlying genetic control (Paper III). In addition, modulation of host-gene expression may prolong and maintain the migratory phenotype. Importantly, all *Toxoplasma* strains tested to date express this trait (Paper III). Transmigration of DCs and macrophages is related to the parasite genotype, with

type II exhibiting the strongest induction, followed by type III, and finally type I (Paper III and IV) [374]. This type of distribution among closely related sub-species is not typical of phenotypes developing in parallel due to biological pressure. Rather, it is coherent with common genes expressing different alleles. This suggests that the genetic control underlying this trait derives from the discrete recombination event giving rise to the three clonal lineages that to day dominate the *Toxoplasma* population in large parts of the world [40, 41, 43, 44]. Importantly, these three lines have emerged relatively recently in evolution, and within-type genetic variation is therefore extremely rare [41, 387]. Furthermore, features such as increased oral infectivity [42] and modulation of immune responses [55], that thrived as a consequence of recombination, has been crucial for the success of all three genotypes. Characteristic of these traits is that they prevail in the most successful *Toxoplasma* strains, i.e. type II strains [42, 65]. In line with this, migratory activation by type II strains is superior to that exhibited by type I or type III (Paper III and IV). Collectively, these studies suggest that the trait of induced host cell migration by *Toxoplasma* has prospered, or even been refined since the three archetypical strains emerged. The fact that it is more prominently expressed by the most successful strains and has not vanished during the course of evolution, indicates its significance for the propagation of *Toxoplasma*.

The discovery of the induced migration exhibited by tachyzoites-infected cells is exciting for several reasons. First, interstitial migration and positioning of leukocytes during inflammatory conditions and host immune responses to pathogens and tumours are vital. Elucidation of the molecular mechanisms governing tachyzoite-induced cell migration may provide new insights into how migratory patterns of leukocytes are controlled. Thus, manipulation of these pathways for therapeutic purposes presents a potential way of regulating the immune response and ultimately affect disease outcome. Second, manipulation of the host migratory machinery by pathogens is an as yet unappreciated means of escaping the immune system and disseminate in the host. The precise contribution and extent of this mechanism to the propagation and immune evasion of different pathogens remains to be clarified. Finally, the subversion of host cell motility by *Toxoplasma* may partly explain the wide dissemination after adoptive transfer of DC-borne tachyzoites (Paper I), and may therefore be a means by which this particular parasite propagates in the host. This will be discussed further in the next section.

4.2 DISSEMINATION BY *TOXOPLASMA GONDII*

The enhanced migration observed in tachyzoite-infected DCs and macrophages encouraged us to study the dissemination of the parasite *in vivo*. Our primary idea was to simply convert our *in vitro* transwell to an *in vivo* setting. Inoculating parasites ip forces them to cross mesothelium, basement membrane and endothelium and access the circulation in order to reach e.g. the spleen. Comparing adoptive transfer of immature DCs, tachyzoite-infected DCs and LPS-stimulated DCs in this model, revealed a substantial enrichment of infected DCs to secondary lymphoid organs. (Paper I). Additional assessments by means of BLI and plaquing assays revealed exacerbation of infection and increased dissemination to the spleen, MLN and brain, indicating DC-assisted parasite dissemination (Paper I). To further investigate the impact of parasite-

induced DC migration on the dissemination of *Toxoplasma*, we pre-treated infected DC with PTX. Inhibition of DC migration reversed the observed enhanced dissemination of parasites and led to reduced overall parasite tissue burdens, similar to mice inoculated with free parasites (Paper I). The sensitivity to PTX by infected DC indicates that DC migration underlies the increased parasite dissemination after adoptive transfer (Paper I). However, the exact contribution of parasite induced migration is difficult to determine considering the importance of chemokine signaling *in vivo* [376]. Yet, increased dissemination was not simply a result of an intracellular advantage over free parasites as adoptive transfer of infected macrophages or lymphocytes did not exacerbate infection (Paper IV). Further experiments evaluating adoptive transfer of DC-borne parasites revealed that this method of infection favors dissemination of type II and type III parasites compared to type I. In addition, it promotes rapid and wide tissue distribution, with high parasite tissue burdens, ultimately resulting in an exacerbation of infection (Paper I and III).

