Influence of IL-12 cytokine family members on T cell differentiation in experimental cerebral malaria

Dissertation

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submitted to the University of Hamburg,
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Hamburg, September 2012

Genehmigt vom Fachbereich Biologie
der Fakultät für Mathematik, Informatik und Naturwissenschaften
an der Universität Hamburg
auf Antrag von Frau Professor Dr. I. BRUCHHAUS
Weiterer Gutachter der Dissertation:
Priv.-Doz. Dr. T. JACOBS
Tag der Disputation: 15. Juni 2012

Hamburg, den 31. Mai 2012

Professor Dr. J. Fromm
Vorsitzender des Promotionsausschusses
Biologie

Declaration on oath *Eidesstattliche Versicherung*

I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids.

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Hamburg, date Signature
Hamburg, den *Unterschrift*

Acknowledgements

My first thanks go to Dr. Thomas Jacobs for great supervision. You gave me the chance to pursue my studies independently, while granting me support whenever I needed it.

I would like to thank Prof. Iris Bruchhaus for her willingness to supervise and evaluate my thesis.

Many thanks to the people in my lab for creating the atmosphere I loved to work in. I want especially to thank Christiane Steeg for what must have been shiploads of answered questions, many of them just asked because to ask you is always faster and easier than and at least as reliable as to look it up somewhere.

I want to thank Elena Helk for so many productive discussions about experimental setups, flow cytometry, γδ T cells, neutrophils, papers, festivals, coffee and everything else we shared.

For her willingness in proofreading this thesis over and over and her support during the final phase, special thanks go to Melanie Piédavent.

In the end I would especially like to thank my closest family for their strong support along the entire road.

Contents

Abbreviations

General abbreviations

-/-	Heterozygous deficient
APC	Antigen presenting cells
bp	Base pairs
BCR	B cell receptor
CD	Cluster of differentiation (nomenclature of surface antigens)
CTLA-4	Cytotoxic T lymphocyte antigen-4
CM	Cerebral malaria
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EBI3	Epstein-barr induced gene 3
ECM	Experimental cerebral malaria
ELISA	Enzymelinked immunosorbent assay
FITC	Fluorescein isothiocyanate
GPI	Glycophosphatidylinositol
HRP	Horseradish peroxidase
IFNγ	Interferon γ
Ig	Immunglobuline
IL	Interleukine
LPS	Lipopolysaccharide
NK cell	Natural Killer cell
NK T cell	Natural Killer T cell
MHC	Major histocompatibility complex
pRBC	Parasitized red blood cells
PCR	Polymerase chain reaction
PE	Phycoerythrine
PerCp	Peridinin chlorphyll protein
p.i.	Post infection
PRR	Pattern recognition receptors

RBC	Red blood cells
ROS	Reactive oxygen species
TCR	T cell receptor
TGFβ	Transforming growth factor β
TNF	Tumor necrosis factor
TLR	Toll-like receptor
WHO	World health organization
X-Gal	5-bromo-4-chloro-indolyl-β-d-galactopyranoside

Abbreviations of nucleobases

A	Adenine	C	Cytosine
G	Guanine	T	Thymidine
U	Uracil		

One- and three-letter-code of amino acids

A	Ala	Alanine	C	Cys	Cysteine
D	Asp	Aspartic acid	E	Glu	Glutamic acid
F	Phe	Phenylalanine	G	Gly	Glycine
H	His	Histidine	I	Ile	Isoleucine
K	Lys	Lysine	L	Leu	Leucine
M	Met	Methionine	N	Asn	Asparagine
P	Pro	Proline	Q	Glu	Glutamine
R	Arg	Arginine	S	Ser	Serine
T	Thr	Threonine	V	Val	Valine
W	Trp	Trytophane	Y	Tyr	Tyrosine

1 Introduction

1.1 The immune response to infection

In his publication 'On the Aetiology of splenic fever, based on the life-history of the *Bacillus anthracis*' in 1876 Robert Koch was the first scientist to completely and consistently connect an infectious disease to a pathogenic microorganism (Koch, R. 1876). The following success of Louis Pasteur to develop vaccines against cholera in chicken in 1880 and rabies in 1885 encouraged scientists to start the search for the mediators of protection. Since then many pathogens were identified: a variety of different bacteria, fungi, viruses and parasites, each of them causing an infectious disease with a different clinical picture. The investigation on the protective mechanisms of the host revealed detailed information on the complex defenses of the immune system.

1.1.1 Innate immunity - first line defense

The cells of the innate immune system are the first to encounter a pathogen after it was able to cross the epithelial barriers into the body of the host. Throughout the tissue phagocytic cells reside that are able to recognize conserved pathogenic surface structures (pathogen associated surface patterns, PAMP) by pattern recognition receptors (PRR), such as toll like receptors (TLRs). TLR4 for instance recognizes lipopolysaccharide (LPS) in the membrane of gram-negative bacteria and TLR2 recognizes different microbial GPI anchors (Carpenter, S. and O'Neill, L.A.J. 2007). Dendritic cells, macrophages and neutrophils express these pattern recognition receptors and with their help they are able to recognize many different pathogens (Michelsen, K.S. et al. 2001). The pathogen is taken up into a phagosome, which acidifies and afterwards fuses with a lysosome to build the phagolysosome, where antimicrobial peptides and enzymes degrade the ingested material. Neutrophils are very short-lived cells that destroy ingested microorganisms by a process called respiratory burst, during which reactive oxygen species (ROS) are produced and released into the phagosome. These do not only destroy the pathogen but fatally harm the neutrophil as well (Hampton, M.B. et al. 1998; Wang, J.P. et al. 2008). All phagocytes start to release cytokines and chemokines upon recognition of pathogens by their PRR. These activate

epithelial cells to upregulate adhesion molecules, activate inflammatory cells throughout the body and build a gradient, which guides inflammatory cells to the site of infection (Iwasaki, A. and Medzhitov, R. 2004). For example natural killer cells (NK cells) are attracted, which are able to recognize the alteration of the cell surface of an infected cell. If such an alteration of a cell is recognized the NK cell releases pore-forming and cytotoxic substances from its granules, and the infected cell is killed (Newman, K.C. and Riley, E.M. 2007).

