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HOST MOLECULAR DETERMINANTS OF *TOXOPLASMA GONDII* DISSEMINATION

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Cover image: Three dimensional reconstruction of human dendritic cells infected by RFP-expressing *Toxoplasma gondii* tachyzoites. Cells were fixed in glutaraldehyde, stained with Alexa Fluor 488 Phalloidin and imaged by optical sectioning on a Zeiss AxioImager Z1 Apotome (100x Plan Apochromat objective). Picture modified by Fernanda Torre.

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I would like to dedicate this thesis to the living memory of Professor **Marie Skłodowska-Curie**, whose death 80 years ago was directly linked to her ground breaking research on radioactivity.

She was the first woman to become professor at the University of Paris, the first woman to be awarded the Nobel Prize and the first and only person to date to be awarded it twice in multiple science categories.

A true pioneer in translational work, she contributed to the development of radiological cancer treatments and constructed mobile diagnostic X-ray units, which she herself put to use near the battle front of the first world war.

To me, she represents the scientific endaevor at its very best; the pursuit of new knowledge, the relentlessness in the face of adversity and the vision of a better world for all people. Her words from the opening of the Radium Institute in Warsaw 29 May 1932 are as true today as they were then:

"*Therapy should be permanently backed up by scientific research without which no progress is possible. Moreover, the search for pure knowledge is one of the important needs of mankind.*"

Karolinska Institutet

Institutionen för Medicin, Huddinge

Host Molecular Determinants of *Toxoplasma gondii* Dissemination

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ABSTRACT

Humans and animals acquire the parasite *Toxoplasma gondii* through contaminated food or water, upon which the parasite rapidly disseminates in the host. In immunocompetent individuals, this acute phase is transient and leads to chronic colonization of preferentially the central nervous system (CNS). The latent chronic infection may reactivate in immunocompromised individuals, causing potentially lethal encephalitis. It has been established that dendritic cells (DCs) are exploited by *T. gondii* as "Trojan horses" to facilitate dissemination in the host. *T. gondii* infected DCs exhibit a hypermigratory phenotype that is a major determinant for enhanced dissemination of the parasite. The host cell pathways involved in the onset and maintenance of this phenotype are largely unknown. This thesis has investigated potential mechanisms of host cell subversion, addressing dissemination during the acute phase of infection and reactivated infection in the brain upon immunosuppression. The main findings of the thesis are listed below.

Significant $CD8^+$ T cell infiltration and activation of CNS resident microglia and astrocytes was observed in close vicinity of reactivated *T. gondii* foci in mouse brain. Cortical microglia exhibited hypermigration upon *T. gondii* infection *in vitro*, as previously shown for DCs. Infected microglia and astrocytes showed increased sensitivity to T cell mediated killing, allowing parasite transmission to surrounding cells. Thus, resident and infiltrating cells may act as Trojan horses, potentiating local *T. gondii* dissemination in the encephalitic brain.

We developed a motility assay in which cells are tracked while migrating in a collagen matrix. In this assay, *T. gondii* infected DCs displayed increased velocities as early as 10 min post invasion. This "hypermotility" coincided with the redistribution of actin and integrins. Infected DCs also chemotaxed along a gradient of CCR7 ligand CCL19. Collectively, this series of cellular events contributes to a shift in the migratory capacity of *T. gondii* infected DCs, expanding the concept of the hypermigratory phenotype.

We showed that DCs express functional γ-aminobutyric acid (GABA) receptors and are capable of producing and secreting GABA. *T. gondii* infection enhanced GABA secretion, while inhibition of GABA production, transport or GABA receptors abolished the hypermigratory phenotype. This autocrine GABAergic activation was crucial for DCs to act as Trojan horses in *T. gondii* dissemination *in vivo*.

This thesis describes the expression of the voltage dependent Ca^{2+} channel $Ca_V1.3$ in murine DCs. We found that GABAergic activation was linked to $Ca_V1.3$ and that selective blockade or knockdown of the latter abolished *T. gondii* induced hypermotility in DCs. This fast-acting signaling axis may allow rapid parasite manipulation of host cell migration.

In summary, we have shown that *T. gondii* exploits non-canonical migration-related host signaling pathways to enhance its dissemination. Several cell types are likely involved as Trojan horses at different stages of the infection process, e.g. DCs, T cells and microglia. This work also illustrates that the study of parasite-host interactions may further our understanding of basic cellular mechanisms, such as motility, and uncover novel signaling pathways in the host.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Toxoplasma gondii är en intracellulär parasit som kan infektera alla varmblodiga djur, inklusive uppskattningsvis 30 % av världens befolkning (15-25 % i Sverige). Parasiten sprids genom smittad mat eller vatten och orsakar potentiellt livslång latent infektion i hjärnan. De flesta upplever milda till obefintliga symptom vid smittotillfället men personer med nedsatt immunförsvar riskerar att drabbas av livshotande toxoplasmos i hjärnan. Att smittas under graviditet innebär också risk för att parasiten infekterar fostret, vilket kan leda till missfall eller allvarliga fosterskador.

Vi studerar de tidiga stadierna av infektionsförloppet då parasiten sprids i kroppen och når viktiga organ som hjärnan och livmodern. Under denna fas infekterar parasiten bl.a. vita blodkroppar (immunceller) och använder dem som ”trojanska hästar” för att färdas i kroppen på ett snabbare och säkrare sätt. *Toxoplasma* manipulerar cellerna och förmår dem att röra sig fortare (bli ”hypermigratoriska”), vilket kräver sofistikerad kommunikation mellan parasit och värdcell. Det senare skulle sannolikt kunna utgöra en angreppspunkt för behandling av *Toxoplasma*-infektion. Målet med min avhandling har varit en mer detaljerad beskrivning av spridningsförloppet vid akut infektion och i den immunnedsatta hjärnan, med fokus på värdcellers rörelseförmåga och intracellulära kommunikation.

Vi har tidigare modellerat sjukdomsförloppet vid immunnedsättning hos en bärare av *Toxoplasma* med hjälp av möss som behandlats med ett kortison-besläktat preparat. I modellen blossar den latenta infektionen upp, vilket orsakar kraftig inflammation i hjärnan och attraherar immunceller till platsen. I den föreliggande studien visade vi att en grupp celler nära den aktiva infektionen i hjärnan, s.k. mikroglia, också blev hypermigratoriska när de infekterades. När immunceller förstörde *Toxoplasma*-infekterade celler undkom parasiten för att sedan infektera själva immuncellerna, vilket potentiellt bidrar till parasitens spridning.

Vi har visat att ett signalsystem som hämmar signalöverföring i hjärnan, det s.k. GABA-systemet, även används av de immunceller vi studerar. GABA:s fysiologiska funktioner i dessa celler är fortfarande okända men våra resultat indikerade att systemet utnyttjas av *Toxoplasma* för sin spridning. Blockering av systemet hämmade infekterade immuncellers rörelseförmåga och samma blockering ledde i våra djurförsök till fördröjd spridning av parasiten i värddjuret.

I en detaljerad undersökning av immunceller direkt efter kontakt med parasiter, kunde

vi se att cellerna radikalt ändrade sitt utseende och rörelsemönster inom 10 minuter efter infektion. Denna tidsrymd kan jämföras med immuncells-aktivering vid inflammation som är i storleksordningen timmar, eftersom cellens uttryck av gener förändras. Transport av information i celler kan även ske i form av flöden av molekyler och joner. Genom att visualisera dessa flöden i immunceller identifierade vi en ny jonkanal med koppling till GABA-systemet. I likhet med detta ledde blockering till förlust av rörelseförmåga hos infekterade immunceller. Aktivering av jonkanaler kan innebära mycket snabb intracellulär överföring av information, vilket stämmer överens med att immuncellerna reagerade snabbt på *Toxoplasma*-infektion.

Sammanfattningsvis har vi visat att *Toxoplasma* utnyttjar intracellulär kommunikation för att förändra sina värdcellers beteende och sannolikt öka sina möjligheter till överlevnad och spridning. Vi kan också konstatera att våra studier lett oss till upptäckten av en ny signalväg i immunceller som kan utgöra en hittills outforskad länk mellan hjärnan och immunförsvaret. Att undersöka en parasit som utvecklats tillsammans med däggdjur under miljontals år kan därmed vara en viktig källa till kunskap om oss själva.

LIST OF PAPERS

The results discussed in this thesis are presented in three publications and one manuscript. The papers are referred to in the text by roman numerals.

I. Dellacasa-Lindberg I, Fuks JM, Arrighi RB, Lambert H, Wallin RP, Chambers BJ, Barragan A. Migratory Activation of Primary Cortical Microglia upon Infection with *Toxoplasma gondii*. 2011. Infect. Immun. Aug;79(8):3046-52.

II. Fuks JM*, Arrighi RB*, Weidner JM*, Kumar Mendu S, Jin Z, Wallin RP, Rethi B, Birnir B, Barragan A. GABAergic Signaling Is Linked to a Hypermigratory Phenotype in Dendritic Cells Infected by *Toxoplasma gondii*. 2012. PLoS Pathog. 8(12):e1003051.
*Contributed equally

III. Weidner JM, Kanatani S, Hernandez-Castañeda MA, Fuks JM, Rethi B, Wallin RP, Barragan A. Rapid Cytoskeleton Remodelling in Dendritic Cells Following Invasion by *Toxoplasma gondii* Coincides with the Onset of a Hypermigratory Phenotype. 2013. Cell Microbiol. Oct;15(10):1735-52.

IV. Fuks JM, Rebellato P, Varas-Godoy M, Uhlén P, Barragan A. $Ca_V1.3$ Signaling Mediates $GABA_A$ Receptor-Induced Migratory Activation of Dendritic Cells Infected by *Toxoplasma gondii*. Manuscript.

CONTENTS

AIMS OF THE THESIS

Main hypothesis

Toxoplasma gondii modulates motility-related host cell signaling to disseminate in the organism.

The work has been carried out according to 3 specific aims:

1) To further characterize *T. gondii* infected immune cells and their role in parasite dissemination during acute and reactivated infection *in vitro* and *in vivo*.

2) To investigate the role of GABAergic signaling in dendritic cells in relation to *T. gondii* infection and the hypermigratory phenotype.

3) To study the role of Ca^{2+} signaling in dendritic cells infected by *T. gondii*, specifically focusing on migratory mechanisms.

LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ALCAM	Activated Leukocyte Cell Adhesion Molecule
Arp	Actin Related Protein
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
Ca_V	Voltage Dependent Ca^{2+} Channel
CCL	C-C Motif Chemokine
CCR	C-C Chemokine Receptor
CD	Cluster of Differentiation
CDC	Cell Division Control Protein
CNS	Central Nervous System
CRAC channel	Ca^{2+} Release-Activated Ca^{2+} Channel
CXCL	Chemokine (C-X-C motif) Ligand
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ESA	Excreted-Secreted Antigens
Fln	Filamin
GABA	γ-Aminobutyric Acid
GAD	Glutamate Decarboxylase
GAT	GABA Transporter
GPCR	G-Protein Coupled Receptor
GPI	Glycosylphosphatidylinositol
GRA	Dense Granule Protein
GTP	Guanosine Triphosphate
HIV	Human Immunodeficiency Virus
HSPC	Hematopoietic Stem and Progenitor Cell
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IP_3	Inositol Trisphosphate
IVIS	*In Vivo* Imaging System
LFA	Lymphocyte Function-Associated Antigen
LPS	Lipopolysaccharide

MAPK	Mitogen Activated Protein Kinase
MHC	Major Histocompatibility Complex
MIC	Microneme Protein
MMP	Matrix Metalloproteinase
MyD88	Myeloid differentiation primary response gene (88)
NF-κB	Nuclear Factor κB
NK cell	Natural Killer cell
PGE	Prostaglandin E
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein Kinase C
PV	Parasitophorous Vacuole
RON	Rhoptry Neck Protein
RyR	Ryanodine Receptor
shRNA	Short Hairpin RNA
TLR	Toll-Like Receptor
RNA	Ribonucleic Acid
SDF	Stromal Cell Derived Factor
SOCE	Store-Operated Ca^{2+} Entry
STIM	Stromal Interaction Molecule
T_H	T Helper Cell
TNF	Tumor Necrosis Factor
TRPM	Transient Receptor Potential Melastatin
VCAM	Vascular Cell Adhesion Molecule
VDCC	Voltage Dependent Ca^{2+} Channel
WASp	Wiscott-Aldrich Syndrome Protein

1) DENDRITIC CELL MIGRATION IN HEALTH AND DISEASE

Since their initial description in lymphoid organs (278), dendritic cells (DCs) have been recognized as a vital component of the immune system. DCs exhibit a unique combination of immune surveillance and migratory capacity. By identifying potentially dangerous agents and initializing an immune response, DCs mediate a link between the innate and adaptive immune system (46). Below, some key aspects of DC functions are introduced, with an emphasis on the role of migration in these processes.

1.1) The Role of DCs in the Immune Response

There is considerable heterogeneity among DCs and several ways of categorizing different subsets have been proposed. Based on lineage, a distinction can be made between conventional DCs, including tissue resident subsets, and non-conventional DCs, e.g. inflammatory DCs (153). Tissue residing DCs, e.g. in gut mucosa and skin, sample the local environment by pinocytosis (soluble antigens), or phagocytosis (non-soluble antigens) (85, 207, 277). Inflammatory DCs derive from monocytes and are recruited to sites of inflammation where they perform similar functions as tissue resident DCs (268).

DCs express a broad range of pattern recognition receptors, e.g. Toll-like receptors (TLRs) and nucleotide-binding and oligomerization domain (NOD)-like receptors, which recognize conserved molecular patterns associated with infection and tissue damage. Well studied examples include gram-negative bacterial lipopolysaccharide (LPS, TLR4) and double stranded or endogenous RNA (TLR3) (48). TLR triggering induces transient DC arrest and enhances uptake of antigen (87), which is then processed to peptides and presented on the cell surface on major histocompatibility complex (MHC) molecules.

DCs having undergone this process are commonly referred to as “mature” and will leave the periphery, enter the lymphatics and migrate to secondary lymphoid organs (62). T lymphocytes (T cells) that have not been exposed to antigen circulate continuously and encounter mature DCs in the lymph nodes, whereupon antigen loaded MHC molecules interact with T cell receptors (202). Recognition of a presented peptide by the T cell receptor in this “immunological synapse”, along with DC co-stimulatory signals such as CD86 and CD40, induces differentiation into effector T cells. Generally, exogenous antigens are loaded on MHC class II and presented to $CD4^+$ T cells and endogenous antigens are presented on MHC class I to $CD8^+$ T cells (125). Presentation of exogenous antigens on MHCI, so-called cross-presentation, can also occur (259).

1.2) Migratory Activity of DCs

Migration can be considered a hallmark feature of DCs, as several of their core functions (e.g. antigen sampling and trafficking to lymph nodes) depend on motility. As such, migration is a highly regulated process that adapts to external and internal factors (244). All motile cells polarize along the axis of migration, extending a 'leading edge' in the direction of movement and retracting the 'trailing edge' at the opposite end. Mechanisms of cell migration can be situated on a scale between adhesion-dependent (mesenchymal) and adhesion-independent (amoeboid) locomotion (126, 164). The integrin family of transmembrane receptors is a major contributor to cell adhesion and adhesion-mediated signal transduction. They are heterodimers of one α subunit (18 described) and one β subunit (8 described) (127). Binding specificity is determined by the subunit composition and includes components of the extracellular matrix (ECM) and surface receptors on other cells (126). Integrins are anchored to the intracellular actin-binding proteins talin and filamin (Fln), providing a direct link between extracellular stimuli and the actin cytoskeleton (52, 204).

Mesenchymal migration operates by consecutive cycles of attachment and detachment, during which the cell exerts force on the extracellular substrate in order to move along or penetrate through it. Cell attachment and force transmission is mainly through integrins (260). Podosomes are transient actin bundle structures that are considered focal points of cell-ECM interactions, degradation and invasion in DCs, macrophages and other myeloid cells. They coordinate several actin-related proteins and integrins in a ring around the actin core with a diameter of about 500 nm and a half-life of 10 min (307). A recent study also proposed a role for podosomes in forming protrusions involved in DC antigen sampling (15).

Highly motile cells, such as DCs and T cells, employ amoeboid migration when migrating in interstitial tissues. This rapid mode of migration is not proteolytic and depends less on cell-matrix interactions, being powered by a focused flow of actin (163, 330). The type of migration employed depends on the extracellular environment and activation status of the cell. Lämmermann and colleagues have shown that long-distance interstitial DC migration does not depend on integrins, in contrast to cell surface attachment, crossing of tissue barriers and concerted cell movement (162, 163, 165). Also, macrophages migrating in a 3D matrix employ a podosome-dependent proteolytic mechanism or podosome independent amoeboid migration, depending on the density and composition of the matrix (306).

1.3) Chemotaxis

Chemotaxis is defined as cell migration directed by gradients of chemoattracting

molecules. Many of these are chemotactic cytokines (chemokines), which control the migration patterns of various cell types in the body. Chemokine receptors are G protein coupled receptors (GPCRs) that relay the directional information encoded in chemokine gradients, likely through polarized expression towards the leading edge of migrating cells (208). The expression of chemokine receptors is dynamic, as exemplified by DCs, where maturation induces down-modulation of C-C chemokine receptor (CCR) 5 and up-regulation of CCR7 (251), which mediates a switch in chemokine responsiveness (73). As a result, CCR7 ligands C-C motif chemokine ligand (CCL) 19 and CCL21 selectively recruit mature DCs to secondary lymphoid organs where T cell interactions occur (93, 133, 248, 255, 322). The expressed set of chemokine receptors thus determines the directionality of movement in tissue (135).

1.4) Molecular Mediators of DC Migration

Rho family GTPases cycle between the active (GTP-bound) and inactive (GDP-bound) form as important regulators of central cellular processes, most prominently actin dynamics (317). Rho GTPases Cdc42, Rac1 and Rac2 play important roles in chemotaxis (7, 295, 321) and it was recently shown that DC trafficking to lymph nodes *in vivo* is abolished in $Cdc42^{-/-}$ cells (165). It has been proposed that Rac1 and Rho confers directionality sensing in chemotaxis while Rac2 and Cdc42 regulate actin dynamics (239, 285). CCR7 ligation induces RhoA dependent activation of the integrin lymphocyte-associated antigen (LFA)-1, inducing a state of high affinity for the ligand intercellular adhesion molecule (ICAM)-1 (101). This process has been implicated in leukocyte rolling and diapedesis during transendothelial migration (113). Also, DC ICAM-1 binds LFA-1 on T cells during immunological synapse formation, increasing interaction affinity and providing an additional co-stimulatory signal for T cell activation (147).

At the center of cell migration is the force generation necessary for the dynamic formation of membrane protrusions. According to present models, membrane protrusions are formed by polymerization of monomeric actin into filamentous (F) actin or by hydrostatic pressure caused by contraction of the actin network (115, 155, 166). Actin contraction is mainly mediated by type II myosin motor proteins (216), which have been proposed to be essential for DC passage through narrow gaps and pores (88, 163). Polymerization of actin is catalyzed by Rho GTPase activated nucleation factors, such as formins and the actin-related protein (Arp) 2/3 complex, producing un-branched and branched actin filaments respectively. The polymerization process is precipitated by profiling-mediated recruitment and activation of monomeric actin (231). Cdc42 regulates Arp2/3 via activation of Wiskott-Aldrich Syndrome protein (WASp), which is required for the formation of podosomes in macrophages and DCs (39, 174, 186). WASp deficient DCs also display impaired migration to lymph nodes and as a result, reduced T cell priming (35, 63). Flns

participate in crosslinking actin filaments and Lamsoul and colleagues recently showed that FlnA expression increases in DCs upon maturation and is associated with podosome formation and proteolytic degradation (167).

The diaphanous-related (Dia) formin mDia1 has been shown to be involved in DC transmigration and *in vivo* chemotaxis (297). In tumor cells, Rho GTPases differentially regulate the mode of migration, RhoA promoting an amoeboid contraction dependent phenotype while Rac1 is associated with mesenchymal migration (252). mDia1 positive feedback on RhoA has also been shown to promote matrigel invasion in a metastasizing cell line (146). The relevance of Rho GTPases in the context of infection is illustrated by the fact that they are targeted by many bacterial infections including *Yersinia*, *Salmonella*, *Clostridium* and *Escherichia* (139, 169, 242, 312).

2) TOXOPLASMA GONDII

Toxoplasma gondii is an obligate intracellular protozoan parasite belonging to the apicomplexa family. *T. gondii* has a broad range of hosts, as any warm-blooded vertebrate can serve as intermediate host and it can survive and replicate in virtually any nucleated cell (3). It is studied both as a pathogen in its own right and as an important model organism for other apicomplexan parasites, such as *Plasmodium* and *Cryptosporidium* (145). *T. gondii* is a global zoonotic pathogen, causing infection on all populated continents (217). Seroprevalence varies greatly between countries, e.g. 14,0 % in Stockholm, Sweden and 74,7 % in Recife, Brazil (84, 232).

2.1) Life Cycle and Population Structure

As definitive hosts, felines permit parasite gametogenesis and sexual recombination, followed by formation of environmentally stable oocyts, which are then shed with the feces. An ingested oocyst will rupture in the small intestine and release sporozoites that invade cells of the intestinal epithelium and differentiate into tachyzoites (78). During the acute phase of infection, tachyzoites disseminate throughout the host, reaching distant and immunopriviledged sites such as the brain, eyes, testes and placenta. This dissemination entails the crossing of multiple biological barriers, such as the intestine, the blood brain barrier (BBB), the blood-retina barrier and the placenta (18). Tachyzoites eventually develop into cyst-forming and slow-dividing bradyzoites (235), which is the parasite stage associated with latent chronic infection, and may reside within the host for a long period of time (282). Tissue cysts are preferentially formed in the central nervous system (CNS) and in muscle tissue, but are also observed in visceral organs. Ingestion of tissue cysts by a feline through predation of an infected intermediate host completes the life cycle of *T. gondii* (78).

