Functional analysis of Ly6C^{hi} inflammatory monocytes

and Ly6G^{+} neutrophil granulocytes

in chronic cerebral toxoplasmosis

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Publications

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Other Publications

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Fu T, Znalesniak EB, Kalinski T, Möhle L, **Biswas A,** Salm F, Dunay IR, Hoffman W. (2015) TFF Peptides Play a Role in the Immune Response Following Oral Infection of Mice with *Toxoplasma gondii*. Eur J Microbiol Immunol. Sep 18; 5(3).

Montagna GN, **Biswas A,** Hildner K, Matuschewski K, Dunay IR. (2015) Batf3 deficiency proves the pivotal role of CD8α^{+} dendritic cells in protection induced by vaccination with attenuated *Plasmodium* sporozoites. Parasite Immunol. Oct 8; 37(10).

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Abstract

Chronic cerebral toxoplasmosis is characterized by the activation of resident cells and infiltration of immune cells from the periphery to the central nervous system (CNS). The bone marrow (BM) derived myeloid cells comprise a heterogeneous population where the subtypes have diverse functions. However, how these cells respond to cerebral toxoplasmosis regarding differentiation and effector functions is not fully understood. The overall aim of this thesis was to investigate the recruitment, function and differentiation of monocytes and neutrophils in murine cerebral toxoplasmosis.

This study showed that the infiltrating $Ly6C^{hi}CCR2^{+}$ inflammatory monocytes play a crucial role in host defense. $Ly6C^{hi}CCR2^{+}$ monocytes regulate the host defense by the production of both pro-inflammatory and anti-inflammatory mediators. We confirmed the recruited monocytes further develop into two distinct subpopulations. (i) The $Ly6C^{int}CCR2^{+}F4/80^{neg}$ monocytes, which upregulated standard dendritic cell (DC) markers suggesting antigen presentation properties. (ii) The $Ly6C^{neg}F4/80^{hi}$ monocytes, which displayed elevated phagocytic capacity while upregulating triggering receptor expressed on myeloid cells – 2 (TREM2). We also showed recruitment of $Ly6C^{hi}$ monocytes is regulated by P-selectin glycoprotein ligand-1 (PSGL-1).

Our study identified that the infiltrating neutrophils are a non-lymphoid source of interferon-(IFN-) γ, the cytokine known to be the major mediator of resistance to *Toxoplasma gondii* (*T. gondii*). Furthermore, we also detected neutrophil-dependant monocyte recruitment to the CNS. Finally, we described that $Ly6G^{+}$ neutrophils are a heterogeneous population where one subset $Ly6G^{+}CD62L^{hi}CXCR4^{+}$ with high cathelicidin-related antimicrobial peptide (CRAMP) expression promotes monocyte recruitment, while the other subset $Ly6G^{+}CD62L^{lo}CXCR4^{+}$ with high IFN-γ production establishes inflammatory response.

Collectively, the present studies revealed that the BM derived myeloid cells display vast degree of heterogeneity with a repertoire of effector functions crucial in parasite control and immune regulation in chronic cerebral toxoplasmosis.

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Abbreviations

A	
Ag	Antigen
APC	Antigen-presenting cell
B	
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophages
BSA	Bovine serum albumin
BBB	Blood brain barrier
C	
CCL-	C-C motif ligand type-
CXCR-	CXC-Motif-Chemokine receptor type-
CXCL-	CXC-Motif-Chemokine ligand type-
CX_3CR1	CX3C chemokine receptor 1
CRAMP	Cathelicidin-related antimicrobial peptide
CMP	Common Myeloid Progenitor
cMoP	Committed monocyte progenitor
CCR-	c-c Chemokine receptor type-
CD	Cluster of differentiation
CSFE	Carboxyfluorescein diacetate succinimidyl ester
CNS	Central nervous system
C_T	Threshold cycle
D	
DC	Dendritic cell
E	

ESL-1	E-selecting ligand-1
ERK	Extracellular-signal-regulated-kinase
ECM	Experimental cerebral malaria
F	
fMLP	Formyl-Methionyl-Leucyl-Phenyalanine
G	
Gr-1	granulocyte-receptor-1
Groβ	growth regulated oncogene-β
GMP	Granulocyte macrophage progenitor
H	
HSC	Haematopoetic stem cell
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPRT	Hypoxanthine guanine phosphoribosyltransferase
I	
IFN-	Interferon-
IL-	Interleukin-
i.p.	Intraperitoneal
i.v.	Intravenous
IκBs	Inhibitors of NF-κB
iNOS	inducible Nitric Oxide Synthase
ILC	Innate Lymphoid Cell
Ig-	Immunoglobulin-
ICAM-	Intercellular cell adhesion molecule-
IRF-	Interferon regulatory factor-
K	
KC	Keratinocyte-derived chemokine
L	

L. monocytogenes	*Listeria monocytogenes*
Ly-	Lymphocyte antigen-
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
M	
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MIP-1	Macrophage inflammatory protein-1
M-CSF	Macrophage colony-stimulating factor
MDP	Macrophage and dendritic cell progenitor
MHC-	Major histocompatibility complex-
MyD88	Myeloid differentiation primary response gene (88)
MMP-	Matrix metalloproteinase-
N	
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NOD	Nucleotide-binding oligomerization domain-containing protein
NK	Natural killer
NADPH	Nicotinamide adenine dinucleotide phosphate
P	
PFA	Paraformaldehyde
PBS	Phosphate buffered saline
PMN	Polymorphonuclear leukocytes
PSGL-1	P-selectin glycoprotein-1
PBMC	Peripheral blood mononuclear cell
P	*Plasmodium*
PGE_2	Prostaglandin E_2

PGD_2	Prostaglandin D_2
R	
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
S	
SDF-1	Stromal cell-derived factor-1
STAT-	Signal transducer and activator of transcription-
T	
T. gondii	*Toxoplasma gondii*
TE	*Toxoplasma* encephalitis
TGF-β	Transforming growth factor beta
Th	T helper
TLR	Toll like receptor
TNF	Tumor necrosis factor
Tip-	TNF and iNOS producing-
TCR	T cell receptor
TREM2	Triggering receptor expressed on myeloid cells – 2
V	
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4
W	
WNV	West Nile Virus

1. Introduction

The parasite *T. gondii* has a high worldwide seroprevalence of 30% - 70% (Montoya & Liesenfeld 2004; Robert-Gangneux & Darde 2012). It can infect any nucleated cell of warm-blooded animals including humans. The parasite evades the host's immune system by concealing itself in migratory immune cells eventually crossing biological barriers such as blood brain barrier (BBB) (Courret et al. 2006a). The parasites remain lifelong within infected neurons in the form of latent cysts (Courret et al. 2006a; Pittman & Knoll 2015; Da Gama et al. 2004). Reactivation of latent cysts upon immunosuppression leads to *Toxoplasma* encephalitis (TE) (Luft et al. 1983; Chaudhry et al. 2014; Barsoum 2006; Luft et al. 1992; Derouin & Pelloux 2008; Brown et al. 2005; Havelaar et al. 2007). In immunocompetent individuals the immune system is constantly stimulated with parasite antigens (Ags) leading to prolonged basal levels of inflammation thus establishing chronic cerebral toxoplasmosis (Montoya & Liesenfeld 2004). Previously, chronic cerebral toxoplasmosis was considered to be asymptomatic. However studies have shown that chronic cerebral toxoplasmosis have an impact on the behaviour of the host (Beste et al. 2014; Parlog et al. 2014). In cerebral toxoplasmosis the role of adaptive immune cells has been extensively studied in experimental models such as mice (Hunter et al. 1994; Orellana et al. 1991; Gazzinelli & Sher 2014; Yarovinsky 2013), whereas the role of myeloid derived innate immune cells is not fully understood. Hence, we investigated the function of the myeloid derived mononuclear cell subsets during chronic cerebral toxoplasmosis.

1.1 *Toxoplasma gondii*

Toxoplasma gondii is a facultatively heteroxenous (a parasite that has more than one obligatory host in its life cycle), polyxenous (a parasite that can infect more than one species) protozoan that causes toxoplasmosis (Dubey et al. 1998). Nicolle and Manceaux in North Africa and Splendore in Brazil first described the parasite in 1908 (Dubey et al. 1998). It belongs to the phylum Apicomplexa which includes intracellular parasites such as *Plasmodium (P.)* (the causative agent of malaria) and Eimeria (the causative agent of chicken coccidiosis) (Dubey et al. 1998).

1.1.1 Life-cycle of *Toxoplasma gondii*

The life cycle of *T. gondii* can be divided into a sexual cycle and an asexual cycle (Figure 1). The sexual cycle takes place only within domesticated or wild cats, the definitive host. The asexual cycle can take place within all warm-blooded animals including humans, the intermediate host. During its life cycle, *T. gondii* converts into various biological stages: motile and fast replicating tachyzoites, fast replicating merozoites (within intestinal epithelial cells), slow replicating bradyzoites (within tissue cysts) and sporozoites (within oocysts) (Dubey et al. 1998). After ingestion of an infected host the tissue cysts or oocysts enter the gut of the cat. The tissue cysts or oocysts are released following acid digestion in the feline stomach. Following the release they penetrate the epithelial cells of the small intestine where they differentiate into tachyzoites and schizont stages. The rapidly dividing tachyzoites disseminate to various tissues whereas the schizont develop into merozoites in the enterocytes, the first sexual stage. The merozoites undergo proliferation to form macrogametes (females) and microgametes (males) that fuse to form oocysts. The oocysts form thick impermeable walls and are shed in the cat's faeces (Pittman & Knoll 2015). Following excretion, the oocysts undergo mitosis and meiosis to produce eight haploid sporozoites within the cyst wall, which maintains the infectivity of the oocysts for an extended period of time (Robert-Gangneux & Darde 2012). Ingestion of sporulated oocysts or encysted bradyzoites (by consuming undercooked meat, unwashed vegetables or contaminated water) by the intermediate host such as humans is the most common route of infection. Once ingested, the sporozoites or the bradyzoites are released in the stomach in a similar manner as in the feline stomach. Bradyzoites or sporozoites then differentiate into tachyzoites (Bohne et al. 1993; Pittman & Knoll 2015). The rapidly dividing tachyzoites infect migratory cells such as macrophages and DCs and disseminate into various tissues such as muscles and brain via the bloodstream (Ueno et al. 2014). The strong immune response from the host leads to a stage conversion into semi dormant, slowly dividing bradyzoites, in the chronic stage. Cysts containing bradyzoites can persist lifelong within slowly dividing striated muscle including heart and tissue of immune privileged sites such as the CNS (Dubey et al. 1998). The ingestion of meat contaminated with tissue cysts by the cat starts a new cycle of infection.

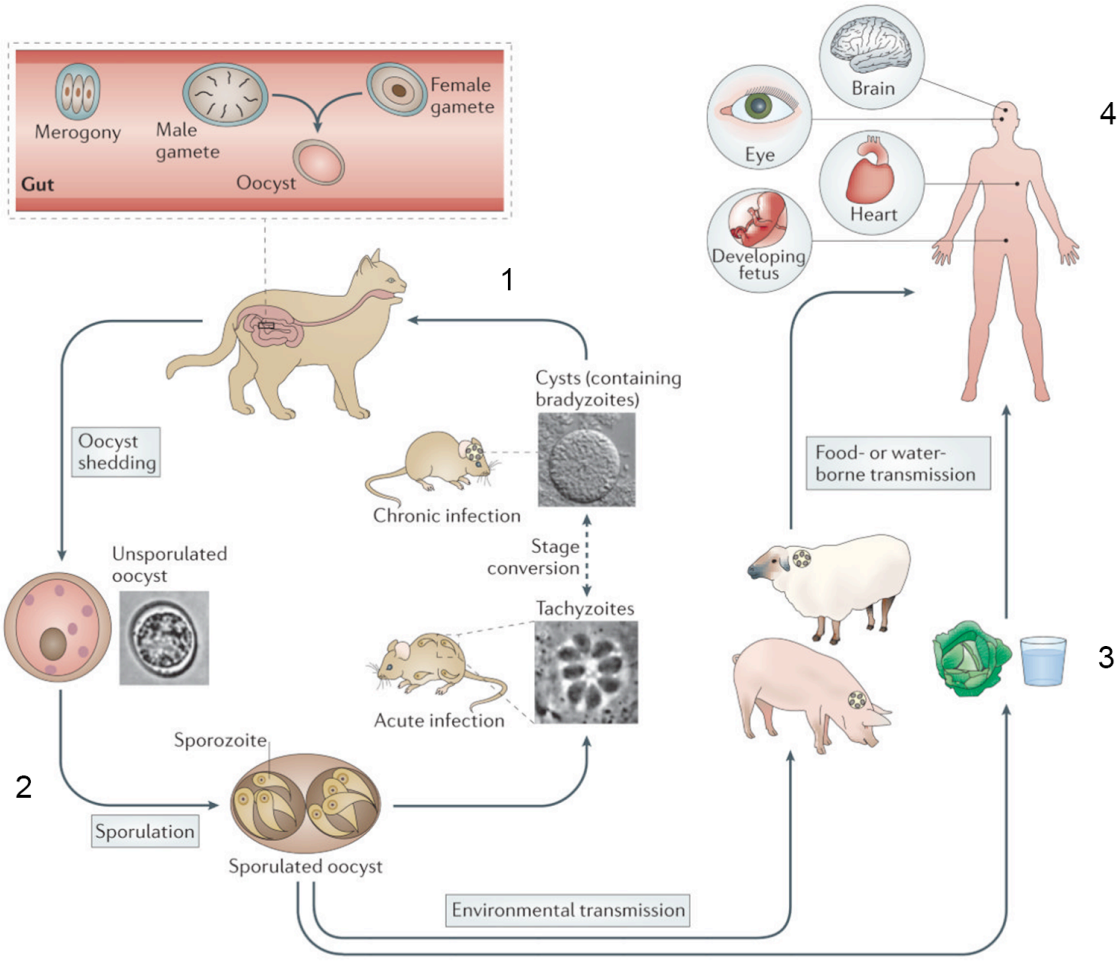


Figure 1. Life cycle of *T. gondii*.

The life cycle of *T. gondii* is divided into a sexual and an asexual cycle. (**1**) The sexual cycle occurs in the intestine of the definitive host, the cat that sheds the oocysts in the faeces after consumption of preys containing infective tissue cysts. (**2, 3**) These oocysts undergo sporulation to infective oocysts that can contaminate food and water, providing a route of infection for intermediate hosts such as humans and other warm blooded animals triggering the asexual life cycle. (**4**) In the intermediate host, the asexual cycle takes place which is characterized by tachyzoites disseminating systemically to all tissues throughout the body which then undergo stage conversion to encysted bradyzoites that reside within tissues life-long leading to chronic infection (adapted from Hunter & Sibley 2012).

1.2 Immune system

The immune system is a network of biological structures and processes within an organism with specialized roles in defence against disease or injury caused by abnormal host cells or foreign invaders such as bacteria, viruses, parasites and fungi (Parkin & Cohen 2001; Chaplin 2010). In

order to control *T. gondii* the host's immune system is crucial to recognize the parasite and its immunogenic molecules that persist systemically throughout the host tissues. There are two fundamentally distinct responses to invading organisms, namely the innate response and the adaptive response (Figure 2).

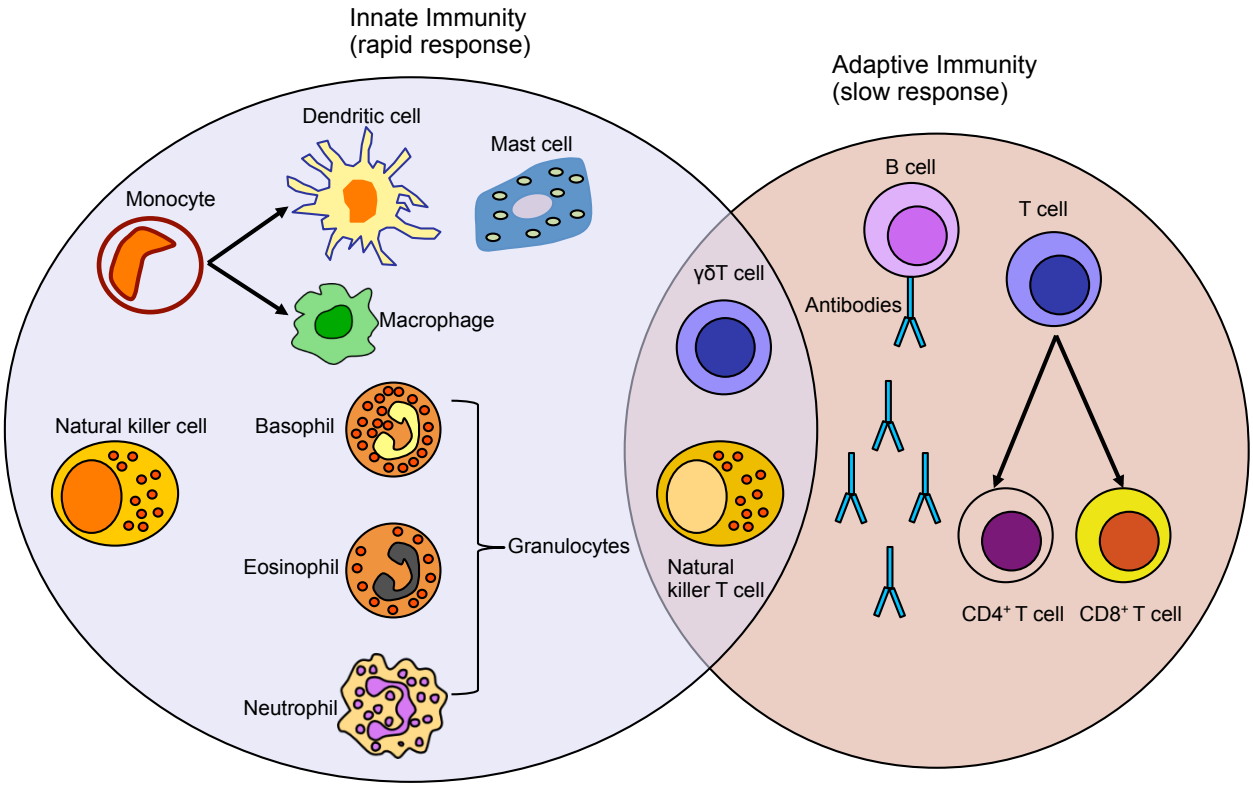


Figure 2. The innate and adaptive immune response.

The innate immune response forms the first line of defence against infection. It consists of granulocytes (basophils, eosinophils and neutrophils), mast cells, monocytes, macrophages, dendritic cells and natural killer cells. The adaptive immune response is slower to develop, but manifests increased antigen specificity and memory. It consists of antibodies, B cells, $CD4^+$ and $CD8^+$ T lymphocytes. Natural killer T cells and γδT cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity (adapted from Dranoff 2004).

1.2.1 Innate immune system

The innate (natural) immune response occurs between innate immune cells that express receptors on their surface such as Toll like receptors (TLRs) that recognize general danger- or pathogen-associated- molecular patterns expressed on the invading microorganism. When a microorganism breaches the physical barrier of the host, innate immune cells comprising of neutrophils,

eosinophils, basophils, mast cells, monocytes, DCs, macrophages and natural killer (NK) cells form the first line of defence against the invading organism. Their main feature is to respond to the pathogen, instantly and in a generic way, which means they do not confer long-lasting immunity. Innate immune cells are critical in host defence and impaired innate cell function may cause chronic susceptibility to infection. Furthermore, innate immune cells are important in activating adaptive immune cells (Iwasaki & Medzhitov 2015).

1.2.2 Adaptive immune system

The adaptive (acquired) immune response forms the second line of defence with increased Ag specificity. Upon primary infection the adaptive response occurs later as it needs the coordination and expansion of specific adaptive immune cells. The adaptive immune system includes B lymphocytes and T lymphocytes with each cell bearing unique receptors that recognize distinct Ags. Ag specificity allows tailored responses to pathogens and pathogen-infected cells. Immunological memory is a unique feature of the adaptive immune system where adaptive immune cells are retained for subsequent encounters with the same pathogen. Both innate and adaptive immune responses are important in conferring protection against *T. gondii* (Dunay et al. 2008; Mordue & Sibley 2003; Elia D. Tait et al. 2010; Liu et al. 2006; Dunay et al. 2010; Yarovinsky 2013; Mashayekhi et al. 2011; Klose et al. 2014).

1.2.3 Innate immune cells

1.2.3.1 Neutrophil granulocytes

Neutrophils are important members of the innate immunity and are also called polymorphonuclear cells (PMNs). They are short-lived (1-2 days) and the most abundant BM derived white blood cells in circulation. Neutrophils constitute up to one-third of the total cells produced by HSCs daily (Figure 3). The development of the neutrophil precursors as well as the terminal differentiation of neutrophils is coordinated by a complex network of transcription factors, growth factors, cytokines, microRNAs and other regulatory factors (Nathan 2006; Borregaard 2010; Bardoel et al. 2014; Dahl et al. 2003; Laslo et al. 2006).

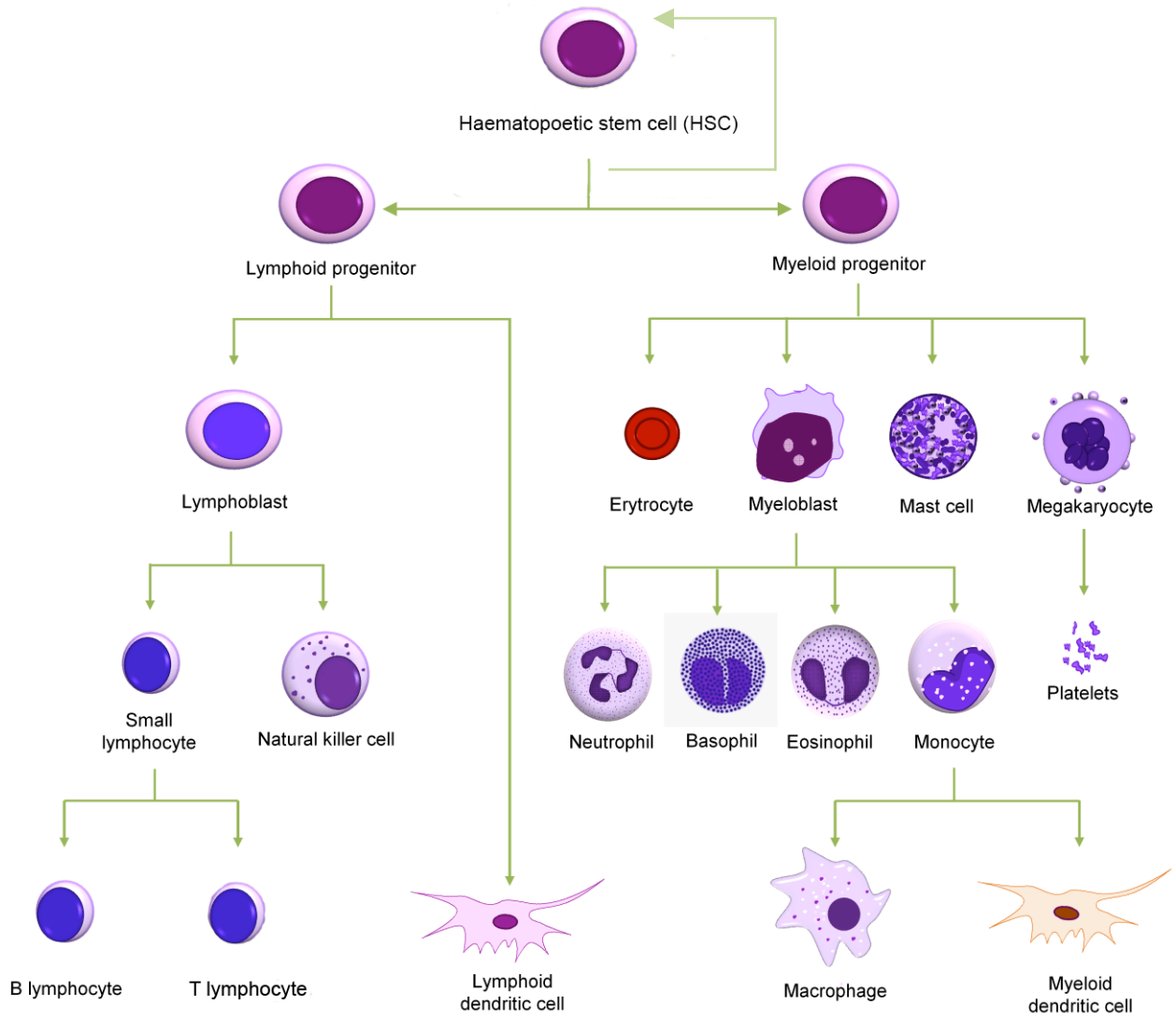


Figure 3. Development of immune cells from haematopoetic stem cells (HSCs).

The comprehensive diagram illustrates the development of immune cells from haematopoetic stem cells (HSCs). HSCs give rise to 2 different lineages through a gradual process of differentiation - common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). CMP give rise to innate immune cells. CLP give rise to adaptive immune cells including natural killer cells, which are also innate immune cells. (adapted from Herrera-carrillo & Berkhout 2015).

In homeostatic condition, production of neutrophils is largely determined by their cell death rate in the periphery (Stark et al. 2005; von Vietinghoff & Ley 2009; Zarbock et al. 2006) and also by their density in the BM (Cain et al. 2011). The egress of neutrophils from the BM depends on the balance of the CXC-motif-chemokine receptors type- 4 and 2 (CXCR4, CXCR2) and their ligands stromal cell-derived factor-1 (SDF-1), keratinocyte-derived chemokine (KC) and growth

regulated oncogene-β (Groβ) respectively (Eash et al. 2010). Murine neutrophils are characterized by expression of CD45 (member of protein tyrosine phosphatase family found on all leukocytes), CD11b (integrin α_M, a cell adhesion molecule that complexes with CD18 to form Mac-1/complement receptor 3) and Ly6G (Lymphocyte antigen 6 complex, locus G) (Rose et al. 2012).

When the homeostatic condition is altered such as during inflammation and injury neutrophils forms the first line of defence. Inflammation induces the secretion of pro-inflammatory molecules such as tumor necrosis factor (TNF), IL-1β and IL-17 that result in the expression of adhesion molecules on the luminal surface of blood vessels. These adhesion molecules include P-selectin, E-selectin as well as members of the integrin family vascular cell adhesion molecules (VCAMs) and intercellular cell adhesion molecules (ICAMs). The corresponding ligands P-selectin glycoprotein-1 (PSGL-1), L-selectin, E-selecting ligand-1 (ESL-1) and CD44 which are expressed on the tips of neutrophil microvilli initiate the migration of neutrophils (Buscher et al. 2010). Reaching the site of infection, neutrophils can respond through various mechanisms, such as phagocytosis, degranulation, secretion of antimicrobial proteins, cytokines, chemokines and generation of neutrophil extracellular traps (NETs) (Bardoel et al. 2014). Neutrophils that are recruited to the site of infection, first undergo transcriptional burst and secrete cytokines such as, IL-1β (Sica et al. 1990), TNF (Tecchio et al. 2012) and IFN-γ (Sturge et al. 2013) and chemokines such as IL-8 (Zeilhofer & Schorr 2000), MCP-1 (Yoshimura & Takahashi 2007) and MIP-1α (Kasama et al. 1994) to recruit other immune cells and for their own reinforcement. Furthermore, neutrophils can undergo NETosis. NETosis is an active cell death during which the chromatin gets decondensed and is released in the form of NETs. NETs consist of antimicrobial proteins such as neutrophil elastase, cathepsin G and histones. They trap and kill microbes and can activate other immune cells (Bardoel et al. 2014; Borregaard 2010). Neutrophils are also involved in the egress of monocytes which has been shown in numerous studies in humans and mice (Mokart et al. 2008; Shiohara et al. 2004; O. Soehnlein et al. 2008). There are several mechanisms by which neutrophil - mediated monocyte emigration occurs. Neutrophils secrete proteins such as CRAMP and cathepsin G, facilitating enhanced monocyte adhesion (Taekema-Roelvink et al. 2001; De Yang et al. 2000; O. Soehnlein et al. 2008; Soehnlein 2012; Sarawuth Wantha et al. 2013). Neutrophils also promote *de novo* synthesis of monocyte chemoattractants

such as MCP-1 (Yoshimura & Takahashi 2007). In the presence of appropriate stimuli such as TNF (Marin 2002), neutrophils secrete monocyte-attracting chemokines themselves. Neutrophil granule proteins also enhance the production of reactive oxygen species (ROS) which leads to effective pathogen clearance (O. Soehnlein et al. 2008).