The transmission of *Toxoplasma* depends on predation of infected mammals and birds by felines. It is therefore essential for the parasite to establish a long lasting infection in the host. Dissemination of *Toxoplasma* during the initial phase of infection is reliant on many factors. Initially, the parasite needs to transverse the intestinal barrier in order to access the host tissues and multiply. The observation that bradyzoites [22] and sporozoites [31, 388] entering the intestine, converted to tachyzoites in the sub epithelial tissue within 4-18 h, suggests that the initial penetration of the intestinal epithelium is achieved by the infective stages of the parasite. This penetration may be the result of direct invasion and basolateral exit of enterocytes [24], paracellular penetration, or leukocyte sampling [113].

Partly dependent on transversal route, the parasite must tackle the humoral or cellular defences of the innate immune system. Although extracellular tachyzoites are resistant to lysis in non-immune human serum [368], they are sensitive to antibody mediated lysis [160], and thus depend on cell invasion for protection. Consequently, the transfer of the parasite into the circulation is dependent on either direct tissue penetration by motile parasites or cell-mediated transfer. Direct tissue penetration by an extracellular parasite is highly possible as *Toxoplasma* may travel extracellularly by the use gliding motility [389, 390]. However, while type I parasites exhibit a potent transmigratory ability that likely facilitates passage across biological barriers, this trait is essentially absent in type II and type III strains [56]. Thus, endothelial passage of tachyzoite is likely linked to the parasite genotype. In line with this, we found the leukocytes in the circulation to be preferentially associated with type II parasite rather than type I. In addition, adoptive transfer of cell-borne parasites substantially increased parasite loads in the circulation for type II and III, in contrast to only a slight increase for type I (Paper III). Furthermore, we found the extracellular fraction of type I parasite to predominate in the spleen soon after ip infection (Paper III). Although it can not be excluded that this finding is experimentally related, e.g. due to increased lysis of type I-infected cells, it may reflect extracellular dissemination. These findings indicate that type I parasites may enter the blood as free parasite. By contrast, we found the intracellular fraction of type II tachyzoites to predominate during early dissemination (Paper III), and type II and type III parasite are often found intracellularly in circulation soon after infection [243, 370, 391, 392]. In addition,

adoptive transfer of DC-borne parasites favored dissemination of type II and type III more than type I (Paper III), indicating greater dependence on cell-mediated transfer of these strains. The rapid appearance of parasitemia after infection [24, 370, 388, 393], in combination with the predilection of the bradyzoites and sporozoites to convert inside leukocytes of the lamina propria [24], may further indicate a cell-mediated transfer of tachyzoites into the circulation. It also raises the question of whether bradyzoites and sporozoites can induce cell migration, something currently under investigation in our lab. In summary, tachyzoites cross the biological barriers protecting the circulation either by direct penetration or via cell-mediated transfer, and this may differ between strains.

Several leukocyte cell types, including macrophages [243, 385] and DCs [242, 243], have been reported to assist parasite transfer into the circulation. *Toxoplasma* preferentially infects these cell types *in vitro* [394] as well as *in vivo* [242], and DCs ($CD11c^+$) in particular are often preferentially parasitized early during infection (Paper III) [242, 243]. In addition, several groups have shown that tissue specific DCs transport tachyzoites into the circulation shortly after infection [242, 243]. In line with this, we found $CD11c^+$ cells to be highly infected 36 h post ip inoculation of tachyzoites (Paper III), and adoptive transfer of DC-borne type II and type III parasites accelerated primarily early dissemination compared to inoculation of free parasites (Paper I and III). By contrast, adoptive transfer of macrophages decelerated parasite dissemination [385] and did not exacerbate infection (Paper IV). Thus, there are substantial data showing that DCs mediate early parasite transfer into the circulation, and this concept is further supported by the inherent retrograde migratory behavior of DCs during infection [174]. Redundancy of transfer pathways are likely considering that infection kinetics are related to strain type (Paper III) [56, 57, 69], infection stage [24] and host cell type [385]. Therefore, the significance of DC-mediated parasite transfer deserves to be evaluated. This issue could be addressed by the use of transgenic mice expressing the diphtheria toxin receptor under the control of the CD11c promotor [232]. Importantly, eliminating $CD11c^+$ cells, e.g. DCs, will affect several immunological parameters which may disrupt or bias obtained dissemination data [121, 148, 149]. Therefore, careful evaluation of kinetics and the use of comparative controls will be essential to interpret such an experiment.