1.1.2 The activation of adaptive immunity

The adaptive immunity consists mainly of two cell types, B cells and T cells, which are able to adapt their defensive response specifically to pathogens. This adaption occurs by their unique receptors, which are highly variable and diverse.

B cells are responsible for the humoral immune response. They produce and secrete antibodies, which are soluble molecules that bind specifically to structures of pathogens. These immunoglobulins consist of a heavy and a light chain, linked by disulfide bonds, with hypervariable regions at the antigen binding site. The constant region of the heavy chain can be recognized by different other cells of the immune system. By their binding antibodies are able to neutralize microbial toxins and mark pathogens for uptake by macrophages. The B cell receptor (BCR) is a membrane bound form of the specific immunoglobulin that is secreted by a B cell. It binds its antigen, which is then phagocytosed and degraded by the B cell. The degradation products are presented on the B cell surface, where they can be recognized by $CD4^+$ helper T cells. If they recognize a presented foreign structure they transmit a signal to the B cell that leads to activation. The B cell starts to proliferate and secrete a specific antibody. This antibody is a soluble form of the BCR and therefore possesses the same antigen binding site (Parker, D.C. 1993). Additionally the process of affinity maturation of the BCR is started, generating an even stronger specificity for the pathogen structure (Janeway, C.A. et al.).

T cells mediate the cellular immune response. They can be separated into cytotoxic $CD8^+$ T cells, which kill infected cells, and $CD4^+$ T helper cells, which assist other cells, for example macrophages in killing phagocytosed pathogens and B cells in recognizing their specific antigen (Doherty, P.C. et al. 1997). The T cell receptor (TCR) is in its structure related to the B cell receptor, the extracellular domain, composed of α and β chain, resembles the extracellular region of the B cell receptor. But in contrast to the B cell

receptor it can only recognize its specific antigen bound to major histocompatibility complex (MHC) molecules on the surface of another cell. The coreceptor CD4 recognizes MHC-II, whereas the coreceptor CD8 recognizes MHC-I. MHC-II is only present on antigen presenting cells (APC), like B cells, dendritic cells and macrophages. The phagolysosome of these cells, containing fragments of digested pathogens, fuses with vesicles carrying MHC-II molecules. Peptides of 18-20 amino-acids in length are loaded onto membrane-bound MHC-II, which travels to the cell surface. There these peptides can be recognized by the T helper cells, which in turn activate B cells to produce antibodies, or macrophages to kill ingested pathogens. MHC-I is expressed on every nucleated cell in the body and is constantly loaded by a variety of peptides from the cytosol. In case of an infection with an intracellular pathogen products of this pathogen are present in the cytosol of the cells and their degradation products are loaded onto MHC-I. The $CD8^+$ T cell recognizes foreign peptides on MHC-I and is able to eliminate the infected cell. There are different ways of elimination. One is the exocytosis of cytotoxic molecules that were before kept in granula in the T cell. Another way is through receptor ligand pairs, which induce apoptosis, like interaction of Fas ligand on the T cell with Fas receptor on the infected cell (Janeway, C.A. et al.).

1.1.3 $CD4^+$ T cell activation and differentiation

Naïve T cells have to be activated to armed effector T cells before they can exert their function. At this point, dendritic cells and, to a lesser extent, macrophages are the connection between the innate and the adaptive immune system. The ligation of their PRR enhances not only the presentation of material from the phagosome on MHC-II but also the upregulation of costimulatory molecules on the cell surface and the secretion of cytokines. Activated dendritic cells travel into the draining lymph node where they meet naïve T cells. The binding of the TCR of the $CD4^+$ T cell to its specific antigen on MHC-II on the dendritic cell transmits the first signal of T cell activation. The second signal, which is necessary to fully activate the T cell, is given by ligation of costimulatory receptor ligand pairs. The molecules CD80 and CD86 on the APC bind the costimulatory CD28 on the T cell surface. CD80 and CD86 are upregulated on the APC surface only after recognition of PAMP by PRR. This ensures, that $CD4^+$ T cells recognizing self structures presented on MHC-II cannot be activated, as the second activation signal is missing (Banchereau, J. and Steinman, R.M. 1998).

There are different tasks for the $CD4^+$ T cells during the immune response and these vary depending on the invading pathogen. Therefore $CD4^+$ T cells differentiate during activation into one of the T helper cell lineages. This differentiation is supported by the surrounding cytokine milieu which is defined by the type of activation the APC experienced through its PRR. In addition to the shaping function of the IL-12 family cytokines, already differentiated T cells suppress the generation of other lineages by their effector cytokines. First only two lineages of differentiated $CD4^+$ T cells were identified, called T_H1 and T_H2. The cytokine IL-12 supports the generation of T_H1 cells (Hsieh, C.S. et al. 1993), which produce the signature cytokines IFNγ and TNFα (Szabo, S.J. et al. 2000). They mediate cellular immunity by activating macrophages and supporting the activation of cytotoxic $CD8^+$ T cells. T_H1 cells are of special importance for protection of the host from obligate intracellular pathogens (Gazzinelli, R.T. et al. 1994). IL-4 promotes the generation of T_H2 cells. They are defined as producers of IL-4 and IL-10 and are associated with the humoral immune response as they exert mainly B cell help (Seder, R.A. and Paul, W.E. 1994). The activated B cells secrete antibodies, which are necessary for the defense against extracellular pathogens (Svetic, A. et al. 1993). T_H 17 cells, which produce IL-17 as their main effector cytokine, are one of the newest identified subsets (Harrington, L.E. et al. 2005). They differentiate by a cytokine milieu containing IL-6, TGFβ and IL-23 (Aggarwal, S. et al. 2003; Veldhoen, M. et al. 2006). At first they were found to exert pathology in different settings of autoimmunity (Komiyama, Y. et al. 2006), but their role is assumed to be the defense against extracellular bacteria and fungi (Gaffen, S.L. et al.). A fourth set of $CD4^+$T cells are the regulatory T cells (Treg), characterized by their expression of CD25 and their secretion of IL-10 (Asseman, C. et al. 1999; Shevach, E.M. et al. 2006). Their differentiation requires TGFβ and in contrast to the other lineages they are not directly engaged in host defense but regulate other effector T cells (Yamagiwa, S. et al. 2001). Thereby they can prevent injury of the host by an overreactive T cell response. Most of the information about T cell differentiation, especially for the more recently identified subsets, originates from *in vitro* studies, though. To which extent T cells are influenced by the different aspects of their environment during infection *in vivo* has still to be extensively investigated.