1.2.3.1.1 Neutrophil granulocytes in peripheral infections and inflammation

Several studies describe the specific tasks of neutrophils in the periphery. On one hand they are required to fight intracellular pathogens such as *Listeria monocytogenes* and *P. falciparum* (Carr et al. 2011; Joos et al. 2010; Hemmer et al. 2010). On the other hand they contribute to disease progression for example in the case of atherosclerosis (Drechsler et al. 2010).

During listeriosis, neutrophils produce pro-inflammatory cytokines such as TNF and IFN-γ, which play a protective role. However, the protective role of neutrophils is organ specific. They are important for the clearance of the bacteria in the liver, but in the spleen their role is more prominent following high dose infection (Carr et al. 2011).

In *Plasmodium* (*P*) infection, neutrophils display enhanced phagocytic capacity by taking up opsonised parasites and malaria pigment (hemozoin) which triggers the release of ROS (Joos et al. 2010). This effector function of neutrophils is important for host resistance but can also ameliorate the disease by damaging endothelial cells (Hemmer et al. 2010).

Neutrophils play a role in the development and progression of experimental atherosclerosis as specific depletion of neutrophils reduced the size of atherosclerotic plaques. Neutrophils are recruited to the inflamed arteries by the co-ordination of chemokine receptor type-1, 2, 5 (CCR1, CCR2, CCR5) and CXCR2 secreted by activated endothelial cells and platelets. The neutrophils cause more inflammation by producing large amounts of free radicals such as nicotinamide adenine dinucleotide phosphate (NADPH) (Drechsler et al. 2010).

1.2.3.1.2 Neutrophil granulocytes in central nervous system infections and injury

Under steady state conditions, neutrophils cannot cross the BBB (Andersson et al. 1992). However, during inflammation, pro-inflammatory mediators secreted by brain resident cells such as microglia and astrocytes make the BBB permeable by activating the endothelial cells (Liu et al. 2015; Perez-de-Puig et al. 2015; Simmons et al. 2014). During injury and infection it is well

documented that neutrophils receive danger signals from brain resident cells. These signals are in the form of chemokines MCP-1 (Yoshimura & Takahashi 2007), MIP-1α (Kasama et al. 1994), MIP-3α and MIP-3β (Mantovani A 2001), cytokine such as IL-17 (Simmons et al. 2014) and activated endothelial cells. These signals help circulating neutrophils to cross the leaky BBB (Del Rio et al. 2001; Joos et al. 2010; Hemmer et al. 2010; Bai et al. 2010; Simmons et al. 2014).

Neutrophils play important roles in the pathogenesis of cerebral infection and injury. During stroke, neutrophils accumulate in the regions of cerebral infarction (Price et al. 2004). Neutrophils increase the damage by a number of mechanisms such as excessive ROS production, release of pro-inflammatory cytokines such as IL-6, TNF, IL-1β and chemokines such as MCP-1, IL-8 and MIP-1α to recruit other immune cells (Emerich et al. 2002; Price et al. 2004; Buck et al. 2008). However, there is no clear correlation between neutrophil infiltration and infarct formation.

In experimental cerebral malaria (ECM), excessive production of IFN-γ by neutrophils accumulating in the brain leads to overt inflammation and characteristic neurological signs of ECM. Selective depletion of $Ly6G^+$ neutrophils prevents neurological symptoms and increases survival. Furthermore, transfer of neutrophils back to the mice confers the susceptibility and thus links the infiltrating neutrophils to the pathogenesis of ECM (Porcherie et al. 2011).

During West Nile Virus (WNV) encephalitis, neutrophils are recruited to the brain through the chemokine ligand type-1, 2 (CXCL1, CXCL2). Neutrophils have a dual role during the course of infection. Depletion of $Ly6G^+$ cells prior to WNV challenge leads to lower viremia and enhanced survival suggesting that neutrophils initially assist in the progression of the infection. After WNV challenge, depletion of $Ly6G^+$ cells leads to increased viral burden and reduced survival rate suggesting that they also contribute to the control of the infection (Bai et al. 2010).

1.2.3.2 Inflammatory monocytes

Alongside neutrophils, monocytes also form a part of the innate immune cells. Monocytes belong to the mononuclear phagocyte system that also includes classical macrophages and classical DCs. Unlike macrophages and DCs that reside in tissues during steady state, monocytes are called in from the blood circulation on demand, mostly associated with inflammation (Dunay

& Sibley 2010; Serbina et al. 2012). Monocytes are derived from HSCs in the BM (Figure 3). The earliest defined precursor is the common myeloid progenitors (CMPs) (Akashi et al. 2000). CMPs sequentially give rise to granulocyte macrophage progenitors (GMPs) which give rise to neutrophils, a common macrophage dendritic cell progenitor (MDP) (Fogg 2006) and finally the committed monocyte progenitor (cMoP) (Hettinger et al. 2013). cMoP give rise to two phenotypically and functionally distinct subsets, discriminated by their Ly6C expression: Ly6C^{hi} and Ly6C^{neg} monocytes (Auffray et al. 2009; Geissmann et al. 2010). Ly6C^{hi} monocytes are characterized by high expression of the chemokine receptor CCR2, the adhesion molecule CD62L and low expression of the fractalkine receptor CX_3CR1 (Auffray et al. 2009; Geissmann et al. 2003; Sunderkotter et al. 2004). Ly6C^{hi} monocytes are also referred to as 'inflammatory' because they are selectively recruited to sites of inflammation and infection (Woollard et al. 2010; Serbina et al. 2003; Sponaas et al. 2009). On the other hand, Ly6C^{neg} monocytes are characterized by low expression of CCR2, CD62L and high expression of CX_3CR1 (Auffray et al. 2009; Geissmann et al. 2003; Sunderkotter et al. 2004). These cells patrol in the vasculature in steady state, quickly enter the inflamed tissue and contribute to the early inflammatory response before it is dominated by Ly6C^{hi} monocytes (Auffray et al. 2007). Murine monocytes constantly express CD45, CD11b and F4/80 (macrophage marker). Exit of monocytes from the BM is controlled by CCR2 ligands CCL2 and CCL7 (Thompson & Van Eldik 2009), which are secreted by many nucleated cells in response to inflammatory signals (Struyf et al. 1998; Thompson & Van Eldik 2009; Shi & Pamer 2011).

During steady state, alongside Ly6C^{neg} monocytes a limited proportion of Ly6C^{hi} monocytes is present within the parenchyma of a range of tissues, where they function similarly to classical DCs and are able to present Ags to T cells (Jakubzick et al. 2013; Tamoutounour et al. 2013).

Upon inflammation, pro-inflammatory cytokines and microbial products direct Ly6C^{hi} monocytes to the sites of infection by upregulating adhesion molecules such as P- and E-selectin, ICAM1, VCAM1 (Tedder et al. 1995; León & Ardavín 2008) on the inflamed endothelium. These adhesion molecules bind to the ligands CD62L, PSGL-1, very late antigen-4 (VLA-4) and lymphocyte function-associated antigen-1 (LFA-1) expressed on monocytes (Shi & Pamer 2011; Engelhardt 2009). One of the effector functions of Ly6C^{hi} monocytes is to produce pro-inflammatory cytokines such as TNF, IL-6, IL-12, IL-1β, IL-1α and also the anti-

inflammatory cytokine IL-10 (Grainger et al. 2013; Dunay & Sibley 2010; Dunay et al. 2008b). However environmental milieu and nature of the invading pathogen determine further effector functions of Ly6C^{hi} monocytes: DC-like phenotype with Ag presentation and T cell priming properties (Serbina et al. 2003; De Trez et al. 2009) and macrophage-like phenotype with tissue repairing properties, (Figure 4) (Shechter et al. 2009; Tacke et al. 2007).

1.2.3.2.1 Inflammatory monocytes in peripheral infections and inflammation

Inflammatory monocytes play an important role in peripheral infections and inflammation. The Ly6C^{hi} monocytes can give rise to various effector phenotypes depending on the context of inflammation such as in listeriosis, in acute *P. chabaudi* infection and in atherosclerosis. In listeriosis, the BM derived Ly6C^{hi} monocytes differentiate into TNF and inducible nitric oxide synthase (iNOS) producing dendritic cells (Tip – DCs) in the spleen. They also express CD11c and major histocompatibility complex-class II (MHC II), the two markers of conventional DC. Lack of CCR2 in mice and the subsequent absence of Ly6C^{hi} monocytes leads to uncontrolled bacterial replication and host death (Serbina et al. 2003).

In acute *P. chabaudi* infection, Ly6C^{hi} monocytes are recruited to the spleen and produce TNF and iNOS. Absence of Ly6C^{hi} monocytes leads to higher parasitemia. They do not express CD11c and MHC II and are incapable of presenting *P. chabaudi* specific peptide Ag to CD4^{+} T cells. Although they do not acquire a DC phenotype, they can produce iNOS, ROS and actively phagocytose parasites (Sponaas et al. 2009).

Ly6C^{hi} monocytes participate critically in cardiovascular diseases such as atherosclerosis, similarly to neutrophils. CCR2 and CCR5 are required for the recruitment of Ly6C^{hi} monocytes to the plaques. They are involved in the progression of atherosclerosis by producing matrix metalloproteinase-9 and 2 (MMP-9, MMP-2) and free oxygen radicals NADPH which degrade the structural proteins of the aortic wall (Tacke et al. 2007).

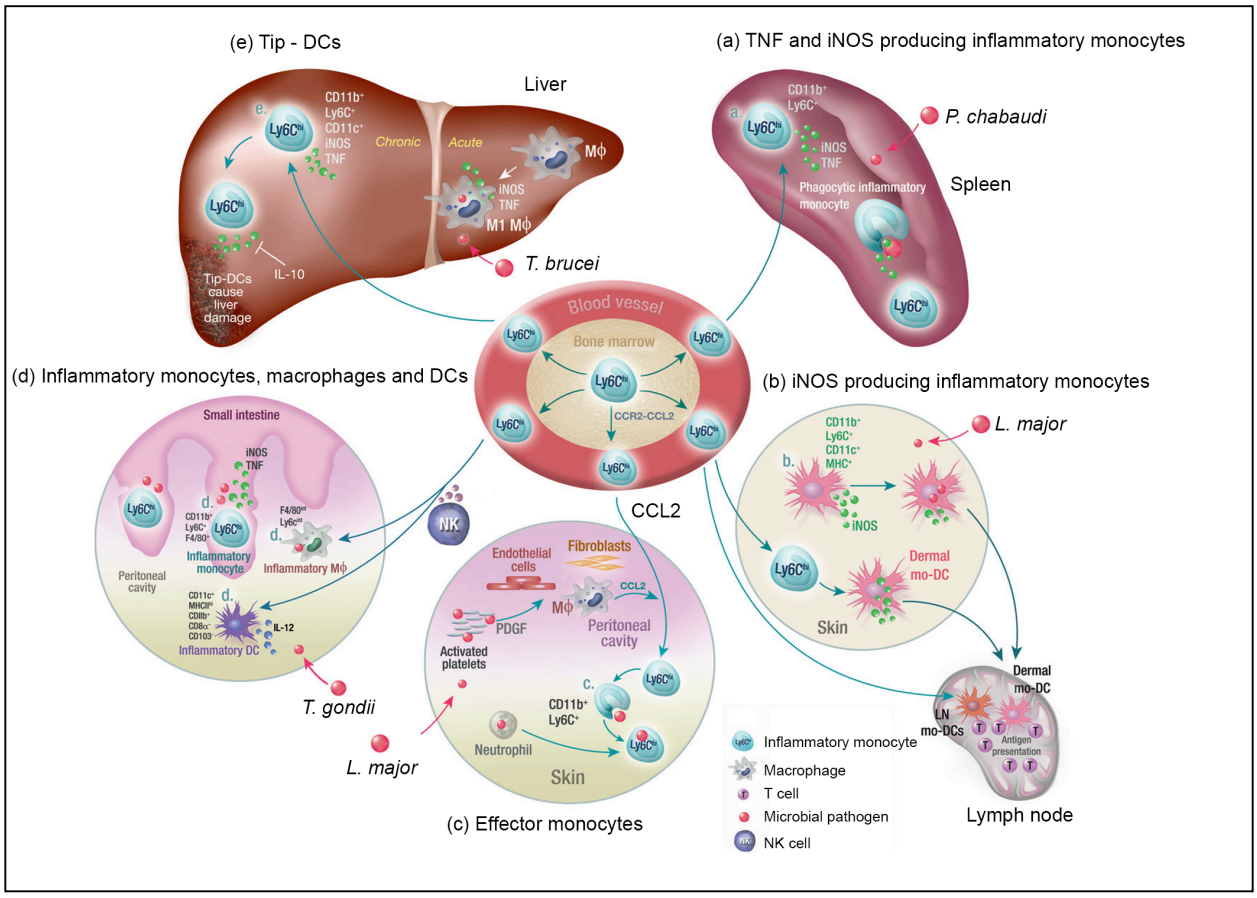


Figure 4. Monocyte heterogeneity in parasitic infections.

During parasitic infections Ly6C^{hi} monocytes egress from the BM in a CCR2-dependent manner into the circulatory system. Depending on the invading pathogen and the environmental conditions Ly6C^{hi} monocytes give rise to monocyte derived DCs or monocyte derived macrophages (adapted from Sheel & Engwerda 2012).

1.2.3.2.2 Inflammatory monocytes in central nervous system infections and injury

New experimental approaches such as mixed BM chimeras have led to the conclusion that BM derived monocytes do not contribute to the maintenance of most peripheral tissue macrophage population in adults during steady state (Hashimoto et al. 2013; Jakubzick et al. 2013; Ginhoux & Jung 2014). However, the role of BM derived monocytes in healthy CNS is still unknown.

In the inflamed CNS, the resident activated microglia produce chemokines such as MCP-1, MIP-1α and MIP-1β to recruit monocytes and lymphocytes (Häusler et al. 2002; Sargsyan et al. 2009). Availability of new surface markers and fate mapping helped to describe the functional differences between monocyte-derived macrophages and microglia (Ritzel et al. 2015). Like in

the periphery, BM derived monocytes can give rise to various effector phenotypes upon invading the CNS. They can differentiate into DC-like monocytes or macrophage-like monocytes depending on the invading pathogen or the environmental milieu that educates them (Mildner et al. 2013). This functional macrophage heterogeneity has been demonstrated in several models of neurodegenerative diseases, injury and infectious diseases (London et al. 2011; Shechter et al. 2009; Gliem et al. 2012; Schumak et al. 2015; Getts et al. 2008).

BM derived monocytes infiltrate the ischemic brain during stroke. They can be protective or detrimental on the stroke process depending on the phenotype of the cell and their number. Infiltrating inflammatory Ly6C^{hi} monocytes can produce ROS, cytokines and MMPs that can cause disruption of the BBB, leading to haemorrhages and continuous influx of myeloid cells. However, macrophage-like Ly6C^{neg} monocytes differentiated from Ly6C^{hi} monocytes are critical in preventing haemorrhagic infarct in permanent stroke models (Dimitrijevic et al. 2007; Gliem et al. 2012).

In cerebral malaria depletion of Ly6C^{hi} monocytes reduces the neuropathology, IFN-γ levels and IFN-γ producing CD8^{+}T cells. This suggests a possible role of Ly6C^{hi} monocytes in the progression of CM (Schumak et al. 2015).

Ly6C^{hi} monocytes contribute to the immunopathogenesis of WNV induced encephalitis since inhibition of the same leads to reduced morbidity and mortality (Getts et al. 2008). The descendants of Ly6C^{hi} monocytes - DC/macrophage – like monocytes might play role in the disease progression but the precise mechanism is still under intense investigation.

1.2.3.3 Dendritic cells

Dendritic cells bridge the gap between innate and adaptive immune responses. They are derived from lymphoid and myeloid progenitors (Figure 3). They are found mainly in the non-lymphoid tissues and organs such as skin, heart, liver, lung and mucosal surfaces (Lewis & Reizis 2012). DCs initiate, stimulate, regulate T cell responses, including Ag-specific T lymphocytes, T_H1/T_H2 modulation, regulatory T cell responses and peripheral T cell deletion (Steinman et al. 2003; Lanzavecchia & Sallusto 2001). Based on their origin and tissue location they express either

CD4 or CD8 or integrin α E (CD103) or integrin α M (CD11b) and integrin α X (CD11c) or Ly6C that promote immune tolerance and T_H1 immune response (Shortman & Liu 2002).

During steady state, immature DCs constantly survey the periphery for pathogens with TLRs that recognize parasites and their specific molecules (Shortman & Naik 2007; Coban et al. 2005; Plattner et al. 2008).

Upon interaction with parasite molecules, DCs undergo maturation and activation. This includes upregulation of molecules that play a role in costimulation of and Ag presentation to naïve T cells, such as CD80, CD86, CD40 and MHC II (Banchereau & Steinman 1998; Banchereau et al. 2000; Nimchinsky et al. 2002). However, these effector functions of mature DCs can be impaired or modulated by some pathogens affecting the outcome of the immune response.

1.2.3.3.1 Dendritic cells in peripheral infections and inflammation

Several studies describe the distinct effector functions of mature DCs in the periphery that are regulated by the context of the inflammation caused , such as during malaria, *listeriosis, leishmaniasis* and atherosclerosis. During parasitic infections such as malaria, soluble factors secreted from *P. yoeii*-infected erythrocytes inhibit maturation of DCs in a dose-dependent manner. This suggests that the inhibition peaks during late infection when there is maximum parasite burden. The infected erythrocytes also inhibit IL-12 production by DCs, the cytokine required to activate cytotoxic T cells (Orengo et al. 2008).

In atherosclerosis the chemokines CCL2, CCL5 and the chemokine receptor CX_3CR1 recruit DCs to the plaques. The interaction between modified autoantigens and TLR4 and TLR2 found on DCs activates them to produce effector molecules such as MMP9 to degrade the atherosclerotic plaque. They also trigger recruitment of cytotoxic T cells by producing IL-12 (Niessner & Weyand 2010).

During infection induced inflammatory reactions, DCs can be complemented from the circulating inflammatory monocytes (Auffray et al. 2009; Varol et al. 2007; Naik et al. 2006; Serbina et al. 2003; Randolph et al. 1999). Several studies have shown the potential of monocyte derived DCs to induce T cell responses against pathogens (Leon 2007; Serbina et al. 2003). During *listeriosis* and *leishmaniasis* monocyte derived DCs formed at the infection site can

effectively induce T_H1 response or have microbicidal functions by producing TNF and iNOS (Leon 2007; Serbina et al. 2003). Thus, DCs links innate immune response to the adaptive immune response by activating T cells. Additionally, circulating monocytes that adopt infection and tissue dependent effector functions can also complement them.

1.2.3.3.2 Dendritic cells in central nervous system infections and injury

In steady state, it is well established that DCs are found within the meninges and the choroid plexus. Their presence suggests that DCs may play a role in immune surveillance, possibly presenting Ags to the T cells to activate them (P. 2013)(Matyszak & Perry 1996; Benjamin D Clarkson et al. 2012; Karman et al. 2004; Agostino et al. 2013). A detailed characterization of the DCs present in the healthy CNS is still missing due to the low yield following isolation. During neuroinflammation, DCs from these areas accumulate in the parenchyma and also are complemented from the periphery (Fischer & Reichmann 2001; Hesske et al. 2010).

During stroke, it has been shown that in the early phase (24 h post stroke injury) 80% of $CD11c^+$ DCs are CNS resident, whereas 72 h later there is a 1 : 1 distribution of peripheral and resident DCs. The recruited DCs were concentrated near the infarct zone, whereas the resident population was near the penumbral zone associated with infiltrating T cells, suggesting their immunoregulatory function (Felger et al. 2010).

In *Borrelia burgdorferi*-driven encephalitis, soluble factors such as TNF and IL-1β differentially influences the function and phenotype of monocyte derived DCs. This in turn influence IFN-γ production which is crucial to fight the infection (Pashenkov et al. 2002).

In cerebral malaria, the DCs of lymphoid origin play a dominant role in the pathogenesis as depletion of $CD11c^+CD8^+$ DCs lead to a complete protection from severe brain pathology. Absence of $CD11c^+CD8^+$ DCs prevents excessive IFN-γ production by cytotoxic CD8+ T cells and reduced levels of the protease granzyme B. The specific deletion also reduced parasitized erythrocytes in the brain (Piva et al. 2012).

1.2.3.4 Macrophages

Macrophages are myeloid derived immune cells see (figure 3). They are placed throughout the body tissues where they engulf and digest dead cells, foreign substances, microbes and cancer

cells by phagocytosis. Macrophages are identified by their specific expression of proteins such as CD11b, F4/80 and CD64 (Khazen et al. 2005). Macrophages belonging to the mononuclear phagocyte system have two distinct origins. The majority of macrophages reside in tissues, established prenatally, such as microglia (Wolf et al. 2013). These cellular compartments maintain themselves by self-renewal, independent of hematopoietic input. In addition, macrophages can develop during adulthood from tissue-infiltrating monocytes upon infection and during inflammatory reactions (Mildner et al. 2013; Varol et al. 2015).

During steady-state, activities of these phagocytes include patrolling tissues for potential pathogens by expressing vast range of sensing molecules such as scavenger receptors, pattern recognition receptors (TLRs and NODs) and non-specific defence by ingesting aged neutrophils and dead cells.

During early stages of infection, macrophages can recruit lymphocytes by secreting cytokines such as TNF and IL-1β. During late stages of infection, macrophages can repair damaged tissues by curbing overt inflammatory reactions by secreting anti-inflammatory cytokines such as IL-10 and TGF-β and lipid mediators such as PGE_2 and PGD_2 (Ariel et al. 2012; Eming et al. 2007; Mildner et al. 2013; Varol et al. 2015).

1.2.3.4.1 Macrophages in peripheral infections and inflammation

Several studies have showed that macrophages play important roles in peripheral infections and inflammation. *L.monocytogenes* is detected by the macrophages through their pattern recognition receptors such as TLRs and NODs. This activates pro-inflammatory mediators to recruit monocytes, which then differentiate into Tip-DCs. These Tip-DCs contribute to clearance of the infection (Leber et al. 2008; Corr & O'Neill 2009; Serbina et al. 2003).

Macrophages are the main component of atherosclerotic plaques. Pro- and anti-inflammatory factors secreted from the lesion site leads to activation of downstream TLRs-NF-κB signalling on macrophages, which then recruit monocytes. The recruited monocytes differentiate into IL-10 expressing tissue-remodelling macrophages which decrease plaque formation (Tacke et al. 2007; Gui et al. 2012).

In *P. chabaudi* infection, splenic macrophages can sense and promote immune responses through TLR9, myeloid differentiation primary response gene (88) (MyD88) and interferon regulatory factor-7 (IRF7) but they are not required to control parasitemia (Kim et al. 2012).

1.2.3.5 Microglia

Microglia are resident macrophages of the brain and the spinal cord. They are seeded in the CNS before birth from the embryonic yolk sac and maintain themselves without any BM – derived myeloid cell replenishment during adulthood (Ginhoux et al. 2010; Ajami et al. 2007). Being resident cells, microglia are crucial for homeostatic maintenance and form the first active immune response in the CNS (Tremblay et al. 2011; Hanisch & Kettenmann 2007). Microglia express surface markers including CD45, CD11b and F4/80. Microglia are extremely plastic, undergoing structural changes, which enables them to perform numerous immunological functions as well as maintain homeostasis.

During steady state, they have a ramified morphology and express little or no immunogenic molecules such as MHC II. Upon CNS insults including microbial invasion they convert to an amoeboid morphology. Amoeboid microglia are highly phagocytic, upregulate surface receptors such as CD45 and MHC II and produce multiple secretory products to contribute to immune defence (Kierdorf & Prinz 2013; Rock et al. 2004). Activated amoeboid microglia express chemokine receptor such as CX_3CR1 and the ligand CXCL1 to communicate with neurons (Meucci et al. 2000; Harrison et al. 1998). To regain homeostasis after damage, microglia clear cellular debris by phagoctosing damaged brain cells through TREM2, a receptor expressed on microglia (Colonna 2003). Apart from chemokines, microglia also secrete pro-inflammatory cytokines such as IL-1 (Chauvet et al. 2001), IL-6 (Erta et al. 2012), TNF (Chao et al. 1992), IL-12 (Aloisi et al. 1997) and anti-inflammatory cytokines such as IL-10 (Ledeboer et al. 2002). Microglia can also stimulate the recruitment of peripheral immune cells such as neutrophils, monocytes and lymphocytes to fight against disease causing agents.

1.2.3.5.1 Microglia in central nervous system infections and injury

Being CNS resident, microglia forms the first line of defence against infections and injury. In the ischemic brain after a stroke, microglia are activated and initiate effectors molecules. These molecules includes pro-inflammatory mediators TNF, IL-6, IL-1β, proteolytic enzymes MMP-9

and MMP-3 and recruit immune cells from the periphery. Microglia also act as a scavenger for damaged cells by upregulating TREM2 expression (Schilling et al. 2005).

CM is characterized by parasitized erythrocytes with endothelial cells that disrupt BBB causing microhemorrhages and forming glial aggregates. Microglia is involved in the pathogenesis by releasing TNF that initiates cerebral complications associated with disease (Medana et al. 1997).

In acute WNV infection, microglia produce lower amounts of the pro-inflammatory mediator nitric oxide (NO), express lower levels of MHC II and have significantly less capacity to process Ags and stimulate T cells compared to infiltrating monocytes (Getts et al. 2008; Cho & Diamond 2012).

1.3 Immune response in acute toxoplasmosis

Ingestion of *T. gondii* causes a primary infection. The parasites that survive the gastric process cross the intestinal epithelium and perpetuate proliferation (Barragan 2002). The intracellular localization of the parasites largely protects itself from cellular antimicrobial factors, but nevertheless a strong immune response is triggered during the acute stage (Munoz et al. 2011; Tait & Hunter 2009). The first line of defence against *T. gondii* is characterized by the recruitment of innate immune cells such as neutrophils, monocytes and DC to the gut, where all of these cell types are implicated in resistance to the parasite (Dunay et al. 2008; Dunay et al. 2010; Mordue & Sibley 2003; Bliss et al. 2001; Elia D. Tait et al. 2010; Del Rio et al. 2001; Sturge et al. 2013; Mashayekhi et al. 2011). The innate immune response to *T. gondii* is defined by the ability to produce IL-12 that stimulates immune cells such as innate lymphoid cells (ILCs), natural killer (NK) cells, neutrophils and T cells to produce IFN-γ (Gazzinelli & Sher 2014; Yarovinsky 2013; Klose 2013). $Ly6C^{hi}$ monocytes, $CD8\alpha^{+}$ DCs, plasmacytoid DCs and neutrophils have been shown in many studies to produce IL-12 but $CD8\alpha^{+}$DCs are the most important contributor (Robben et al. 2005; Mashayekhi et al. 2011; Pepper et al. 2008; Bliss et al. 2001). In addition, DCs sense parasite molecules such as *T. gondii* profilin and glycosylphosphatidylinositols through their TLRs to produce IL-12 (Yarovinsky et al. 2005; Pifer & Yarovinsky 2011). Alongside, $Ly6C^{hi}Gr1^{+}$ monocytes also produce high amounts of TNF, iNOS and ROS, which directly contribute to controlling the parasite levels in the host (Dunay et al. 2008; Dunay & Sibley 2010).

1.3.1 Neutrophil granulocytes

When *T. gondii* successfully breaches the epithelial barrier, the signals generated by the parasite activate local endothelial cells that guide the circulating neutrophils to ward the site of infection (Denkers et al. 2012). Recently it has been shown that neutrophils store IFN-γ in their granules, since their promyelocyte stage (Sturge et al. 2015). This IFN-γ forms a very important non-lymphoid source in acute *Toxoplasma* infection (Sturge et al. 2013). The neutrophil-derived IFN-γ is required for TLR independent host protection against *T. gondii*, unlike NK and T cell that require the TLR adaptor protein MyD88, for the production of IFN-γ (Sturge et al. 2013). However, the role of neutrophils on parasite replication was unclear, as earlier reports of increased parasite replication upon neutrophil depletion used RB6-8C5 monoclonal antibody (mAb) (Bliss et al. 2001). The anti-granulocyte (Gr-1) RB6-8C5 mAb depleted also the inflammatory monocytes due to their common expression of Gr-1 (Ly6C/G), making the earlier findings complicated (Del Rio et al. 2001; Bliss et al. 2001). Selective depletion of neutrophils revealed that they are not associated with an increased susceptibility to acute *Toxoplasma* infection, but rather caused considerable tissue damage (Dunay et al. 2010). However, mice deficient of CXCR2, the major chemoattractant for the neutrophils, were more susceptible compared to the RB6-8C5 mAb depleted mice, upon *T. gondii* infection (Del Rio et al. 2001). However this study was done on mice with BALB/c background unlike the 1A8 depletion study which was done on C57BL/6 (Dunay et al. 2010). This shows factors such as mouse strain, host species and parasite lineage could influence the role of neutrophils during *T. gondii* infection. Although the neutrophils may not play a crucial role against acute *T. gondii* infection, it has been shown recently that the parasite activates the MEK-extracellular-signal regulated kinase (ERK) pathway in neutrophils, which leads to NET formation. This could be an innate mechanism leading to entrapment and killing of the parasite (Denkers et al. 2012). Collectively, the aforementioned studies show that neutrophils play a broad role (protective and pathogenic to some extent) in acute toxoplasmosis.