The initial crossing of the endothelial barrier to enter the circulation is a crucial event for the parasite as it enables latency and the possibility of propagation. Once in the blood or lymphatic system, an intracellular parasite will be protected from a multitude of microbicidal cellular and humoral factors designed to kill extracellular pathogens [395]. Thus, there is a strong prerequisite for a tachyzoite that enters the blood to be intracellular, or alternatively to quickly invade a leukocyte or an immune restricted site, once in the circulation. This raises the question of how, and when during the dissemination process, normally immune restricting barriers are breached, e.g. the BBB or the placenta. There is little known about the mechanisms by which *Toxoplasma* facilitates relocation across these barriers. However, translocation can be accomplished in essentially three ways [296]. First, powered by its own motility [389], the parasite may actively invade [396] the apical part of the epithelial cell and exit through the basolateral side, so called transcellular traversal. Second, the parasite may use an adhesion-dependent paracellular pathway, similar to that used by leukocytes, to traverse

without disrupting the integrity of the epithelial layer [372]. Thirdly, the parasite may exploit leukocyte assisted transfer, i.e. the Trojan horse mechanism [243]. The two first pathways entail an extracellular parasite, while the latter requires the parasite to be intracellular. While type I tachyzoites exhibit a potent transmigratory ability [56] and extracellular presence *in vivo* (Paper III), type II parasites are quickly eliminated after iv injection [391] and is characterized by an association to leukocytes after oral and ip inoculation (Paper III) [243]. Thus, whereas the transcellular and paracellular pathways may be used by type I parasites, the Trojan horse mechanism seems more likely for type II. Corroborating this notion, adoptive transfer of tachyzoite-infected DCs resulted in an accelerated dissemination of type II tachyzoites to the brain and testis (Paper I), indicating that infected DCs traffic to these organs. Similar to DCs, monocytes have been shown to transport tachyzoites to the brain [243], and T cells may shuttle parasites in the circulation, albeit at a later stage of infection (Ellen Robey, personal communication). In addition, neutrophils [397] as well as NK cells (Paper II) have been suggested to contribute to parasite dissemination. The collective impression from these studies is that DCs and monocytic cells are primary targets of *Toxoplasma* invasion while lymphocytes take on an increasing quantity of the parasite burden as the infection progresses. Yet, the relative significance and distinct roles of individual cell types in the dissemination of *Toxoplasma* are far from clear. In addition, potential strain-dependent differences further complicate the interpretation of the collected results. However, the collected data discussed in this section suggest that different cell types contribute to parasite dissemination in an infection progression-dependent manner. Biological adjustment by *Toxoplasma* to the actions of the innate immune system, and the innate migratory pathways of immune cells, could partly explain strain differences and host cell preferences during parasite dissemination. Nevertheless, these processes also involve complex parasite-induced mechanism, such as induced hypermotility (discussed in the previous section) and immune evasion events (discussed in the next section), to establish latency and assure propagation.