1.1.4 The role of IL-12 family cytokines in T cell differentiation

These cytokines of the IL-12 family are closely related to the subject of $CD4^+$ T cell

differentiation. Until now four different IL-12 family cytokines have been discovered. They belong to the type I cytokines and are evolutionary related to IL-6. The subunits of the receptor for IL-6 are IL-6Rα and gp130, the latter is an abundant molecule and a shared component of many different receptors (Mihara, M. et al.). The IL-12 family cytokines are composed of two subunits which are noncovalently linked. The smaller subunit is similar to IL-6, whereas the larger subunit resembles a soluble form of the IL-6Rα. Hereby the subunits work like a system of building blocks, where a small subunit and a large subunit are combined to form a cytokine, and replacement of one subunit would result in a different family member. For successful secretion of the cytokines the expression of both subunits in one cell is necessary. The receptors are dimeric as well and built of one common and one unique subunit (Kastelein, R.A. et al. 2007).

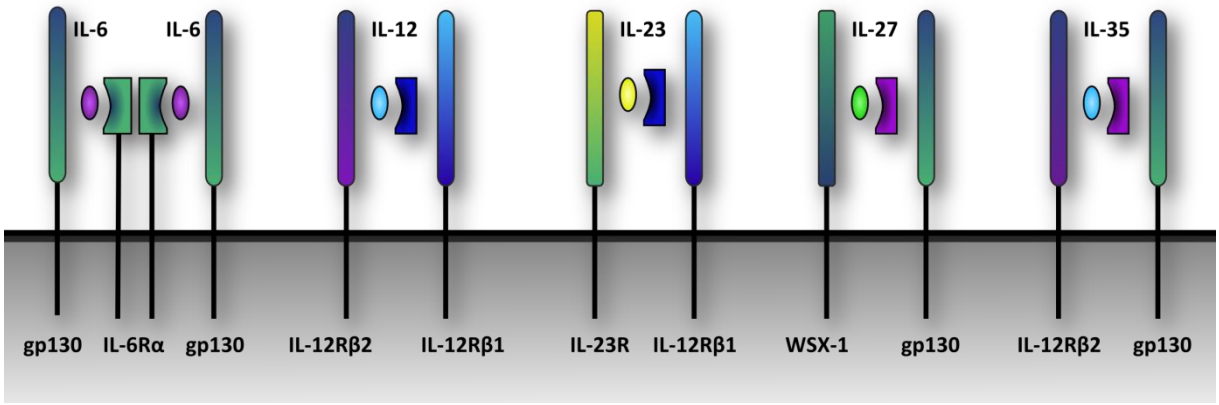

Figure 1-1: IL-6 and the IL-12 cytokine family

The IL-12 family cytokines are dimeric cytokines, composed a smaller type 1 cytokine like subunit and a larger subunit that resembles a soluble form of the IL-6Ra. The receptors are dimeric as well and built of one common and one unique subunit.

IL-12 was the first discovered cytokine of this family. Its large subunit is IL-12p40, which is shared with the cytokine IL-23. The small subunit is IL-12p35, which is also the small subunit of IL-35. Together the subunits build the 70 kDa cytokine IL-12p70 (Gately, M.K. et al. 1991). The receptor subunits are IL-12Rβ1 and IL-12Rβ2, the former is shared with the IL-23R, the latter with the receptor of IL-35. The cytokine IL-12 is expressed by APC upon their activation via PRR and is part of the cytokine mileu that leads to a T_H1 differentiation of $CD4^+$ T cells (Hsieh, C.S. et al. 1993). The importance of these cells in the immunity against obligate intracellular pathogens has been shown in infections with *L. major*, *M. tuberculosis* and different viruses (Flynn, J.L. et al. 1995;

Heinzel, F.P. et al. 1993; Wysocka, M. et al. 1995).

Some of the effects that were originally ascribed to IL-12, because of the involvement of the IL-12p40 unit, have later been shown to originate from IL-23. This cytokine results from a combination of the IL-12p40 subunit with the small subunit IL-23p19 (Oppmann, B. et al. 2000). During experimental autoimmune encephalomyelitis (EAE), the murine model of multiple sclerosis, it was first described, that neutralization of IL-12 prevents disease (Leonard, J.P. et al. 1995). Later it could be demonstrated, that IL-23p40 deficient mice are resistant, whereas IL-12p35 deficient mice are still susceptible to EAE. This demonstrated the importance of IL-23 for the generation of the pathogenic T_H17 cell subset (Becher, B. et al. 2002; Langrish, C.L. et al. 2005). IL-23, like IL-12, is produced by APC after ligation of PRR and binds on the T cell surface to the receptor dimer of the IL-23R and the IL-12Rβ1 subunit, which is shared with the IL-12R (Parham, C. et al. 2002).

The cytokines IL-12 and IL-23 are strongly proinflammatory cytokines, supporting the generation of $CD4^+$ T effector cells of the T_H1 and T_H17 lineage, respectively. IL-27, the third member of the IL-12 family, is build from different subunits as the other two: the Epstein-Barr-induced gene 3 (EBI3) is the large and IL-27p28 is the small subunit (Pflanz, S. et al. 2002). Though expressed by APC as well, IL-27 acts differently on lineage differentiation of $CD4^+$ T cells. It was demonstrated to be necessary for early T_H1 differentiation in *L. major* infection (Takeda, A. et al. 2003; Yoshida, H. et al. 2001) but suppresses T_H1 as well as T_H2 at later time points in different models (Holscher, C. et al. 2005; Miyazaki, Y. et al. 2005; Rosas, L.E. et al. 2006). Additionally, IL-27 suppresses pathologic T_H17 responses in a model of EAE (Batten, M. et al. 2006). Apart from being inevitable for early T_H1 differentiation it seems to have a regulatory function, which helps avoiding extended and pathological immune activation.