1.3.2 Inflammatory monocytes

The sequence at which immune cells are recruited during primary infection with *T. gondii* consists of initial extravasation of neutrophils followed by emigration of monocytes from the

BM. Ly6C^{hi} monocytes play a vital role in controlling parasite replication (Dunay et al. 2008; Dunay & Sibley 2010). There is uncontrolled replication of the parasite in the peritoneum and other tissues of transgenic mice that lack the receptor CCR2 needed for the mobilization of inflammatory monocytes (Robben et al. 2005). The mice that survived the initial phase of infection had severe CNS pathology and succumbed within 30 days, suggesting a role of inflammatory monocytes in the brain (Benevides et al. 2008). Alongside their pro-inflammatory signature (producing IL-1α, IL-1β, TNF, iNOS, IL-12 and IL-6), Ly6C^{hi} monocytes also produce IL-10, an immunoregulatory cytokine, thus showing their dual capacity to control parasite burden and reducing collateral damage to tissue (Grainger et al. 2013). It was shown that the differentiation of Ly6C^{hi} monocytes into F4/80 expressing macrophages and CD11c and MHC II expressing DCs, depends on NK cell-derived IFN-γ (Goldszmid 2012). Thus, Ly6C^{hi} monocytes differentiate into a spectrum of effector cells with distinct antimicrobial properties playing an essential role in innate immune defence and contribute to adaptive immune response.

1.3.3 Dendritic cells

In the periphery, interaction of *T. gondii* profilin with TLR11 presented on DCs leads to robust production of IL-12 (Denkers & Striepen 2008; Yarovinsky et al. 2005). Studies, aimed to define which DC subset is the major contributor to IL-12 production, found, that mice lacking the basic leucine zipper transcription factor ATF-like 3 (Batf3) which cannot develop CD8α^{+}DCs, succumbed to *T. gondii*. Batf3$^{-/-}$ mice suffered from a severe IL-12 defect, reduced CD8^{+} T cell response and increased parasite burden (Mashayekhi et al. 2011).

1.3.4 Macrophages

During acute infection, intracellular *T. gondii* disrupts the NF-κB, STAT1 and MAPK signalling pathways of peritoneal macrophages. These pathways are important for pro-inflammatory cytokine production. Hence disruption of these pathways results in suppression of their potent microbicidal activity (Denkers et al. 2004). However, the macrophages recruit BM derived inflammatory monocytes to the periphery. The inflammatory Ly6C^{hi} monocytes disrupt the parasitophorous vacuole formed by the parasite within the host cells by secreting IRG proteins that release the parasite into the cytoplasm where they are killed by immunomodulatory molecules (Denkers et al. 2012). Collectively, in acute *Toxoplasma* infection macrophages can

sense their local conditions and consequently adopt specialized functions or recruit circulating monocytes on demand.

1.4 The central nervous system

The CNS is comprised of the brain, the spinal cord, optic nerves and the retina. They are composed of glial cells, neurons and blood vessels (Purves 2004). The immune system in the CNS functions differently as it is separated from the systemic immune system by the BBB and blood- cerebrospinal fluid barrier (BCSFB). The integrity of the BBB is maintained by tight junctions between brain endothelial cells, the basal lamina of these cells and astrocyte endfeet processes (Ransohoff et al. 2003; Takeshita & Ransohoff 2008). Thus, pathogens, circulating immune cells and factors within the blood cannot readily access the 'immune-privileged' CNS (Ransohoff & Engelhardt 2012). However the complex neuroimmunological interactions which occur during homeostatic and pathologic conditions as observed in many studies, have challenged the concept of 'immune-privilege' (Ransohoff & Engelhardt 2012). It is now accepted that apart from resident glial cells, there are immune cells that provide immune surveillance. These immune cells can also drain from the brain and interact with the peripheral immune system through classical lymphatic vessels (Louveau et al. 2015).

1.5 Immune response in the cerebral *T. gondii* infection

Following oral ingestion of *T. gondii*, the parasite rapidly disseminates throughout the body via blood stream within 24 hours (Dubey et al. 1998; Barragan 2002). Several studies have indicated that the parasite can transmigrate within motile immune cells such as macrophages (Da Gama et al. 2004) and DCs (Courret et al. 2006a; Lambert et al. 2006). The parasite, which has tropism towards CNS, has developed specific molecular strategies to invade it. In the CNS the parasite infect the resident cells such as neurons, astrocytes and microglia within 7-10 days post-infection (Fischer et al. 1997; Halonen et al. 1998). Once inside the brain, the strong immune response from the host forces a stage conversion of the parasite from tachyzoites to the slowly dividing bradyzoites that ultimately reside in tissue cysts and, establish a chronic infection. During this stage the host immune system is constantly stimulated with parasite Ags. This prevents re-activation of the latent infection by maintaining the CNS immunologically active (John et al. 2011; Fischer et al. 1997; Sa et al. 2015). In the brain, *T. gondii* infected resident cells such as

astrocytes and microglia mount a strong response against the infection by upregulating chemoattractants such as MCP-1, MIP-1α, MIP-1β to recruit peripheral immune cells (Strack et al. 2002). Immune cells from the periphery inundate the CNS during the chronic stage of *T. gondii* infection. Neutrophils being the sentinels of the immune system invade foremost, followed by the BM derived monocytes and DCs (John et al. 2011; Fischer & Reichmann 2001). Brain resident immune cells are capable of activating the adaptive immune response by secreting cytokines such as TNF, IL-1, IL-10, TGF-β and IFN-γ (Fischer et al. 1997; Suzuki et al. 2005) and upregulating transcription factor NF-κB (Molestina & Sinai 2005) that can activate other immune regulators. The adaptive immune response plays a critical role in the prevention of cerebral toxoplasmosis by secreting the cytokine IFN-γ without which the survival can be highly compromised (Suzuki 2002). NF-κB and CD40-40L signalling is important for optimal IFN-γ production by $CD4^+$ and $CD8^+$ T cells which invade the CNS (Caamaño et al. 2000; Reichmann et al. 2000). The role of brain resident cells and recruited adaptive immune cells has been extensively studied in cerebral toxoplasmosis. However, the role of recruited myeloid cells in cerebral toxoplasmosis still remains uncertain.

1.5.1 Microglia

The microglia forms the major effector cells in preventing *T. gondii* tachyzoite proliferation in the brain. Studies have shown that IL-6, TNF and IFN-γ abrogate *T. gondii* multiplication in a dose-dependent manner by reducing the entry of the parasite into microglia. However, once inside the cell the cytokine treatment has little or no effect on the tachyzoite replication (Rock et al. 2004). Microglia also secrete chemokines such as MCP-1, MIP-1α and MIP-1β to recruit peripheral immune cells for a robust immune response (Strack et al. 2002). It was reported that apart from T cells, microglia produce IFN-γ suggesting they play crucial role in limiting parasite replication (Sa et al. 2015; Kang & Suzuki 2001). Microglia, apart from initiating a pro-inflammatory environment also secrete IL-10 to suppress the host tissue inflammation. This reduces neuronal damage but also facilitates the persistence of the parasite by suppressing the CNS immune response (Deckert-Schlüter et al. 1997; Deckert-Schluter et al. 1998).

1.5.2 Myeloid derived innate immune cells

During cerebral toxoplasmosis, it has been shown by John B. et al 2011 that 95 % of the DCs are derived from the periphery. These DCs are mature, capable of presenting Ags to naïve T cells, major producers of IL-12 and direct T_H1 immune response (John et al. 2011; Fischer & Reichmann 2001). Conversion of BM derived monocytes into DCs can be a source to the infiltrating DCs in the CNS but it is yet undefined.

The particular function of other myeloid derived innate immune cells such as the neutrophils and the $Ly6C^{hi}$ inflammatory monocytes in cerebral toxoplasmosis is yet to be addressed.

2. Aims

The myeloid derived innate immune cells namely Ly6C^{hi} inflammatory monocytes and Ly6G^{+} neutrophils play crucial roles in acute toxoplasmosis. However, their specific functions in chronic cerebral toxoplasmosis are still unknown. Therefore, to gain more insight I did phenotypic and functional analysis of these myeloid derived innate immune cells.

2.1 To study the role of Ly6C^{hi} inflammatory monocytes in cerebral toxoplasmosis.

In the first part of the thesis my aim was to investigate the phenotype and particular function of the recruited Ly6C^{hi} monocytes along the course of a low *T. gondii* cysts dose induced chronic infection. At first I studied the heterogeneous myeloid derived cell populations infiltrating the brain. To understand their specific function, the myeloid derived Ly6C^{hi} inflammatory monocytes were depleted. To gain insight into the inflammatory mediators expressed and secreted by Ly6C^{hi} inflammatory monocytes and their subsets, comprehensive surface and intracellular flow cytometric analysis was completed. Furthermore, I did adoptive transfer experiments to study the fate of Ly6C^{hi} inflammatory monocytes infiltrating the brain. Finally, I studied the adhesion molecules that play a role in the recruitment of Ly6C^{hi} monocytes to the CNS.

2.2 To study the role of Ly6G^{+} neutrophil granulocytes in cerebral toxoplasmosis.

In the second part of the thesis my aim was to investigate the phenotype and function of the recruited Ly6G^{+} neutrophils during cerebral toxoplasmosis. At first I characterized the Ly6G^{+} neutrophils in the peripheral blood and in the brain following low *T. gondii* cysts dose induced chronic infection. I did a detailed surface and intracellular flow cytometric analysis to gain insight into the Ly6G^{+} neutrophils. To understand their specific function, Ly6G^{+} neutrophils were depleted. I also functionally characterized the recruited Ly6G^{+} neutrophil subsets in cerebral toxoplasmosis.

3.Materials and methods

3.1 Materials

3.1.1 Chemicals used for animal experiments

Table 1. Chemicals used for animal experiments

Isoflurane	Baxter, Illinois, USA
4 % paraformaldehyde (PFA)	Affymetrix, California, USA
PBS	Lonza, Basel, Switzerland

3.1.2 Materials for cell culture

All cell culture work was carried out under a laminar flow hood. The sterile cell culture media were prewarmed in a water bath at 37 °C before use. Cells were cultured in an incubator at 37 °C, 5 % CO_2 and 60 % of water vapour. The cell culture plastic materials were purchased from Sigma-Aldrich (Missouri, USA).

Table 2. Reagents for cell culture.

Cell culture medium	DMEM (Life Technologies, California, USA), 10 % fetal calf serum (FCS, Life Technologies, California, USA), 100 U Penicillin / streptomycin (PAA LaboratoriesGmbH, Pasching, Austria)
PBS	Lonza, Basel, Switzerland
HBSS	Life Technologies, California, USA
RBC Lysis Buffer	eBioscience, San Diego, USA

HEPES	Sigma-Aldrich, Steinheim, Germany
Percoll	GE Healthcare, Braunschweig, Germany
Permeabilisation Buffer	Biolegend, San Diego, USA
Brefeldin A	Biolegend, San Diego, USA
Monensin	Biolegend, San Diego, USA
Glucose	Sigma-Aldrich, Steinheim, Germany
Cytochalasin D	Sigma-Aldrich, Steinheim, Germany
Well chamber	Nalge Nunc International, New York, USA
Trypan blue	Sigma-Aldrich, Steinheim, Germany
Ethanol (70 %, 98 %)	Pharmacy, University, Magdeburg

3.1.3 Materials for molecular biology

TaqMan Gene Expression Assays with RNA-to-Ct-1-Step Kit were obtained from Life Technologies, California, USA.

Roche FastStart Essential DNA Green Master, Basel, Switzerland was used for SYBR Green I-based real time PCR.

All the primers were bought from TIB Molbio, Berlin, Germany

Table 3. Materials for molecular biology.

AllPrep DNA/RNA/Protein Mini kit	Qiagen, Hilden, Germany
RNAse free Eppendorf tubes	Eppendorf, Hamburg, Germany

Bashing Beads Lysis Tubes	Zymogen, California, USA
β-mercaptoethanol	Roth, Karlsruhe, Germany
AllProtect Tissue Reagent	Qiagen, Hilden, Germany
Sterile distilled water	Berlin Chemie AG, Berlin, Germany

3.1.4 Antibodies for immunological analysis

Table 4. Antibodies for flow cytometric analysis.

Antibody	Clone
BV510-anti-mouse CD45 mAb (rat)	(30-F11)
APC-anti-mouse CD11b mAb (rat)	(M1/70)
FITC-anti-mouse CD11c mAb (hamster)	(V418)
FITC- anti-mouse F4/80 mAb (rat)	(BM8)
FITC-anti-mouse Ly6G (rat)	(1A8)
FITC-anti-mouse Ly6C (rat)	(HK1.4)
PE-anti-mouse CCR2 (mouse)	(475301)
FITC-anti-mouse CD86 (mouse)	(GL1)
PerCP-Cy5.5-anti-mouse CD80 (mouse)	(16-10A1)
FITC-anti-mouse MHC ClassI (mouse)	(AF6-88.5-5-3)
PE - anti-mouse MHC ClassII (rat)	(M5/114.15.2)

PE - anti-mouse TREM2 (rat)	(237920)
PE - anti-mouse CD64 (rat)	(X54-5/7.1)
APC-anti-mouse CX3CR1 mAb (rat)	(Polyclonal)
PerCP-Cy5.5-anti-mouse CXCR2 (rat)	(SA045E1)
APC-anti-mouse CXCR4 mAb (rat)	(L276F12)
PE - anti-mouse IL-1α (rat)	(ALF-161)
PE - anti-mouse IL-1β (rat)	(NJTEN3)
PE - anti-mouse IL-6 (rat)	(MP5-20F3)
PE - anti-mouse NOS2 (rat)	(CXNFT)
PE - anti-mouse IL-12p40 (rat)	(C17.8)
PE - anti-mouse IL-10 (rat)	(JES5-16E3)
PE - anti-mouse IFN-γ (rat)	(XMG1.2)
FITC - anti-mouse TNF (rat)	(MP6-XT22)
FITC - anti-mouse LFA-1 (rat)	(M17/4)
PE - anti-mouse PSGL-1 (rat)	(2PH1)
FITC -anti-mouse CD62L (rat)	(MEL-14)
PE -rat-IgG2b isotype control	(eB149)

PE -rat-IgG2a isotype control	(eBR2a)
FITC -rat-IgG2a isotype control	(eBM2a)
PE -rat-IgG1 isotype control	(eBRG1)
FITC -rat-IgG1 isotype control	(eBRG1)
FITC -hamster-IgG isotype control	(eBio299Arm)
PE -hamster-IgG isotype control	(eBio299Arm)
anti-FcγIII/II receptor	(93)

Cells were stained with Fixable Viability Dyes Zombie NIR APC-Cy7 (Biolegend, San Diego, USA) and Zombie Violet BV421 (Biolegend, San Diego, USA) prior to fixation to exclude dead cells.

All antibodies obtained from eBioscience or Biolegend or R&D Systems and used at a concentration of 0,5 to 1µg/ $1x10^6$ cells following titration.

Table 5. Kits used for immunological analysis.

Total ROS Detection Kit	ENZO Life Sciences, Farmingdale, USA
CFSE Labelling Kit	Invitrogen, Karlsruhe, Germany
Lightning-Link™ Tandem Conjugation Kit	Innova Biosciences, Cambridge, UK
Bond Polymer Refine Detection Kit	Leica Biosystems, Wetzlar, Germany

Antigen Peptide

Toxoplasma lysate antigen (TLA) was prepared by sonification of tachyzoites obtained from the peritoneal cavities of BALB/c mice infected 3 days prior with 10^6 tachyzoites of the virulent BK strain of *T. gondii* (kindly provided by K. Janitschke, Robert Koch-Institut, Berlin, Germany).

For *in vitro* restimulation of cells, TLA was prepared as described above; optimal concentrations of TLA were determined prior to use.

Table 6. Depleting antibodies for flow cytometric analysis.

anti-CCR2 (MC21 clone) mAb	Mathias Mack, University of Regensburg
anti-Ly6G (1A8 clone) mAb	BioXCell, New Hampshire, US
anti-mouse PSGL-1 (4RA10) mAb	BioXCell, New Hampshire, US
Rat IgG2a mAb	BioXCell, New Hampshire, US
Rat IgG mAb	eBioscience, San Diego, CA

Table 7. Antibodies for histological analysis.

Primary Antibody	Dilution	Company
Iba1	1 : 250	Dako, Denmark
CD3	1 : 250	Dako, Denmark
Toxo	1 : 200	Dianova, Germany
Mac-1	1 : 200	Abcam, Denmark

Table 8. Materials for immunofluorescence.

Dilution Buffer	PBS (containing 2 % normal goat serum (Vector Laboratories), 1 % BSA, 0.1% gelatine, 0.05 % Tween20 and 0.05 % sodium azide)
Primary antibodies	

CCR2	GenWay Biotech, San Diego, USA
Ly6C	GenWay Biotech, San Diego, USA
Secondary antibodies	
Alexa 488	GenWay Biotech, San Diego, USA
Alexa 594	GenWay Biotech, San Diego, USA
ProLong Gold containing DAPI	Thermo Scientific, Massachusetts, USA

Table 9. Instruments.

Table Centrifuge	Biofuge PICO, Massachusetts, USA
Nanodrop	Thermo Scientific, Massachusetts, USA
Shaker	Peqlab, Erlangen, Germany
Pipette	Eppendorf, Hamburg, Germany
Centrifuge	Heraeus, Hanau, Germany
Incubator	Thermo Scientific, Massachusetts, USA
FACS Canto II	BD Biosciences, Heidelberg, Germany
FACS Vantage DIVA	BD Biosciences, Heidelberg, Germany
PCR machine	Peq lab, Erlangen, Germany
Pipette boy	Eppendorf, Hamburg, Germany

Thermomixer compact	Eppendorf, Hamburg, Germany
Neubauer counting chamber	Lauda-Königshofen, Germany
Coverslip (for Neubauer counting chamber)	Polysciences, Warrington, USA
Fluorospheres	Invitrogen, Karlsruhe, Germany
Syringe	BD Discardit II, Heidelberg, Germany
Needle	Braun, Frankfurt, Germany
Strainer	Thermo Scientific, Massachusetts, USA
Fluoroscence Microscope	Carl Zeiss, Jena, Germany

3.1.5 Animals

Age and sex matched C57BL/6 wildtype (WT) mice, obtained from Janvier (Cedex, France) were used. All animals were kept under conventional conditions in an isolation facility throughout the experiments. All animal experiments were in compliance with the German animal protection law in a protocol approved by the Landesverwaltungsamt Sachsen-Anhalt (University of Magdeburg).

3.2 Methods

3.2.1 Experimental treatment of mice

3.2.1.1 Infection of mice with ME49 strain

T. gondii cysts of type II ME49 strain were harvested from the brains of female NMRI mice infected i.p. with *T. gondii* cysts 5 – 6 months earlier. Brains obtained from infected mice were mechanically homogenized in 1 ml sterile PBS. The cyst numbers were counted in a 10 µl brain suspension using a light microscope. Three cysts were administered i.p. in a total volume of 200 µl/mouse. Control mice were mock-infected with sterile PBS.

3.2.1.2 Depletion of inflammatory monocytes

Monocytes were depleted by administering anti-CCR2 mAb (clone MC-21). Mice were injected i.p. with 75 µg (40) mAb on days 20, 22, 24, 26, and 28 post-infection. Twenty-four hours after the last treatment on day 29, the mice were sacrificed. Rat IgG was used as a control mAb.

3.2.1.3 Depletion of neutrophils

Neutrophils were depleted by administering 500 µg anti-Ly6G mAb i.p. The antibody was administered on alternate days starting from day 12 to day 23. Twenty-four hours after the last treatment, the mice were sacrificed. Rat IgG2a was used as a control mAb.

3.2.1.4 Anti-PSGL-1 treatment

To assess the transmigration capacity of the cells, 2 mg/kg rat anti-mouse PSGL-1 was administered i.p. every alternate day from day 16 to day 26 post-infection. Twenty-four hours after the last treatment on day 27, the mice were sacrificed. Isotype-matched rat IgG was administered to the controls.

3.2.2 Ex vivo methods

3.2.2.1 Sacrifice and organ isolation

Animals were anaesthetized with isoflurane. Blood was obtained using a 26 guage needle and 1 ml syringe from the posterior vena cava and stored in FACS buffer for further studies. After the blood isolation, mice were transcardially perfused with ice cold PBS to remove blood from the tissues.

For FACS analysis, organs were stored in ice-cold DMEM media. For RT-PCR, organs were stored in ice-cold AllProtect Tissue Reagent for 24 h and then transferred to -20 °C for further studies. For histology and immunofluoroscence, organs were stored in 4 % PFA at 4°C or snap frozen in an embedding medium at -80 °C in the presence of 2-methylbutane and stored at -80 °C.

3.2.2.2 Parasite number

To determine the total *T. gondii* cyst burden in the brains of infected mice, full brains were obtained and mechanically homogenized in 1 ml sterile PBS and the total cyst numbers were

determined using light microscope. To confirm *T. gondii* cyst numbers, five to six coronal slides per mouse (same brain region) were analysed by microscopy n = 4 mice per group. Two researchers performed the blinded analysis.

3.2.2.3 Preparation of peripheral blood suspension

Peripheral blood obtained from the mice was diluted in the presence of FACS Buffer and washed for 10 min at 400 × g. The erythrocytes in the blood were lysed using RBC Lysis Buffer for 15 min at 4 °C. The lysed cells were washed in PBS for 10 min at 400 × g. The cell pellet was resuspended in the FACS Buffer in the desired concentration for further analysis.

3.2.2.4 Preparation of bone marrow cells suspension

Femurs and tibiae were carefully cleaned from the adherent soft tissue. The tip of each bone was cut and the marrow was harvested by inserting a syringe 26 gauge needle on one end and flushing with PBS. Cells were passed through the 70 μm strainer and washed in PBS for 10 min at 300 × g. The erythrocytes in the BM cells were lysed using RBC Lysis Buffer for 15 min at 4 °C. The lysed cells were washed in PBS for 10 min at 300 × g. The cell pellet was resuspended in the PBS in the desired concentration. Cell numbers were calculated accordingly for further analysis.

3.2.2.5 Isolation of brain lymphocytes

Brains were homogenized in a buffer containing 1 M HEPES (pH 7.3) and 45 % glucose and then sieved through a 70 μm strainer. The cell suspension was washed and fractionated on Percoll gradient. The cells were resuspended with 10 ml of 75 % Percoll. Then, 10 ml of 25 % Percoll was loaded and lastly 5 ml of PBS was added on the top. The cells were centrifuged for 25 min at 800 × g without brake. The cells in the interphase that comprised of mononuclear cells were washed with PBS for 10 min at 400 × g. The cell pellet was washed again with FACS Buffer and finally resuspended in the FACS Buffer in the desired concentration. Cell numbers were calculated accordingly for further analysis.

3.2.2.6 Counting of cells

All cells were counted using a Neubauer counting chamber according to manufactures' protocol. 4 % Trypan blue was used to count only the living cells. Based on the counting the desired cell concentration was adjusted with FACS buffer or DMEM media.

3.2.2.7 Adoptive transfer

Isolated BM cells from 4-weeks *T. gondii* (ME49)–infected C57BL/6 mice were stained with the desired fluorescent conjugated Abs as indicated in Table (4). $CD11b^{+}Ly6C^{hi}$ monocytes were sorted and labelled with 5 mM CFSE. 1×10^{6} $CD11b^{+}Ly6C^{hi}$ monocytes were injected i.v. into 4-weeks *T. gondii* (ME49)–infected C57BL/6 mice. Forty-eight hours later, the recipient mice were sacrificed and the brain mononuclear cells were isolated and analyzed by flow cytometry.

3.2.2.8 Carboxyfluorescein diacetate succinimidyl ester (CSFE) labelling of cells

The sorted $CD11b^{+}Ly6C^{hi}$ monocytes were resuspended in prewarmed PBS with 0.1 % BSA at a final concentration of 1×10^{6} cells/ml. 2 µl of 5 mM stock CFSE solution per ml of cells was added for a final working concentration of 10 µM. The cells were then incubated at 37 °C for 10 min. The staining was quenched by adding 5 volumes of ice-cold culture medium to the cells and incubated for 5 min on ice. The cells were then pelleted and resuspended in PBS.

3.2.3 Analysis of immune response

3.2.3.1 Survival

The mice were monitored daily for symptoms related to chronic cerebral toxoplasmosis. The development of cerebral toxoplasmosis was scored according to the following symptoms: 0 = no symptoms, 1 = ruffled fur, 2 = hunched back, 3 = wobbly gait. The mice were monitored till day 60 p.i. following which they were sacrificed due to ethical reasons.

3.2.3.2 Surface staining

For extracellular staining, 1×10^{6} mononuclear cells isolated from different organs were resuspended in 80 µl of FACS Buffer. 2 µg of anti-FcγIII/II receptor antibody per 10^{6} cells and Live-Dead Dye (diluted 1 : 1000 with FACS Buffer) diluted in 10 µl of FACS Buffer were added to the cell suspension and incubated at 4 °C for 20 min to block unspecific staining. Thereafter, cells were either stained with fluorochrome-conjugated antibodies or isotype controls against cell surface markers as indicated in Table (4) for 30 min at 4 °C. The stained cells were washed in FACS Buffer for 10 min at 400 × g. The cell pellet was resuspended in 4 % PFA for 20 min at 4

°C. Cells were finally washed one more time and then resuspended in 100 µl of FACS Buffer and measured.

3.2.3.3 Intracellular re-stimulation assay

For intracellular cytokine staining, single-cell suspensions from brain (1×10^6 cells per well) were stimulated in 96-well flat bottom plates in the presence of TLA (5 µg/ml) to increase cytokine production. The Golgi transport inhibitors Brefeldin A (10 µg/ml) and Monensin (10 µg/ml) were added 2 h later to increase the protein concentration in Golgi complex. After 4 h, cells were washed in 200 µl of FACS Buffer. The cell pellet was resuspended in 80 µl of FACS Buffer and then coupled with 2 µg of anti-FcγIII/II receptor antibody per 10^6 cells and Live-Dead Dye (diluted 1 : 1000 with FACS Buffer) diluted in 10 µl of FACS Buffer for 15 mins in ice. Cells were then surface stained in FACS Buffer for 30 min on ice, washed twice in FACS buffer and fixed in 4 % PFA. The fixed cells were permeabilized by resuspending in 200 µl BD Perm/Wash ™ Buffer (diluted 1 : 10 with distilled H_2O). Cells were washed twice with the BD Perm/Wash ™ Buffer for 10 min at 400 × g. To measure the cytokine expression, cells were stained with the following antibodies as indicated in Table (4) for 45 min in BD Perm/Wash ™ Buffer. Matched isotype controls and unstimulated cells were used as negative controls. Cells were then washed once in FACS Buffer for 10 min at 400 × g and resuspended in 100 µl of FACS Buffer. Cells were acquired using a flow cytometer and flow cytometric data were analysed using FlowJo software (Version 9.6.4 TreeStar).

3.2.3.4 Phagocytosis Assay

The isolated mononuclear cells were cultured in 12-well chambers at a density of $4 * 10^5$ cells/ml. A total of 50 ml FluoSpheres latex beads were added after 1hour pre- treatment with cytochalasin D, and cells were incubated under standard culture conditions for 8 hours. Samples were acquired and analysed with FlowJo software (Version 9.6.4; Tree Star).

3.2.3.5 ROS detection Assay

Single-cell suspensions from brain were stained for the extracellular proteins with the antibodies as indicated in Table (4) in FACS buffer for 30 min in ice. Total ROS Detection Kit, according to the manufacturer's instructions was used to measure the ROS production.

3.2.3.6 Immunohistochemistry

Brains were removed and were stored in 4 % PFA at 4°C or snap frozen in an embedding medium at -80 °C in the presence of 2-methylbutane and stored at -80 °C. The frozen brain were sectioned coronally at 4mm. Frozen sections were acetone fixed for 10 min and then rehydrated and blocked by incubation for 10 min in diluent. Sections were incubated with primary Abs Ly6C and CCR2 in diluent for 60 min, rinsed in wash buffer (PBS containing 0.5 % FBS), and incubated for 60 min with secondary Abs conjugated to Alexa 488 or Alexa 594. For negative controls, primary Abs were substituted by isotype-matched controls of the same species. Sections were rinsed in wash buffer and mounted in Vectashield containing ProLong Gold containing DAPI. Slides were examined with a Zeiss epifluorescence microscope equipped with an AxioCam CCD camera and Axiovision v4.0 software for image capture. Images were processed with similar linear adjustments for all samples in Photoshop 4.0 (Adobe, San Jose, CA).