4.3 IMMUNE EVASION AND SEQUESTRATION BY *TOXOPLASMA GONDII*

Toxoplasma is a very successful parasite. The secret of its success depends on ingenious factors such as the ability to form environmentally stable oocysts, infect a diverse repertoire of human and animal hosts, and withstand climate variations. However, cellular and molecular interactions during the dissemination process of the parasite underlay the establishment of chronic infection, an essential requisite for propagation. Tachyzoites traveling through the mammalian host is a process which certainly involves numerous cell interactions and the chance of catching a secure undeviating ride is probably very limited. Therefore, it is conceivable that *Toxoplasma* has developed multiple pathways to manipulate the immune system, avoid detection and resist elimination.

Intracellular immune evasion mechanisms

Toxoplasma can invade any nucleated cell type powered by an active process termed ‘gliding motility’ [64, 389]. Upon invasion, the parasite establishes a protective parasitophorous vacuole (PV) within the host cell cytosol which is resistant to anti-

microbial actions in the absence of IFN-γ [398, 399]. Moreover, parasite-infected cells exhibit diminished responses to IFN-γ [400], and reduced transcription of important microbicidal genes, including iNOS and p47 GTPases [401]. These effects are likely mediated by parasite induced upregulation of suppressor of cytokine signaling (SOCS) proteins [402] which leads to transcriptional blocking or degradation of STAT1 [400-402]. In addition, infected macrophages and DCs fail to produce TNF and IL-12 [67, 238], indicating subversion of NF-κB signaling. Indeed, NF-κB activation and translocation is suppressed in infected macrophages [403, 404], an affect likely mediated through parasite-induced inhibition of the NF-κB subunit p65/RelA [405]. In addition, phosphorylation and subsequent translocation of the IL-10-responsive gene activator STAT3 have recently been shown to suppress pro-inflammatory cytokines in infected macrophages [67]. Whereas *Toxoplasma* clearly blocks intracellular anti-microbial pathways, the parasite appears to exert opposing effects on the death program of the host cell. While *Toxoplasma* sometimes trigger cell death [51, 53, 290, 406], it may during other conditions inhibiting apoptosis [395, 407, 408]. Although systemic versus cellular effects, and the use of different parasite strains or host cell lines, may explain some of these contradictory results, the precise role/impact of apoptosis during the different phases of *Toxoplasma* infection is not yet clear [290, 395]. However, *Toxoplasma* has been reported to inhibit host cell apoptosis by interfering with the caspase cascade [407], via degradation of poly(ADP-ribose) polymerase [407], or through increased expression of anti-apoptotic molecules [409]. Collectively, these studies show that *Toxoplasma* actively interferes with host cell signaling pathways to inhibit intracellular defense mechanisms, modulate cytokine secretion, and regulate cell death.

Host cell preference

Many of the studies on molecular manipulation of infected cells have been performed in macrophages or DCs. This is logical considering the microbicidal effector functions of these cell types and the availability of excellent *in vitro* cultivation systems. When assessing DC-mediated dissemination of *Toxoplasma*, an important aspect of our investigation involved evaluation of infection level of different leukocyte subpopulations to determine host cell preference. Our investigations showed that myeloid cells in general ($CD11b^+$), and DCs in particular ($CD11c^+$), were preferentially parasitized in the spleen, blood, MLN and in the intraperitoneal cavity shortly after infection (Paper II and III). This was in line with what other groups had observed and was thus not unexpected [243]. The permissiveness of these cells to infection by *Toxoplasma in vitro* and *in vivo* has been attributed to factors such as *in vivo* distribution, early recruitment, and tolerance to rapid tachyzoite division [242, 243, 394]. Interestingly, when analyzing the infection level of different lymphocyte subpopulations *in vivo*, we observed that NK cells were preferentially infected over T cells and B cells (Paper II). This had not been reported previously and appeared peculiar to us since NK cells are generally believed to home to the infection site and limit parasite burden [283] via early IFN-γ production [62]. While cell-mediated cytotoxicity is a hallmark of NK cells, studies on the consequence of cytotoxicity directed at *Toxoplasma* infected cells were limited [289]. Instead, the notion that cytotoxicity plays a limited role during toxoplasmosis is probably derived from the overwhelming evidence for IFN-γ mediated immunity and parasite mediated inhibition