IL-35 is the most recently discovered IL-12 family cytokine. It contains, as IL-27, the large subunit EBI3 and additionally the small subunit IL-12p35, which it shares with IL-12 (Devergne, O. et al. 1997). Surprisingly, it is not produced by APC, like the other IL-12 family cytokines, but by regulatory T cells (Collison, L.W. et al. 2007). But like the other family members it targets $CD4^+$ T cells. It activates and induces regulatory T cells and suppresses the T_H17 differentiation in favor of T_H1 (Collison, L.W. et al.; Niedbala, W. et al. 2007). The receptor for IL-35 has been discovered very recently. It combines the

IL-12Rβ2 subunit, which is part of the IL-12 receptor, as well, with gp130, which is shared with the IL-27 receptor and other cytokine receptors apart from the IL-12 family (Collison, L.W. et al. 2012).

1.1.5 IL-17

T_H17 cells are directly influenced by IL-12 family cytokines. They are activated by IL-23 and regulated by IL-27 and even more by IL-35. Their roles in autoimmunity as well as in the immune response against extracellular bacteria and fungi have been mainly ascribed to their effector cytokine IL-17.

Two members of the IL-17 family are expressed by T_H17 cells, IL17A and IL-17F (Harrington, L.E. et al. 2005). These two cytokines are the closest relatives of the IL-17 family with a sequence identity of about 50 %. The receptor for IL-17A is the IL-17RA, which can be ligated by IL-17F as well, though with reduced affinity. The high affinity receptor for IL-17F is IL-17RC (Shen, F. and Gaffen, S.L. 2008). In this thesis the use of the term IL-17 always refers to IL-17A as it has been described as the most inflammation-related cytokine of the family. It is able to trigger innate immune mechanisms by activating the secretion of proinflammatory chemokines and cytokines, antimicrobial peptides and acute-phase proteins (Iwakura, Y. et al. 2008). Additionally, it promotes granulopoeisis, leading to neutrophil mobilization (Fossiez, F. et al. 1996; Laan, M. et al. 1999).

The production of IL-17 by T_H17 cells links the adaptive immune response to the activation of innate mechanisms. Though T cells are the most common source of IL-17, it can be produced by other cells, as well, like NK cells. Furthermore, unconventional T cells are prominent producers of IL-17. NK T cells and γδ T cells both express a TCR but are rather part of the innate immune compartment than of the adaptive immunity (Cua, D.J. and Tato, C.M.).

1.1.6 γδ T cells - an innate T cell population

The TCR of conventional T cells is composed of an α chain and a β chain which undergo rearrangement during development of T cells. This rearrangement creates the diversity of TCRs and their ability to recognize many different structures of pathogens. Additionally to these αβ T cells unconventional T cells have been discovered. Two examples are NK T cells and γδ T cells. NK T cells express an invariant TCR that

recognizes structures bound to the MHC-related surface molecule CD1d. Additionally, they express surface receptors that were before described to characterize NK cells, like NK1.1 (MacDonald, H.R. 2002). γδ T cells develop earlier than αβ T cells during ontogeny from the thymus. They combine a γ chain and a δ chain in their T cell receptor (Raulet, D.H. 1989). Although the combination of their TCR chains would allow a high variety, the repertoire of γδ TCR is limited. They have been shown to account only for a very small part of the T cells in lymphoid tissues, like spleen, lymph nodes and blood. But they are far more abundant in peripheral tissues, especially in epithelia. Their TCR is able to bind ligands presented on MHC and MHC-related surface molecules, but some ligands are even recognized without MHC presentation (Bonneville, M. et al.). Several studies even show activation of γδ T cells without involvement of the TCR. The TCR independent activation can occur via PRR like TLR and NOD-like receptors (Martin, B. et al. 2009). The ability of a pool of preexistent γδ T cells to respond to danger signals without the need for costimulation and clonal expansion locates them at the borders between innate and adaptive immunity. The activated γδ T cells exert T cell responses until the specific adaptive immune response is fully mounted. They can mediate cytotoxicity, promote B cell responses and produce T cell cytokines. In some of these functions the γδ T cells resemble very different αβ T cell subsets (Qin, G. et al. 2009; Zheng, B. et al. 2003). Like $CD4^+$ αβ T cells they have been shown to frequently express the T_H1 cytokine IFNγ or the T_H17 cytokine IL-17, contributing to the activation of other effectors. In this regard, several studies have demonstrated that it is possible to distinguish between subsets of γδ T cells that produce IFNγ and those that produce IL-17. IFNγ producing γδ T cells express the surface molecule CD27, which they need for survival. IL-17 producing γδ T cells do not express CD27 but CCR6, which is not found on the IFNγ expressing subset (Haas, J.D. et al. 2009; Ribot, J.C. et al. 2009).

1.2 Malaria

Malaria is still one of the three most dangerous infectious diseases worldwide, together with tuberculosis and HIV. In 2010 the WHO estimated 216 million cases and 660,000 deaths; 86% of the deaths were children under 5 years of age. The disease is caused by intracellular protozoan parasites of the *Plasmodium spp*. In humans five different parasites are known. *Plasmodium falciparum* is the most dangerous of them and caused 91% of the malaria cases in 2010 (WHO 2011).

The life cycle of the parasite is divided into two stages that are located in different hosts. Sporozoites are transmitted into the blood of the human host with the saliva during the blood meal of an infected mosquito of the *Anopheles spp*. In humans the infection divides again into two stages. During the liver stage the sporozoites travel through the blood into the liver and infect hepatocytes. Within seven days several thousand merozoites develop in one infected hepatocyte. They are released into the blood stream and start the blood stage. During blood stage the merozoites infect erythrocytes. After about 48 hours 16 to 32 daughter merozoites develop in one erythrocyte. They are released and infect new erythrocytes. Some of the parasites within erythrocytes differentiate to sexual stages, gametocytes. They can be taken up during the bite of a *Anopheles* mosquito and start the sexual cycle within the mosquito (Hafalla, J.C. et al.).