3.2.3.7 Histology

Brains were removed and immersed in 4 % paraformaldehyde for several days and sectioned coronally at 4 mm. Paraffin-embedded, 4-mm–thick sections were de-paraffinized and conventionally stained with hematoxin and eosin (H&E). Primary Abs against macrophage 1 Ag to label microglia and mononuclear cells and against *T. gondii* were used. Slides were developed using the Bond Polymer Refine Detection kit. For the evaluation, whole tissue sections were digitized at 230 nm resolution using a MiraxMidi Slide Scanner (Zeiss MicroImaging).

3.2.3.8 Semi-quantitative Real Time (RT) PCR

Isolation of RNA and DNA from the brains of infected (treated with isotype control or depleting antibody) mice was performed with Qiagen AllPrep DNA/RNA Mini Kit. The DNA and RNA purity was determined by absorbance at 230, 260, 280 nm in a NanoDrop device. The SuperScript reverse transcriptase kit with primers was used to transcribe mRNA into cDNA as described by the manufacturer. Semi-quantitative RT-PCR for *T. gondii*, IL-1α, IL-1β, IL-12, IL-6, IFN-γ, TNF, iNOS, IL-10, hypoxanthine guanine phosphoribosyltransferase (HPRT) and mouse argininosuccinate lyase gene (Mm.ASL) was performed using individual Taqman® gene

expression assay. Amplification was performed with a GeneAmp 5700 sequence detection system. Quantification was performed with the $\Delta\Delta C_T$ threshold cycle (C_T) method with HPRT or Mm.ASL as the housekeeping gene. Data are expressed as the increase in the level of mRNA or DNA expression in treated mice over untreated mice.

3.2.3.9 Statistical analyses

Data were analysed by Student's t test or Mann-Whitney t test by two groups or one-way ANOVA for several groups, followed by Tukey's post-test with GraphPad Prism 6 (San Diego, CA). In all cases, results were presented as mean ± SD and were considered significant with $p < 0.05$.

4. Results

4.1 Experimental model for chronic cerebral toxoplasmosis.

Mouse strain and parasite lineage influence the course of chronic cerebral toxoplasmosis. To answer our questions, we selected C57BL/6 mice (H-2^b haplotype of MHC). This mice strain develops cerebral toxoplasmosis, thus enabling us to study the role of myeloid cells employed during the course of the infection. We infected the mice with 2-3 cysts of Type II (ME-49) strain. We selected this strain because after successful infection, type II strain develops cysts in the brain (Dunay IR 2015). Besides the strain type, the cyst number also influences the course of the infection from chronic progressive to TE. Hence, this strain and low dose of infection was selected to gain insight into the ongoing inflammation in the brain. The recruitment of the $Ly6G^+$ neutrophils and the $Ly6C^{hi}$ inflammatory monocytes starts from week two and three post-infection, respectively. Hence, to study the specific role of $Ly6G^+$ neutrophils and $Ly6C^{hi}$ inflammatory monocytes, the respective cell populations were depleted when they begin to extravasate into the brain. The mice were sacrificed week four post-infection to carry out further experiments.

4.2 Functions of $Ly6C^{hi}$ inflammatory monocytes in cerebral toxoplasmosis.

4.2.1 Myeloid cells are recruited upon chronic *T. gondii* infection.

Cerebral toxoplasmosis is associated with activation of resident immune cells and peripheral cell recruitment to the CNS; however the engrafted myeloid immune cells subsets have not been fully characterized yet. Accordingly, we investigated the characteristic features and fate of the newly described $Ly6C^{hi}$ monocytes following extravasation in the brains of infected mice.

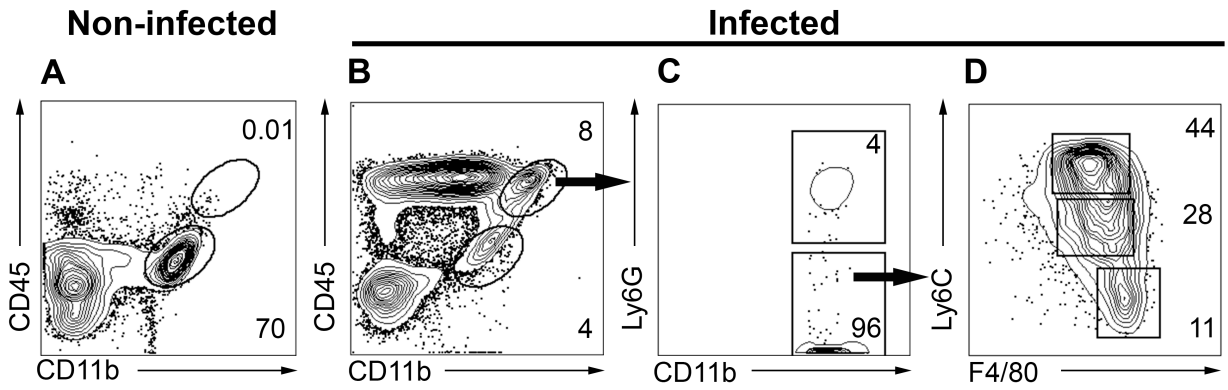


Figure 5. Myeloid cell recruitment and activation of microglia.

Leukocytes were isolated from brains of non-infected (**A**) and *T. gondii*-infected (**B - D**) C57BL/6 mice and analysed by flow cytometry. Following the basic forward scatter (FSC) - side scatter (SSC) gating; the singlet cells were selected for further characterization. (**A**) shows resident microglia ($CD11b^{+}CD45^{lo}$, lower gate), (**B**) shows activated microglia ($CD11b^{+}CD45^{int}$, lower gate) and the myeloid population ($CD11b^{+}CD45^{hi}$, upper gate). (**C**) The myeloid population consists of neutrophils ($CD11b^{+}Ly6G^{+}$, upper gate) and monocytes ($CD11b^{+}Ly6G^{-}$, lower gate). (**D**) The monocytes can be further divided according to their expression of Ly6C and F4/80: $Ly6C^{hi}F4/80^{int}$, $Ly6C^{int}F4/80^{int}$ and $Ly6C^{neg}F4/80^{hi}$. Numbers represent percentage of parent population. Data shown here is the representative of 4 independent experiments (n = 4); results are shown as mean ± SD.

We observed an ingress of the $CD45^{hi}$ population in the brains of C57BL/6 mice 4 weeks after low dose *T. gondii* infection (Fig. 5B), while; in non-infected controls the major cell population was resting resident microglia, ($CD45^{lo}CD11b^{+}$; Fig. 5A) supporting previous reports (Strack et al. 2002). Further phenotyping of the cell subsets (Fig. 5B) subdivided the $CD45^{hi}$ population into $CD45^{hi}CD11b^{-}$ (ungated) comprising mainly of recruited lymphocytes and the $CD45^{hi}CD11b^{+}$ population (upper gate; 8.0 ± 1.06 % of the parent population) encompassing myeloid derived cells, namely monocytes, neutrophils, macrophages and DCs. Brain resident activated microglia expressed elevated levels of CD45 upon infection ($CD45^{int}CD11b^{+}$ gated, lower gate; 4.0 ± 0.31 % of the parent population; Fig. 5B).

The Ly6G (1A8) Ab (Daley et al. 2008; Dunay et al. 2010) was used to distinguish monocytes (96.0 ± 2.11 % of the $CD45^{hi}CD11b^{+}$; Fig. 5C) from $Ly6G^{+}$ neutrophils (4.0 ± 2.02 % of the $CD45^{hi}CD11b^{+}$). Relative expression of Ly6C and F4/80 was used to further differentiate between the myeloid cell subsets (Fig. 5D). Based on these surface markers, we identified three distinct myeloid cell subpopulations: $Ly6C^{hi}F4/80^{int}$, $Ly6C^{int}F4/80^{int}$ and $Ly6C^{neg}F4/80^{hi}$. Brain resident microglia were persistently negative for all Ly6 Ags. These data suggest that during chronic *T. gondii* infection, alongside with the activation of resident microglia, a heterogeneous

population of myeloid cells infiltrate the CNS and they can be further divided into three distinct subsets based on the Ly6C expression.

4.2.2 Ly6C^{hi}CCR2^{+} cells localize near the inflammatory foci during cerebral toxoplasmosis.

Immunofluorescence analysis of brain sections of *T. gondii* infected mice revealed accumulation of Iba1^{+} cells (ionizing calcium-binding adaptor molecule-1). The protein is exclusively expressed on macrophages / microglia and is upregulated upon activation (Fig. 6E) in the meninges as well as in the cortex compared to non-infected controls (Fig. 6A). Closer examination suggested robust cell activation of Iba1^{+} ramified microglia with fewer ramifications, bigger soma, marked increase of average surface area and rather amoeboid morphology (Fig. 6E´). Importantly, inflammatory foci of infected brains contained Ly6C (Fig. 6F, 6F´) and CCR2 (Fig. 6G, 6G´) positive cells, suggesting Ly6C^{hi}CCR2^{+} monocytes (Fig. 6H, 6H´). Those amoeboid shaped inflammatory cells were mainly located directly or adjacent to the lesions in the infected cortex close to the vessels, and only occasionally in the meninges. These observations further indicate that Ly6C^{hi}CCR2^{+} monocytes are recruited to the CNS upon *T. gondii* infection, which is in accordance with previous studies describing rapid monocytes egress from the BM to the CNS during inflammatory conditions (Getts et al. 2008; Ransohoff & Cardona 2010).

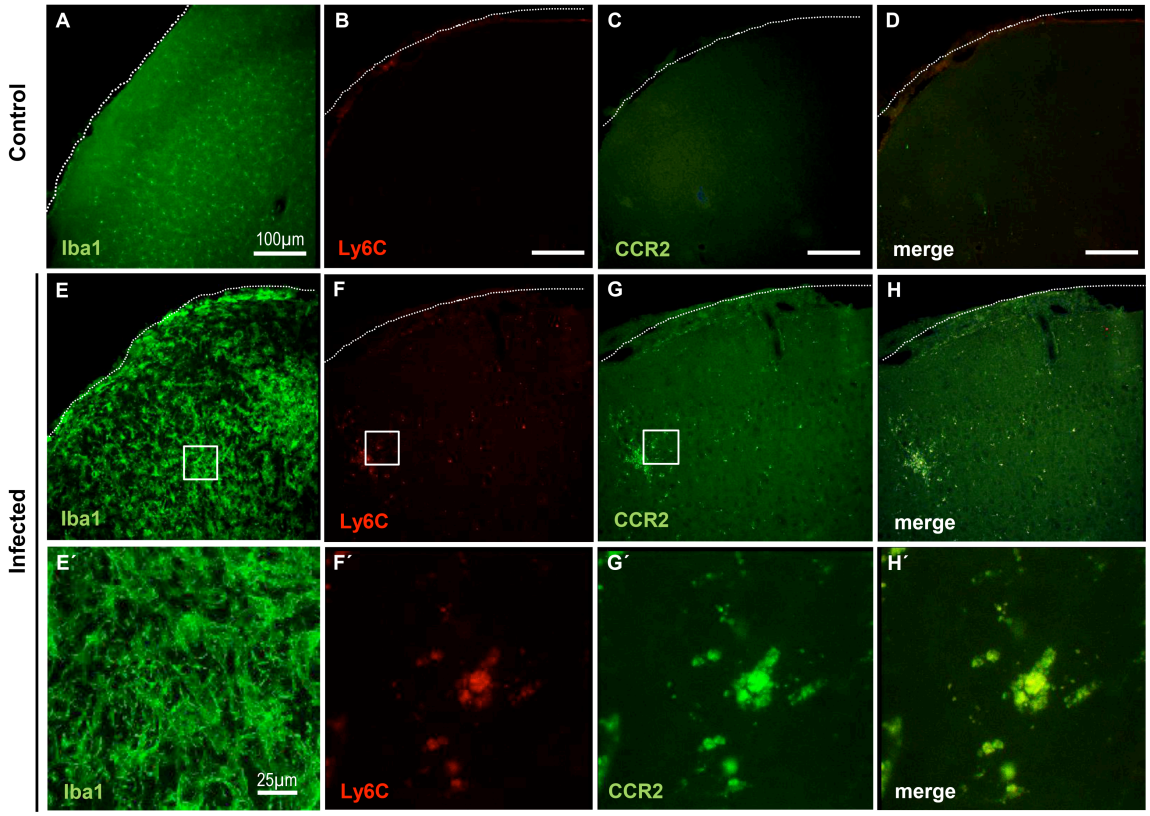


Figure 6. Immunofluorscence staining of microglia and monocytes in brain slides.

We show the cortex and the cortical meninges in non-infected control (**A - D**) and infected (**E - H**') brains. In the non-infected brain (**A**), Iba^{+} microglia have a ramified morphology whereas in the infected brain (**E**) they display rather amoeboid morphology with bigger soma. The Ly6C and CCR2 staining are negative in the control brains (**B - C**), but positive in the parenchyma of the infected brains (**F - G**) displaying the recruitment of inflammatory monocytes during *T. gondii* infection (**H**). Five to six coronal slides per mouse were analysed n = 4 mice per group. Scale bars: 100 µm in (**A - H**) and 25 µm in (**E**' - **H**').

4.2.3 Ablation of Ly6C^{hi} monocytes upon cerebral toxoplasmosis is detrimental.

To evaluate the contribution of Ly6C^{hi} monocytes in the parasite control, we took advantage of the new depleting anti-CCR2 Ab (MC-21) (Ginhoux & Jung 2014; Ransohoff & Cardona 2010). CCR2 is highly expressed on monocytes hence blockade of CCR2 with a monoclonal antibody (mAb) is crucial to understand the exclusive functions of monocytes. Thus, we applied the anti-CCR2 mAb and isotype control IgG mAb every alternate day from 20 days post-infection (dpi) to 28 dpi, to two groups of infected mice respectively. 24 hours after the last Ab treatment, mice

were sacrificed and the successful depletion of Ly6C^{hi} monocytes in the blood was confirmed (2.0 ± 0.13 % to 0 %; Fig. 7A left, B, D).

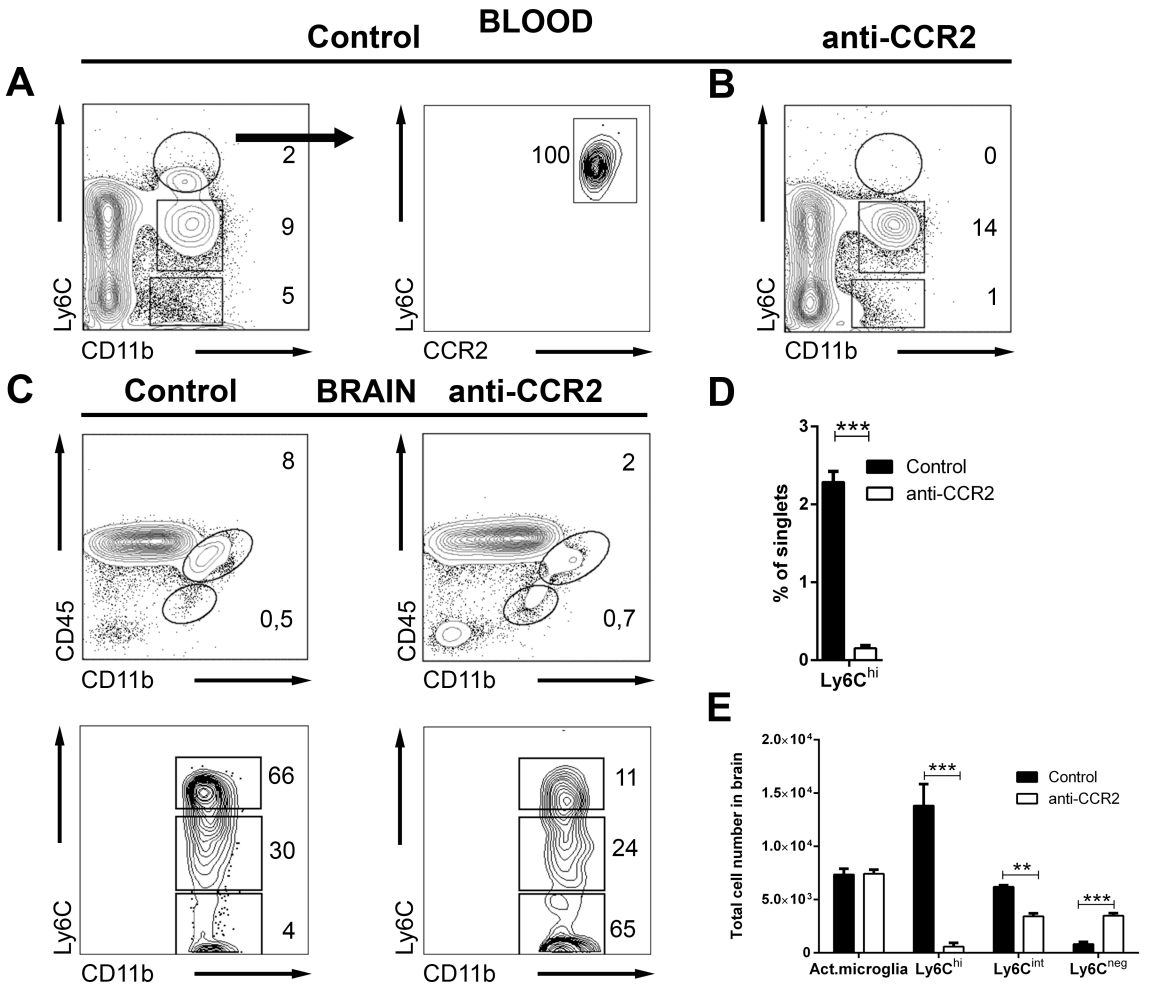


Figure 7. Selective depletion of CCR2^{+}Ly6C^{hi} monocytes.

C57BL/6 mice were infected with *T. gondii* and from day 20 to day 28 post infection alternatively treated with either IgG mAb or anti-CCR2 to deplete inflammatory monocytes. After the standard FSC - SSC gating, single cells were selected for further characterization. (**A** left, **B**) displays the representative plots to define Ly6C^{hi} inflammatory monocytes (upper gate), Ly6C^{int} neutrophils (middle gate) and Ly6C^{neg} resident monocytes (lower gate) in the blood. The coexpression of Ly6C and CCR2 on inflammatory monocytes (Ly6C^{hi}) is shown further (**A**, right). (**D**) The bar graph represents the percentage of Ly6C^{hi} in the blood. (**C**) After the basic FSC - SSC and singlet gating, upper plots show the gating of activated microglia (CD11b^{+}CD45^{int}) and the myeloid population (CD11b^{+}CD45^{hi}) in the brain. Lower plots display the monocyte subsets (from the myeloid gate): Ly6C^{hi}, Ly6C^{int} and Ly6C^{neg}. (**E**) The bar graphs represent the total cell number of the respective subset in the brain. Numbers in the plots represent percentage of parent population. Data shown here are representative of 5 independent experiments with n = 4 mice for each group; results are shown as mean ± SD. Significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were determined using the Student's t test. (Act. microglia; Activated microglia).

Importantly, a reduction of recruited myeloid cells (8.0 ± 1.5 % to 2.0 ± 0.28 %) in the brains of anti-CCR2 treated mice was observed (Fig. 7C, upper panel). This observation was further confirmed with a significant decrease of $Ly6C^{hi}$ monocytes (lower panel; 66.0 ± 8.6 % to 11.0 ± 6.2 %) and slightly in the $Ly6C^{int}$ population (30.0 ± 2.7 % to 24.0 ± 1.6 %) (Fig. 7C, lower panel). In addition, a rise of the $Ly6C^{neg}$ population was observed (4.0 ± 0.7 % to 65.0 ± 4.8 %) which might have entered the brain before depletion occurred (before day 20) (Fig. 7C, lower panel). Alterations in the absolute cell numbers revealed a significant reduction of $Ly6C^{hi}$ ($p < 0.001$), and $Ly6C^{int}$ ($p < 0.01$) cell counts. Moreover there was an increase in the $Ly6C^{neg}$ ($p < 0.001$) cell subset, whereas the microglia compartment remained unaltered (Fig. 7E).

Next, we investigated whether the selective ablation of $Ly6C^{hi}$ monocytes had an impact on brain pathology and survival of chronically *T. gondii*-infected mice. Histological examination of infected anti-CCR2 treated mice displayed higher frequency of inflammatory foci (Fig. 8B, D), extensive activation of Mac-1 (Macrophage antigen – 1; expressed on macrophages) on microglia and on mononuclear cells, and infiltration of immune cells into the cortex (Fig. 8D and F). Notably, we observed increased numbers of *T. gondii* cysts in infected anti-CCR2 treated mice compared to untreated infected control brains (Fig. 8H, I). Most importantly, depletion of $Ly6C^{hi}$ monocytes resulted in decreased survival rates, as by day 60 all anti-CCR2 treated mice succumbed to the infection, whereas all untreated infected mice survived (Fig. 8J). Together, these results demonstrate that $Ly6C^{hi}CCR2^{+}$ monocytes carry out fundamental functions in parasite control during cerebral toxoplasmosis, and their depletion exacerbates the outcome of the infection.

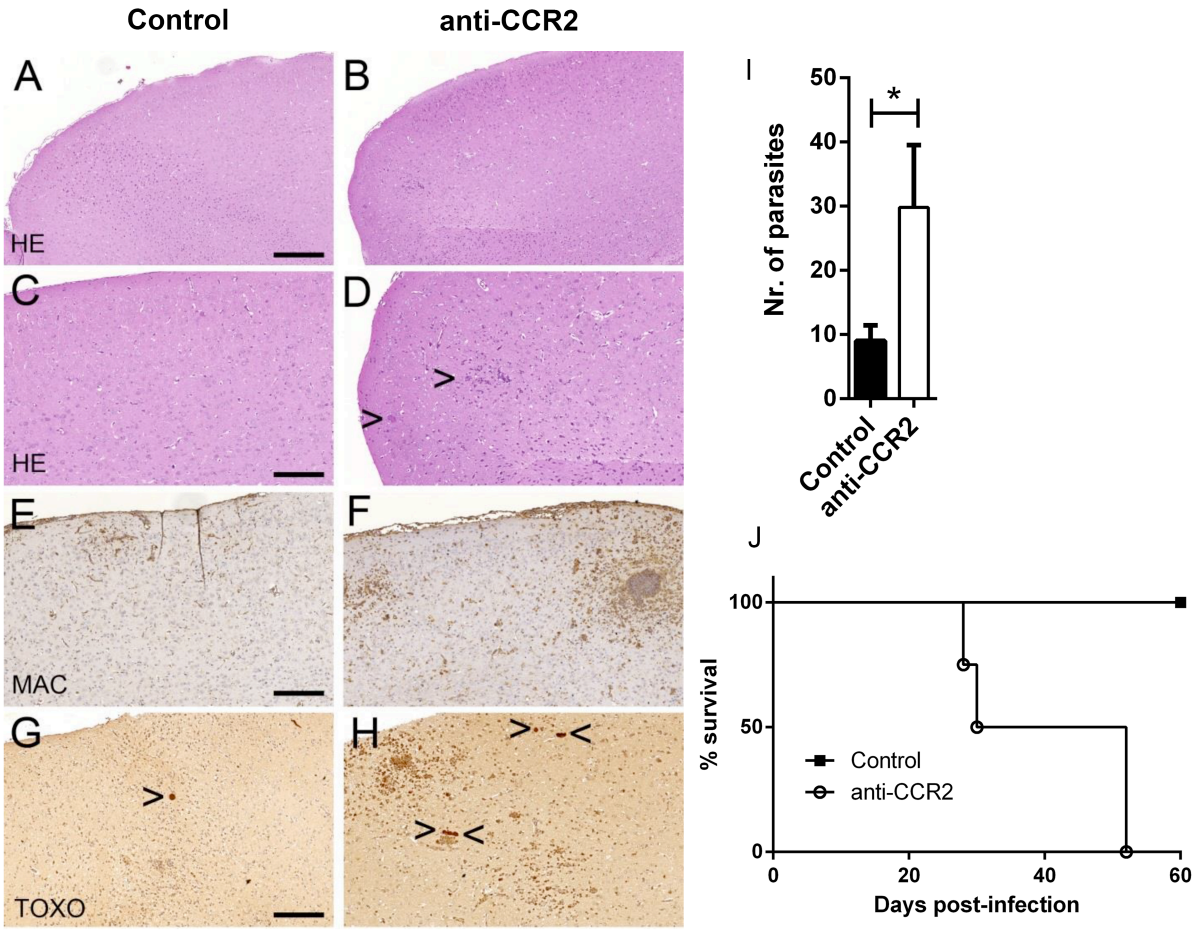


Figure 8. Increased parasite load and decreased survival in anti-CCR2 treated *T. gondii*-infected mice.

(**A - H**) Immunohistological representation of control (left panel) and anti-CCR2 (right panel) brains shows more inflammation in H&E staining (**D**, arrows), increased habitation of mononuclear cells (**F**) and more parasites (**H**) in the anti-CCR2 treated brain. (**I**) The bar graph is the quantification of the cyst burden in the control and anti-CCR2 treated brains. (**J**) Survival curve of the *T. gondii*-infected, control and anti-CCR2 treated mice were monitored from day 0 until day 60. Five to six coronal slides per mouse were analysed n = 4 mice per group. Total cyst count was determined from brain lysates n = 4 mice per group. The survival experiment was repeated 2 times with n = 8 - 10 mice per group. Significant differences (* $p < 0.05$) were determined using the Student's t test.

4.2.4 Characterization of mononuclear cell subsets upon cerebral *T. gondii* infection.

The heterogeneity of monocytes and macrophage and its multifunctionality in the CNS has been intensively studied (Mildner et al. 2013; London et al. 2013; London et al. 2011; Ransohoff & Cardona 2010). These mononuclear cells share over 50 characteristic surface markers, making their discrimination between the subsets a complex assignment (Ransohoff et al. 2003; Chan et

al. 2007). Therefore, to distinguish between the distinct myeloid cell subsets in the brain upon *T. gondii* infection we first compared the expression of specific surface markers. Cells from brains of infected and control mice were isolated, and comprehensive analysis was performed by flow cytometry.

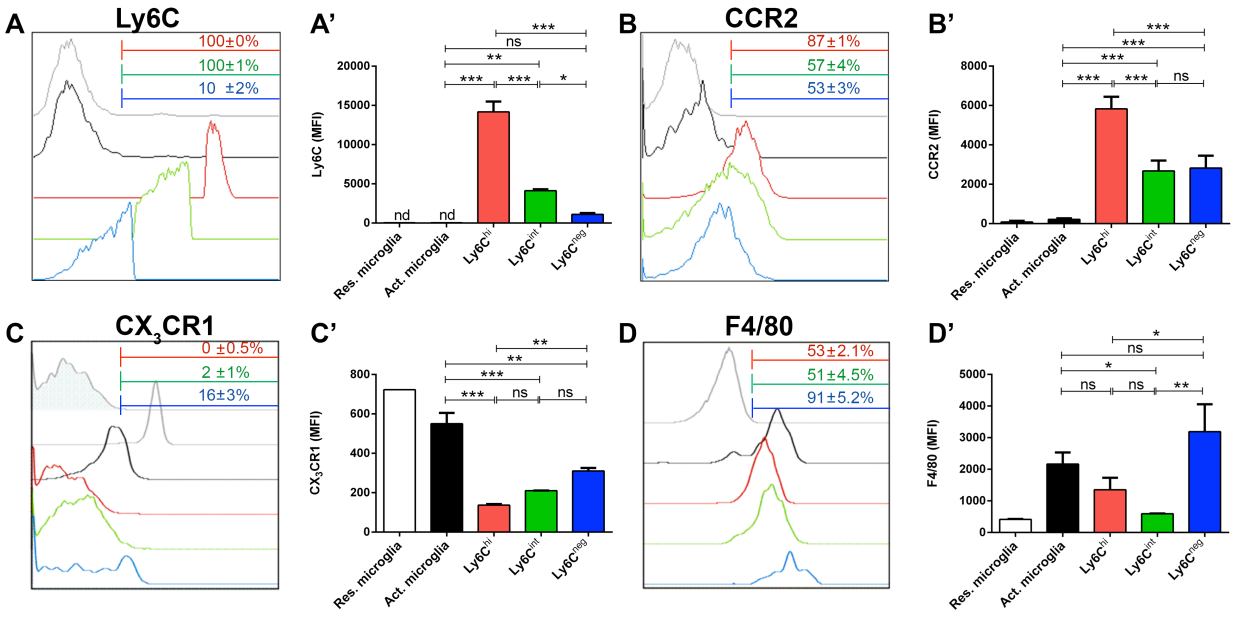


Figure 9. Phenotypic characterisation of mononucelar cell populations in the brain.