While *T. gondii* is the only species assigned to the *Toxoplasma* genus, early population genetics studies identified three dominant clonal lineages (types I, II and III) with distinct virulence and species distributions (120, 270). Genotyping has identified type II as the most prevalent in human infections (119, 120), although discrepancies have been observed in patients with certain clinical manifestations (106, 143). These and similar observations, along with analysis of a wider range of host species, has revealed a more complex population structure with several “atypical”, and often disease-causing, strains represented in the gene pool (326). Still, the canonical three types remain the best characterized and are used experimentally to represent some of the genetic diversity within the *T. gondii* population structure. In this thesis, the three types have been represented by the strains RH-LDMluc (type I), PTGluc and PRU-RFP (type II) and CTGluc (type III) (69, 116, 222).

2.2) Toxoplasmosis

Infection via ingestion of tissue cysts in infected meat or oocysts from environmental contaminations generally produces mild or no symptoms in otherwise healthy human hosts. Likewise, the establishment of the chronic phase is not associated with clinical manifestations in immunocompetent individuals (196). However, epidemiological studies have linked *T. gondii* seropositivity to a higher incidence of psychiatric conditions such as self-directed violence, violent suicide attempts and schizophrenia (219, 303). The most serious clinical outcomes are seen in immunocompromised patients, e.g. AIDS patients or organ transplant recipients on immunosuppressive drugs, where latent tissue cysts may reactivate in the CNS. The switch from latent bradyzoites to rapidly dividing tachyzoites causes potentially fatal toxoplasmic encephalitis (10, 334). Infection during pregnancy can result in miscarriage, still birth or, particularly when infected during the first or second trimester of pregnancy, congenital toxoplasmosis (196). Congenital infection is associated with birth defects such as chorioretinitis, intracranial calcification and other CNS abnormalities (112).

2.3) Lytic Cycle

Tachyzoite motility is based on a parasite intrinsic mechanism termed “gliding motility”, powered by actin polymerization and a myosin motor protein, which are also necessary for host cell invasion (75, 192, 249). Invasion is a multi-step process involving the orientation of the tachyzoite apical end towards the host cell membrane followed by sequential discharge of secretory organelles termed micronemes, rhoptries and dense granules (40, 199). Several microneme proteins (MICs) contain adhesion domains that have been proposed to mediate initial generic binding to the host cell (42). Rhoptry neck proteins (RONs) and MICs are involved in the formation of a tight junction between the host cell and parasite, which the parasite passes through using mainly actinomyosin propulsion (4, 26, 81). This “moving complex” forms when RON2 is injected into the host cell membrane, where it interacts with apical membrane antigen (AMA) 1 and RONs 4, 5 and 8 (26, 156, 157). *T. gondii* profilin-like protein (toxofilin) has been proposed to contribute to tachyzoite invasion by depolymerizing host actin and facilitating entry (70).

Upon active invasion, the parasite establishes an intracellular compartment termed the parasitophorous vacuole (PV) that is non-fusogenic due to exclusion of proteins required for recruitment of endo/lysosomes (197, 198). However, Zhao and colleagues recently demonstrated that even phagocytosed tachyzoites are capable of actively “invading” the host cell from the phagolysosome, thus forming a PV (343). The parasite also blocks autophagy-mediated degradation by activating the epidermal growth factor receptor (EGFR) cascade (201). The PV recruits host organelles such as endoplasmic reticulum

(ER) and mitochondria and allows for parasite replication, salvaging of nutrients from the host and export of parasite effector molecules that subvert host cell functions (56, 94, 123).

Parasite egress from the host cell employs several of the molecular mediators involved in invasion (118, 269). Permeabilization of the PV membrane and host cell plasma membrane is induced, followed by active parasite exit, although the role of gliding motility in natural egress is unclear (31). The factors triggering egress are incompletely understood, although sensing of host cell ion balance, likely indicative of the extracellular environment, plays an important role (98, 179, 191). Egress is also triggered by host cell exposure to $CD8^+$ T lymphocyte killing mechanisms, i.e. Fas receptor-Fas ligand interaction or perforin, protecting the parasite from immune destruction (227). Tomita and colleagues reported that the time between invasion and egress *in vivo* is considerably shorter than *in vitro*, which was attributed to premature egress induced mainly by inflammatory macrophages (302).

2.4) Immune Response to *T. gondii*

DCs play a key role in immunity against *T. gondii* as important producers of the pro-inflammatory cytokine interleukin (IL)-12 (5). Indeed, depletion of DCs abolishes IL-12 production during *T. gondii* infection, severely impairing host survival (176). IL-12 promotes a T helper cell type 1 (T_H1) response by inducing T cell differentiation and stimulating their secretion of interferon (IFN)-γ (344). Early studies identified production of IFN-γ as a major protective factor in *T. gondii* infection (205, 289). Beiting and colleagues recently showed that infection with the closely related apicomplexan parasite *Neospora caninum* activates TLR3 and an IFN-α/β response, which is actively suppressed in favor of an IFN-γ response during *N. caninum*/*T. gondii* co-infection (21). Several immune cells contribute to the production of IFN-γ at different stages of *T. gondii* infection, including neutrophils, natural killer (NK) cells and T cells, enhancing growth restriction and destruction of intracellular parasites (335). NK cells are also able to kill extracellular tachyzoites and infected cells, which however does not appear to significantly contribute to parasite control (149). IFN-γ receptor KO mice are unable to control even low doses of a low virulent type III parasite, rapidly exhibiting high parasite loads in visceral organs and in the brain (69). In mice, toxofilin is recognized by TLR11 and TLR12, inducing MyD88 mediated IL-12 production (148, 336). Toxofilin has been implicated in parasite motility and invasion and is secreted during these early processes (70, 178, 229). Additionally, TLR2 and TLR4 are activated by glycosylphosphatidylinositol (GPI)-linked surface proteins and the endosomal TLR7 and TLR9 by *T. gondii* RNA and DNA (9, 64, 200). Humans lack functional expression of TLR11 or TLR12 and as these have been considered crucial for parasite control in mice, the details of innate immune detection of *T. gondii* in humans are less well understood (100, 335).

Protective immunity against *T. gondii* is characterized by the maintenance of balance between inflammatory and anti-inflammatory mediators. T_H1 cells produce auto-regulatory IL-10, which down-modulates production of inflammatory cytokines (e.g. IL-12) by DCs and macrophages (212). IL-10 also protects the small intestine from necrosis during *T. gondii* infection (290). Loss of the T_H2 associated cytokines IL-4 and IL-33 increase parasite loads and the levels of IFN-γ and pro-inflammatory mediators, causing aggravated pathology (138, 241, 292). Production of IL-27 during inflammatory conditions of high IL-12 has anti-proliferatory effects that dampen T cell expansion and associated pathology (124). Adequate T cell responses are also necessary for long-term control of *T. gondii* latent infection, as seen in depletion studies and in patients with AIDS or on immunosuppressive therapy (90, 99, 223). Antibody responses against *T. gondii* contribute to parasite control by blocking parasite invasion and opsonizing extracellular parasites, increasing killing by phagocytosis and the complement cascade (57, 254, 310).

T. gondii control in the CNS is dependent on infiltrating immune cells and resident brain cells such as microglia and astrocytes (41). Microglia have been described as CNS resident macrophages and are involved in homeostatic processes, e.g. tissue remodeling and immune functions, e.g. antigen presentation and cytokine production (234). Astrocytes are the most abundant brain cell type and form part of the blood brain barrier (BBB, Chapter 3.2) (329). Neurons, astrocytes and microglia are permissive to parasite proliferation and stage conversion *in vitro*, although in brain tissue, cysts are preferentially found in neurons (41, 89, 193). Microglia and astrocytes produce IFN-γ, in addition to infiltrating $CD8^+$ and $CD4^+$ T cells observed upon parasite infection in the CNS (286, 319). $CD8^+$ T cell can also directly eliminate *T. gondii* cysts (291). Several resident cell types have been proposed to recruit T cells to the infected brain (141). Blood vessel endothelial cells up-regulate ICAM-1, vascular cell adhesion molecule (VCAM) and MHCII in response to IFN-γ (65). Several chemokines are up-regulated in the encephalitic brain, including CCL2 and CXCL10 by astrocytes and CCL5 and CXCL9 by microglia. Also, CCL5-expressing leukocytes have been observed in close vicinity of *T. gondii* cysts in the brain (280). IL-10 production by microglia and infiltrating T cells contributes to immunomodulation similarly to the periphery (66, 258).

3) TROJAN HORSE DISSEMINATION OF *T. GONDII*

3.1) Dissemination During Acute Stage Infection (paper III)

The route of dissemination of *T. gondii* has long been the subject of investigation and debate, yet is only partially understood. Extracellular tachyzoites are migratory and can penetrate ECM and polarized cell monolayers, which is dependent on MIC2-ICAM-1 interactions and intrinsic gliding motility (17, 19, 96, 108). The parasite is detected in mesenteric lymph nodes, spleen and brain within hours to days of infection via oral, intraperitoneal or intragastrical administration (50, 79, 107, 284, 339). Recent work has shown that intracellular tachyzoites are the major contributors to dissemination and that $CD11b^+$ and $CD11c^+$ cells are parasitized early and to a greater extent relative to other cell types (47, 58, 305). Trafficking to secondary lymphoid organs is also a key function of DCs, making them a potentially attractive target cell for parasite subversion as a Trojan horse (Chapter 2.1). Upon invasion by a *T. gondii* tachyzoite, DCs exhibit a "hypermigratory phenotype" characterized by enhanced transmigration through porous membranes and penetration of polarized monolayers, as well as increased parasite dissemination *in vivo* (see Chapter 6.2 for definitions). Infected DCs thus contribute to the shuttling of parasites within the host and allow more rapid access to restricted organs such as the brain (160, 161). Early studies showed that the hypermigratory phenotype is abolished by pertussis toxin treatment but is independent of MyD88-mediated TLR activation and signaling via chemokine receptors CCR5 and CCR7 (160). This should also be viewed in the context that knockouts of TLRs 1, 2, 4, 6 or 9 did not impact parasite loads in mice inoculated with extracellular tachyzoites (116). The induction of hypermigration has been confirmed for a number of *T. gondii* isolates belonging to types I, II and III. Interestingly, type II strains consistently induced a higher degree of transmigration in DCs than type I strains, type III strains being intermediate (161). Also, infection of DCs with *N. caninum* induces an increased rate of transmigration across an *in vitro* model of the placental barrier, as well as potentiates parasite dissemination *in vivo* (53).