1.2.1 Malaria blood stage: immune response and pathology

Because of the high replication rate only very few parasites are necessary to complete the liver stage and establish a patent infection. The brief encounter of immune cells with parasites, before they invade into hepatocytes, is not sufficient to mount an effective immune response. Later, during blood stage, the distribution of the parasite throughout the blood leads to a strong activation of the immune response. This is characterized by high amounts of IL-12, which activate NK cells and support the development of T_H1 cells among the $CD4^+$ T cells. Their production of IFNγ, together with the IFNγ produced by the T_H1 supported $CD8^+$ T cells, activates for example macrophages to kill ingested parasites and parasitized red blood cells (pRBC). Development of antibodies against the erythrocytic stages is beneficial but not sufficient, as the parasite possesses a huge family of variable surface antigens. The ability to switch between the expression of different genes enables the parasite to evade humoral immune responses (Hafalla, J.C. et al.).

But the strong activation of the immune system during blood stage can cause severe pathologies, which are known to often accompany the infection with *P. falciparum* in humans. Special to *P. falciparum* in contrast to other malaria parasites is the extended modification of the erythrocyte surface. Parasite derived molecules in knob like structures on the surface of the pRBC mediate adhesion to endothelia, a process called sequestration. This enables the parasite to stay in the smaller vessels in the tissue and avoid clearance during a passage through the spleen (Beeson, J.G. and Brown, G.V. 2002).

The high concentration of proinflammatory cytokines in the blood can lead to suppression of hematopoiesis in the bone marrow, leading to slow replacement of destroyed erythrocytes. Together with pRBC many uninfected RBC are cleared from the periphery. These effects might lead to the pathology of severe anaemia (Chang, K.H. and Stevenson, M.M. 2004). Another pathology, which is found particularly in children in endemic areas, is the cerebral malaria (CM). CM is characterized by a massive inflammation in the otherwise immuneprivileged area of the brain, followed by cerebral symptoms ranging from neuronal dysfunction to coma and death (Grau, G.E. et al. 1989). In the small vessels of the brain parasites sequester to activated endothelial cells and are soon accompanied by $CD4^+$ and $CD8^+$ T cells (Baptista, F.G. et al.; McQuillan, J.A. et al.). The high concentration of cytokines and inflammatory cells leads to an impairment of the blood-brain-barrier (Grab, D.J. et al.). It is still discussed, if the endothelial cells are activated by sequestering pRBC or if a previous activation of endothelia by inflammatory mediators is necessary to facilitate sequestration (van der Heyde, H.C. et al. 2006). Furthermore, most of the evidence suggests, that neither pRBC nor T cells actually leave the vessels and migrate into the brain parenchyma (Lacerda-Queiroz, N. et al.; Medana, I.M. and Turner, G.D. 2006).

1.2.2 The mouse model of experimental cerebral malaria (ECM)

The infection of C57Bl/6 mice with the murine pathogen *Plasmodium berghei* ANKA is a widely used model for cerebral malaria in humans. The mice develop only a low parasitaemia, but cerebral symptoms between days 7 and 10 after infection, resulting in death (de Souza, J.B. et al.). The immune system is not able to control the parasitaemia, in the surviving mice the parasitaemia rises slowly and the infection cannot be cleared. In this mouse model the pathologic inflammation is promoted by IFNγ producing $CD8^+$ T cells that adhere to the brain endothelium. They are supported by a T_H1 polarized $CD4^+$ T cell response and mice deficient for IFNγ signaling are protected against ECM (Amani, V. et al. 2000). Depletion of either $CD4^+$ or $CD8^+$ T cells prevents cerebral malaria, as well (Hermsen, C. et al. 1997). It has been very recently demonstrated, that a lack of IL-17 does not alter the pathology, excluding a contribution of T_H17 cells (Ishida, H. et al.).

1.3 Aim

The experimental cerebral malaria (ECM) is the mouse model for the pathology of cerebral malaria as it occurs in humans infected with *Plasmodium falciparum*. In this model the involvement of activated $CD4^+$ T cells is believed to contribute to pathology. Mice deficient for signaling of IFNγ, the effector cytokine of T_H1, are protected from cerebral malaria, whereas mice deficient for IL-17, the T_H17 effector cytokine are susceptible. IL-12 family cytokines are involved in shaping the direction of $CD4^+$ T cell polarization. The APC derived cytokine IL-12 activates the T_H1 response whereas IL-23 activates the T_H17 response. IL-35, a regulatory cytokine produced by regulatory T cells, activates further regulatory T cells and suppresses IL-17 release.

The aim of the thesis is to clarify further if the interplay of the IL-12 family cytokines IL-35 and IL-23 is able to influence the mainly IL-12 driven proinflammatory immune response during ECM.

The role of IL-35 *in vivo* could be investigated by the application of recombinant IL-35 during infection. A knock-out of the cytokine is not applicable, as the subunits are shared with other IL-12 family cytokines. Knock-out of the EBI3 gene would create a deficiency for IL-27 as well; knock-out of p35 a deficiency of IL-12. A specific antibody against IL-35 is not commercially available, nor is purified recombinant IL-35. Therefore a recombinant fusion molecule of murine single chain IL-35 with a human IgG1 should be constructed and purified. The molecule should be tested for activity *in vitro* and applied in *P. berghei* ANKA infection *in vivo*. Hereby, the influence of the induction of regulatory mechanisms and the regulation of the T_H17 polarisation could be analyzed.

To investigate the influence of IL-23 on the $CD4^+$ T cell polarization during ECM, IL-17 deficient mice were analyzed and compared to IL-12p35 deficient mice, which lack the influence of IL-12, during infection. In further experiments the relation between IL-23 release by APC, the activation of IL-17 and the positive influence of IL-17 on anti-parasite immunity should be investigated.