To measure the expression of surface and activation markers on leukocytes in the brain infected and non-infected mice were analysed by flow cytometry (**A – D'**). A similar gating strategy was followed as in Fig. 5 (A - D). (**A – D**) Representative histograms showing expression levels of surface markers by cell population. Bars mark the cells positive for the particular marker. Numbers above bars display the percentage of cells positive for the marker of the concerned population: resident microglia ($CD11b^{+}CD45^{lo}$, grey), activated microglia ($CD11b^{+}CD45^{int}$, black), inflammatory monocytes ($CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{hi}$, red), $Ly6C^{int}$ monocytes ($CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{int}$, green) and $Ly6C^{neg}$ monocytes ($CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{neg}$, blue), isotype control (tinted). (**A'** – **D'**) Bar graphs represent the median fluorescence intensity for the specific marker MFI ± SD (n = 3). Data are representative of 3 independent experiments with n = 4 mice per group. One-way ANOVA analysis followed by Tukey's *post hoc* test was performed for multiple comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (Res. microglia; resident microglia, Act. microglia; Activated microglia).

Ly6C is expressed on the surface of BM derived myeloid cells such as monocytes, neutrophils, DCs and a small subset of lymphocytes (Lee et al. 2013). Thus we observed Ly6C expression on monocytes whereas non-infected and infected microglia that are independent from haematopoietic input were negative (Fig. 9A, 9A').

CCR2, expressed on monocytes, is a receptor for monocyte chemokine protein-1 (MCP-1) that has been established to play the determining role for egress of inflammatory monocyte from BM

(Dunay & Sibley 2010; Geissmann et al. 2010). As expected, Ly6C^{hi} monocytes expressed high levels of CCR2, and its expression on Ly6C^{int} and Ly6C^{neg} population was lower, whereas CCR2 on microglia was absent. This observation initiated our hypothesis that the Ly6C^{int} and Ly6C^{neg} subpopulation has differentiated eventually from the infiltrating Ly6C^{hi} monocyte subset (Fig. 9B, 9B').

The fractalkine receptor CX_3CR1 is expressed on microglia in the brain and in the spinal cord. The ligand of the fractalkine receptor, CX_3CL1 is solely expressed on selected neurons. The CX_3CR1 - CX_3CL1 axis is crucial in steady state and in pathological settings to maintain microglial functions such as phagocytosis, synaptic pruning and secretion of inflammatory mediators such as TNF (Mizuno et al. 2003; Zujovic et al. 2000; Takahashi et al. 2005). Hence, we observed that the microglia cells expressed high amounts of CX_3CR1 with or without infection as described previously (Mildner et al. 2013; Varol et al. 2015), while the receptor on recruited myeloid cells was present in low levels (Fig. 9C, 9C').

F4/80 is a well-characterized membrane protein known to identify macrophages and microglia. Although broadly represented, its expression levels are influenced by the maturation stage, developmental processes and the type of macrophage (Gordon et al. 2011). As expected, F4/80 was expressed by activated microglia and by myeloid derived Ly6C^{neg} cells (Fig. 9D, 9D').

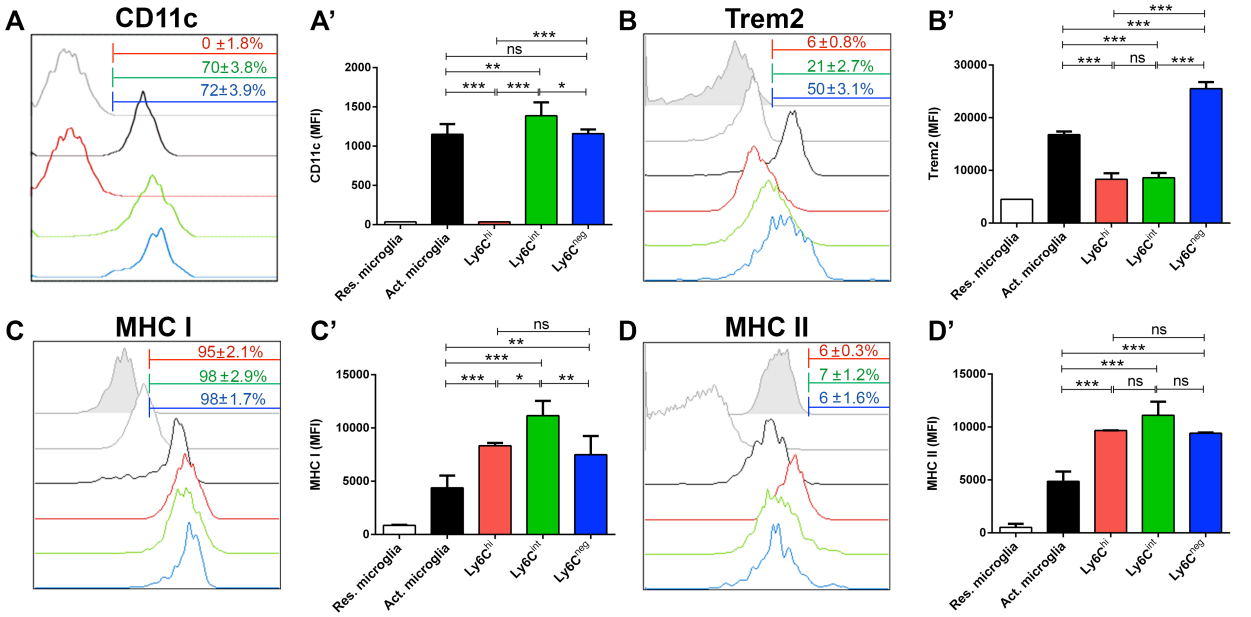


Figure 10. Phenotypic characterisation of mononuclear cell populations in the brain.

To measure the expression of surface and activation markers on leukocytes in the brain infected and uninfected mice were analysed by flow cytometry (**A – D'**). A similar gating strategy was followed as in Fig. 5 (A - D). (**A – D**) Representative histograms showing expression levels of surface markers by cell population. Bars mark the cells positive for the particular marker. Numbers above bars display the percentage of cells positive for the marker of the concerned population: resident microglia ($CD11b^{+}CD45^{lo}$, grey), activated microglia ($CD11b^{+}CD45^{int}$, black), inflammatory monocytes ($CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{hi}$, red), $Ly6C^{int}$ monocytes ($CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{int}$, green) and $Ly6C^{neg}$ monocytes ($CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{neg}$, blue), isotype control (tinted). (**A' – D'**) Bar graphs represent the median fluorescence intensity for the specific marker MFI ± SD (n = 3). Data are representative of 3 independent experiments with n = 4 mice per group. One-way ANOVA analysis followed by Tukey's *post hoc* test was performed for multiple comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (Res. microglia; resident microglia, Act. microglia; Activated microglia).

CD11c an integrin glycoprotein broadly expressed on myeloid derived DCs induces cellular activation by regulating cytotoxic T cell responses (Bevan 2004). We observed a significant upregulation of CD11c on activated microglia cells, in line with previous studies highlighting their activation status (John et al. 2011). Furthermore, expression of CD11c was high on $Ly6C^{int}$ and $Ly6C^{neg}$ myeloid derived cells, implying their Ag presenting capability (Fig. 10A, 10A').

The surface marker TREM2 on microglia promotes phagocytosis of apoptotic neurons and cellular debris without eliciting inflammatory response (Ravichandran & Lorenz 2007; Napoli & Neumann 2009). We observed higher levels of TREM2 on activated microglia and on myeloid derived $Ly6C^{neg}$ cells (Fig. 10B, 10B'), pointing towards their phagocytic capacity. The $Ly6C^{hi}$ and $Ly6C^{lo}$ subpopulations expressed low levels of TREM2, implying little phagocytic activity.

The cell surface protein MHC I and II act as 'chaperons' to present intracellular peptides to T cell receptors (TCRs) as potential foreign Ags to initiate acquired immune response. Parallel to their elevated CD11c expression, Ly6C^{int} cells upregulated MHC I and II indicating their efficacy to initiate adaptive immune responses by Ag presentation (Fig. 10C, 10C', 10D, 10D').

Collectively, the extensive phenotypic characterization of the myeloid cell subsets reveals that these cells are distinct from microglia and they express different levels of specific surface markers suggesting well-defined functions.

4.2.5 Characterization of mononuclear cell subsets in the periphery during cerebral *T. gondii* infection.

Next we elucidated the surface markers of the cell subsets in the blood before they entered the infected CNS. Cells from the peripheral blood of infected and control mice were isolated, and comprehensive analysis was performed by flow cytometry.

The surface marker Ly6C known to identify myeloid derived blood monocytes is influenced by their activation status (Geissmann et al. 2003). Hence, the inflammatory Ly6C^{hi} monocytes expressed high amount of Ly6C whereas the resident Ly6C^{neg} monocytes known to patrol and maintain tissue homeostasis expressed less Ly6C (Fig. 11C, 11C').

As expected, the BM-derived Ly6C^{hi} monocytes that have entered the circulation recently expressed high amount of CCR2 whereas the resident monocytes Ly6C^{neg} that are patrolling the periphery with or without inflammation were negative (Fig. 11D, 11D').

CX_3CR1 and F4/80 were predominantly expressed on resident Ly6C^{neg} monocytes suggesting macrophage phenotype (Fig. 11E, 11E', 11F, 11F').

The surface Ag CD11c was meagrely expressed on all investigated populations in the blood, however, the highest appearance was observed on Ly6C^{hi} monocytes (Fig. 11G, 11G').

Similarly, MHC I was upregulated on Ly6C^{hi} inflammatory monocytes upon *T. gondii* infection and MHC II was mainly expressed on resident Ly6C^{neg} monocytes during infection (Fig. 11H, 11H', 11I, 11I').

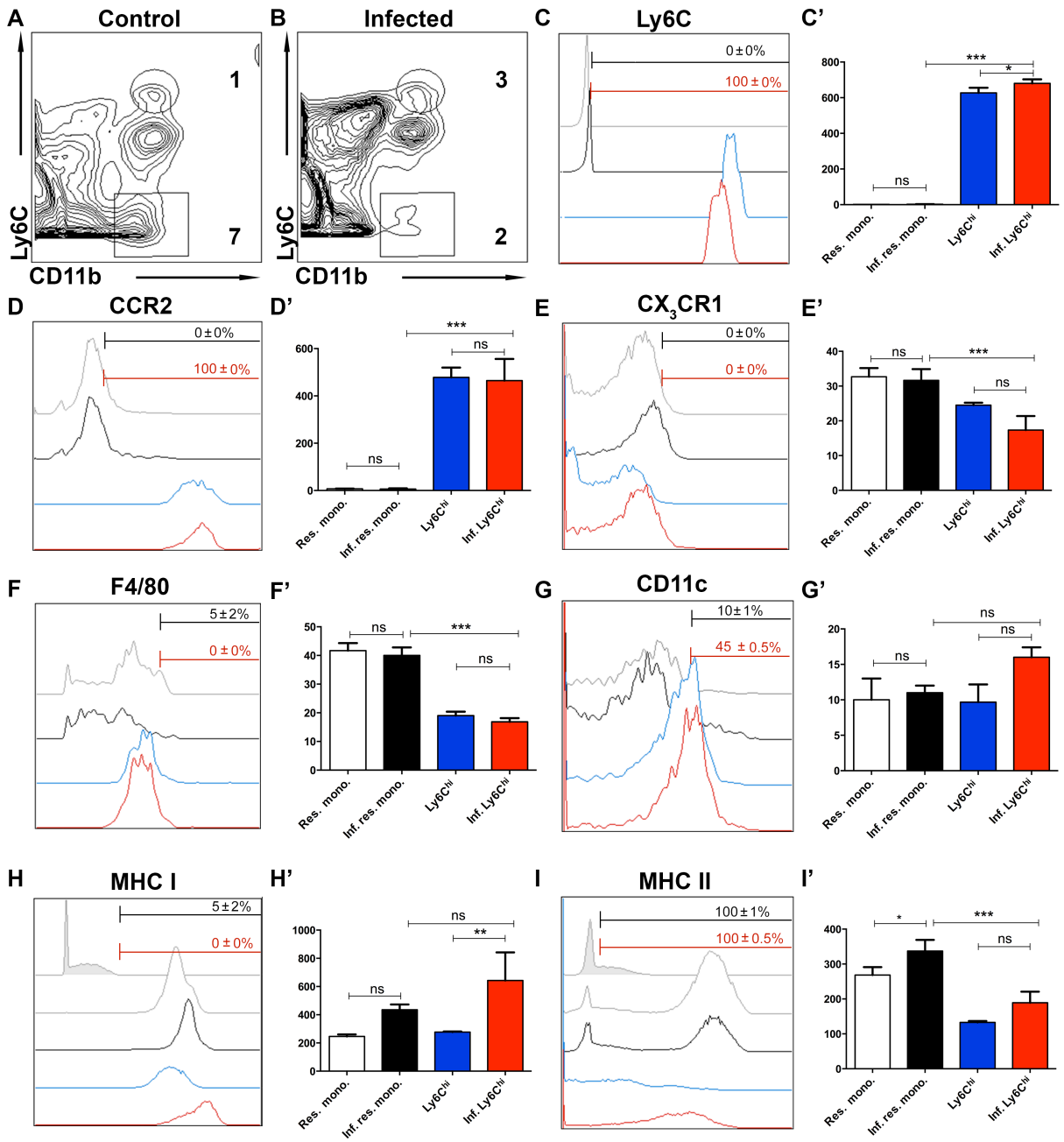


Figure 11. Phenotypic characterisation of monocytes in the blood.

(**A** – **B**) After the standard FSC - SSC gating and doublet exclusion, monocytes were gated in control and infected blood based on their CD11b and Ly6C expression: $CD11b^{+}Ly6C^{hi}$ (upper gate) for inflammatory monocytes and $CD11b^{+}Ly6C^{neg}$ (lower gate) for resident monocytes. (**C** – **I**') Expression of surface and activation markers in the blood monocytes, were analysed by flow cytometry. (**C** – **I**) Representative histograms showing expression levels of surface markers by cell population. Bars mark the cells positive for the particular marker. Numbers above bars display the percentage of cells positive for the marker of the concerned population: infected resident monocytes ($CD11b^{+}Ly6C^{neg}$, grey, untinted), infected inflammatory monocytes ($CD11b^{+}Ly6C^{hi}$, black, untinted). (**C**' – **I**') Bar graphs represent the median fluorescence intensity for the specific marker MFI ± SD (n = 3). Data are representative of 2 independent experiments. One-way ANOVA analysis followed by Tukey's *post hoc* test was performed for

multiple comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (Res. mono; resident monocytes, Inf. res. mono; infected resident monocytes, Inf. Ly6C^{hi}; infected Ly6C^{hi}).

4.2.6 Unique cytokine profile of myeloid cells subsets in cerebral toxoplasmosis.

In cerebral toxoplasmosis we observed an influx of immune cells from the periphery. To gain an insight into the cytokines released by these three distinct myeloid derived cell subsets to engage specific effector mechanisms, we performed intracellular flow cytometry analyses.

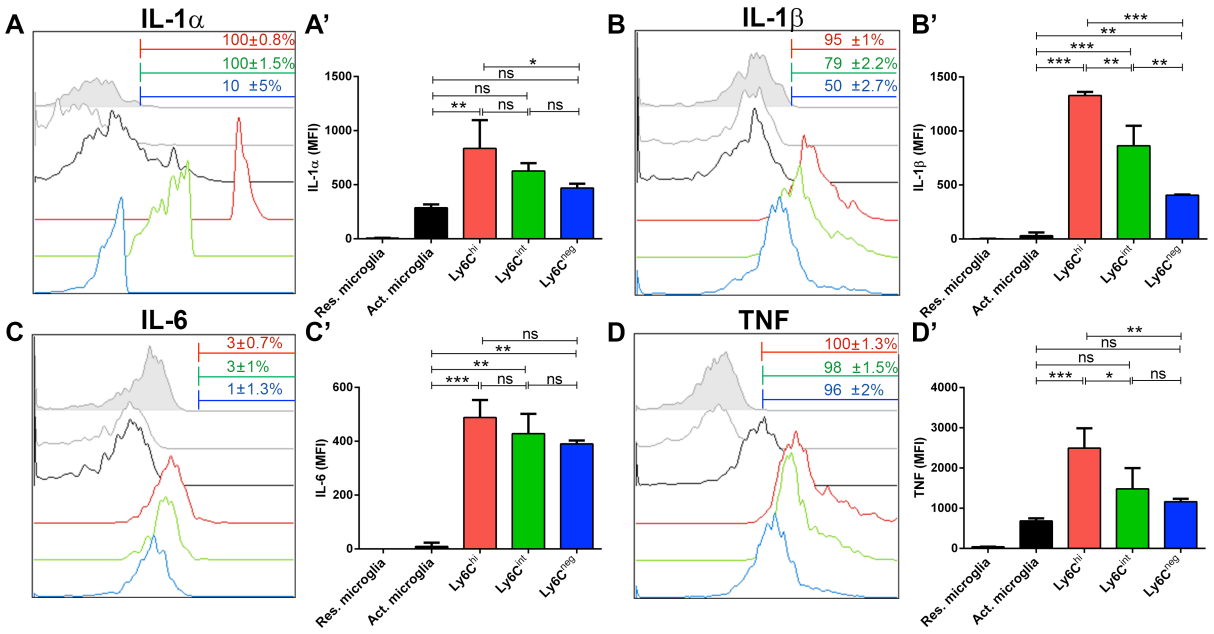


Figure 12. Cytokine production of the cell subsets in the brain.

Intracellular cytokine production by cells isolated from brains of non-infected and infected mice after *in vitro Toxoplasma* lysate antigen stimulation (**A – D'**). The cells were gated as shown in the representative plots of Fig.5 (A - D). (**A – D**) Representative histograms showing expression levels of surface markers by cell population. Bars mark the cells positive for the particular marker. Numbers above bars display the percentage of cells positive for the marker of the concerned population: resident microglia (CD11b^{+}CD45^{lo}, grey), activated microglia (CD11b^{+}CD45^{int}, black), inflammatory monocytes (CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{hi}, red), Ly6C^{int} monocytes (CD11b^{+}CD45^{hi}Ly6G^{-} Ly6C^{int}, green) and Ly6C^{neg} monocytes (CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{neg}, blue), isotype control (tinted) (**A – D**). (**A' – D'**) Bar graphs represent the median fluorescence intensity (MFI) of the respective fluorochrome for a particular cytokine, MFI ± SD (n = 3). Data are representative of 3 independent experiments with n = 4 mice per group. One-way ANOVA analysis followed by Tukey's *post hoc* test was performed for multiple comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (Res. microglia; resident microglia, Act. microglia; Activated microglia).

We detected that Ly6C^{hi} monocytes were able to produce high amounts of pro-inflammatory mediators such as IL-1α, IL-1β, IL-6 and TNF (Fig. 12A – 12D'). The pro-inflammatory cytokines secreted during chronic toxoplasmosis is in line with the inflammatory profile of

myeloid derived cells during acute toxoplasmosis, necessary to limit the infection (Dunay & Sibley 2010; Grainger et al. 2013).

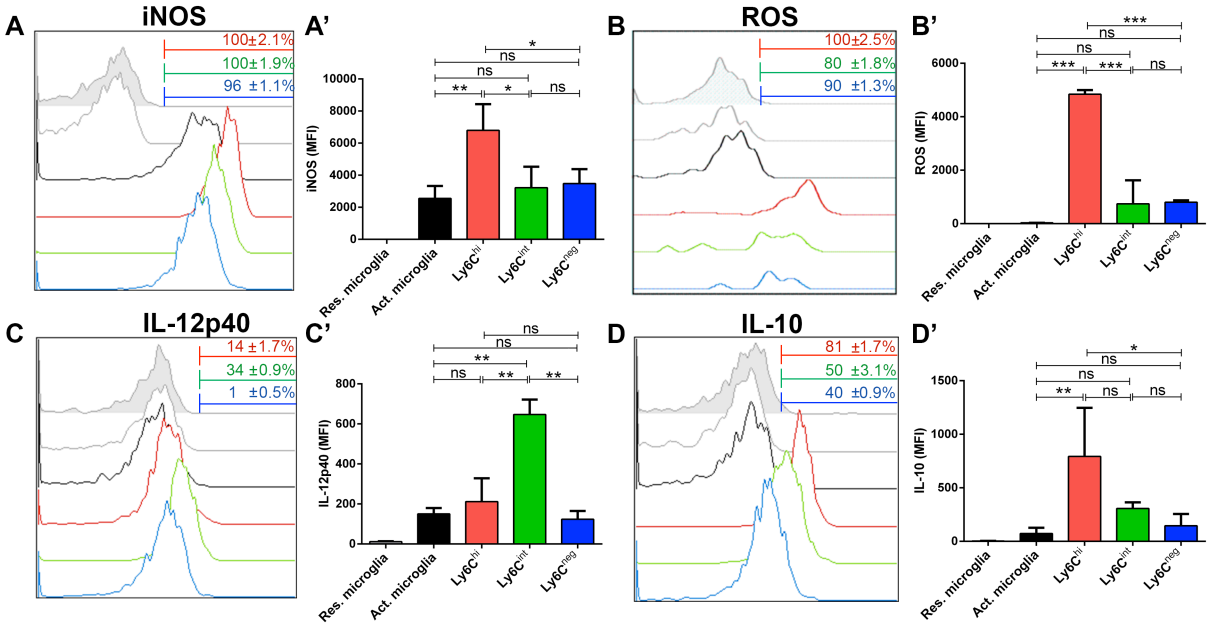


Figure 13. Cytokine production of the cell subsets in the brain.

Intracellular cytokine production by cells isolated from brains of uninfected and infected mice after *in vitro Toxoplasma* lysate antigen stimulation (**A – D'**). The cells were gated as shown in the representative plots of Fig. 5 (A - D). (**A – D**) Representative histograms showing expression levels of surface markers by cell population. Bars mark the cells positive for the particular marker. Numbers above bars display the percentage of cells positive for the marker of the concerned population: resident microglia ($CD11b^{+}CD45^{lo}$, grey), activated microglia ($CD11b^{+}CD45^{int}$, black), inflammatory monocytes ($CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{hi}$, red), $Ly6C^{int}$ monocytes ($CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{int}$, green) and $Ly6C^{neg}$ monocytes ($CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{neg}$, blue), isotype control (tinted) (**A – D**). (**A' – D'**) Bar graphs represent the median fluorescence intensity (MFI) of the respective fluorochrome for a particular cytokine, MFI ± SD (n = 3). Data are representative of 3 independent experiments with n = 4 mice per group. One-way ANOVA analysis followed by Tukey's *post hoc* test was performed for multiple comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (Res. microglia; resident microglia, Act. microglia; Activated microglia).

The enzyme inducible nitric oxide synthase (iNOS) known to have a direct effect on the parasite replication (Hunter & Sibley 2012) was secreted at a maximum rate by $Ly6C^{hi}$ monocytes (Fig. 13A, 13A'). The reactive oxygen species (ROS) was secreted explicitly by $Ly6C^{hi}$ monocytes, suggesting their strong potential to eliminate parasites (Fig. 13B, 13B'). The $Ly6C^{int}$ and $Ly6C^{neg}$ population produced lesser amounts of cytokines; however mainly $Ly6C^{int}$ cells secreted IL-12, suggesting their occupation to shape the adaptive immune system (Fig. 13C, 13C'). Activated microglia did not significantly contribute to cytokine production in the model of low dose *T. gondii*-induced chronic infection in the brain. Interestingly, alongside their pro-inflammatory and

anti-parasitic functions, Ly6C^{hi} cells also expressed the immunoregulatory cytokine IL-10 upon *in vitro* stimulation, implicating their possible dual feature to maintain tissue homeostasis to counterbalance the ongoing CNS inflammation (Fig. 13D, 13D'). Thus, Ly6C^{hi} monocytes and their subsets have a mixed phenotype ranging from inflammatory to regulatory profile necessary to control cerebral toxoplasmosis.

4.2.7 Ly6C^{neg} myeloid cells show strong phagocytic capacity.

To examine the phagocytic properties of microglia and recruited myeloid cell subsets, we performed an *ex vivo* phagocytosis assay. Therefore, the respective cell subsets were isolated, sorted and then incubated with fluorescent latex beads.

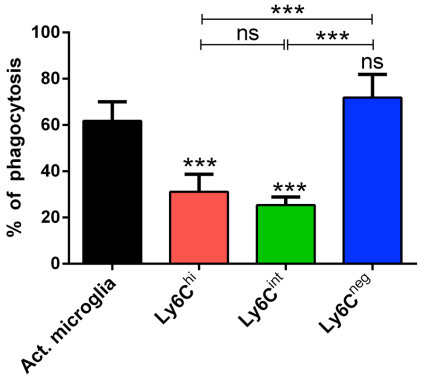


Figure 14. Phagocytic capacity of the mononuclear cell subsets in the brain.

The uptake of fluorescent latex beads by brain mononuclear cells *ex vivo* was measured by flow cytometry. After the standard FSC - SSC gating and singlet gating, CD11b^{+} cells were further gated as shown in Fig. 5 (A – D). Bar graphs show the percentage of phagocytosis of the latex beads by the respective population. Data are representative of 3 independent experiments with n = 4 mice. One-way ANOVA analysis followed by Tukey's *post hoc* test was performed for multiple comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (Res. microglia; resident microglia, Act. microglia; Activated microglia).

While recruited Ly6C^{hi} and Ly6C^{int} cells exhibited low phagocytic capacity (31.1 ± 3.6 % and 25.0 ± 2.2 %, respectively), Ly6C^{neg} cells demonstrated prominent phagocytic ability (80.2 ± 5.6 %), alongside with those of activated resident microglia (60.2 ± 1.2 %), implying that both act like macrophages (Fig. 14). This observation correlates with our previous data (Fig. 10B, B') where Ly6C^{neg} cells and activated resident microglia had the highest expression of TREM2.

4.2.8 Ly6C^{hi} cells engraft and differentiate in the brain upon adoptive transfer.

To confirm that recruited Ly6C^{hi} monocytes further differentiate to the previously described Ly6C^{int} and Ly6C^{neg} cells following extravasation in the CNS, we conducted adoptive transfer experiments. We injected 1 × 10^6 sorted CFSE-labelled Ly6C^{hi}CD11b$^+$ cells isolated from the BM of *T. gondii*-infected mice; intravenously into *T. gondii*-infected recipient mice. The cells were injected when the ongoing inflammation had already affected the BBB permeability and the recruitment of the inflammatory cells to the CNS reached its peak (data not shown).

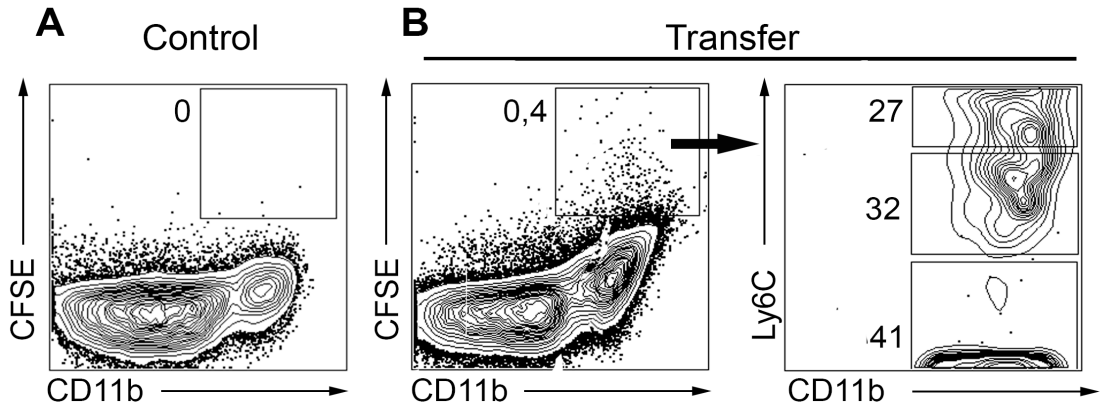


Figure 15. Adoptively transferred CFSE$^+$Ly6C^{hi} monocytes are recruited to the brain.

Sorted CFSE$^+$CD11b$^+$Ly6C^{hi} monocytes from infected wildtype BM were injected intravenously into *T. gondii*-infected mice. 48 h later, the brain mononuclear cells were isolated. After FSC - SSC and singlet gating, transferred CFSE$^+$CD11b$^+$ cells were analysed in infected mice that did not (Control, **A**) or did receive CFSE – labelled cells (Transfer, **B**). CFSE$^+$ cells showed heterogeneous Ly6C expression; numbers represent percentage of the parent population. Data are representative of 2 independent experiments with n = 4 mice per group; results are shown as mean ± SD.