In order to better understand the signaling pathways involved in the onset of the hypermigratory phenotype, we resolved to study the early events following parasite invasion in DCs. We developed a motility assay where DCs migrate through a collagen matrix cast on a chamber slide and are followed by live imaging (Chapter 6.1). Using fluorescent parasite lines, cell tracking thus allows differentiation between infected and non-infected DCs and monitoring of individual DCs on a time scale of minutes post infection. The onset of the hypermigratory phenotype was previously related to an increased rate of DCs transmigration at 3 h post incubation with *T. gondii* (160). We

showed that infected DCs display a significantly increased motility as early as 10 min post incubation with tachyzoites. In agreement with this finding, the onset and maintenance of hypermotility was independent of *de novo* protein synthesis by the host cell and by the parasite, for at least 6 h (Paper III). This suggests that the proteins necessary for a rapid migratory response are present in DCs at the point of invasion. Koshy and colleagues recently showed that *T. gondii* is capable of injecting rhoptry proteins into cells it does not productively invade (150), however we did not observe any migratory activation in the non-infected bystander population. Using the recently described drug 4-bromophenacyl bromide (237), we showed that parasite attachment without rhoptry secretion was insufficient to induce hypermotility (Paper III). Parasite invasion was thus a requirement for hypermotility, in line with previous results that neither exposure to parasite lysate, excretory/secretory antigen (ESA), infected DC supernatant or phagocytosis of heat-killed parasites is sufficient to induce the hypermigratory phenotype in DCs (160, Paper II, Paper III). Collectively, these results indicate that the molecular trigger(s) of hypermotility are not secreted prior to invasion and are not present in the extracellular environment.

The onset of hypermotility coincided with a dramatic rearrangement of the DC cytoskeleton, including the dissolution of adhesive podosome structures, rounding of the cell body, the appearance of veils and displacement of CD18 and CD11c integrins (Paper III). In this context, we interpret the cytoskeletal alterations observed in *T. gondii* infected DCs as indicative of a shift in migratory capacity and mode of migration. Indeed, previous reports indicate that RhoA dependent loss of podosomes is a prerequisite for fast amoeboid migration in mature DCs (39, 308, 309). Podosome dissolution has previously been linked to prostaglandin signaling (308) and *T. gondii* infection in macrophages induces PGE2 production (221). However, we saw no effect on podosome dissolution or hypermotility when DCs were treated with inhibitors of cyclooxygenase, the PGE2 synthesizing enzyme (Paper III).

3.2) Chronic Stage and Reactivated Infection (paper I)

Reactivated (recrudescent) toxoplasmosis in the CNS is the most common cause of focal brain lesions in patients infected with HIV, leading to significant morbidity and mortality (223). In contrast to the acute phase, where peripheral organs carry high parasite loads, parasite replication during the recrudescent phase is mainly localized to the CNS (69). The CNS is separated from the circulating blood by the BBB, which controls the transport of nutrients and signaling molecules as well as trafficking of immune cells. Structurally, the BBB consists of the tight junction linked capillary endothelium, surrounded by the perivascular ECM of the basement membrane and pericytes. Lining microglia and cellular processes from astrocytes and neurons modulate BBB properties, such as permeability and transport (Fig 1) (1). Parasite dissemination within the CNS during the recrudescence

process is poorly understood, though it is likely of importance for disease progression. CNS resident microglia have been proposed to act as Trojan horses in an *ex vivo* model system for CNS infection by *Listeria monocytogenes*, carrying the bacteria inside the tissue (238). Similar observations have been made for monocytes that migrate to the brain in an IFN-γ dependent manner (76, 77). During toxoplasmic encephalitis, microglia up-regulate the ICAM-1 ligands LFA-1 and macrophage-1 antigen (Mac-1), as well as ICAM-1 itself (67). Human immunodeficiency virus (HIV)-1 has also been reported to use a Trojan horse mechanism to enter the CNS (211, 225). HIV-1 trans-activator protein induces secretion of CCL2 and other chemokines in microglia, eliciting migration through autocrine activation of CCR2 (60, 83). This was recently confirmed by overexpression of CCR2 in primary microglia (134). These results may also play a role in the *T. gondii* recrudescence process since HIV is a common underlying infection in patients with toxoplasmic encephalitis (311).

In order to study parasite-host cell interactions during recrudescent toxoplasmosis we used a combined approach of a mouse model of chronic *T. gondii* infection and primary cultures of glial cells. The recrudescence model is based on chronic infection with a non-virulent, cyst forming and luciferase expressing strain (CTG luc) of *T. gondii*. Infection progression is monitored through bioluminescence imaging and when the chronic stage is reached, the animals are immunosuppressed by dexamethasone treatment (69). The distribution of reactivated cysts thus produced resembles those observed in human patients (206).

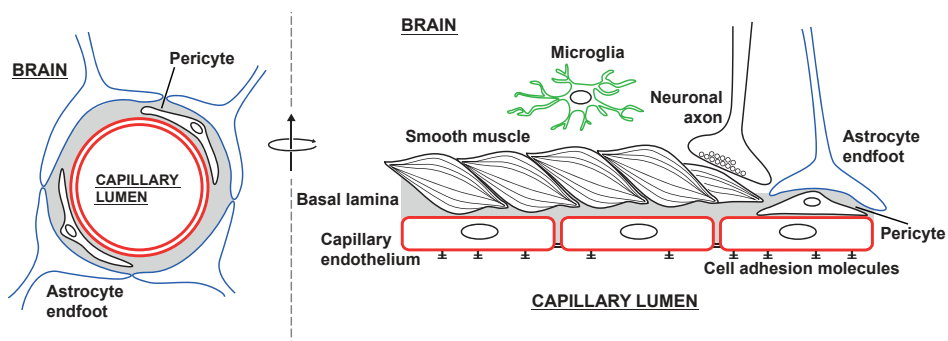

FIGURE 1. Schematic representation of the blood-brain barrier. Transvers (left) and longitudinal (right) cross sections are shown. The capillary endothelium is depicted in red, astrocytes in blue and microglia in green.

Immunohistochemical analysis of recrudescent brain tissue showed significant activation of astrocytes and microglia in and around foci of replicating parasites (Paper I). Given the important roles ascribed to these CNS native cells during *T. gondii* infection (Chapter 2.4),

we proceeded to investigate their role in the recrudescence process. To directly assay the ability of microglia and astrocytes to function as Trojan horses, we compared their *in vitro* transmigration capacity and observed that microglia responded to infection with increased transmigration, as shown for DCs, while astrocytes did not. Infection also enhanced the capacity of microglia to penetrate astrocyte monolayers (Paper I).

Characterizing microglia maturation markers, we showed that *T. gondii* infection induces moderate up-regulation of CD54 (ICAM-1) and MHCI, while expression of co-stimulatory CD86 and MHCII was maintained (Paper I). Since IFN-γ is secreted by many cell types, including microglia (286), during immune responses against *T. gondii* (Chapter 2.4), we compared maturation marker expression of IFN-γ-stimulated infected and non-infected microglia. We observed a down-modulation of the IFN-γ response in the infected cells for all four markers as early as 6 h post infection. To establish whether this modulation of molecules involved in T cell interactions (Chapter 1.1) affected parasite spread, we employed a T cell killing assay. Infected microglia and astrocytes displayed increased sensitivity to $CD8^+$ T cell mediated killing, which correlated with increased numbers of infected T cells (Paper I). The data suggest that *T. gondii* escapes the host glial cell targeted for killing, only to infect the effector T cell, as previously shown for infected DCs and NK cell or T cell mediated killing (51, 226, 227). In accordance with these and previously published results (257), we observed recruitment of $CD8^+$ T cells to replicative parasite foci, which puts them in close proximity of activated (and potentially infected) microglia and astrocytes in the CNS of encephalitic mice (Paper I).

T. gondii infected microglia display a similar migratory activation as infected DCs, suggesting an extension of the Trojan horse concept to this resident CNS cell type, as was previously done with macrophages (159). It is tempting to speculate that these similarities are connected to the shared origin of microglia, macrophages and DCs in myeloid progenitors during embryonal development (102). The results also highlight the role of host-parasite interactions in the actual pathology of the infection. Zhang and colleagues recently showed that activated microglia significantly contribute to neuronal damage during toxoplasmic encephalitis. The neurotoxic activity was connected with the production of pro-inflammatory cytokines and induction of iNOS (342). A similar role has been described in cell death during neuronal development, where microglia also participate in clearance of dying cells and debris (187, 316). *T. gondii* infection induces NF-κB dependent matrix metalloproteinase (MMP) secretion in astrocytes, causing fibronectin degradation *in vitro* (181, 182). Release of chemokines and growth factors from dying neurons attracts microglia and stimulates phagocytosis (209, 210). Microglia also exhibit immunomodulatory activities during chronic and acute *T. gondii* infection (Chapter 2.4). This, in combination with our finding that activated microglia and astrocytes are found in close vicinity of foci of replicating tachyzoites (Paper I), suggests that neuronal damage

in recrudescent encephalitis is a consequence of a disturbed host-parasite balance, where both the parasite and the immune system contribute to tissue destruction.

3.3) DC Chemotaxis in *T. gondii* Dissemination (paper II and III)

The rapid dissemination of *T. gondii* to secondary lymphoid organs, along with a reported link between infection and chemotactic responses in DCs (71) prompted us to investigate the role of chemotaxis in the hypermigratory phenotype. We found that DCs chemotax along a gradient of CCL19 at 24 h post *T. gondii* infection, to the same extent as LPS stimulated DCs. The velocity at which infected DCs chemotaxed was however significantly higher than that of LPS-matured DCs (Paper II), which is in line with previous results stating that the hypermigratory phenotype is regulated independently of chemotaxis (160). In agreement with these findings, infected DCs also up-regulated CCR7 and down-regulated CCR5 (Paper II, Paper III).

Interestingly, a down-modulation of CCR5 was observed in the non-infected bystander DCs in the infected population, while these cells did not exhibit chemotaxis or upregulation of CCR7 (Paper II, Paper III). It is tempting to speculate that since the down-modulation of CCR5 occurs shortly after infection compared to the up-regulation of CCR7 (Paper III), they contribute to different aspects of *T. gondii* dissemination. The loss of CCR5 and the onset of hypermotility could allow parasitized DCs to rapidly leave the periphery, while CCR7 expression directs them to secondary lymphoid organs, a hot spot of immune cell interactions.

It has been known for some time that soluble *T. gondii* cyclophilin-18 induces chemotaxis and IL-12 production in DCs via CCR5 ligation (6). Also, treating DCs with ESA or concentrated recombinant *T. gondii* dense granule protein (GRA) 5 has been shown to upregulate CCR7 expression and chemotaxis (72, 224). These events collectively contribute to a shift in the migratory patterns of DCs (Chapter 1.2). It is not surprising therefore, that the process of altering the chemokine receptor profile in DCs is complex and highly controlled, and that the individual steps may be independently regulated.