2 Material

2.1 Laboratory equipment

Agarose gel electrophoresis system	*PeqLab Biotechnology, Erlangen*
Analytical scales	*Sartorius AG, Göttingen*
Benchtop rotator 'Duomax 2030'	*Heidolph Instruments, Schwalbach*
Benchtop microcentrifuge	*Eppendorf, Hamburg*
Benchtop microcentrifuge	*Eppendorf, Hamburg*
Camera for DNA gel pictures	*Kaiser Fototechnik, Berlin*
Centrifuge 'Megafuge 1.0 R'	*Heraeus Instruments, Hanau*
CO_2 incubator	*Thermo Scientific, Waltham, USA*
Class II biological safety cabinet 'LaminAir HB 2448'	*Heraeus Instruments, Hanau*
ELISA reader 'MRX II'	*Dynex Technologies, Berlin*
Flow cytometer 'FACS Calibur'	*BD Bioscience, Heidelberg*
Flow cytometer 'Accuri C6'	*BD Bioscience, Heidelberg*
Incubated shaker	*B. Braunb Biotech International, Meisungen*
Inverse microscope 'Nikon TMS'	*Nikon Instruments, Badhoeverdorp, Netherlands*
IVC 'Blue Line IVC Sealsafe'	*Tecniplast, Valencia, Italy*
Microbial incubator	*Heraeus Instruments, Hanau*
Microscope 'Olympus CK2'	*Olympus, Hamburg*
Microscope 'Axiostar plus'	*Zeiss, Oberkochen*
Microwave	*Panasonic, Wiesbaden*
Nano-drop photometer 2000 c	*Thermo Scientific, Waltham, USA*
Pestle for reaction tubes (1.5 and 2.0 mL)	*Eppendorf, Hamburg*
pH meter HI 221	*Hanna Instruments, Kehl am Rhein*
Pipettes	*Gilson, Middleton, USA*
Pipette controller 'Swiftpet+'	*Abimed, Langenfeld*
Photgraphic processing apparatus "Curix 60"	*Agfa, Cologne*

Power supply for agarose gel elektrophoresis	*BioRad, Munich*
Power supply for PAA gel electrophoresis	*Consort, Turnhout, Belgium*
Power supply for western blot	*Biotec-Fischer, Reiskirchen*
PAA gel electrophoresis system	*peqLab Biotechnology, Erlangen*
Quantitative real time PCR Cycler 'Rotor Gene'	*Qiagen, Hilden*
Shaker 'Thermomixer Comfort'	*Eppendorf, Hamburg*
Thermocycler Px2	*Thermo Scientific, Waltham, USA*
Vortex 'MS1 Minishaker'	*IKA Labortechnik, Staufen*

2.2 Glass- and plasticware

10 mL plastic column	*Mo Bi Tec, Goettingen*
Cell culture plates (6-, 24- and 96-well)	*Greiner bio-one, Frickenhausen*
Cell strainer 'Falcon'	*BD Biosciences, Bedford, USA*
Centrifuge tubes (15 and 50 mL)	*Greiner bio-one, Frickenhausen*
Cryotubes (1.8 mL)	*Nunc, Roskilde, Danmark*
Dialysis tube	*Roth, Darmstadt*
Disposable cuvette 'UVette'	*Eppendorf, Hamburg*
Disposable chirugical scalpel	*Braun, Melsungen*
Disposable pipette (5, 10 and 25 mL)	*BD Bioscience, Heidelberg*
DuoSet ELISA for IFNγ and IL-17	*R&D Systems, Wiesbaden*
Reaction Tubes (0.5, 1.5 and 2.0 mL)	*Eppendorf, Hamburg and Sarstedt, Nümbrecht*
ELISA Microtiterplate 'microlon'	*Greiner bio-one, Frickenhausen*
FACS Tubes	*Sarstedt, Nümbrecht*
Glass Pipettes (5, 10 and 20 mL)	*Brand, Wertheim*
Neubauer Counting Chamber (0.1 mm x 0.0025 mm^2)	*Hecht-Assisten, Sondheim*
Needle (27G x 3/4")	*Braun, Melsungen*
Petri Dish	*Sarstedt, Nümbrecht*
Pipet Tips	*Sarstedt, Nümbrecht*
Syringes (1, 5 and 10 mL)	*Braun, Melsungen*
Sterile Filters 0.2 µm	*Sarstedt, Nümbrecht*

2.3 Chemicals

All chemical substances were, unless stated otherwise, obtained from the companies *Merck (Darmstadt)*, *Roth (Karlsruhe)* and *Sigma-Aldrich (Deisenhofen)*.

2.4 Material for molecular biology

2.4.1 Olginucleotide Primer

All primers used for PCR were obtained from MWG Biotech (Ebersberg). Oligo(dT)$_{18}$ primer for reverse transcription was obtained from Fermentas (St Leon-Roth). Recognition sequences for restriction enzymes are underlined.

Primer	Sequence	Restriction enzyme
Oligo(dT)$_{18}$	TTTTTTTTTTTTTTTTTT	
ebi3 fw	CCCCGAATTCCACTGAAACAGCTCTCGTGGCT	*Eco*RI
ebi3 rev	TCCATGGAGAACCACCACCACCAGAACCACCAC CACCAGAACCACCACCACCCTTATGGGGTGCAC TTTCTACTTGCCCA	*Nco*I
p35 fw	GGCCCCATGGATTCCAGTCTCTGGACCTGCCA	*Nco*I
p35 rev	CGGAGATCTCAGATAGCCCATCACCCTGTTGA	*Bgl*II
qPCR RPS9 fw	CCGCCTTGTCTCTCTTTGTC	
qPCR RPS9 rev	CCGGAGTCCATACTCTCCAA	
qPCR IL-23p19 fw	GCCAAGGTCTGGCTTTTTAT	
qPCR IL-23 rev	AGGGAGGTGTGAAGTTGCTC	

2.4.2 Plasmids

pDrive	*Qiagen, Hilden*
pFuse-hIgG1-Fc2	*Invivogen, San Diego, USA*

2.4.3 Enzymes

Unless stated otherwise all enzymes were used with the buffer according to the protocol included by the manufacturer.