of apoptosis [289, 395]. For these reasons, we hypothesized that NK cell-mediated cytotoxicity, directed towards *Toxoplasma*-infected cells, lay behind the preferential infection of NK cells. In our lab, Emma Persson *et al*., were simultaneously investigating the impact of cytotoxic pathways on the stability of infected cells. They had observed that death receptor ligation in *Toxoplasma*-infected cells lead to rapid egress of parasites with the ability to infect surrounding cells [290]. This was an intriguing finding because it pointed to a previously undescribed means of immune evasion. While Emma Persson and colleagues worked on understanding the mechanism behind this phenomenon, Catrine Persson and I focused on investigating the fate of the egressing parasites.

Cell-mediated cytotoxicity

DCs are highly parasitized shortly after infection, and mediate early *Toxoplasma* dissemination (Paper I and III) [242, 243]. Similarly, NK cells rapidly home to the infection site and produce cytokines [126, 283]. Thus, these two cell types are likely to interact during the initial phase of infection. Furthermore, NK cell-DC interactions *in vivo* are important for host defense and development of adaptive immunity [231]. In addition, NK cells readily lyse immature DCs *in vitro* and *in vivo*, while leaving mature cells intact (Paper II)[410, 411]. This dichotomy is presumed to be caused by increased levels of classical- and non-classical MHC class I molecules on activated DCs [411, 412]. However, intracellular pathogens, which need to protect themselves from cell mediated cytotoxicity, frequently modulate this family of molecules to avoid clearance by NK cells and $CD8^+$ T cells [413]. Interestingly, while *Toxoplasma* is a highly adapted micro-organism, readily modifying the immune response by several means, including actively inhibiting MHC class II expression in infected murine APCs [238, 400, 414], the parasite does not appear to have a profound effect on MHC class I expression [414]. Thus, the expression of MHC class I on the host cell is likely not unfavorable to the parasite and may even be beneficial for propagation (Paper II). Indeed, maturation induced by heat-killed *Toxoplasma* tachyzoites resulted in upregulation of MHC class I and II, and unchanged susceptibility of DCs to NK cell-mediated lysis *in vitro* (Paper II, and unpublished observation**)**. By contrast, DCs infected with live parasites were hypersensitive to NK cell-mediated lysis, and this could not be abrogated by blocking parasite replication (Paper II). Although the immature phenotype of tachyzoite-infected DCs may explain the lack of resistance to NK cell-mediated killing [238], the enhanced susceptibility to the latter requires an additional explanation. Since *Toxoplasma* infection render classical and non-classical MHC class I molecules unchanged (unpublished observation) [414], the increased sensitivity of infected DCs to NK cell-mediated cytotoxicity may be caused by an activating receptor expressed on the infected cell surface. Thus far, the only activating receptor we have looked at is Rae-1, which is not expressed on either infected or uninfected DCs. Notably, Boysen *et al*., showed that *Neospora*-infected bovine fibroblasts had increased susceptibility to autologous NK cell cytotoxicity involving the activating receptor NKp46 [415]. Even though the involvement of this receptor could not explain the increased cytotoxicity to infected targets, it suggests that activating receptors are involved in this mechanism. Nevertheless, this matter awaits further investigation.

Immune cell sequestration by Toxoplasma

The increased susceptibility to cytotoxicity of infected human and mouse DCs, and the difference in infection rate between NK cells and T cells *in vitro* and *in vivo*, encouraged our further investigation (Paper II) [290]. Apoptotic cell death is associated with elevated levels of intracellular calcium [416]. In addition, Ca^{2+} is an important factor in parasite-induced egress from the host cell [417, 418]. Indeed, the causal mechanism leading to parasite egress upon death signals induced by FasL, TRAIL, or perforin, was demonstrated to include Ca^{2+} fluxes [290]. Subsequently we showed that the ability of NK cells to lyse infected DCs *in vitro* were completely dependent on perforin (Paper II), confirming results from other studies [279, 410, 419]. When assessing the infection rate of the different cell populations, the perforin$^{-/-}$ NK cells demonstrated a substantially reduced level of infection compared to wt NK cells. This difference could be reversed by adding the egress-promoting agent dithiothreitol (DTT) to perforin$^{-/-}$ NK cell cultures, indicating that the parasites infecting the NK cells originated from the DCs. Consequently, using real-time confocal microscopy, we recorded NK cell-mediated lysis of infected DCs and the subsequent transfer of parasites to effector NK cells (Paper II). Thus, a perforin-dependent mechanism mediates parasite invasion of NK cells in cultures with infected DCs.