In summary, we have built on previous observations that DCs become hypermigratory upon *T. gondii* infection, by showing that they also migrate at higher velocities in a collagen matrix (hypermotility) (160, Paper III). This migratory activation coincides with morphological alterations, including redistribution of actin and integrins, and differential expression of chemokine receptors (Paper II, Paper III). Thus, it appears that the hypermigratory phenotype progresses over time and that DCs acquire traits at different stages of this process, acting in conjunction or synergistically. How each trait specifically contributes to parasite dissemination *in vivo* will be the subject of further studies.

3.4) *T. gondii* Dissemination to the CNS

In a detailed investigation of *T. gondii* infected mice, an increased number of DCs was found around plaques of replicating parasites in infected villi in the small intestine, while very few DCs were detected inside plaques (105). The authors propose an early DC migration wave out of parasite plaques as a potential cause of this observation, which goes well in line with our findings concerning the early onset of the hypermigratory phenotype of DCs (Paper III). Also, Coombes and colleagues observed influx of neutrophils into the small intestine of *T. gondii* infected mice. When infected, these neutrophils remained motile and could carry parasites across the intestinal epithelium into the lumen (55).

While the mechanisms by which tachyzoites enter the CNS are still largely unknown, three hypothetical models have been proposed (158): extracellular tachyzoites disseminate and traverse of the BBB (i), a Trojan horse is used for transmigrating across the BBB (ii) or Trojan horse mediated dissemination is followed by induction of host cell lysis at the BBB and extracellular tachyzoite transmigration (iii). Although the involvement of (i) and (iii) has not been disproven, much recent investigation has focused on potential molecular mediators of (ii) (190). Brain endothelium upregulate ICAM-1 and VCAM-1 during toxoplasmic encephalitis and IFN-γ stimulation (67, 154). Severity of toxoplasmic encephalitis also correlates with increased VCAM-1 and activated leukocyte cell adhesion molecule (ALCAM) expression and higher permeability of the BBB (273). In line with this, Wang and colleagues have shown that $CD8^+$ T cell recruitment into the CNS is severely impaired in *T. gondii* infected IFN-$\gamma^{-/-}$ mice, which was rescued with recombinant IFN-γ treatment. Also, blocking α4 integrin/VCAM-1 binding abolishes recruitment of $CD8^+$ and $CD4^+$ T cells (246, 318). Up-regulation of CCL21 has been observed in filamentous structures in the encephalitic brain, associated with $CD8^+$ T cells (328). The putative attraction of DCs by these structures was not addressed in this study. Lachenmaier and colleagues showed that *T. gondii* infected antigen presenting cells shuttles parasites across an *in vitro* BBB model and carries parasites to the brain when injected intravenously (154). DCs also enter the encephalitic brain through a mechanism dependent on LFA-1 but independent of chemokine receptors CCR2, CXCR3, CX3CR1, CCR5 and CCR6 (137). *T. gondii* infected DCs migrate across retinal vascular endothelium in a CAM dependent manner (95). Infected monocytes under shear stress have been shown to adhere to and transmigrate through human endothelial monolayers in a Mac-1/ICAM-1 dependent manner (109, 304). *T. gondii* C18 induces CCR5 dependent macrophage migration and infection with a C18 overexpressing *T. gondii* strain enhanced recruitment to the intraperitoneal cavity (128, 129). Infected macrophages also exhibit altered adhesion properties and trafficking to mesenteric and cervical lymph nodes (61).

These studies lend support to model (ii), stressing the importance of CAMs, cytokines

and chemokines in modulating immune cell trafficking to the CNS. They also indicate the potential involvement of immune cell types other than DCs as Trojan horses, e.g. T cells, monocytes, macrophages and neutrophils. The contribution of the three hypothetical modes of CNS entry awaits thorough *in vivo* evaluation and may differ with *T. gondii* strains, since the types differ in tachyzoite migratory capacity and induction of the hypermigratory phenotype (19, 161).

4) THE GABAERGIC SYSTEM IN CELL MIGRATION

4.1) GABA in the CNS and in the Periphery

γ-aminobutyric acid (GABA) is a small molecule that was initially described as a plant metabolite before its role as the main inhibitory neurotransmitter in the CNS was discovered in the 1950s (91). Several prokaryotes, e.g. lactic acid bacteria, also produce GABA as part of their metabolism (172). GABA is derived from glutamate by the enzyme glutamate decarboxylase, stored in vesicles and released by so-called GABAergic neurons into the cleft of inhibitory synapses, from where it is cleared by the GABA transporter family (GAT) of plasma membrane solute carriers (38, 262). GABA signals through ionotropic ($GABA_A$) and metabotropic ($GABA_B$) receptors expressed throughout the CNS. $GABA_A$ receptors are heteropentameric Cl^- channels assembled as combinations of 19 different subunits, however only a small subset of the potential combinations are observed *in vivo* (272). $GABA_B$ receptors are G_iPCRs and thus signal via adenylate cyclase (36). Outside the CNS, GABA receptors are found in a variety of compartments, especially endocrine tissues including the pancreas, adrenal gland, prostate and uterus (103, 214). Mounting evidence also suggest that GABA, and other neurotransmitters such as dopamine, adrenaline, acetylcholine, serotonin and glutamate, play important roles in the immune system (170).

4.2) The GABAergic System in Immune Cells

Although the role of GABA as an immunomodulator is not well understood, several types of immune cells have been shown to express components of the GABAergic system (recently reviewed in (136)). T cells were the first immune cells for which evidence of GABA receptor activity was presented (23) and the role of GABA has mainly been investigated in the context of T cell activation and proliferation (30, 299, 320). In connection with these results, GABA has been implicated in the severity of autoimmune inflammation in models of diabetes, multiple sclerosis and rheumatoid arthritis (27, 300, 301). Other studies have proposed that macrophages, neutrophils and T cells themselves produce GABA (74, 236, 281).

GABA/GABA receptor activity has been linked to cell migration in the CNS, where its role depends on the mode of migration, cell type involved, stage of maturation and region of the brain (185). This complexity is mirrored in the few studies performed so far on immune cells, restating the fact that immune cell migration is highly context dependent. Neutrophils express $GABA_B$ receptors that co-localize with Akt and, when activated, induce phosphorylation of Akt and chemotaxis (236). In adult hematopoietic stem and progenitor

cells (HSPCs), SDF-1α-induced chemotaxis was blocked by GABA and $GABA_B$ receptor agonists, which has also been shown for $GABA_A$ receptors in monocytes (263, 327). GABA itself, however, acted as a chemoattractant for umbilical cord HSPCs (338). The role of GABA in DC migration has not previously been addressed.

4.3) GABAergic Signaling in the Hypermigratory Phenotype (paper II)

Investigating potential molecular mediators of the *T. gondii*-induced hypermigratory phenotype, we showed by ELISA that murine and human DCs are capable of producing and secreting GABA into the extracellular milieu (Paper II). This confirms previous results that DCs express GAD65 (27). Upon *T. gondii* infection we observed up to a 5-fold increase in supernatant GABA concentration that was sensitive to inhibition of GAD or GAT. This response was observed upon infection with representative strains from types I, II and III. No increase in GABA production was seen in the non-infected bystander population (Paper II).

Next, we showed by whole cell patch clamping that DCs respond to GABA by producing $GABA_A$ receptor currents and that this response is blocked by $GABA_A$ specific inhibitors. We detected up-regulation of GAT4 at 2 h post infection and increased GABA production at 3 h post infection (Paper II), at which time DCs are also hypermigratory (160). In line with this observation, blocking the GABAergic activation of infected DCs using inhibitors of GABA synthesis, secretion or $GABA_A$ receptors abrogated the hypermigratory phenotype in these cells. Antagonizing $GABA_B$ receptors produced a minute decrease in infected DC transmigration, suggesting that $GABA_A$ receptor activity accounts for the majority of the migratory induction (Paper II). Together with the induction of DC GABA production upon infection, this suggested an autocrine/paracrine GABA signaling cascade, similar to what has been proposed for neuronal migration (184).

GABA production by DCs should also be viewed in the context of immune cell interactions, since it may affect other immune cells expressing GABA receptors, such as T cells (30, 299). It would be interesting to study the impact of increased DC GABA secretion upon *T. gondii* infection on T cell function, as both cell types are key players in protective immunity against the parasite (Chapter 2.2) as well as potential Trojan horses (227, Paper I).

Finally, adoptive transfers showed that GABAergic inhibition of infected DCs abrogated the Trojan horse disseminatory advantage *in vivo* to a similar extent as DC immobilization (160, Paper II). This indicates a central role for GABAergic signaling in the hypermigratory phenotype.

MacRae and colleagues recently described a metabolic GABA shunt in *T. gondii* tachyzoites, suggesting that GABA plays a role in the energy metabolism of the parasite (183). In accordance with our results (Paper II), the authors did not detect parasite secretion of GABA, indicating that the increase in GABA seen in DC supernatant upon *T. gondii* infection is due to stimulated production in the DC itself.

4.4) Cell Motility and Migration – What is the Role of GABA? (paper II & III)

Studies evaluating the invasive and metastasizing capacity of cancer cell lines have shown both positive (13, 131, 177, 274, 340) and negative (49, 122, 298) effects of GABA receptor stimulation. A common feature in these studies is the emphasis on the role of MMPs and MAPK activation in invasion and metastasis. Further studies will address the potential role of GABAergic signaling in DC MMP activity and invasive capacity. We interpret the varying experimental outcomes presented in studies performed on neuronal, immune and metastasizing cells as indicative of differential regulation by GABA of different modes of cell migration.

The observation that non-infected bystander DCs are not hypermigratory, despite being exposed to the same elevated GABA concentration as the infected population (160, Paper II) suggests that GABAergic activation is necessary but not sufficient for the *T. gondii* induced hypermigratory phenotype. Accordingly, stimulation of GABA receptors in non-infected DCs does not significantly alter their transmigration rate. GABAergic inhibition reduced the motility of non-infected DCs but not their transmigration capacity (Paper II). Also, GABA deprived DCs underwent similar morphological changes as untreated DCs upon *T. gondii* infection (Paper III). These observations implicate parasite subversion of separate signaling pathways in DCs, collectively promoting the hypermigratory phenotype. This interpretation is corroborated by the rapid onset of hypermotility (Paper III), while our data also show that the hypermigratory phenotype is transcriptionally regulated over time (Paper II).

We showed that GABAergic inhibition did not abolish directionality in chemotaxing infected DCs but reduced the velocity at which they migrated (Paper II). GABA did not act as a chemoattractant to naïve, LPS-matured or infected DCs (Paper II), in contrast to embryonic neurons, neutrophils and HSPCs (20, 236, 338). These results suggest that GABAergic activation and chemotactic signaling are independently regulated but operate in conjunction in infected DCs, where the former gives the signal for hypermotility and the latter provides directionality of movement. A similar mechanism has been shown for gonadotropin releasing hormone-1 neurons, in which $GABA_A$ receptors and the chemokine receptor CXCR4 interact to guide the neurons to form an organized pattern in

the developing brain (43).