Restriction enzymes

Enzyme	Recognition sequence	Manufacturer
*Bgl*II	5'-AGATCT-3'	*MBI Fermentas, St Leon-Roth*
*Eco*RI	5'-GAATCC-3'	*MBI Fermentas, St Leon-Roth*
*Nco*I	5'-CCATGG-3'	*MBI Fermentas, St Leon-Roth*

Other enzymes

'Revert Aid' reverse transcriptase	*MBI Fermentas, St Leon-Roth*
T4 DNA ligase	*MBI Fermentas, St Leon-Roth*

2.4.4 Reagents

Agarose	*Biomol, Hamburg*
Ampuwa H2O	*Fresenius, Graz, Austria*
Ampicilline	*Sigma, Deisenhofen*
dNTPs	*MBI Fermentas, St Leon-Roth*
Dithiothreitol (DTT)	*Gibco BRL, Eggesheim*
DNA gel extraction kit 'QIAquick'	*Qiagen, Hilden*
DNA standard '100 bp DNA Ladder plus'	*MBI Fermentas, St Leon-Roth*
Ethidiumbromide	*Sigma, Deisenhofen*
LB-agar	*Sigma, Deisenhofen*
LB-broth	*Sigma, Deisenhofen*
Maxima™ SYBR Green qPCR Master Mix	*MBI Fermentas, St Leon-Roth*
Nucleo Spin Plasmid Kit 'Mini'	*Macherey & Nagel*
PCR Cloning Kit	*Qiagen, Hilden*
Plasmid Kit Plus 'Maxi'	*Qiagen, Hilden*
TaqMan Gene Expression Assay for IL-23p19	*Applied Biosystems, Carlsbad, USA*
TaqMan Gene Expression Assay Master Mix	*Applied Biosystems, Carlsbad, USA*
trizol	*Invitrogen*
X-Gal	*MBI Fermentas, St Leon-Roth*
Zeocin	*Invivogen, San Diego, USA*

2.4.5 Culture Media, Solutions and Buffers

Ampicillin

25 mg/mL	in H_20

Followed by sterile filtration

dNTP Mix

2 mM each	dATP, dGTP, dCTP, dTTP

5 x KCM

0.5 M	Potassium chloride
0.15 M	Calcium chloride
0.25 M	Magnesium chloride

Ad 30 mL H2O

Followed by sterile filtration

LB-Agar Plates

35 g LB-Agar ad 1 L with ddH2O

Followed by sterilization in an autoclave. Addition of desired antibiotics (ampicillin or Zeocin) after cooling to less than 55°C. 20 mL were poured per petri dish and the dishes were dried at room temperature.

LB Broth

20 g LB-Broth ad 1 L with ddH2O

Followed by sterilization in an autoclave

TBE Buffer

0.89 M	Tris-base
0.89 M	Boric acid (H_3BO_3)
20 mM	EDTA
pH 8.0	

1 M MES

19.5 g	Morpholino ethane sulfonic acid

In 100 mL H_2O, sterile filtered, store at -20 °C

1 M MOPS

11.51 g	3-(N-morpholino) propane sulfonic acid

In 50 mL H_2O, sterile filtered, store at 4 °C

TFB1

5 mL	1 M MES
6.045 g	$RbCl_2$
0.735 g	$CaCl_2$ x $2H_2O$
4.94 g	$MnCl_2$ x $4H_2O$

H_20 ad 500 mL, pH 5.8, sterile filtered, store at 4 °C

TFB2

1 mL	1 M MOPS
1.102 g	$CaCl_2$ x $2H_2O$
0.120 g	$RbCl_2$
15 mL	Glycerol

H_2O ad 100 mL, pH 6.5, sterile filtered, store at 4 °C

X-Gal Stem Solution

20 mg/mL	5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-Gal)
	in dimethylformamide

2.5 Material for Biochemistry

2.5.1 Reagents

Bovine serum albumine (BSA)	*Serva Fenbiochemika, Heidelberg*
Bradford test solution 'Coomassie Plus'	*Pierce Biotechnology, Rockford, USA*
Bromophenol blue	*Sigma, Deisenhofen*
ECL detection solution	*Amersham Pharmacia, Freiburg*
Milk powder	*Roth, Karlsruhe*
PVDF membrane 'Immobilon-FL'	*Millipore, Billerica, USA*
protein G sepharose '4 Fast Flow'	*GE Healthcare, Uppsala, Sweden*
Protein gel standard 'PAGE Ruler Unstained Protein Ladder	*MBI Fermentas, St. Leon-Roth*
Protein gel standard 'PAGE Ruler Prestained Protein Ladder	*MBI Fermentas, St. Leon-Roth*
Tween 20	*Sigma, Deisenhofen*
Whatman Paper	*Schleicher& Schuell, Dassel*
X-ray Film	*Agfa, Cologne*

2.5.2 Solutions and buffers

Affinity chromatography: elution buffer

0.1 M	Glycine
pH 2.7	

ELISA blocking solution

1 %	BSA
in PBS	

ELISA substrate buffer

100 mM	Sodiumhydrogen-phosphate
pH 5.5	

ELISA substrate solution

200 µL	TMB stem solution
1.2 µL	H_2O_2
12 mL	ELISA substrate buffer

ELISA stop solution

2 M	Sulphuric acid (H_2SO_4)

ELISA TMB stem solution

30 mg	Tetramethylbenzidine
5 mL	DMSO

SDS PAGE 10 % APS

100 mg	Ammonium persulfate
1 mL	H_2O

SDS PAGE 4x lower

18 g	Tris base
4 mL	10 % SDS
Ad 100 mL H_2O, pH 8.8	

SDS PAGE 4x upper

6.06 g	Tris base
4 mL	10 % SDS
Ad 100 mL H_2O, pH 6.8	

SDS PAGE seperating gel

2.5 mL	4x lower
3.5 mL	30 % Acrylamide
4 mL	H_2O
100 µL	10 % APS
12.5 µL	TEMED

SDS PAGE stacking gel

1.25 mL	4x upper
0.8 mL	30 % Acrylamide
3 mL	H_2O
50 µL	10 % APS
5 µL	TEMED

SDS PAGE 5x loading dye

50 mM	Tris
2 %	SDS
5 %	Glycerine
10 %	DTT
Several Drops	bromophenol blue

SDS PAGE 10x running buffer

31 g	Tris base
140 g	Glycine
10 g	SDS
Ad 1 L H_2O	

Silver stain developing solution

6 g	Natriumcarbonate (NA_2CO_3)
5 µL	Natriumthisulfate stem solution
50 µL	Formaldehyde
120 mL	ddH_2O