In addition to perforin, TRAIL is important for NK lysis of DCs *in vivo* [279]. Therefore, perforin$^{-/-}$ mice were treated with anti-TRAIL and anti-FasL antibodies and inoculated with DC-borne parasites or free tachyzoites. While the level of infected T cell was unchanged in the antibody-treated knockout animals, a significant reduction of the number of infected NK cells was observed. Yet, this reduction did not reach background levels (Paper II). This result shows that cytotoxicity is involved in the infection of NK cells *in vivo* but also implicates other modes of infection. Importantly, cytotoxicity-dependent infection of NK cells was evident after 48 h post inoculation of DC-borne parasite. Therefore, the innate cytotoxicity by NK cells may characterize interactions between infected DCs and NK cells early during infection. By contrast, T cells require priming to execute cytotoxicity, and T cell-mediated cytotoxicity is unlikely to distinguish interactions between infected DCs and T cells shortly after infection. However, Emma Persson and colleagues used an elegant system to mount specific cytotoxic T cell responses against infected cells. When comparing primary and secondary immune responses, infection of activated CD8 T cells was increased approximately 7 times in primed compared to naïve mice, and a strong correlation was found between T cell activation and infection [290]. Interestingly, using two-photon microscopy, Ellen Robey and colleagues have observed transfer of parasites from macrophages to T cells in lymph nodes approximately 9 days after infection (personal communication). Collectively, these studies suggest that cytotoxic lymphocytes may acquire their infection when killing infected cells. This means of infection represents a way for the parasite to avoid elimination and maintain dissemination even though the parasite has been recognized by the immune system. Although infected NK cells and T cells are not induced to migrate (Paper IV), they are not paralyzed by infection and may thus contribute to parasite dissemination. In addition, infected NK cells are resistant to killing by other NK cells and the intracellular defenses of this cell type may not be as well developed

as in DCs or macrophages (Paper II). Thus, parasite transfer from DC to NK cell may actually benefit the parasite by providing refuge in an otherwise very hostile environment. I have previously described this type of immune cell sequestration mechanism as a delinquent changing car to avoid the police. The great ability of *Toxoplasma* to manipulate host cell conduct and escape immunity possibly calls for a more daring simile. Thus, upon exposure *Toxoplasma* may dump its first getaway car and instead hijack the police vehicle. Intriguing is the notion that this may be a 'planed' event to favor dissemination of the parasite. After all, the police vehicle seems to be a much more bullet proof means of transportation. Alternatively, this mechanism may be used by the parasite to circumvent early clearance, as a way to escape from the inflammatory site or simply for immune evasion during the course of infection.

To double-cross the immune response

This means of parasite transfer raises questions about the immune response to *Toxoplasma*. While intracellular parasites may manipulate pro-inflammatory and anti-microbial pathways, the vast majority of leukocytes involved in the immune response are probably not infected, and display normal IFN-γ responses [401]. In fact, *Toxoplasma* infection leads to vast production of pro-inflammatory cytokines and selective parasite molecules triggers production of IL-12 [62, 119, 420]. Consequently, it has been speculated that the strong Th1 response induced by the parasite may be evolutionary functional to prevent intermediate host mortality and thereby promoting the maintenance of the *Toxoplasma* life cycle [119]. However, a strong IL-12 response also promotes cell-mediated cytotoxic immunity which, considering the possibility of parasite transfer, may aid the dissemination and propagation of the parasite. Therefore, diverse benefits may accompany *Toxoplasma*-induced IL-12 production during infection. On the other hand, immune regulatory and anti-inflammatory factors, such as IL-10, TGF-β and lipoxin, induced by *Toxoplasma* infection, balance the Th1 responses, deactivate macrophages and controling overwhelming inflammatory responses [270, 395, 420-423]. In addition, B cells are crucial for long term resistance to *Toxoplasma* [162], and antibodies have been shown to primarily block invasion of new host cells [159]. Therefore, humoral immunity may be important to limit cytotoxicity-induced parasite transfer. Collectively, these studies suggest that *Toxoplasma* strikes a delicate balance, concurrently protecting its intracellular entity and limiting host pathology.