In vivo migration exposes DCs to a complex and dynamic environment, including varying concentrations of GABA in different organs and compartments (29, 92), which likely requires constant adaptation (Chapter 1.2). The presence of low ambient GABA production by non-infected DCs in our *in vitro* cultures raises the possibility of a concentration dependent GABA receptor response. This has been proposed for extrasynaptic $GABA_A$ receptors in the CNS, where the $GABA_A$ channel conductance depends on the extracellular GABA concentration (175). It would be interesting to experimentally address the impact of varying extracellular GABA concentrations on DC function in organs where GABAergic signaling is prevalent, such as the brain or the pancreas.

4.5) GABAergic Activation During Toxoplasmosis

Future experiments will examine whether the GABAergic activation described here is generalizable to other cell types and parasite stages. One of the most tempting scenarios to explore would be neurons harboring bradyzoites in the chronically infected brain, keeping in mind the role of GABA as a neurotransmitter. The 'fatal attraction' hypothesis proposes that *T. gondii* actively subverts host innate fear responses in order to increase the probability of spread by predation (felines in particular as definitive hosts of *T. gondii*, (323)). Alterations of GABAergic signaling in the brain has been linked to changes in mood, anxiety level and fear responses (37, 140, 203). Also, astrocytes produce GABA, which reduces secretion of inflammatory cytokines in microglia (168). This, together with the fact that microglia exhibit a hypermigratory phenotype upon *T. gondii* infection (Paper I), adds an additional layer to the roles of glial cells during toxoplasmosis. Further studies will address whether astrocytes respond to infection with GABAergic activation and the potential effects of GABA on microglial migration. Recent microarray data show alterations in neurotransmitter and neuropeptide systems in neuroepithelioma cells infected by *T. gondii* (332). While no significant changes in transcription of GABA related genes were detected, these systems are also post-translationally regulated (324). Finally, *T. gondii* has been shown to produce dopamine through an endogenous tyrosine hydroxylase, which is also detected in brain tissue cysts, and by up-regulating microRNA-132, which alters host cell dopamine metabolism (233, 333). Collectively, these results describe a complex interplay between the rodent host and the tparasite, which should be further characterized in relation to host behavior.

5) CALCIUM (Ca^{2+}) SIGNALING IN IMMUNE CELL FUNCTION

5.1) Ca^{2+} - A Ubiquitous Second Messenger

The Ca^{2+} ion is one of the most ubiquitous second messengers in cellular signaling (130). Signals are relayed through so called 'on' reactions where Ca^{2+} entry from the extracellular space or Ca^{2+} release from intracellular stores located in the ER cause transient increases in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). These are then translated into cellular responses by Ca^{2+} sensitive proteins, after which 'off' reactions restore the ambient $[Ca^{2+}]_c$ via ion transporters and pumps (25). The modes of Ca^{2+} signaling are determined by the initiating stimuli and range from singular peaks in $[Ca^{2+}]_c$ to oscillatory patterns of specific frequencies (275).

Ca^{2+} release from intracellular stores and extracellular Ca^{2+} entry can be triggered by multiple kinds of stimuli and are often intertwined. Phospholipase C is activated by GPCRs and cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messengers diacyl glycerol and inositol 1,4,5-trisphosphate (IP_3). IP_3 activates the IP_3 receptor in the ER, which releases Ca^{2+} through the receptor pore (195). Intracellular store release can also function as amplification of extracellular Ca^{2+} entry, particularly via Ca^{2+} sensitive ryanodine receptors (RyRs) on the ER (8). Inversely, intracellular stores can activate influx of extracellular Ca^{2+}, a process known as store-operated Ca^{2+} entry (SOCE). The main SOCE mediator described is the Ca^{2+} release activated Ca^{2+} (CRAC) channel, consisting of the Ca^{2+} binding stromal interaction molecule (STIM) in the ER membrane and the plasma membrane Ca^{2+} channel Orai. When the intracellular stores are released, STIM interacts with Orai, which triggers influx of extracellular Ca^{2+} (24).

5.2) Ca^{2+} Signaling in Cell Migration

Cell migration is intimately linked to Ca^{2+} fluxes through several key signaling pathways (260). A gradient of $[Ca^{2+}]_c$ is observed along the axis of migrating cells, likely ensuring differential activity of Ca^{2+} dependent effector proteins at the leading edge and rear (261). Chemotaxis is a Ca^{2+} dependent process, since chemokine receptor ligation induces influx of extracellular Ca^{2+} and release of intracellular stores in DCs (218, 256). Similar observations have also been made in microglia for CCR5 engagement (114, 265). Blockade of Ca^{2+} permeable transient receptor potential melastatin (TRPM) 2 or the IP_3 receptor, abolishes DC chemotaxis and homing to lymph nodes (283). Also, potassium channel $K_{Ca}3.1$ and Cl^- channel Ano6, both of which are Ca^{2+} induced, are involved in CCR7 mediated chemotaxis (264, 296). TRPM4 is a Na^+ permeable channel that regulates $[Ca^{2+}]_c$ by altering

the electrochemical driving force of Ca^{2+} entry. Barbet and colleagues showed that loss of this regulation in $TRPM4^{-/-}$ mice abrogates DC migration and trafficking to lymph nodes (16). TRPM4 deficient mast cells are also deficient in migration and displayed impaired actin recruitment (266). Zebrafish primordial germ cells migrate via a plasma membrane blebbing mechanism (Chapter 1.2) dependent on local STIM induced $[Ca^{2+}]$ increases (32). Microglial podosomes co-localize with several Ca^{2+} related proteins and inhibition of CRAC channels interferes with podosome formation and transmigration (271).

5.3) Membrane Potential and Voltage Dependent Ca^{2+} Channels

The membrane potential (E_m) is defined as the electric potential difference between the cytosolic side and the extracellular side of the plasma membrane. Cells maintain an electrochemical gradient across the plasma membrane, mainly by active separation of K^+ and Na^+ through the K^+/Na^+ ATPase, resulting in a high intracellular K^+ concentration ($[K^+]_i$) and high extracellular Na^+ concentration ($[Na^+]_e$). The resulting unequal distribution of charge is known as the resting potential (245). Mathematically, the membrane potential can be expressed by the Goldman-Hodgekin-Katz equation for k positively charged monovalent ions and l negatively charged:

$$E_m = -\frac{RT}{F}\ln\left(\frac{\sum_k P_{M_k^+}[M_k^+]_i + \sum_l P_{N_l^-}[N_l^-]_i}{\sum_k P_{M_k^+}[M_k^+]_e + \sum_l P_{N_l^-}[N_l^-]_e}\right)$$

where R denotes the ideal gas constant, F Faraday's constant, T the temperature in degrees Kelvin and P_x the membrane permeability of ion x. Considering only K^+, Na^+ and Cl^- gives:

$$E_m = -\frac{RT}{F}\ln\left(\frac{P_{K^+}[K^+]_i + P_{Na^+}[Na^+]_i + P_{Cl^-}[Cl^-]_e}{P_{K^+}[K^+]_e + P_{Na^+}[Na^+]_e + P_{Cl^-}[Cl^-]_i}\right)$$

Decreasing the absolute value of E_m below the resting potential is termed "depolarization", while an increase is termed "hyperpolarization". Experimentally, depolarization of the cell membrane can be induced by increasing $[K^+]_e$, which reduces the electrochemical driving force of K^+ diffusing out of the cell (Fig 1A&B). In excitable cells (neuronal, muscle and endocrine cells), E_m changes are induced during an action potential, which is the basic mode of information transfer in the CNS (245).

The impact of Cl^- channels, e.g. $GABA_A$ receptors, on E_m depends on the intracellular Cl^- concentration ($[Cl^-]_i$), as has been shown in the developing CNS. In pre-natal neurons $[Cl^-]_i$ is high and triggering $GABA_A$ receptors will thus induce Cl^- efflux and depolarization (Fig

1C), while in mature neurons the reverse is true (Fig 1D). This developmental switch is driven by alterations in the Cl^- homeostasis of the cells via differential expression of the Cl^- transporters NKCC1 (Cl^- uptake) and KCC2 (Cl^- extrusion) (reviewed in (22)), and the energy metabolism of the cell (345).

Voltage-dependent Ca^{2+} channels (VDCCs) are plasma membrane proteins that respond to membrane depolarization by a conformational change that opens the channel (44). VDCC nomenclature is based on sequence homology of the pore-forming and voltage-sensing α1 subunit, grouping them into the 3 subfamilies Ca_V1, 2 and 3 (82). Expression of VDCCs has been considered as a hallmark of excitable cells and has therefore been mostly studied in the CNS where the different subfamilies have distinct functions in synaptic transmission (45). However, mounting evidence points towards VDCCs, particularly the Ca_V1 subfamily, playing important roles in cells of the innate and adaptive immune system (Table 1), although few details are known about their function (288).

5.3) VDCC Signaling in Immune Cells

Several B cell subsets exhibit Ca_V1 dependent Ca^{2+} influx upon ligation of the B cells receptor (104, 117).

Murine $CD8^+$ and $CD4^+$ T cells express $Ca_V1.1$, $Ca_V1.2$ and $Ca_V1.4$, although differential expression is observed upon transition from naïve to effector cells (14, 151, 279). Ca_V1 expression also varies between T cell subsets, as T_H2 polarized cells express a wider repertoire than T_H1 polarized cells (240, 253). Activation of Ca_V1 through ligation of the T cell receptor is implicated in T cell survival, expansion and homeostasis (213, 240).

Ligation of the mast cell IgE receptor induces a $Ca_V1.2$ mediated transient $[Ca^{2+}]_i$ increase and reduces apoptosis by blocking cytochrome c release (287, 294). TNF-α secretion by peripheral blood monocytes is blocked upon inhibition of $Ca_V1.2$ and $Ca_V1.3$ and Ca_V1 activity has been linked to monocyte secretion of apolipoprotein A1 (54, 267).

DC maturation is Ca^{2+}-dependent (59, 86, 314) and blocking Ca_V1 in human monocyte-derived DCs inhibits engulfment of apoptotic bodies and IL-12 secretion (230). Curiously, Ca_V1 inhibition in LPS-matured DCs stimulated IL-12 secretion in a MAPK-dependent manner (2). DCs also express RyR1, which is activated by $Ca_V1.2$ and rapidly stimulates MHCII expression (313).