Notably, CFSE labelled Ly6C^{hi} cells were found in the CNS as early as 24 hours after the transfer (data not shown). 48 hours after the transfer, Ly6C^{hi} cells (27.2 ± 3.2 % of CD11b$^+$CFSE$^+$gate) downregulated Ly6C and apparently differentiated into Ly6C^{int} (32.0 ± 1.6 % of CD11b$^+$CFSE$^+$gate) and Ly6C^{neg} cells (41.0 ± 2.0 % of CD11b$^+$CFSE$^+$gate; Fig. 15B). These data clearly show that Ly6C^{hi} monocytes are recruited to the brain upon *T. gondii*

infection, after which they dowregulate Ly6C expression and further develop into Ly6C^{int} and Ly6C^{neg} subsets to carry out particular functions in parasite elimination and host defence.

4.2.9 Migration of Ly6C^{hi} monocytes to the brain is PSGL-1 dependent.

Selective leukocyte homing to the site of inflammation has been shown to be dependent on chemokines and distinct adhesion molecules (Ley et al. 2007; An et al. 2008). To evaluate the role of adhesion molecules in CCR2^{+}Ly6C^{hi} monocytes recruitment to the CNS in toxoplasmosis, we measured the expression of CD62L (L-selectin), LFA-1 (Lymphocyte function-associated antigen 1) and PSGL-1 (P-selectin glycoprotein ligand - 1) on Ly6C^{hi} monocytes and their subsets.

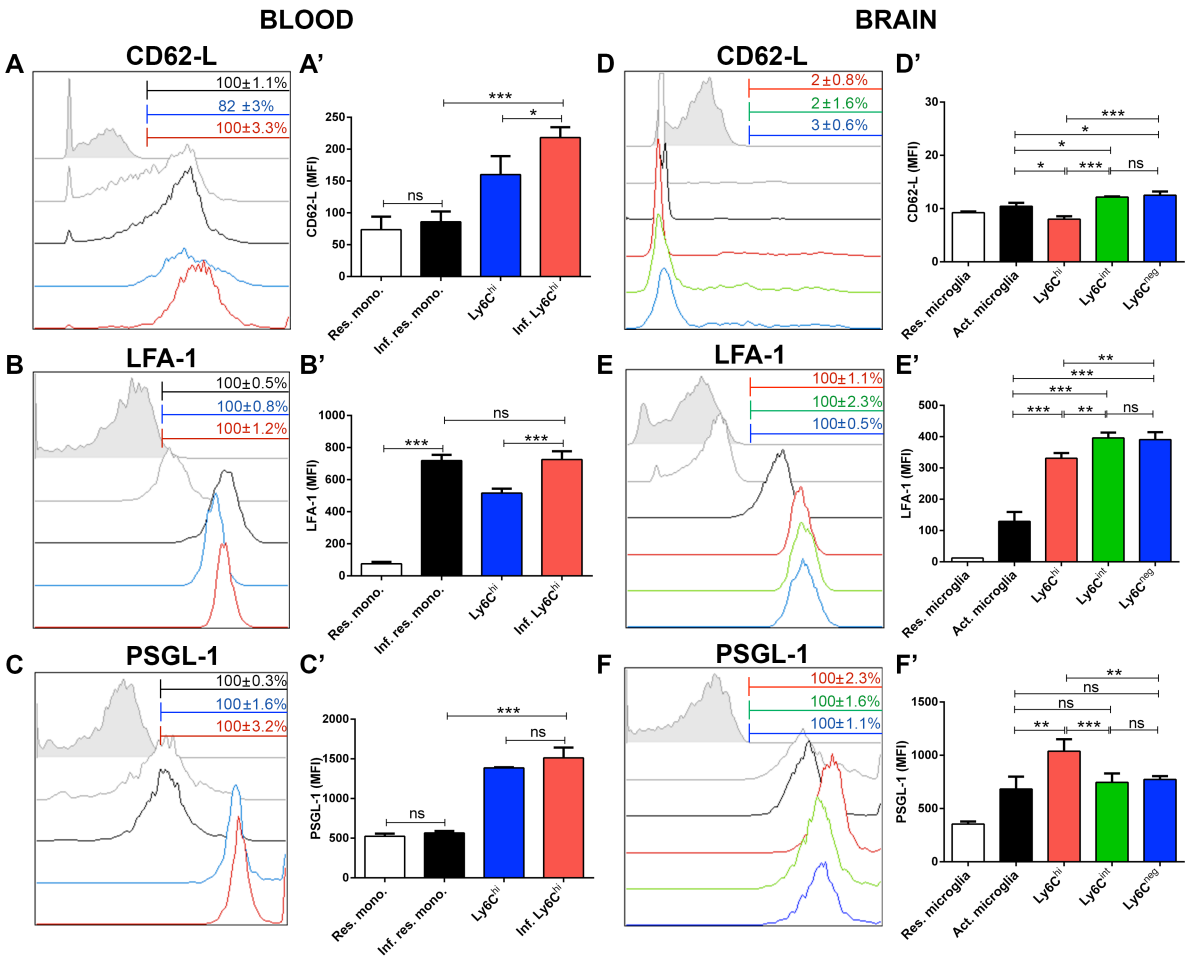


Figure 16. CCR2^{+}Ly6C^{hi} monocytes express PSGL-1.

Cells from the peripheral blood (**A** – **C**') (gating strategy: Fig. 11) and brains (**D** – **F**') (gating strategy: Fig. 5) of non-infected and infected mice were analyzed for the expression of adhesion molecules. (**A** – **F**) Representative histograms showing expression levels of adhesion molecule by cell population. Bars mark the cells positive for the particular adhesion molecule. Numbers above bars display the percentage of cells positive for the adhesion molecule of the concerned population. Blood cell subsets (**A** – **C**') are indicated as followed: resident monocytes (white), resident monocytes from infected mice (black), inflammatory monocytes (blue), inflammatory monocytes from infected mice (red), isotype control, tinted. Brain leukocyte subsets (**D** – **F**') are as follows: resident microglia (CD11b^{+}CD45^{lo}, grey), activated microglia (CD11b^{+}CD45^{int}, black), inflammatory monocytes (CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{hi}, red), Ly6C^{int} monocytes (CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{int}, green) and Ly6C^{neg} monocytes (CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{neg}, blue), isotype control (tinted). (**A**' – **F**') Bar graphs represent the median fluorescence intensity for the antibody, MFI ± SD (n = 5). Data are representative of 2 independent experiments. One-way ANOVA analysis followed by Tukey's *post hoc* test was performed for multiple comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Res. mono; Resident monocytes, Inf. res. mono; Infected resident monocytes, Inf. Ly6C^{hi}, Infected Ly6C^{hi}, Res. microglia; Resident microglia, Act. microglia; Activated microglia.

CD62L expressed exclusively on leukocytes facilitates to leave the bloodstream; make random contacts with the inflamed endothelium; rolling and finally mediates transmigration through the

endothelium to the site of infection (Ley et al. 2007). We observed that CD62L was highly expressed on Ly6C^{hi} inflammatory monocytes in the periphery, and upon entry to the brain; they were shed as the Ly6C^{hi} inflammatory monocytes, become effector cells. Resident monocytes in the blood expressed only low levels of L-selectin (Fig. 16A, 16A', 16D, 16D').

LFA-1 is expressed on all leukocytes. It is essential for leukocyte recruitment to inflamed tissues. Chemokines secreted from the site of infection activate LFA-1 resulting in firm adhesion on endothelial intracellular cell adhesion molecules (ICAMs) (Ley et al. 2007). In line with this, we observed LFA-1 expression both in the periphery and in the brain by the recruited monocytes as well as on Ly6C^{int} and Ly6C^{neg} cells in the CNS. Resident monocytes in the blood upregulated LFA-1 upon *T. gondii* infection, similarly to resident microglia cells in the brain (Fig. 16B, 16B', 16E, 16E').

PSGL-1 expressed on Ly6C^{hi} inflammatory monocytes interacts with P - or E - selectin on the inflamed endothelium (primary tethering) or on a rolling / adhered leukocyte (secondary tethering) (An et al. 2008). We observed that during cerebral toxoplasmosis Ly6C^{hi} inflammatory monocytes expressed significantly higher PSGL-1 compared to resident cells in the blood and in the CNS (Fig. 16C, 16C', 16F, 16F').

To test whether high PSGL-1 levels detected on the surface of Ly6C^{hi} monocytes had a functional role in their migration to the CNS, anti-PSGL-1 Ab treatment was applied.

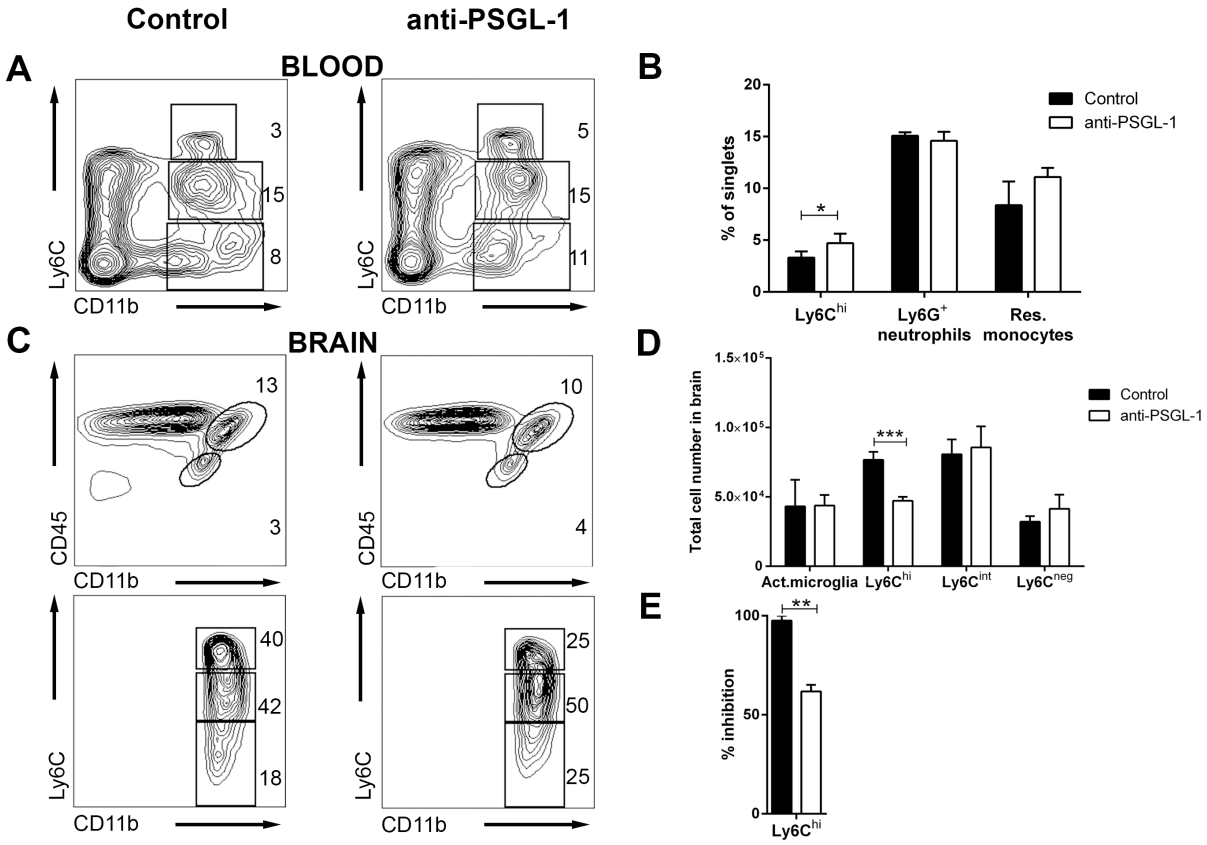


Figure 17. PSGL-1 modulates Ly6C^{hi} monocyte migration to the CNS.

Rat IgG (control, left) or anti-PSGL-1 (2 mg/kg; anti-PSGL-1, right) was administered to *T. gondii*-infected mice i.p. every alternate day from day 16 to day 26 post infection. Flow cytometric analysis of blood and brain was performed 24 h after the last treatment. After the FSC-SSC gating and doublet exclusion, cells were further analysed. (**A**) Representative plots are shown to define Ly6C^{hi} inflammatory monocytes (upper gate), Ly6C^{int} neutrophils (middle gate), Ly6C^{neg} resident monocytes (lower gate) in the blood. (**B**) The bar graphs represent the percentage of singlets of the respective population in the peripheral blood. (**C**) Displays the representative plots to define Ly6C^{hi} inflammatory monocytes in the brain. Upper plots show the gating of microglia (CD11b^{+}CD45^{int}) and the myeloid population (CD11b^{+}CD45^{hi}) in the brain. The myeloid gate is further characterized for the monocyte subsets (lower plots): Ly6C^{hi}, Ly6C^{int} and Ly6C^{neg}. Experiments were repeated twice with n = 4 for each group. (**D**) The bar graphs represent the total cell number of the respective population in the brain. (**E**) The relative inhibition in the recruitment of Ly6C^{hi} monocytes following anti-PSGL-1 treatment was compared to the IgG-treated controls. Numbers were normalised on control brains. The numbers in the representative contour plots are percentage of the parent population. Data are representative of 2 independent experiments with n = 5 mice per group; results are shown as mean ± SD. Significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) in the bar graphs were determined using the Student's t test. Res. mono; Resident monocytes, Act. microglia; Activated microglia.

After 24 hours of the last Ab application, we observed an increased proportion of Ly6C^{hi} monocytes in the peripheral blood (3.0 ± 0.6 % to 5.0 ± 0.92 % of the parent population; Fig. 17A, B) of the treated mice. This implies that PSGL-1 does contribute to the rolling and adhesion of Ly6C^{hi} inflammatory monocytes on the endothelium, hence in the absence of PSGL-1

sufficient transmigration does not occur and cells accumulate in the blood. In the brains of infected anti PSGL-1-treated mice we observed a reduction in the percentage (40.0 ± 1.67 % to 25.0 ± 2.1% of the $CD45^{hi}CD11b^{+}Ly6G^{-}$ population) and the total cell numbers within the $Ly6C^{hi}$ compartment (Fig. 17C, lower panel and 17D respectively), however, no marked difference was detected in the frequencies and total cell numbers of other cell subsets (Fig. 17C, lower panel and 17D respectively). The inhibition of $Ly6C^{hi}$ cell recruitment to the brain was markedly diminished ($p < 0.001$; Fig. 17E) confirming that PSGL-1 is an important mediator for monocyte homing to the CNS.

4.3 Functions of $Ly6G^{+}$ neutrophil granulocytes in cerebral toxoplasmosis.

4.3.1 Rapid influx of neutrophil granulocytes upon cerebral toxoplasmosis.

Chronic cerebral toxoplasmosis induces egress of $Ly6C^{hi}$ monocytes and $Ly6G^{+}$ neutrophils from the BM to the blood. Following our previous characterization of the BM derived monocytes here we analysed the phenotype of the neutrophil granulocytes. CD11b and Ly6C expression were used to identify the inflammatory monocytes ($Ly6C^{hi}$), neutrophils ($Ly6C^{int}$) and resident monocytes ($Ly6C^{neg}$) in the blood (Fig. 18A and C). The $CD11b^{+}$ cells were further gated based on their Ly6C and Ly6G expression (Fig. 18B and D). The neutrophil specific Ab Ly6G (1A8) was used further to separate neutrophils ($Ly6G^{+}$) from monocytes ($Ly6G^{-}$) (Fig. 18B and 18D). Upon infection we observed increased percentage of circulating neutrophils (15.7 ± 1 % to 30 ± 3.06 % of the parent population) in the blood.

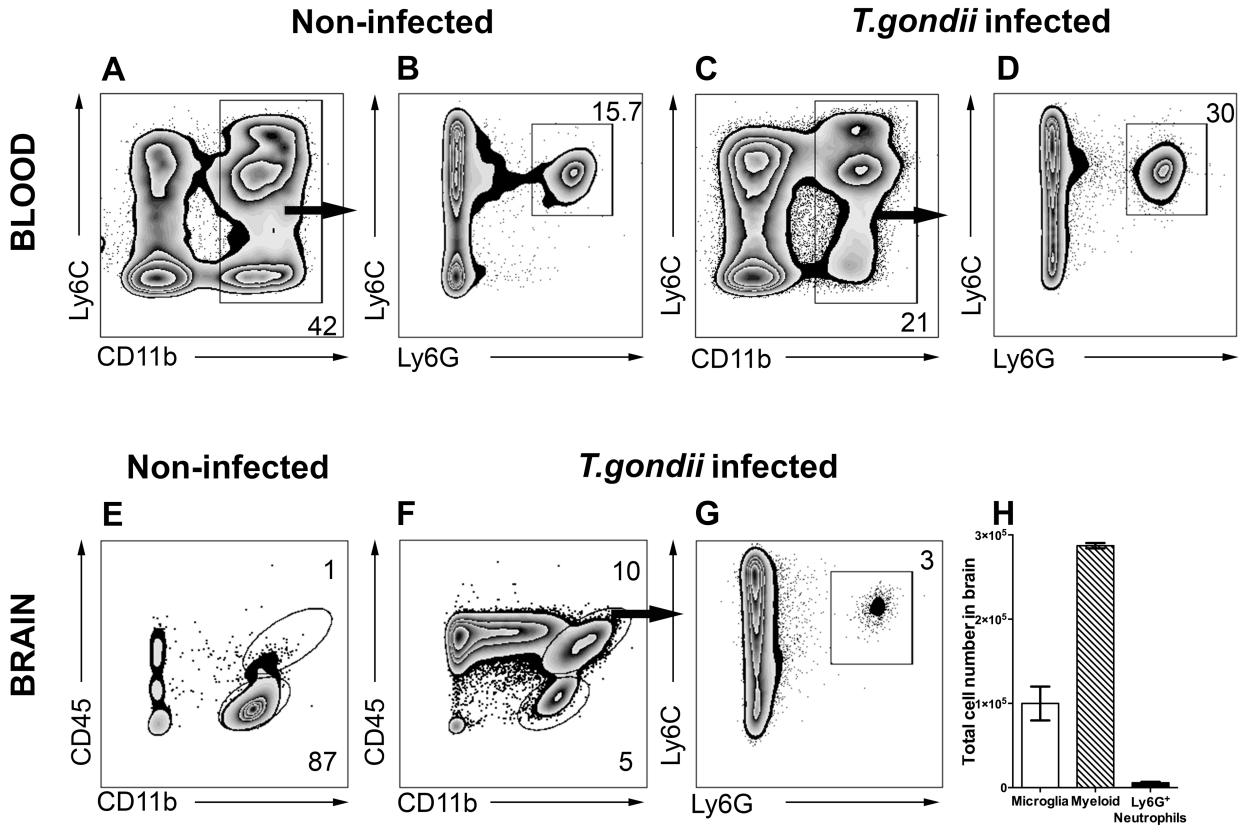


Figure 18. Gating strategy of neutrophils in blood and in brain.

Cells were isolated from non-infected blood and brain (**A - B and E**) and *T. gondii* infected blood and brain (**C - D and F - G**) C57BL/6 mice and analysed by flow cytometry. Following FSC-SSC and singlet gating, live cells were further characterized. Panel (**A and C**) shows gating of $CD11b^+$ cells which are further characterized into $Ly6C^+Ly6G^+$ neutrophils (**B and D**) in the blood. Panel (**E**) shows the resident microglia ($CD11b^+CD45^{lo}$, lower gate). Panel (**F**) shows the percentage of activated microglia ($CD11b^+CD45^{int}$, lower gate) and the myeloid population ($CD11b^+CD45^{hi}$, upper gate). (**G**) The myeloid population consists of neutrophils ($Ly6C^+Ly6G^+$, upper gate) and monocytes ($Ly6C^+Ly6G^-$, ungated). (**H**) The bar graphs represent total $CD11b^+$ cells in the infected brains. Numbers represent mean ± SD, % of parent population. The data shown here is the representative of 3 independent experiments with n = 5 mice.

Alongside the influx of immune cells in the periphery, cerebral toxoplasmosis leads to the activation of brain resident cells and infiltration of peripheral immune cells to the brain (Biswas et al. 2015). The sequence at which the peripheral immune cells enter the brain comprises of initial trafficking of neutrophils, followed by the recruitment of BM-derived monocytes and the lymphocytes. We observed the entry of $CD45^{hi}$ population in the brains of C57BL/6 mice after 4 weeks of low dose *T. gondii* infection (Fig. 18F) while in non-infected controls the main cell population was resting resident microglia ($CD45^{lo}CD11b^+$; Fig. 18E). The recruited cells were $CD45^{hi}CD11b^-$ (ungated; Fig. 18F) comprising mainly recruited lymphocytes and the

$CD45^{hi}CD11b^{+}$ population (upper gate; 10.0 ± 2.1 % of the parent population) including BM-derived myeloid cells, namely monocytes, neutrophils, macrophages and DCs. Upon infection, brain resident activated microglia cells expressed elevated CD45 levels upon activation ($CD45^{int}CD11b^{+}$gated, lower; 5.0 ± 1.06 % of the parent population.).

The Ly6G (1A8) antibody was further used to distinguish $Ly6G^{+}$ neutrophils (Fig. 18G; 3.0 ± 1.02% of the $CD45^{hi}CD11b^{+}$) from monocytes. The immune cells of myeloid origin in the brain during cerebral toxoplasmosis were also quantified (Fig. 18H). The number of infiltrating myeloid cells ($3 \times 10^5 \pm 500$ $CD45^{hi}CD11b^{+}$ cells) in the brain was three times more with respect to activated microglia ($1 \times 10^5 \pm 2000$ $CD45^{int}CD11b^{+}$ cells). Whereas, $CD45^{hi}CD11b^{+}Ly6G^{+}$ neutrophils ($3 \times 10^3 \pm 50$ $CD45^{hi}CD11b^{+}Ly6G^{+}$) cells formed a small yet defined population of myeloid cells. These data suggest that cerebral toxoplasmosis leads to the activation of resident microglia and infiltration of a heterogeneous population comprising of myeloid and lymphoid cells to the CNS.

4.3.2 Phenotypic analysis of infiltrating neutrophils upon cerebral *T. gondii* infection.

We inverstigated certain surface markers, known to mediate immune response, to characterize infiltrating neutrophils and compare it to those on activated microglia during cerebral toxoplasmosis. Flow cytometry was used to characterise the phenotype of immune cells in the brains of infected and control mice.

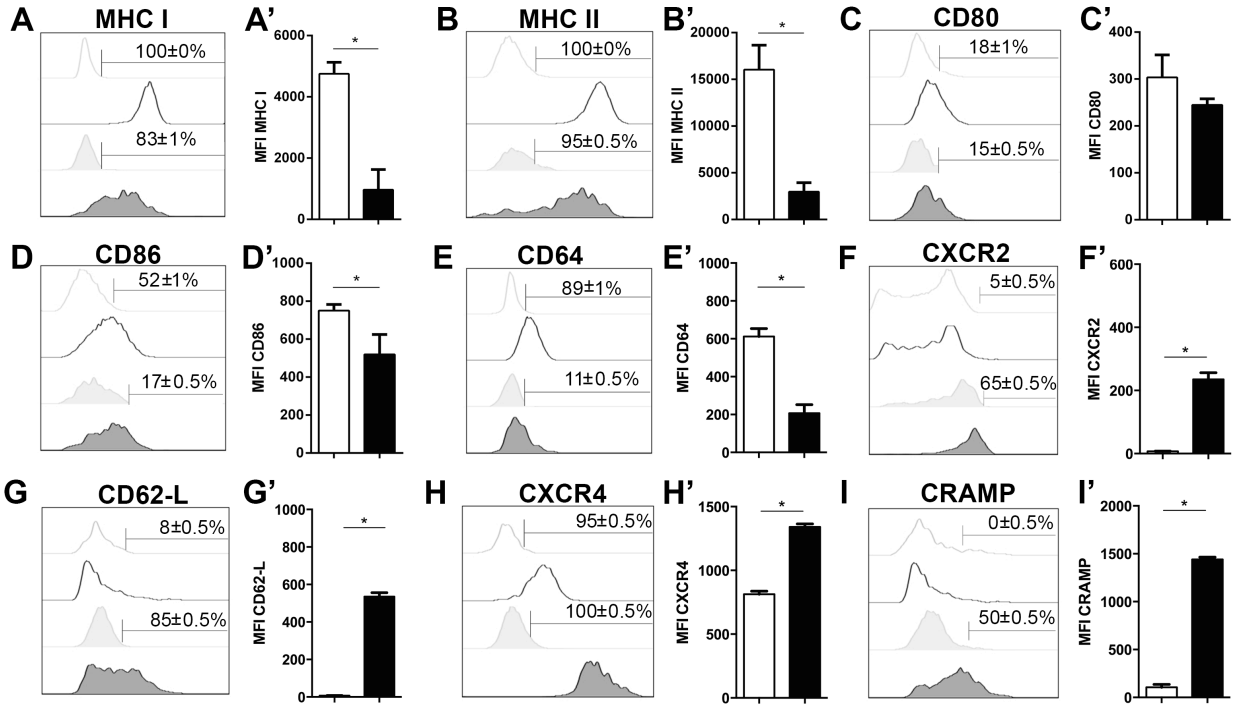


Figure 19. Phenotypic analysis of neutrophils and activated microglia in the brain.

(**A – I'**) Expression of activation markers and chemokine receptors in the infected brain, were analysed by flow cytometry. A similar gating strategy was followed as in Fig. 18 (**F - G**). Activated microglia ($CD11b^{+}CD45^{int}$) and neutrophils ($CD11b^{+}CD45^{hi}Ly6G^{+}Ly6C^{+}$), were assessed for their relative expression. (**A – I**) Histograms show the representative expression level of the surface maker by the cell population. Bars mark the cells positive for the particular cytokine. Numbers above the bars represent percentage of cells positive for the cytokine of the respective population: activated microglia ($CD11b^{+}CD45^{int}$) (without any tint), neutrophils ($CD11b^{+}Ly6G^{+}$) (tinted). (**A' – I'**) Bar graphs represent the median fluorescence intensity for the specific marker MFI ± SD (n = 4) (activated microglia; white bars and neutrophils; black bars). Data are representative of 2 independent experiments with n = 4 mice. Mann-Whitney test was performed for comparisons (* $p < 0.05$).

A large percentage of neutrophils (83 ± 1 % expressed MHC I; 95 ± 0.5 % expressed MHC II) and activated microglia (100 % expressed MHC I; 100 % expressed MHC II) expressed the Ag presenting molecules MHC I and MHC II (Fig. 19A, A', B, B'). We found a small percentage of neutrophils (15 ± 0.5 % expressed CD80; 17 ± 0.5 % expressed CD86) and activated microglia (18 ± 1 % expressed CD80; 52 ± 1 % expressed CD86) expressing co-stimulatory molecules CD80 and CD86 (Fig. 19C, C', D, D'). A small percentage of neutrophils expressed the receptor CD64 (FcγR1) mediating phagocytosis (11 ± 0.5 % expressed CD64) unlike the brain resident macrophages, the activated microglia (89 ± 1 % expressed CD64) (Fig. 19E, E'). CXCR2 was expressed on the neutrophils, (65 ± 0.5 % expressed CXCR2; Fig. 19F, F'). However, a small

percentage of activated microglia expressed CXCR2 (5 ± 0.5 % expressed CXCR2; Fig. 19F, F'). CD62L, known to be important for leukocyte rolling (Rainer 2002) to reach the site of inflammation was expressed on neutrophils (85 ± 0.5 % expressed CD62L; Fig. 19G, G') unlike activated microglia (8 ± 0.5 % expressed CD62L; Fig. 19G, G'). CXCR4 was constitutively expressed on neutrophils (100 ± 0.5 % expressed CXCR4) and microglia (95 ± 0.5 % expressed CXCR4) (Fig. 19H, H'). A large percentage of infiltrating neutrophils expressed the granule protein CRAMP (50 ± 0.5 % expressed CRAMP; Fig. 19I, I') in cerebral toxoplasmosis. The phenotypic characterization of the neutrophils and activated microglia revealed that these cells express specific immune mediators to establish a suitable immune response.

4.3.3 Cytokine production of infiltrating neutrophils upon cerebral *T. gondii* infection.

Several studies have shown that neutrophils not only release preformed mediators (Bardoel et al. 2014) but also secrete a plethora of cytokines that control and regulate immune responses (Sturge et al. 2013). To understand their cytokine profile in the inflamed brain during cerebral toxoplasmosis, intracellular flow cytometry was performed.