5 FINAL REMARKS

Since the pioneering work by Edward Jenner at the end of the 18th century, scientists have been striving towards a better understanding of the immune responses to infection. Although parasites in general belong to the most widespread and devastating human pathogens, their intricate ability to manipulate the immune response has persistently puzzled immunologists, and many gaps still remain in our understanding of host responses to parasitic infections. *Toxoplasma gondii* is a ubiquitous parasite with potentially severe medical implications for infected individuals. In this thesis, I have summarized and discussed my work on dissemination and immune evasion of this pathogen.

I have pursued the hypothesis that *Toxoplasma* enables successful dissemination by using leukocytes as Trojan horses during infection. The initial assumption when I started working in 2003 was that *Toxoplasma* hijacks leukocytes in the peripheral tissue and then travels inside these cells to locations where the parasite is protected from immune attack. With little prior experimental knowledge of *Toxoplasma* dissemination biology, this was basically the limit of my imagination and the best idea I could come up with to explain the rapid accumulation of parasite in immune restricted locations. Although this hypothesis is still encouraging after six years of research and certainly not disproved, many unexpected discoveries by us and others have contributed to a more intricate suggestion of how this process could occur.

Essentially, after oral infection *Toxoplasma* penetrates the epithelial barrier and burrows down to infect cells in the deeper tissue. Upon tachyzoite transformation the parasite enters the circulation mainly inside host cells. DCs carry the parasite to secondary lymphoid organs, preferentially the spleen, where cytotoxic lymphocytes facilitate the distribution of parasites to adjacent cells by targeting the infected DCs. *Toxoplasma* then disseminates throughout the body in various leukocytes as the infection progresses. This model is consistent with a Trojan horse type of mechanism, and a lot of published data support this concept.

While one may speculate on a model for *Toxoplasma* dissemination, several fundamental questions still remain to be answered. First, tachyzoites certainly cross restricted barriers inside leukocytes but the comparative input of this type of transfer and the relative contribution of different leukocyte cell types, is not clear. Second, the implication of immune cell sequestration for parasite immune evasion and dissemination needs to be further evaluated. Third, the increased receptiveness to cytotoxicity, and the acquired hypermotility of infected DCs and macrophages, should be mechanistically assessed to determine if molecular pathways governing these phenotypes may be manipulated to restrict parasite dissemination. Finally, other intracellular pathogens should be explored to determine if induction of host cell hypermotility, as a mechanism for parasite dissemination, is a general phenomenon or specific to *Toxoplasma*.

The process by which *Toxoplasma* manages to disseminate and access normally immune restricted organs, particularly the brain, is important because it is an essential

part of *Toxoplasma* pathology. Restricting the dissemination process would theoretically eliminate the ability of the parasite to establish chronic infection and cause reactivated disease. A similar reasoning may be applied to other diseases caused by intracellular microbes, and much effort has been attributed to elucidate the dissemination pathways of important human pathogens such as HIV or Malaria. Therapies designed to confine these pathogens and impair their dissemination has proven effective, demonstrating the rational to study dissemination biology in a suitable model organism such as *Toxoplasma*. Hopefully, the work presented in this thesis has contributed to narrowing the gaps on our knowledge of parasite dissemination biology and immune evasion.

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