Agonist/antagonist studies have also implicated Ca_V1 activity in immune functions of neutrophils, macrophages, and microglia (12, 114, 243). However, the relevance of these results depends on the number of different inhibitors used, inhibitor concentrations and

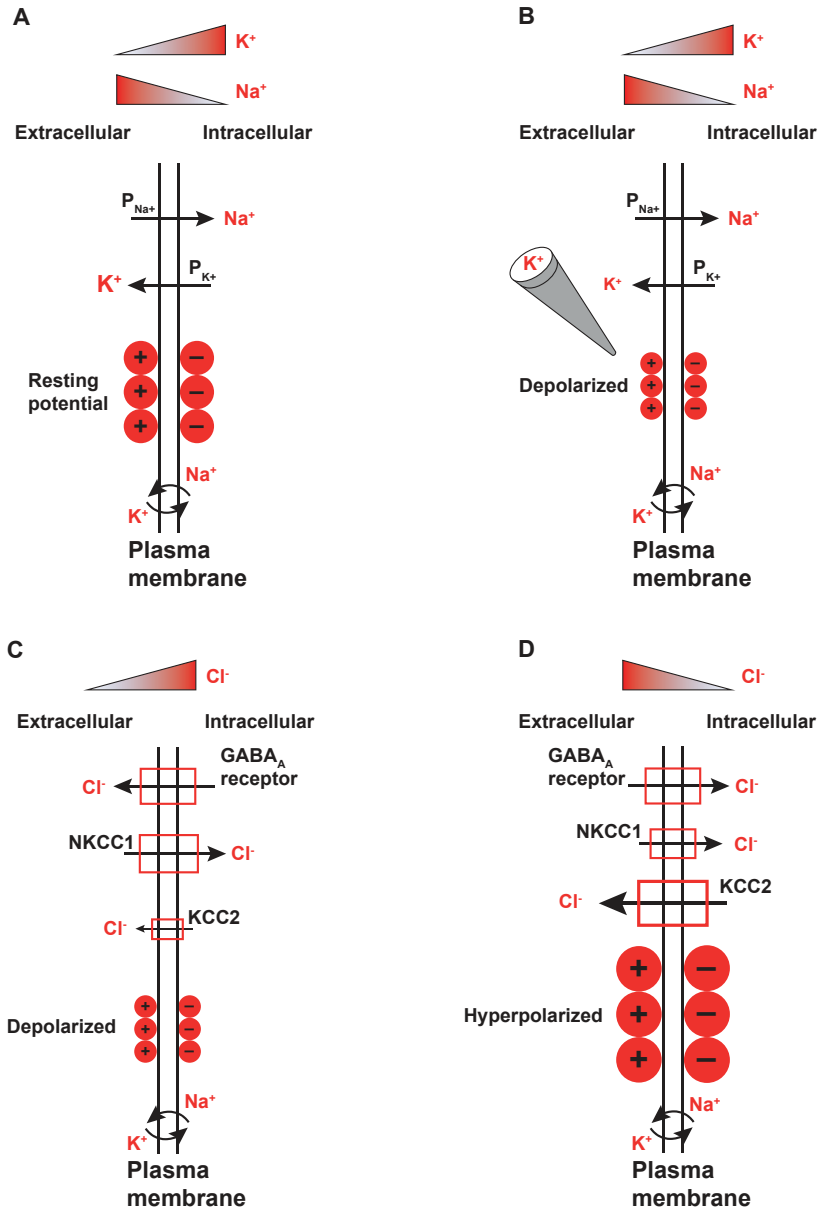

FIGURE 2. Regulation of the plasma membrane potential. The cell maintains a high $[Na^+]i$ and a high $[K^+]_e$ (A). When K^+ is added to the extracellular space, the electrochemical driving force for K^+ diffusion out of the cell is reduced, which depolarizes the cell (B). Low Cl^- extrusion compared to uptake will create an inwards Cl^- gradient and triggering $GABA_A$ receptors will thus mediate depolarization (C). In the reverse situation, $GABA_A$ receptor triggering will hyperpolarize the cell (D).

whether antagonist and agonist data concur and should thus be interpreted carefully. Also, several well-established VDCC inhibitors have off targets that likely become increasingly prominent at higher concentrations (337, 341).

TABLE 1. VDCC expression on immune cells.
Summary of literature reports of VDCCs identified in immune cells. Methods of characterization are abbreviated as Ph (pharmacological), WB (western blot), IF (immunofluorescence) and K (gene knockout or knockdown).

Cell type	Ca_V1 identified	Characterization	Function	References
Neutrophils	Unknown	Ph	IgG-mediated phagocytosis	(243)
NK cells	Unknown	Ph, WB, FACS	Release of cytolytic factors	(346)
DCs	Human: 1.2 Mouse: 1.3	Ph, WB, IF Ph, PCR, IF	IL-12 production, MHCII expression DC hypermotility	(230, 313, Paper IV)
Monocytes	Human: 1.2, 1.3	Ph, PCR, WB	TNF-α production	(54)
Macrophages	Unknown	Ph	NO/ROS production	(12, 132)
Mast cells	Mouse: 1.2	Ph, PCR, K	Anti-apoptotic	(293, 294)
Microglia	Unknown	Ph	CCL5 signaling, Neurotoxicity	(111, 114)
B cells	Rat: 1.3 rat	Ph, FACS	B cell receptor activation	(80, 247)
T cells	Human: 1.2, 1.3, 1.4 Mouse: 1.1, 1.2, 1.4	Ph, PCR, IF, K	NFAT-dependent maturation, cytokine production	(14, 28, 151, 279)

5.5) Ca^{2+} and GABAergic Signaling in *T. gondii* Infected DCs (paper IV)

To investigate the role of Ca^{2+} signaling in the hypermigratory phenotype, we first showed that transmigration and motility of *T. gondii* infected DCs is severely compromised in the absence of extracellular Ca^{2+}. Ni^{2+} blockade of plasma membrane Ca^{2+} channels confirmed this finding (Paper IV). Known links between GABAergic signaling and VDCCs in the CNS (33, 144) prompted us to test whether this concept applies to DCs as well, particularly

since VDCCs have not been previously studied in the context of DC motility.

We confirmed VDCC activity on DCs by high KCl-induced depolarization and showed that inhibiting Ca_V1 by nifedipine or verapamil abrogated hypermotility to the same extent as GABA deprivation (Paper IV). DCs may thus be an interesting exception to the trend that immune cell VDCCs do not respond to artificial depolarization (14, 279, 346). Importantly, hypermotility could not be restored by exogenous GABA in DCs where Ca_V1 was inhibited by nifedipine, in contrast to GABA deprived DCs. Next, we showed that KCl restored hypermotility in GABA deprived DCs, but again not in DCs where Ca_V1 was inhibited (Paper IV). These data indicate that Ca_V1 activity occurs downstream of GABA receptor signaling and is required for *T. gondii* induced hypermotility. Inhibition of purinergic receptors did not reduce infected DC motility, despite inducing Ca^{2+} entry when stimulated with ATP (250, Paper IV). This suggests that Ca^{2+} entry through Ca_V1 specifically is required for hypermotility. While we could not directly observe influx of Ca^{2+} upon stimulation with exogenous GABA, the fact that KCl could substitute GABA in restoring hypermotility in GABA deprived cells indicates that they stimulate the cell in a similar way (Paper IV). This is further supported by previous results that human DCs respond with depolarization to KCl and necrotic cell extracts (313). In our hands, measuring total cytosolic Ca^{2+} changes may also lack sufficient spatial resolution and sensitivity to detect the fluctuations we propose occur downstream of $GABA_A$ receptor activation.

We screened VDCC subtypes by quantitative reverse transcription PCR and found $Ca_V1.3$ to be predominantly expressed, with all other subtypes being low or undetectable. $Ca_V1.3$ was also identified by immunofluorescence, whereas staining patterns in *T. gondii*-infected and non-infected DCs did not differ (Paper IV). This is in contrast to human DCs where $Ca_V1.2$ expression has been reported (313), although these share the highest degree of sequence homology among Ca_V1 (82). To investigate whether $Ca_V1.3$ is the functionally dominant VDCC in murine DCs, we used a recently described inhibitor with high specificity against $Ca_V1.3$ compared to other VDCCs (142). Treatment at a concentration recommended for optimal Ca_V1 selectivity (142) abrogated hypermotility and hypermigration (Paper IV). We then employed RNA interference using short hairpin RNA (shRNA) packaged in a lentiviral delivery system (171). DCs transduced with shRNA targeting $Ca_V1.3$ failed to become hypermotile upon *T. gondii* infection, which could not be rescued by addition of GABA (Paper IV). While we cannot completely exclude participation of other Ca^{2+} entry pathways, such as SOCE (121), the shRNA data together with the relative specificity shown by purinergic receptor inhibition suggest dependence on $Ca_V1.3$. We thus conclude that Ca^{2+} is a second messenger to GABAergic signaling in DCs and that $Ca_V1.3$ is the major contributor to the Ca^{2+} entry necessary for the hypermigratory phenotype.

VDCC triggering allows for very rapid signal transduction (11), which is in line with

some of the proposed functions in immune cells (Table 1) as well as the rapid onset of hypermotility in *T. gondii* infected DCs (Paper III). Migratory activation has been reported in conjunction with cytoskeletal rearrangement in a metastatic breast cancer cell line (97). Both of these processes were sensitive to GABA receptor blockage or Ca_V1 inhibition. Further experiments will address whether the *T. gondii*-induced morphological changes in DCs (Paper III) are dependent on Ca_V1 activity.

Manipulation of host Ca^{2+} signaling pathways by *T. gondii* is not a phenomenon unique to DCs but has been reported in other cell types. Infection in mast cells was recently shown to inhibit antigen-mediated degranulation by reducing Syk kinase phosphorylation of PLCγ and thus inhibiting ER Ca^{2+} release (276). This inhibition requires attachment but not invasion of tachyzoites, in contrast to the onset of hypermotility in DCs (276, Paper III). Haroon and colleagues have shown that *T. gondii* infected neurons exhibit altered responses to glutamate stimulation and that the majority of neurons that carry cysts in the chronically infected mouse brain are functionally silent (110). The authors connected these results with behavioral alterations that occur in chronically infected mice, characterized by the loss of fear of cat odor (110, 315). In macrophages, Ca^{2+} signaling is required for protein kinase C (PKC)-dependent MAPK signaling and IL-12 expression induced by *T. gondii* (188). Tachyzoite invasion also alters the macrophage Ca^{2+} signaling pattern independently of MyD88 and CCR5 (189). As macrophages exhibit hypermigration upon *T. gondii* infection (159) and likely express VDCCs and $GABA_A$ receptors (12, 27), further studies will examine whether this pathway is subverted by the parasite in a similar way as in DCs. Lin and colleagues recently showed that ectopic expression of *T. gondii* GRA1 in a macrophage cell line alters Ca^{2+} signaling in these cells, providing a potential link between parasite invasion and Ca^{2+} signaling modulation (173). *T. gondii* is thus capable of subverting different host Ca^{2+} signaling pathways, in order to inhibit cellular responses or to enhance them.

Ca^{2+} signaling in the *T. gondii* tachyzoite itself has been implicated in several phases of the lytic cycle, most notably the invasion process (180, 228). Recently Pace and colleagues showed that a transient increase in tachyzoite $[Ca^{2+}]_c$ is required for invasion and is partly mediated by nifedipine-sensitive entry of extracellular Ca^{2+} (215). In our experimental setup, all Ca^{2+} signaling inhibitors were added 2-3 h post *T. gondii* challenge, which reduces the probability that the inhibition of hypermotility we observed is due to drug effects on the parasites. Thus, we could also show that the GABAergic activated Ca^{2+} influx upon *T. gondii* infection is necessary not only for the induction but also for the maintenance of hypermotility (Paper IV).