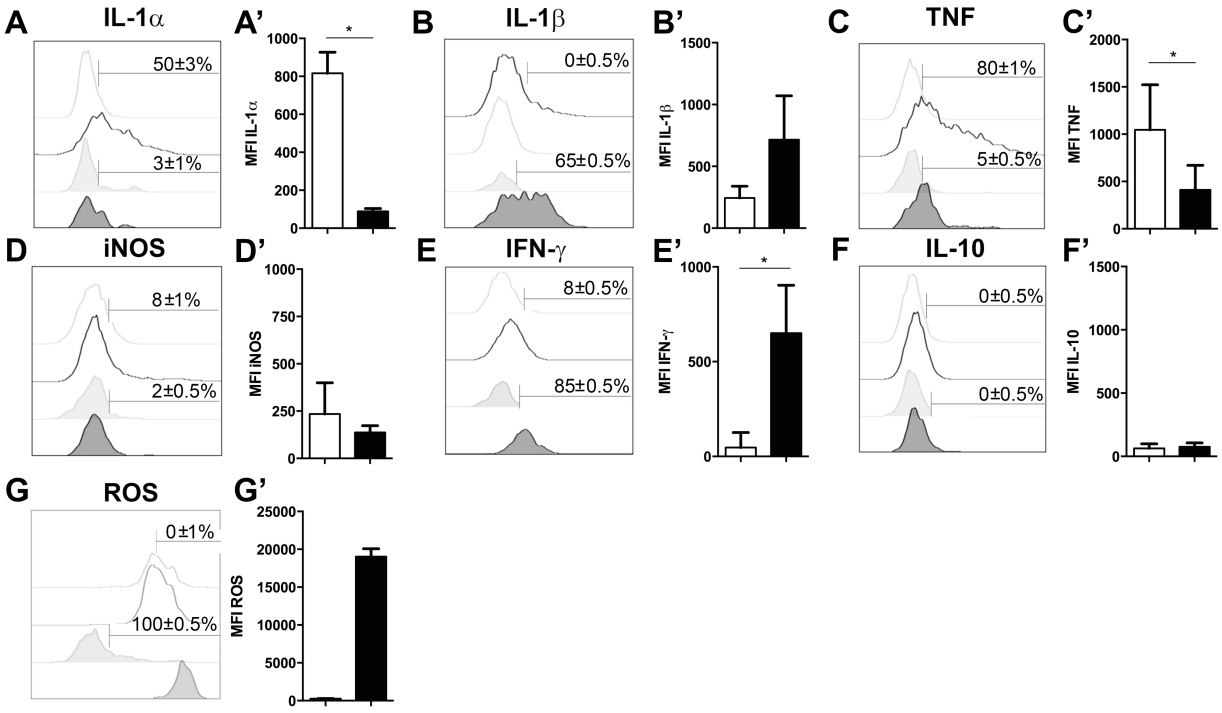


Figure 20. Cytokine production of neutrophils and activated microglia in the brain.

(**A – G**) Intracellular cytokine production by cells isolated from brains of infected mice after *in vitro Toxoplasma* lysate antigen stimulation. The cells were gated as shown in the representative plots of Fig. 18 (**F – G**). (**A – G**) Histograms show the representative expression level of the cytokine by the cell population. Bars mark the cells positive for the particular cytokine. Numbers above the bars represent percentage of cells positive for the cytokine of the respective population: activated microglia ($CD11b^{+}CD45^{int}$) (without any tint), neutrophils ($CD11b^{+}Ly6G^{+}$) (tinted). (**A' – G'**) Bar graphs represent the median fluorescence intensity (MFI) of the respective fluorchrome for a particular cytokine, MFI ± SD (n = 4) (activated microglia; white bars and neutrophils; black bars). Data are representative of 2 independent experiments with n = 4 per group. Mann-Whitney test was performed for comparisons (* $p < 0.05$). White bars and black bars represent activated microglia and neutrophils respectively.

We found that the pro-inflammatory mediators such as IL-1α and TNF were not produced by the neutrophils (3 ± 1 % produced IL-1α; 5 ± 0.5 % produced TNF) unlike the brain resident activated microglia (50 ± 3 % produced IL-1α; 80 ± 1 % produced TNF) (Fig. 20A, A', C, C'). IL-1β was mainly produced by the neutrophils (65 ± 0.5 % produced IL-1β) (Fig. 20B, B'). Interestingly, IFN-γ, which is the main driving factor of the host immune response against cerebral toxoplasmosis, was also produced by the neutrophils (85 ± 0.5 % produced IFN-γ) (Fig. 20E, E') unlike the activated microglia. The cytokine IL-10 and the enzyme iNOS were not produced by neutrophils (0 ± 0.5 % produced IL-10; 2 ± 0.5 % produced iNOS) and activated microglia (0 ± 0.5 % produced IL-10; 8 ± 1 % produced iNOS) (Fig. 20D, D', F, F'). However the neutrophils exclusively produced ROS (100 ± 0.5 % produced ROS) (Fig. 20G, G'). This

analysis revealed that infiltrating neutrophils are a source of immune modulating cytokines during cerebral toxoplasmosis.

4.3.4 Differential production of IFN-γ over the course of cerebral toxoplasmosis.

IFN-γ plays a critical role in the host response to cerebral toxoplasmosis. Although previous studies have suggested NK cells, T cells and microglia to be the source of this cytokine during cerebral toxoplasmosis, the role of neutrophils is not known (Sa et al. 2015; Suzuki 2002). Hence, we investigated the source of IFN-γ and hypothesized neutrophils produce significant levels of IFN-γ during cerebral toxoplasmosis. Since neutrophils are the first immune cells to reach the brain, we investigated 1.5 week and 4 weeks post infection to study the dynamics of the cytokine production over the course of the cerebral toxoplasmosis. We found that during the early phase, $Ly6G^+$ neutrophils were the most dominant producer of IFN-γ (75 ± 0.5 % produced IFN-γ) followed by the activated microglia (47 ± 0.5 % produced IFN-γ). There were meagre numbers of $CD11b^-$ lymphocytes in the brain to make a significant contribution (6 ± 0.5 % produced IFN-γ) (Fig. 21A, A'). With the progression of the infection, there was ingress of $CD11b^-$ lymphocytes to the brain, which then became the major IFN-γ producers (85 ± 0.5 % produced IFN-γ) (Fig. 21B, B'). Our results indicate that $Ly6G^+$ neutrophils are one of the early IFN-γ producers during cerebral toxoplasmosis.

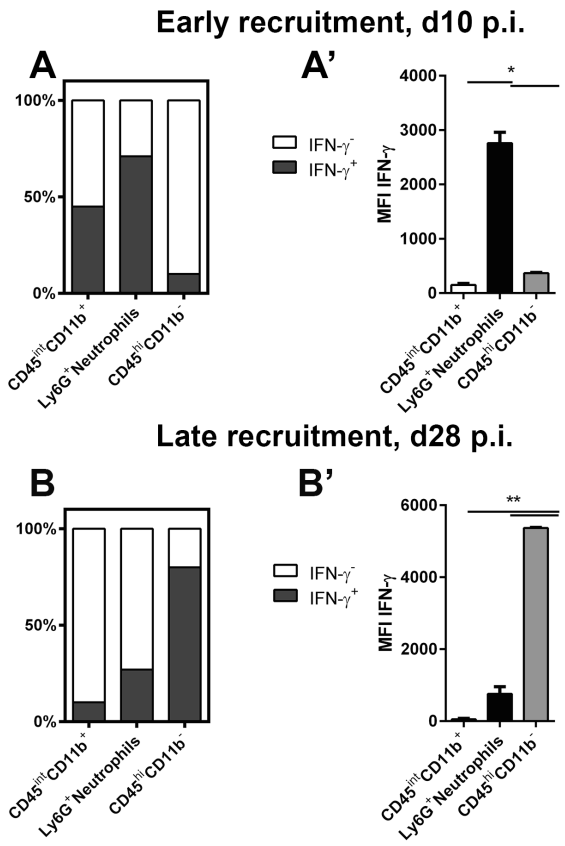


Figure 21. IFN-γ production over the course of cerebral toxoplasmosis.

(**A – B'**) Intracellular cytokine production by cells isolated from brains of infected mice after *in vitro Toxoplasma* lysate antigen stimulation. The cells were gated as shown in the representative plots of Fig. 18 (**F – G**). (**A** – **B**) The fraction of the total cell population expressing IFN-γ was plotted in stacked bar graphs. (**A'** – **B'**) Bar graphs represent the median fluorescence intensity (MFI) of the respective fluorchrome for a particular cytokine, MFI ± SD (n = 4). Data are representative of 2 independent experiments with n = 4 per group. Significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were determined using the Mann-Whitney test. White bars, black bars and grey bars represent $CD45^{int}CD11b^{+}$ activated microglia, $Ly6G^{+}$ neutrophils and $CD45^{hi}CD11b^{-}$ lymphocytes respectively.

4.3.5 Neutrophil depletion in cerebral toxoplasmosis.

Although previous studies (Dunay et al. 2010) have suggested that neutrophils do not play role in controlling acute toxoplasmosis rather cause ileal pathology, their role in cerebral toxoplasmosis is still not fully understood. To investigate the functional role of neutrophils in cerebral toxoplasmosis we took advantage of the depleting anti-Ly6G mAb (1A8). Following our observation that $Ly6G^{+}$ neutrophils are the early IFN-γ producers during cerebral toxoplasmosis,

we started the depletion 1.5 week post infection, when neutrophils were already present in the brain.

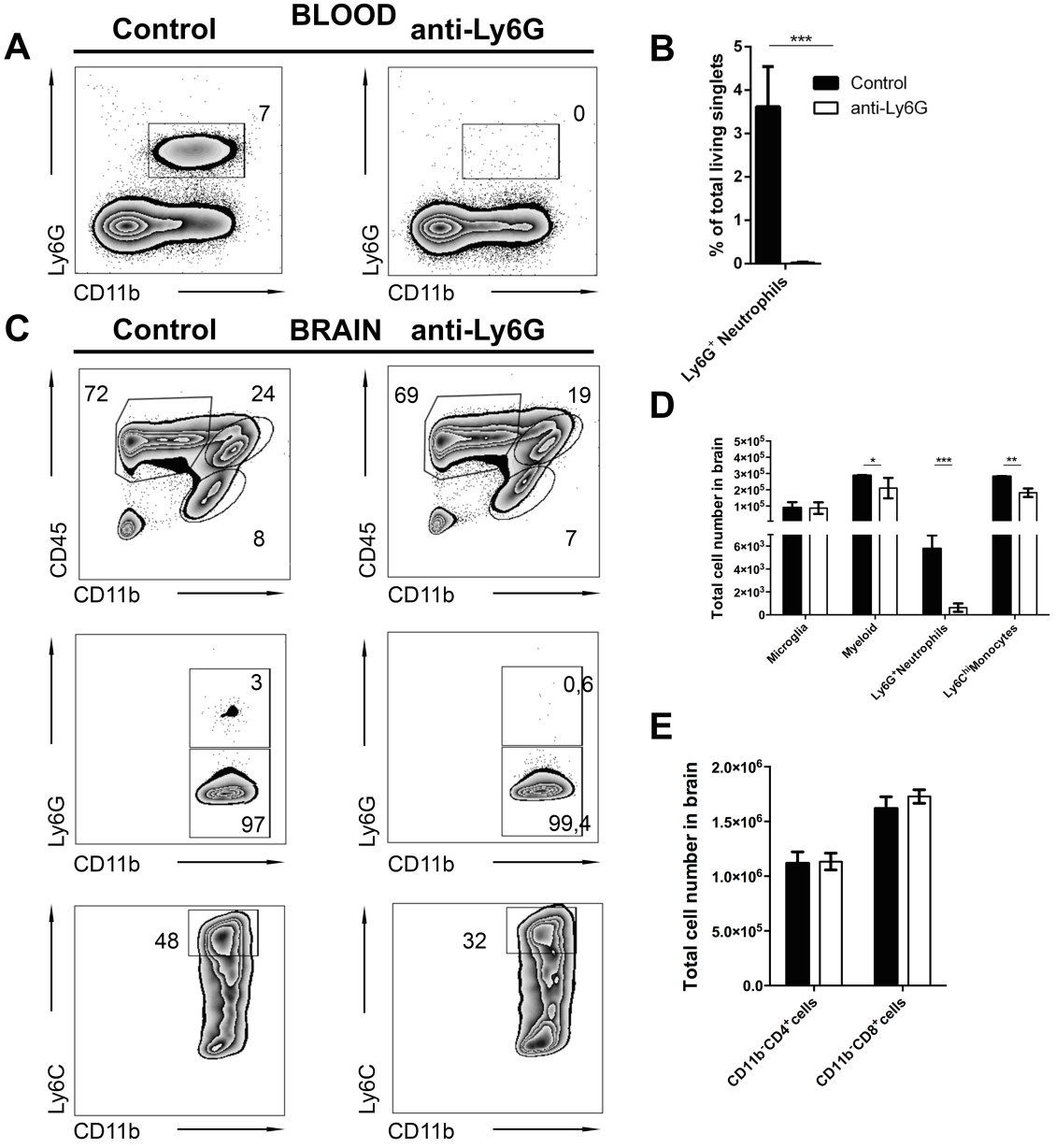


Figure 22. Selective depletion of Ly6G$^+$ neutrophils.

C57BL/6 mice were infected with *T. gondii* and treated with either IgG mAb (**control, left**) or anti-Ly6G (**anti-Ly6G, right**) to deplete neutrophils. After the standard FSC - SSC, singlet gating, live cells were selected for further characterization. Panel (**A**) displays the representative plots to define CD11b$^+$Ly6G$^+$ circulating neutrophils (upper gate) in the blood. (**B**) The bar graph represents the percentage of Ly6G$^+$ in the blood. (**C**) The upper plots show the gating of lymphocytes (CD11b$^-$CD45^{hi}), activated microglia (CD11b$^+$CD45^{int}) and the myeloid population

($CD11b^+CD45^{hi}$) in the brain. Middle plots display the myeloid subsets: $Ly6G^+$(neutrophil) and $Ly6G^-$ (monocyte) in the brain. Lower plots display the inflammatory monocyte subset (from the $Ly6G^-$ gate): $Ly6C^{hi}$. (**D**) The bar graphs represent the total cell number of the respective subset in the brain. (**E**) The bar graphs represent the total cell number of the respective subset gated from the $CD11b^-$ population. Data shown here are the representative of 2 individual experiments with n = 4 mice for each group. The numbers in the representative contour plots is the mean ± SD, % of the parent population. Significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were determined using the Mann-Whitney test.

Thus, we applied the anti-Ly6G mAb and isotype control IgG2a mAb every alternate day from 12 dpi to 23 dpi, to two groups of infected mice respectively. 24 hours after the last Ab treatment, mice were sacrificed and the successful depletion of $CD11b^+Ly6G^+$ monocytes in the blood was confirmed (7.0 ± 0.13 % to 0 %; Fig. 22A, B).

Interestingly, a reduction of recruited myeloid cells (24.0 ± 1.5 % to 19.0 ± 0.28 %) in the brains of anti-Ly6G treated infected mice was observed (Fig. 22C, upper panel). This observation was further confirmed with a significant decrease of $Ly6G^+$ neutrophils (Fig. 22C, middle panel; 3.0 ± 0.5 % to 0.6 ± 0.3 %). Further characterization revealed reduced recruitment of inflammatory monocytes (Fig. 22C, lower panel; 48.0 ± 3.0 % to 32.0 ± 1.3 %) in the brains of anti-Ly6G mice. Alterations in the absolute cell numbers revealed a significant reduction of $CD45^{hi}CD11b^+$ myeloid cells ($p < 0.05$), $Ly6G^+$neutrophils ($p < 0.001$) and $Ly6C^{hi}$ monocytes ($p < 0.01$), whereas the lymphocytes ($CD45^{hi}CD11b^-CD4^+$ and $CD45^{hi}CD11b^-CD8^+$) and microglia ($CD45^{int}CD11b^+$) compartment remained unaltered (Fig. 22D, E).

Next, using RT-PCR we investigated whether the depletion of $Ly6G^+$ neutrophils had an impact on the mRNA levels of parasite burden and cytokine levels of anti-Ly6G treated mice. We found that mAb 1A8 mediated depletion of $Ly6G^+$ neutrophils did not adversely affect the parasite burden (0 fold-change over IgG treated controls; Fig. 23A). However cytokine analysis showed significant reduction in the expression of pro-inflammatory cytokines: TNF (0.4 ± 0.1 fold-change over IgG treated controls, $p < 0.01$; Fig. 23C) and IFN-γ (0.6 ± 0.1 fold-change over IgG treated controls, $p < 0.001$; Fig. 23E) and immunoregulatory IL-10 cytokine (0.4 ± 0.1 fold-change over IgG treated controls, $p < 0.01$; Fig. 23D), whereas IL-1β levels remained unaltered (Fig. 23B). Together these results demonstrate that $CD11b^+Ly6G^+$ neutrophils are not essential in controlling parasite burden during cerebral toxoplasmosis, but they are important for immune during the course of the infection.

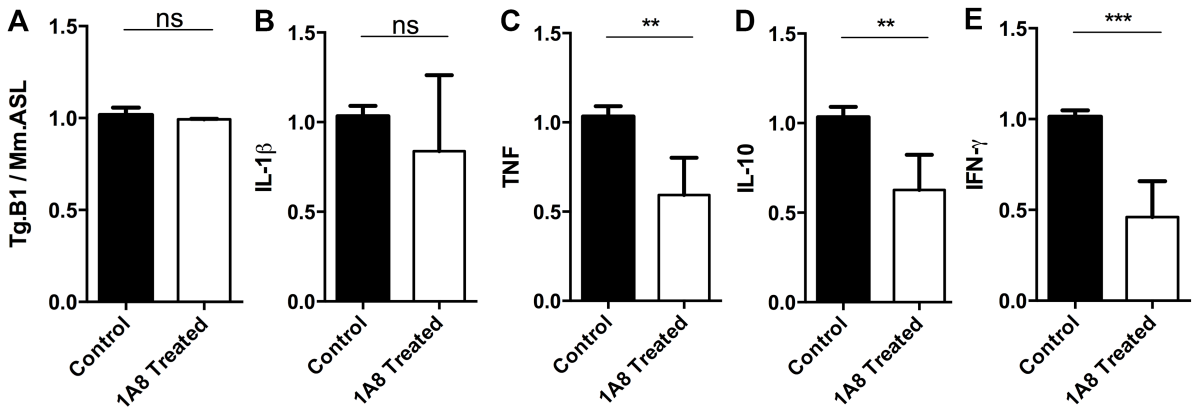


Figure 23. RT-PCR analysis of Ly6G$^+$ neutrophils depleted mice.

Cytokine analysis of the C57BL/6 mice infected with *T. gondii*, treated with either IgG mAb (black bars) or anti-Ly6G (white bars) was done. Fig. 23 (**A – E**) shows the cytokine expression normalised on the control mice. Data shown here is the representative of 2 individual experiments with n = 4 mice for each group. Significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were determined using the Mann-Whitney test.

4.3.6 Emergence of neutrophil subset in cerebral toxoplasmosis.

There have been several studies indicating the presence of different neutrophil subsets with distinct phenotypes and functional profiles in various disease conditions (Tsuda et al. 2004; Pillay et al. 2010; Beyrau et al. 2012). We found that in cerebral toxoplasmosis the infiltrating neutrophils are heterogeneous.

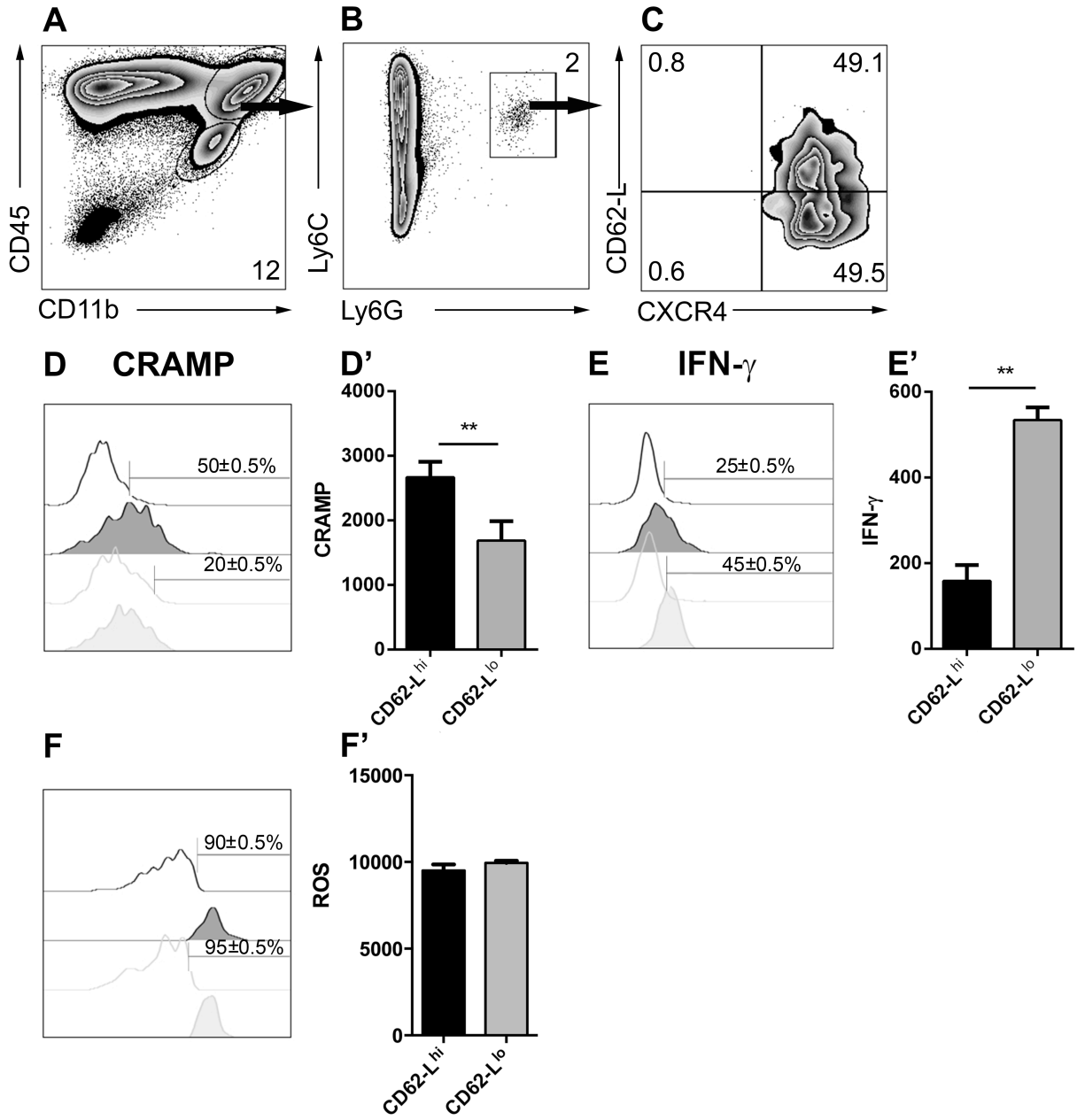


Figure 24. Emergence of neutrophil subsets in the brain.

(**A - B**) Following the initial gating, Ly6G^{+} neutrophils were further characterized based on their CXCR4 and CD62L expression (**C**). The neutrophil subsets CXCR4^{+}CD62L^{lo} and CXCR4^{+}CD62L^{hi} were further studied for their CRAMP, IFN-γ and ROS expression. (**D - F**) Representative histogram shows the CRAMP, IFN-γ and ROS expression of neutrophil subsets CXCR4^{+}CD62L^{hi} (black) and CXCR4^{+}CD62L^{lo} (grey). Bars mark the cells positive for the particular markers. Numbers above the bars represent percentage of cells positive for the marker of the respective population: CXCR4^{+}CD62L^{hi} (black) and CXCR4^{+}CD62L^{lo} (grey). (**D', E' and F'**) Bar graphs represent the median fluorescence intensity (MFI) of the respective fluorochrome for a particular marker, MFI ± SD (n = 4) (CXCR4^{+}CD62L^{hi} (black) and CXCR4^{+}CD62L^{lo} (grey)). The numbers in the representative contour plots is the mean + SD, % of the parent population. Data are representative of 2 independent experiments with n = 4 mice per group. Significant differences (** $p < 0.01$) in the bar graphs were determined using the Mann-Whitney test.

Following the initial gating strategy (Fig. 24A, B), we found that $Ly6G^{+}CXCR4^{+}$ neutrophils expressed different levels of CD62L (Fig. 24C). We found that large percentage of $CXCR4^{+}CD62L^{hi}$ neutrophils expressed CRAMP (50 ± 0.5 % expressed CRAMP) and small percentage of IFN-γ (25 ± 0.5 % produced IFN-γ) whereas small percentage of $CXCR4^{+}CD62L^{lo}$ neutrophils expressed CRAMP (20 ± 0.5 % expressed CRAMP) and large percentage of IFN-γ (45 ± 0.5 % produced IFN-γ) (Fig. 24D-E'). However, the neutrophil subsets produced similar levels of ROS (90 ± 0.5 % of $CXCR4^{+}CD62L^{hi}$ neutrophils produced ROS; 95 ± 0.5 % of $CXCR4^{+}CD62L^{lo}$ neutrophils produced ROS) (Fig. 24F, F'). This data shows the presence of functional heterogeneity in the infiltrating neutrophils during cerebral toxoplasmosis.

5. Discussion

5.1 The role of Ly6C^{hi} inflammatory monocytes in cerebral toxoplasmosis.

The first part of my thesis describes the role of CCR2^{+}Ly6C^{hi} inflammatory monocytes in cerebral toxoplasmosis. Although Ly6C^{hi} inflammatory monocytes have been extensively discussed in recent years in other infections, injury and inflammation models the contribution of Ly6C^{hi} inflammatory monocytes and their subsets in cerebral toxoplasmosis was poorly understood (Shechter & Schwartz 2013; Mildner et al. 2013; London et al. 2013; Satpathy et al. 2013; Epelman et al. 2014).The BM derived CCR2^{+}Ly6C^{hi} inflammatory monocytes are known to have a significant degree of heterogeneity (Shechter & Schwartz 2013; Mildner et al. 2013; Zigmond et al. 2012; London et al. 2013; Serbina et al. 2003). Monocytes are capable of switching their phenotype in response to tissue derived signal and the immunological microenvironment into monocyte – derived macrophage or monocyte – derived DC subsets (Serbina et al. 2003; Zigmond et al. 2012; London et al. 2013; Tacke et al. 2007). In the CNS, previous studies have suggested that alongside the resident microglia cells, recruited BM derived mononuclear cells are important to control CNS inflammation and infection (Getts et al. 2008; John et al. 2011; London et al. 2013; Shechter et al. 2009; Fischer & Reichmann 2001). However, differences in the experimental setups have made it difficult to precisely correlate results and determine the relative importance of the definitive cell subpopulations during inflammation and infection.

The role of BM derived CCR2^{+}Ly6C^{hi} monocytes is well documented in various infectious conditions in the periphery. Dunay et al. have previously shown that inflammatory monocytes are crucial to control acute *T. gondii* infection in the gut, due to production of anti-microbial mediators such as IL-12, TNF and iNOS. The absence of CCR2^{+}Ly6C^{hi} monocytes was followed by uncontrolled parasite replication, extensive tissue damage and rapid death of mice (Dunay et al. 2008b; Dunay et al. 2010; Dunay & Sibley 2010). Serbina et al. 2003 had similar findings in *Listeria monocytogenes* induced infection where CCR2^{+}Ly6C^{hi} monocytes were major producers of TNF and iNOS. In the absence of CCR2^{+}Ly6C^{hi} monocytes mice rapidly succumbed, demonstrating their important role in host defence (Serbina et al. 2003). Alongside their protective functions CCR2^{+}Ly6C^{hi} monocytes can also lead to disease progression. Schumak et

al. 2015 recently observed that Ly6C^{hi} monocytes are substantially involved in brain inflammation and immune cell recruitment to the CNS, leading to experimental cerebral malaria upon *P. berghi* infection (Schumak et al. 2015). Getts et al. 2008 reported, that Ly6C^{hi} monocytes infiltrate the CNS during viral encephalitis, they contribute to viral clearance but they also induce significant immunopathology (Getts et al. 2008). The opposing beneficial and detrimental nature of Ly6C^{hi} monocytes, which depends on the context of infection, warrants further characterization to understand the intricate behaviour.

In the chronic phase of *T. gondii* infection, parasites persist in cysts within immune privileged sites, most importantly the CNS (Courret et al. 2006; Da Gama et al. 2004; Pittman & Knoll 2015). The latent stage is associated with marginal inflammation and cell recruitment to the CNS, which is necessary to provide adequate IFN-γ levels, the major driving force for parasite control. The ongoing inflammation has been shown to cause neurological and behavioural alterations (Havlícek et al. 2001; Beste et al. 2014). In the murine models of chronic cerebral toxoplasmosis the characteristics of T cell subsets, specific mononuclear cells, for example resident microglia and antigen-presenting cells (APCs) have been extensively studied (Clark et al. 2011; Schlüter et al. 2001; John et al. 2011). In contrast the role of the myeloid cell subsets need further investigation (Strack et al. 2002; Schluter et al. 2003; Drogemuller et al. 2008). The brain resident microglia eliminate parasites in an IFN-γ dependent manner, in addition to their efficient phagocytic capacity and cytokine production (Deckert et al. 2006; Sa et al. 2015). The origins of DCs in chronic cerebral toxoplasmosis have been debated. It is unclear whether they differentiate from local resident cells or have a peripheral haematopoietic cell of origin (Deshpande et al. 2007; John et al. 2011). The latter publication has described precisely that CD11c^{+} DC subsets are derived from the BM to generate potential T cell responses. However if the CNS infiltrating CD11c^{+} DC subsets originates from the BM derived CCR2^{+}Ly6C^{hi} monocytes is yet to be identified.

To gain an insight into the role of CCR2^{+}Ly6C^{hi} monocytes we studied recruited myeloid cells within the brain upon cerebral toxoplasmosis. We found that upon low dose *T. gondii* infection brain resident microglia were activated with elevated CD45 expression. There was a massive infiltration of immune cells from the periphery of lymphoid (CD11b^{-}, ungated) and myeloid (CD11b^{+}, gated) origin. Further phenotyping of myeloid cells (CD11b^{+}, gated) revealed,

neutrophils ($CD45^{hi}CD11b^{+}Ly6G^{+}$) and inflammatory monocytes ($CD45^{hi}CD11b^{+}Ly6G^{-}$ $Ly6C^{hi}F4/80^{int}$). Based on Ly6C and F4/80 expression there were two more $CD45^{hi}CD11b^{+}Ly6G^{-}$ myeloid populations present in the CNS, namely $Ly6C^{int}F4/80^{int}$ and $Ly6C^{neg}F4/80^{hi}$. The gating strategy proved that there is an infiltration of heterogeneous immune cells from the periphery. The localization of $Ly6C^{+}$ and $CCR2^{+}$ cells via immunofluorescence revealed that these amoeboid shaped inflammatory cells were in the cortex adjacent to the lesions and nearby to the vessels.

Dunay et al. have detected that $CCR2^{-/-}$ mice were extremely sensitive to acute *T. gondii* infection, with over 90% of the mice succumbing. Therefore they concluded that $CCR2^{+}Ly6C^{hi}$ monocytes were necessary to control the acute *T. gondii* infection and to limit the induced small intestinal pathology (Dunay et al. 2008b). The remaining 10% of infected mice displayed elevated parasite numbers in the CNS, suggesting that these cells might be involved in the chronic phase of the infection. Thus, to study the role of $CCR2^{+}Ly6C^{hi}$ monocytes in the chronic stage, depletion of the cell subset had to be performed after the infection overcame the acute phase and parasites were present in the CNS.

In the present study, we specifically depleted $CCR2^{+}Ly6C^{hi}$ monocytes, taking advantage of the depleting antibody anti-CCR2 (MC – 21). The treatment resulted in increased immunopathology and elevated parasite numbers in the CNS during cerebral toxoplasmosis. Notably, mice could not control the infection and succumbed due to the lack of $Ly6C^{hi}CCR2^{+}$ monocytes. This suggests the critical role of this cell subset in controlling the chronic phase of the infection.

Due to the differential expression of Ly6C and F4/80 among the myeloid compartment we characterized the phenotype of the recruited $CCR2^{+}Ly6C^{hi}$ monocytes, $Ly6C^{int}$ and $Ly6C^{neg}$ myeloid derived cells, and resident microglia. We conducted comprehensive surface stainings with specific markers by flow cytometry. The chemokine receptor CCR2 expressed on the surface of monocytes decreased with the corresponding downregulation of Ly6C expression. Microglia did not express CCR2 showing that they are independent of haematopoietic input. The chemokine receptor CX_3CR1 known to be important in maintaining microglial functions was predominantly expressed on microglia with or without infection and was expressed only in low levels on $Ly6C^{hi}$, $Ly6C^{int}$ and $Ly6C^{neg}$ monocytes (Mizuno et al. 2003; Zujovic et al. 2000;

Takahashi et al. 2005). The macrophage marker F4/80 was expressed mainly by activated microglia cells and by Ly6C^{neg} monocytes suggesting their macrophage like nature. Microglia cells upregulated CD11c upon infection-induced activation, as detected in other studies (John et al. 2011). CD11c expression was also elevated in Ly6C^{int} and Ly6C^{neg} monocytes pointing toward their DC properties. Interestingly, MHC I and MHC II were elevated predominantly on the surface of Ly6C^{int} monocytes, implying that these cells are the previously characterized brain DCs (John et al. 2011). The extensive phenotypic characterization proved that the recruited myeloid cells are distinct from resident microglia.

We also characterized blood monocytes to study their activation status in the ongoing inflammation. Blood monocytes comprises of two distinct populations, the inflammatory $CX_3CR1^{lo}CCR2^{+}Ly6C^{hi}$ and the patrolling $CX_3CR1^{hi}CCR2^{-}Ly6C^{lo}$ monocytes (Geissmann et al. 2003). Correspondingly, in our current experiments $CX_3CR1^{lo}CCR2^{+}Ly6C^{hi}$ monocytes and $CX_3CR1^{hi}CCR2^{-}Ly6C^{lo}$ were present in the blood. Upon *Toxoplasma* infection the common DC marker CD11c and MHC I were upregulated on the surface of Ly6C^{hi} monocytes, suggesting modified activation status even prior to reaching the site of infection.

Following phenotypic analysis, we conducted cytokine analysis produced by mononuclear cells in the CNS by intracellular flow cytometry. Comparing the cytokine profile of the resident and recruited mononuclear cell subsets in the CNS upon cerebral toxoplasmosis revealed that Ly6C^{hi} monocytes produced the highest levels of pro-inflammatory molecules such as IL-1α, IL-1β, IL-6, TNF and iNOS. These results clearly indicate that monocytes are essential to control *T. gondii* infection in the brain, similar to our previous findings in the periphery (Dunay et al. 2008b; Dunay & Sibley 2010). Additionally, the elevated ROS production was observed exclusively by the Ly6C^{hi} monocytes, further suggesting their critical role in the host defence. Ly6C^{int} monocytes were the major producer of IL-12. In line with their CD11c and MHC expression, this suggests their capability to initiate adaptive immune responses. When comparing cytokine production of activated resident microglia cells to CD45^{+}CD11b^{+} myeloid derived cells, the recruited cells contributed in greater extent to the secretion of pro-inflammatory mediators in the model of chronic *T. gondii* infection in the brain. Notably, in addition to their antiparasitic capacity, Ly6C^{hi} monocytes were also able to secrete the regulatory factor IL-10, suggesting a dual function of limiting pathogen expansion and regulating detrimental immunopathology in the

CNS. Our observations are consistent with a recent study by Grainger *et al*., reporting similar dual features of Ly6C^{hi} monocytes in the acute *T. gondii* infection model (Grainger et al. 2013). Additionally, this study also described that monocytes regulate neutrophil function via prostaglandin E2 secretion, hence contributing to our understanding of their complex functions. Similar diverse cytokine profiles of Ly6C^{hi} monocytes ranging from pro – inflammatory to anti – inflammatory were also seen in models of autoimmunity and neurodegeneration (London et al. 2013; Shechter et al. 2009).

In our current study, we also compared the phagocytic capacity of brain resident cells to those of recruited mononuclear cell subpopulations isolated from the CNS of *T. gondii*-infected mice. Activated microglia alongside Ly6C^{neg} myeloid derived cells displayed increased phagocytic potential, unlike Ly6C^{hi} and Ly6C^{int} cells. F4/80 and particularly TREM2 expression was also the highest on activated microglia and Ly6C^{neg} myeloid derived cells, as described recently for TREM2 (Melchior et al. 2010; Takahashi et al. 2007; Turnbull et al. 2006). Thus substantial upregulation of TREM2 on activated microglia and Ly6C^{neg} myeloid derived cells in infected brains suggest the capacity to engulf invading microorganisms and dead tissue remains due to the ongoing inflammation.

The current paradigm suggests that monocytes do not substantially contribute to tissue resident macrophages under steady state conditions, and the brain resident macrophages, microglia, sustain themselves without any BM – derived myeloid cell replenishment during adulthood (Ginhoux et al. 2010; Ajami et al. 2007). However, flexibility of the Ly6C^{hi} inflammatory monocytes allows them to perform distinct macrophage-like functions. The diverse behaviour of the monocyte-derived macrophages was demonstrated in experimental autoimmune uveitis where the kinetics of $CX_3CR1^{lo}Ly6C^{+}$ and $CX_3CR1^{hi}Ly6C^{-}$ changed along the course of the disease (London et al. 2013). Such heterogeneity was also shown in an acute model of colitis where the recruited $CX_3CR1^{int}Ly6C^{hi}$ monocytes promoted inflammation, but over time giving rise to a $CX_3CR1^{int}Ly6C^{lo}$ population that displayed all the trademarks of a DC (Zigmond et al. 2012). Thus, upon reaching the brain Ly6C^{hi} monocytes extravasate and display distinct functions in parasite clearance, Ag presentation and resolution of the inflammation. In cerebral toxoplasmosis, the conversion of monocytes to brain DCs upon recruitment was proposed by a previous study (John et al. 2011). Confirming their plasticity, we also detected that recruited

Ly6C^{hi} monocytes in the brain further differentiate into two distinct population CD11c^{+}Ly6C^{int} brain DCs and Ly6C^{neg}F4/80^{+} macrophages and carry out particular functions in parasite clearance.

Leukocyte transmigration in the blood through the blood-brain barrier upon cerebral inflammation is dependent on adhesion molecules and their receptors. Inflammatory monocytes use the CCR2–CCL2 axis to egress from the BM, but the mechanism of crossing the blood-brain barrier is poorly understood (Shi & Pamer 2011). Therefore, we investigated the involvement of certain key adhesion molecules in the recruitment of Ly6C^{hi} inflammatory monocytes to the CNS. CD62L (L-selectin), which assists immune cells to enter different tissues (Sperandio et al. 2003), was highly expressed on Ly6C^{hi} monocytes in the blood, but was shed upon their entry into the CNS as they turned into effector cells. Consistent with previous studies, the CD62L expression was low on resident monocytes and microglia (Ginhoux & Jung 2014). LFA-1, which can interact with P- and E-selectin on the activated endothelial cells to initiate rolling was present both in the periphery and in the CNS on Ly6C^{hi} monocytes, as well as on differentiated Ly6C^{int} and Ly6C^{neg} monocytes. Previous studies have described elevated LFA-1 expression on resident microglia in cerebral toxoplasmosis and their functional role on recruitment of leukocytes (John et al. 2011). We measured highest PSGL-1 expression on the surface of Ly6C^{hi} monocytes, which led us to the hypothesis that this molecule might play a role in monocyte entry.

The dependence on PSGL-1 for lymphocyte migration is well established (Engelhardt 2009; Sathiyanadan et al. 2014; Hoos et al. 2014; Inoue et al. 2005). However, to date, the function of PSGL-1 in monocyte recruitment has not been sufficiently addressed. One previous study described the role of PSGL-1 in Ly6C^{hi} monocyte homing to the site of atherosclerosis in blood vessels of mice (Sperandio et al. 2003). This study revealed that Ly6C^{hi} monocytes, which are PSGL-1^{hi} and CD62L^{+}, preferentially interacted with P- and E-selectin on activated endothelium or with CD62L on a rolling/adherent leukocyte under flow by secondary tethering. However, the authors speculated that other adhesion molecules such as LFA-1 and VCAM-1 (ligand VLA-4) might not be key factors in monocyte homing, as their ligands were expressed at lower levels on Ly6C^{hi} cells. Furthermore, other studies detected PSGL-1–dependent monocyte migration in the periphery during tumour metastasis and thrombus formation (Inoue et al. 2005; Hoos et al.

2014). Thus, to our knowledge, we investigated for the first time PSGL-1–dependent monocyte recruitment to the CNS. Treatment of mice with anti–PSGL-1 Ab revealed a significant inhibition of Ly6C^{hi} cell recruitment to the brain upon chronic T. gondii infection, confirming that PSGL-1 is critical for monocyte homing to the CNS.

Altogether, our findings combined with emerging evidence from other murine models further highlight the plasticity of recruited Ly6C^{hi} monocytes. Cerebral *T. gondii* infection leads to cytokine production by the infiltrating Ly6C^{hi} monocytes, which play an influential role in the protection of the inflamed brain. The Ly6C^{hi} monocytes further give rise to Ly6C^{int} and Ly6C^{neg} subsets and perform divergent functions such as Ag presentation and phagocytosis. Thus, monocytes and their descendants play multifaceted roles to control cerebral toxoplasmosis. In conclusion, our findings indicate that, during cerebral *T. gondii* infection, Ly6C^{hi} inflammatory monocytes infiltrate the CNS and differentiate into phenotypically and functionally distinct cell subsets and carry out pivotal functions to control the chronic stage.

5.2 The function of Ly6G^{+} neutrophil granulocytes in cerebral toxoplasmosis.

The second part of my thesis aims to characterize the Ly6G^{+} neutrophils in cerebral toxoplasmosis. Neutrophils are the first immune cells to migrate to inflammatory sites and form the initial effector molecules against infection and injury. Neutrophils secrete a repertoire of cytokines that play a critical role in infection and injury (Nathan 2006; Zhou et al. 2003). However, the role of neutrophils in cerebral toxoplasmosis is still unclear. A previous study had concluded that neutrophils is the limiting factor against uncontrolled tachyzoite replication in cerebral toxoplasmosis (Bliss et al. 2001). However, this study was based on depletion of Gr1^{+} cells. Gr1^{+} cells include neutrophils but also monocytes, DCs, macrophages and lymphocytes (Daley et al. 2008; Dunay et al. 2010). Hence the effects seen before cannot be related only to neutrophils.

Therefore we investigated the specific function of Ly6G^{+} neutrophils in cerebral toxoplasmosis. We found that after 4 weeks of low dose infection of *T. gondii* the percentage of Ly6G^{+} neutrophils in the blood increased. In the brain, resident microglia were activated with elevated levels of CD45 expression. There was a massive infiltration of immune cells from the periphery of lymphoid (CD11b^{-}) and myeloid (CD11b^{+}) origin. Further phenotyping of myeloid cells

($CD11b^+$) revealed inflammatory monocytes ($CD45^{hi}CD11b^+Ly6G^-$) and neutrophils ($CD45^{hi}CD11b^+Ly6G^+$). The gating strategy showed that there was an infiltration of $Ly6G^+$ neutrophils from the periphery.

Further we performed phenotypic characterization of the $Ly6G^+$ neutrophils in comparison to the activated microglia. MHC I and MHC II, the molecules known to process and present Ags to T cells to initiate adaptive immune response, were highly expressed by neutrophils and activated microglia, making them efficient Ag presenters (Lei et al. 2001; Kumar & Sharma 2010; Biswas et al. 2015). We detected a small percentage of neutrophils and activated microglia expressing CD80 and CD86, the molecules known to support Ag presentation, and/or- activate T cell (Denkers et al. 2004; Louveau et al. 2015). CD64 (FcγR1) belong to a group of receptors which induce phagocytosis via ITAM-Syk signalling, was not expressed by the neutrophils unlike the activated microglia (Linnartz et al. 2010). The chemokine receptor CXCR2 typically expressed on neutrophils (Liu et al. 2010) was downregulated upon entering the brain. However, CXCR2 detected on oligodendrocytes and neurons (Liu et al. 2010) where it mediates a wide range of functions, was not expressed on the activated microglia. CD62L known to be important for leukocyte rolling (Rainer 2002; Biswas et al. 2015) to reach the site of inflammation was expressed on the neutrophils unlike the activated microglia. CXCL12, the ligand of CXCR4 is constitutively expressed on endothelial cells in the CNS (Wilson et al. 2010; McCandless et al. 2006). We showed that cerebral toxoplasmosis leads to increased expression of CXCR4 on neutrophils and on microglia. CRAMP, a member of the cathelicidin family of antimicrobial peptides stored in neutrophils (Oliver Soehnlein et al. 2008; Mócsai 2013), was highly expressed by the infiltrating neutrophils. Hence by the phenotypic characterization we demonstrated that the neutrophils have a distinct profile.

Following surface characterization, we studied the anti- and pro- inflammatory molecules secreted by $Ly6G^+$ neutrophils and by activated microglia. We detected the production of pro-inflammatory molecules such as IL-1α and TNF by activated microglia. However, IL-1β was mainly produced by neutrophils. The anti- inflammatory cytokine IL-10 and the enzyme iNOS were not produced by either of the cells. Most importantly, we found that the cytokine IFN-γ, which is the main driving factor of the host immune response against cerebral toxoplasmosis, was produced by neutrophils unlike activated microglia. There is increasing evidence that apart

from NK cells and T cells, neutrophils are another important source of this cytokine. In acute pulmonary infection with *Nocardia asteroides* neutrophils produce IFN-γ (Ellis & Beaman 2002). In *Salmonella typhimurium*-induced colitis neutrophils are a cellular source of mucosal IFN-γ production (Kirby et al. 2002; Sturge et al. 2013). Our observation was disparate from Sa et al. where it was reported that microglia are the only CD11b+ cells secreting IFN-γ in cerebral toxoplasmosis (Sa et al. 2015). However the study was conducted in a reactivation model of cerebral *T. gondii* infection which is distinct from our model. They also used a different mouse strain that could influence the immune response (Sa et al. 2015). Interestingly, our observation was in line with Sturge et al. where it was reported that the neutrophils store IFN-γ at promyelocyte stage in the absence of inflammation (Sturge et al. 2015). They also detected that during acute toxoplasmosis neutrophils form an important non-lymphoid source of IFN-γ (Sturge et al. 2013). The neutrophil-derived IFN-γ does not require TLR adaptor protein MyD88-dependent signalling unlike NK and T cells in host protection against *T. gondii* (Sturge et al. 2013). Our observation revealed for the first time that apart from acute toxoplasmosis infiltrating neutrophils produce IFN-γ also during cerebral toxoplasmosis. We also observed that infiltrating neutrophils are the dominant producers of IFN-γ during early stage of cerebral toxoplasmosis. However, during later stages of cerebral toxoplasmosis, infiltrating lymphocytes constitute the major producers of IFN-γ.

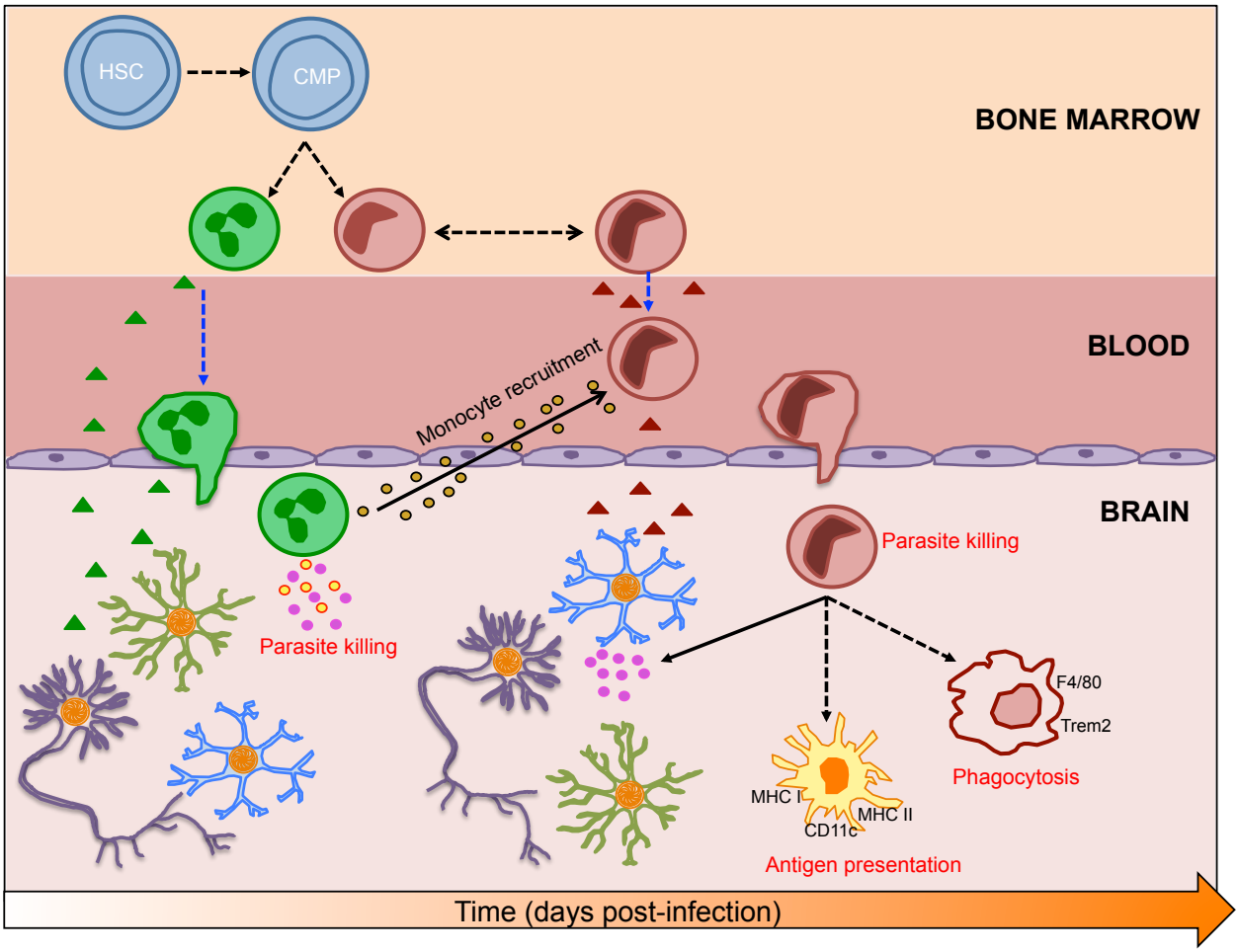
IFN-γ is crucial for survival during *T. gondii* infection. IFN-γ- or IFN-γ-receptor deficient mice show a high susceptibility to the parasite (Sturge & Yarovinsky 2014). Hence neutrophils producing IFN-γ suggested a crucial role played by this cell population in cerebral toxoplasmosis. Taking advantage of the depleting antibody anti-Ly6G (1A8), we specifically depleted $Ly6G^+$ neutrophils. Absence of $Ly6G^+$ neutrophils resulted in a significantly decreased recruitment of $Ly6C^{hi}$ monocytes to the CNS during cerebral toxoplasmosis. However, the depletion had no effect on the recruitment of $CD11b^-CD4^+$ and $CD11b^-CD8^+$ T lymphocytes to the brain. Moreover, the depletion did not alter the parasite burden. These results suggest that the unaltered lymphocyte compartment efficiently complements the IFN-γ producing $Ly6G^+$ neutrophils. However, we detected a significant reduction of TNF, IL-10 and IFN-γ mRNA levels in the brains of neutrophils depleted mice compared to untreated ones. $Ly6C^{hi}$ monocytes in the CNS during cerebral toxoplasmosis have been shown by our previous studies to express

TNF and IL-10 (Biswas et al. 2015). Hence the reduction of TNF and IL-10 levels was corroborated by the reduced recruitment of $Ly6C^{hi}$ monocytes upon $Ly6G^{+}$ neutrophils depletion. However, the reduced IFN-γ levels was most likely due to the complete ablation of $Ly6G^{+}$ neutrophils as there was no change in the lymphocyte compartment.

The $Ly6G^{+}$ neutrophils have been described to influence the recruitment of $Ly6C^{hi}$ monocytes in various infection and injury models (Oliver Soehnlein et al. 2008; O. Soehnlein et al. 2008; Zhou et al. 2003). To gain insight into the mechanistical detail as to how the extravasated neutrophils can recruit $Ly6C^{hi}$ monocytes, we performed a comprehensive analysis. We detected that based on CXCR4 expression neutrophils display a vast degree of plasticity and heterogeneity. $Ly6G^{+}$ neutrophils comprised of $CD62L^{hi}CXCR4^{+}$ and $CD62L^{lo}CXCR4^{+}$ subsets. On one hand the $Ly6G^{+}CD62L^{hi}CXCR4^{+}$ subset expressed high levels of CRAMP and produced low levels of IFN-γ. On the other hand, $Ly6G^{+}CD62L^{lo}CXCR4^{+}$ neutrophils expressed low levels of CRAMP and produced high levels of IFN-γ. Differential expression of CRAMP and IFN-γ elucidated functional heterogeneity of $Ly6G^{+}$ neutrophils. While one subset $Ly6G^{+}CD62L^{hi}CXCR4^{+}$ with high CRAMP, promotes monocyte recruitment, the other subset $Ly6G^{+}CD62L^{lo}CXCR4^{+}$ with high IFN-γ establishes high inflammatory response against cerebral toxoplasmosis.

Altogether our data combined with a growing body of evidence highlight the existence of distinct $Ly6G^{+}$ neutrophils subsets. Cerebral toxoplasmosis leads to the infiltration of $Ly6G^{+}$ neutrophils to the CNS. We report for the first time that $Ly6G^{+}$ neutrophils form an early non-lymphoid source of IFN-γ upon developing neuroinflammation. We detected neutrophil dependent recruitment of $Ly6C^{hi}$ monocytes in cerebral *T. gondii* infection. We also identified subsets within $Ly6G^{+}$ neutrophils performing divergent functions such as monocyte recruitment and secretion of the pro-inflammatory cytokine IFN-γ. The multifaceted action of the neutrophil subsets in boosting the inflammatory response and specifically recruiting the $Ly6C^{hi}$ monocytes display the heterogeneity of infiltrating neutrophils during cerebral *T. gondii* infection.

5.3 Summary of the study.



Chronic cerebral toxoplasmosis is characterized by the activation of brain resident cells and infiltration of immune cells from the periphery. We showed $Ly6G^{+}$ neutrophils that infiltrate the brain first, secreted IFN-γ, the cytokine which is known to be the major mediator of resistance to

T. gondii. Specific depletion of the infiltrating $Ly6G^+$ neutrophils resulted in reduced recruitment of $Ly6C^{hi}$ inflammatory monocytes to the brain. The reduced recruitment of $Ly6C^{hi}$ inflammatory monocytes resulted in decreased levels of IL-10 and TNF in the brain. Finally, we showed $Ly6G^+$neutrophils are a heterogeneous population based on CXCR4 and CD62-L expression. While one subset $Ly6G^+CD62L^{hi}CXCR4^+$ with high CRAMP expression, promotes monocyte recruitment, the other subset $Ly6G^+CD62L^{lo}CXCR4^+$ with high IFN-γ secretion establishes high inflammatory response against cerebral toxoplasmosis.

Following $Ly6G^+$ neutrophils, $Ly6C^{hi}$ inflammatory monocytes extravasate to the brain. We detected that neutrophil-derived granule proteins CRAMP and adhesion molecule PSGL-1, plays a crucial role in the recruitment of $Ly6C^{hi}$ inflammatory to the brain. The recruitment of $Ly6C^{hi}$ inflammatory to the brain is critical as specific depletion of this monocyte subset resulted in elevated parasite load and decreased survival of infected mice. $Ly6C^{hi}$ inflammatory monocytes governed parasite control due to production of pro-inflammatory mediators, such as IL-1α, IL-1β, IL-6, iNOS, TNF and ROS. Interestingly, $Ly6C^{hi}CCR2^+$ monocytes also produced the regulatory cytokine IL-10, revealing their dual feature in host defense. Moreover, we confirmed by adoptive transfer the recruited monocytes gives rise to two subsets of monocyte descendants with distinct phenotypes as $Ly6C^{int}CCR2^+F4/80^{neg}$ antigen presenting cells and as $Ly6C^{neg}F4/80^{hi}$ phagocytic cells. The differentiated $Ly6C^{int}CCR2^+F4/80^{neg}$ subset upregulated MHC I and MHC II molecules suggesting dendritic cell (DC) properties such as interaction with T cells, whereas the $Ly6C^{neg}F4/80^{hi}$ cell subset displayed elevated phagocytic capacity while upregulating TREM2.

Collectively, our findings elucidated that during chronic cerebral toxoplasmosis bone marrow derived myeloid cells, namely $Ly6C^{hi}$ inflammatory monocytes and $Ly6G^+$ neutrophils, are not homogeneous cell populations, but display a vast degree of plasticity and heterogeneity. Understanding the molecular cues that guide the differential fates of myeloid cells *in vivo* should provide attractive targets for the treatment of chronic cerebral toxoplasmosis.

6. References

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Erklärung

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation zum dem Thema:

Functional analysis of Ly6C^{hi} inflammatory monocytes and Ly6G^{+} neutrophil granulocytes in chronic cerebral toxoplasmosis

selbständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weierhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, 26.04.2016

Aindrila Biswas

Curriculum vitae

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