5.6) A Hypothetical Model for *T. gondii* Induced DC Hypermotility (paper IV)

To situate the mechanistic findings presented here in a host-parasite interaction perspective, we propose the following model for the GABA-induced Ca^{2+} signaling upon *T. gondii* infection (Fig 2).

The active invasion of a *T. gondii* tachyzoite rapidly stimulates GABA synthesis (I), likely by GAD65, which is activated by phosphorylation by Ca^{2+} independent PKC isoform ε (324). It remains to be investigated whether *T. gondii* induces this non-conventional PKC, as has been shown for a conventional PKC (188). An alternative hypothesis is that the parasite directly activates GAD65 via effector molecules secreted into the host, as several of these are active kinases (220).

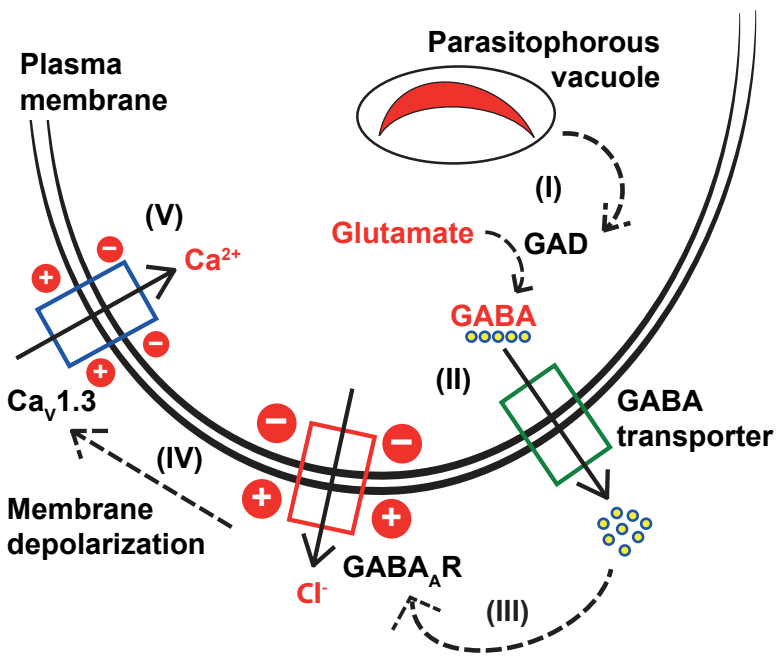

FIGURE 3. Proposed initial steps of migratory activation of DCs upon *T. gondii* infection. Tachyzoite entry induces GAD dependent synthesis of GABA, which is excreted by GAT. Extracellular GABA activates $GABA_A$ receptors in an autocrine manner, causing Cl^- efflux and membrane depolarization. This triggers $Ca_V1.3$ dependent Ca^{2+} entry, which signals a shift in migratory capacity.

Next, GAT mediated secretion of GABA into the extracellular environment occurs (II). The

rapid upregulation of GAT4 upon infection supports the hypothesis that GABA secretion is connected with the onset of the hypermigratory phenotype. Extracellular GABA activates $GABA_A$ receptors in an autocrine manner (III), causing Cl^- efflux, which depolarizes the plasma membrane (IV). This triggers voltage sensitive $Ca_V1.3$ mediated influx of Ca^{2+} (V) and downstream activation of migratory pathways.

6) METHOD DEVELOPMENT AND CONSIDERATIONS

Investigation of the Trojan horse hypothesis of *T. gondii* dissemination requires appropriate assays that model host cell migration under varying conditions (Chapter 3.4). The assays employed in this thesis to quantify cell motility and migration are discussed below. As all *in vitro* models, they represent attempts to mimic different facets of the *in vivo* setting under defined conditions. For more detailed descriptions of the methods and procedures employed in the different studies, the reader is referred to the methods sections of the respective papers.

6.1) Assays to Measure Cell Migration

Motility Assay (paper III & IV)

This assay was developed to allow quantification of cell migration on a single cell level and to distinguish non-infected cells from *T. gondii* infected ones (Fig 4). Infection with fluorescent parasites is done in a 96 well format, after which the cells are embedded in a collagen I matrix and transferred to multiwell glass slides. Live cell imaging is then performed and cells are manually tracked to minimize errors associated with automated tracking solutions. During the optimization phase we defined optimal incubation volumes and times for the different steps, concentration of collagen, imaging intervals and tracking method. We then evaluated the reproducibility of the assay and found that DCs increase their velocity by a factor 2,9±0,9 upon infection in 12 experiments performed over a period of 6 months. Velocities of non-infected DCs in the motility assay are generally lower than what has been observed *in vivo*, although closer to DCs migrating in interstitial tissue than those in lymph nodes (163, 194). We conclude that, while the motility assay underestimates the motile capacity of DCs *in vivo*, it is a robust and useful assay to study the migratory activation of DCs and other cells upon *T. gondii* infection.

Transmigration Assay (paper I, II, III & IV)

The transmigration assay is performed in a chamber divided into a top and bottom compartment by a porous membrane, on which cells are deposited. Counting the number of cells in the lower compartment after a period of incubation gives the transmigration rate. The assay can be made to more closely model a biological barrier by coating the membrane with ECM components, e.g. collagen or matrigel, and/or a monolayer of cells, e.g. endothelial cells, astrocytes or BeWo (53, 160, Paper I). A potential drawback of the assay is the relatively long incubation times and binary output, compared to the motility assay.

Chemotaxis Assay (paper II & III)

From the many assays described in the literature that measure chemotaxis, we prefer a commercially available system set in microfabricated channels on a glass slide (μ-Slide, Ibidi). The arrangement of two perpendicular channels, one containing the cell suspension and the other a chemokine gradient, admits superior control of gradient formation compared to commonly used Boyden chamber systems.

Adoptive Transfers and Bioluminescence Imaging (paper II)

To evaluate the impact of different factors on *T. gondii* Trojan horse dissemination *in vivo*, we adoptively transfer cells infected *in vitro* with parasite strains expressing luciferase. Parasite dissemination and replication is followed through bioluminescence imaging (IVIS, PerkinElmer), allowing repeated observations of the same animals in contrast to plaquing assays (68). On the level of individual organs, however, the plaquing assay remains a more sensitive technique due to the lower detection threshold of replicating parasites (Paper II).

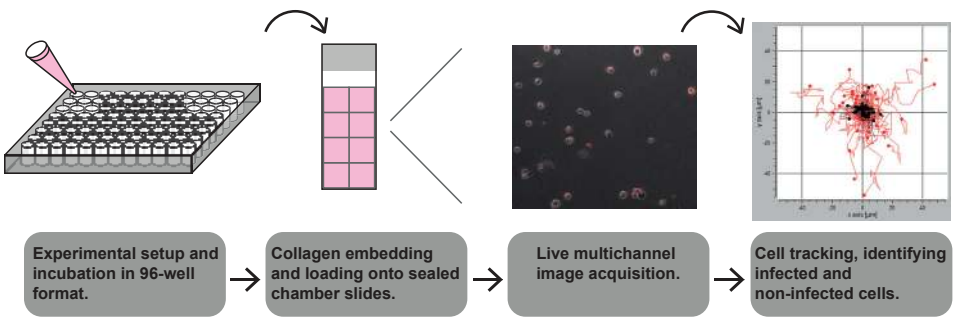

FIGURE 4. Motility assay work flow. Graphical representation of the steps from experimental setup to cell tracking and data analysis.

6.2) Defining the Hypermigratory Phenotype

Using the assays described above allows us to dissect the hypermigratory phenotype observed upon *T. gondii* infection into more precise terms. The increased velocity and transmigration rate observed in the motility and transmigration assays upon infection, we have termed “hypermotility” and “hypermigration” respectively. Also, infected DCs acquire the capacity to chemotax along a CCR7 gradient (Chapter 3.3). Importantly, infection of DCs promotes parasite dissemination *in vivo*, which can be linked to *in vitro* hypermigration, hypermotility and chemotaxis by pharmacological studies (160, 161, Paper II). The hypermigratory phenotype can thus be defined as presenting as subset or all of these traits collectively (325).

7) CONCLUSIONS AND FUTURE PERSPECTIVES

The requirement for tachyzoite dissemination from the small intestine to distant sites where pathology develops is a prerequisite for clinical manifestations of *T. gondii* infection, e.g. chorioretinitis (the eye), congenital toxoplasmosis (the placenta) and toxoplasmic encephalitis (the brain). Having previously confirmed the disseminatory advantage given by the hypermigratory phenotype *in vivo*, we here modeled this process using different *in vitro* approaches. A key step in understanding *T. gondii* dissemination will be the linking of different aspects observed *in vitro*, e.g. morphological alterations, hypermotility, hypermigration and chemotaxis, to corresponding cellular activities *in vivo*. The differential regulation of these traits, indicated by our results, suggests that they could be selectively targeted in therapeutic strategies. This would however require knowledge of which processes actually mediate the enhanced dissemination observed *in vivo*. Similarly, the impact of the proposed Trojan horse mediated local dissemination in the CNS on the clinical course of toxoplasmic encephalitis should be evaluated.

The work presented in this thesis features a GABAergic-Ca^{2+} signaling axis previously undescribed in DCs. While several of its components have been associated to migratory properties in other cell types, the role of this pathway in basal DC migration remains to be studied. As Ca^{2+} signaling modulates cellular motility on many different levels, dissecting the cascades downstream of GABAergic activation is challenging. Simultaneous work to identify *T. gondii* derived factors that participate in the initiation of the hypermigratory phenotype will likely facilitate this task. From a therapeutic perspective, the implication of GABA receptors and VDCCs in *T. gondii* dissemination is interesting, since these have been successfully studied as drug targets. Two compounds that were found to inhibit the hypermigratory phenotype in Paper IV, nifedipine and verapamil, have been used to treat hypertension since the 1970s and 80s respectively.

Work by us and others have described a role for GABA in regulating the immune system. The term ”neuroimmunotransmitter” has been proposed to denominate the growing class of signal transducers that relay information both in the CNS and in the immune system (170). In the specific case of GABA, however, it is important to note that while it has been widely studied as a neurotransmitter in the vertebrate CNS, it has been described in prokaryotes, plants, fungi and protozoa (34, 152, 172, 331). Being a simple γ-amino acid that is interconvertible with key metabolic molecules such as glutamate and oxaloacetate, the apparent evolutionary retention of GABA is not surprising. Whether this ubiquitous presence may enable interactions across species, e.g. between commensal or parasitic organisms and their hosts, remains to be investigated. However, this could provide a new

dimension to the expression of GABA receptors on a variety of immune cells.

Finally, *T. gondii* has been employed as a model for other apicomplexan parasites for over 15 years (145). This thesis has examined potential mechanisms of *T. gondii* dissemination. In the light of the hypermigratory phenotype, this parasite could also be considered as a tool for studying motility and migration of immune cells.

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