Silver stain fixing solution

50 mL	Methanol
12 mL	Glacial acetic acid
38 mL	ddH_20

Silver stain silver nitrate solution

200 mg	Silver nitrate ($AgNO_3$)
75 µL	Formaldehyde (37 %)
100 mL	dd H_2O

Silver stain natriumthisulfate stem solution

430 mg	Natriumthiosulfate-pentahydrate
5 mL	dd H_2O

Western blot blocking solution

4 %	Milk powder
in PBS	

Western blot CAPS buffer

2.213 g	CAPS (3-(cyclohexamine)-1-propane-sulfonic acid
ad 100 mL dd H_20, pH 11	
100 mL	Methanol
800mL	dd H_20

2.6 Material for cell biology

2.6.1 Reagents

Dimethylsulfoxide (DMSO)	*Sigma, Deisenhofen*
Fetal calf serum (FCS)	*PAA, Pasching, Austria*
Gentamicin 200mM	*PAA, Pasching, Austria*
Golgi-Stop	*BD Bioscience, Heidelberg*
GMCSF containing supernatant of transfected AG 8653 myeloma cells	*BNI, Hamburg*
L-glutamine 10 mg/mL	*PAA, Pasching, Austria*

MACS Pan T cell purification Kit	*Miltenyi, Bergisch Gladbach*
Nycoprep	*Progen, Heidelberg*
RPMI 1640 (without L-glutamine)	*PAA, Pasching, Austria*
Transfection reagent 'TurboFect'	*MBI Fermentas, St. Leon-Roth*
Trypane blue	*Sigma, Deisenhofen*
Trypsine-EDTA	*PAA, Pasching, Austria*
Fixing solution 'BD Cytofix/Cytoperm'	*BD Bioscience, Heidelberg*
Permeabilising solution 'BD Perm/Wash'	*BD Bioscience, Heidelberg*

2.6.2 Culture Media, Solutions and Buffers

Freeze solution (RPMI)

10 %	FCS
80 %	RPMI 1640
10 %	DMSO

Red blood cell (RBC) lysis buffer

10 %	0.1 M tris-HCl (pH 7.5)
90 %	0.16 M ammoniumchloride

Cell culture medium

500 mL	RPMI 1640
50 mL	FCS
5 mL	L-glutamine
2.5 mL	Gentamycin

10x Phosphate buffered saline (PBS)

80 g	Sodiumchloride (NaCl)
2 g	Potassiumchloride (KCl)
14.4 g	Disodiumhydrogen-phosphate (NA_2HPO_4)
2.4 g	Potassiumdihydrogen-phosphate (KH_2PO_4)
ad 1 L H_2O,	

FACS buffer

1 %	FCS
0.1 %	Natriumazide (NaN_2)
In PBS	

2.7 Antibodies

Antibody	Clone	Manufacturer
Anti human IgG1, PE labeled	polyclonal	*Jackson, Bar Harbor, USA*
Anti human-IgG1, HRP labeled	polyclonal	*DAKO, Glostrup, Denmark*
Anti mouse CD3, PE labeled	145-2C11	*Biolegend, San Diego, USA*
Anti mouse CD3,purified	145-2C11	*BNI, Hamburg*
Anti mouse CD4, PE labeled	GK1.5	*BD Bioscience, Heidelberg*
Anti mouse CD4,PerCP labeled	GK1.5	*Biolegend, San Diego, USA*
Anti mouse CD8, PerCP labeled	53-6.7	*Biolegend, San Diego, USA*
Anti mouse CD11b, FITC labeled	M1/70.15	*Caltag, Buckingham, UK*
Anti mouse CD27, PE labeled	C6.7F9	*eBioscience, San Diego, USA*
Anti mouse EBI3, purified	355022	*R&D Systems, Wiesbaden*
Anti mouse γδ TCR, FITC labeled	GL3	*Biolegend, San Diego, USA*
Anti mouse γδ TCR, purified	GL3	*BNI, Hamburg*
Anti mouse GR-1, PE labeled	RB6-8C5	*eBioscience, San Diego, USA*
Anti mouse GR-1, purified		*BNI, Hamburg*
Anti mouse IL-17A, APC labeled	17B7	*eBioscience, San Diego, USA*
Anti mouse IL-17F, PE labeled	18F10	*eBioscience, San Diego, USA*
Anti mouse Rat-IgG, HRP labeled	polyclonal	*Jackson, Bar Harbor, USA*

2.8 Bacteria

E. coli DH5αF'	*BNI, Hamburg*
E.coli Top10	*BNI, Hamburg*

2.9 Eucaryotic cell lines

Chinese hamster ovary cells (CHO)	*BNI, Hamburg*

2.10 Parasites

Plasmodium berghei ANKA	BNI, Hamburg

2.11 Mice

Strain	Origin	Abbreviaton used in the thesis
C57Bl/6	*Charles River*	WT
C57Bl/6 p19KO	*FZ Borstel*	IL-23p19$^{-/-}$
C57Bl/6 p35KO	*FZ Borstel*	IL-12p40$^{-/-}$
IL-17KO	*FZ Borstel*	IL-17$^{-/-}$
IL-17AF-KO	*UKE Hamburg*	IL-17A$^{-/-}$IL-17F$^{-/-}$
TLR2KO	*FZ Borstel*	TLR2$^{-/-}$

3 Methods

3.1 Moleucular biology

3.1.1 Isolation of mRNA

For isolation of mRNA from tissue, tissue samples were taken into 1 mL Trizol reagent (*Invitrogen*) and homogenized by grinding with a pestle for Eppendorf tubes. The samples were centrifuged at 10,000 g and 4 °C for 10 min to clear the lysate from cell debris. From the supernatant mRNA was isolated by phenol-chloroform extraction and subsequent ethanol precipitation according to the manufacturer's protocol.

For isolation of mRNA from cultured BMDC, 1x 10^5 BMDC were taken and lysed with 1 mL Trizol reagent (*Invitrogen*). Afterwards mRNA was isolated by phenol-chloroform extraction and subsequent ethanol precipitation according to the manufacturer's protocol.

3.1.2 Reverse transcription of mRNA into cDNA

Retroviral reverse transcriptases are able to use mRNA as matrix for production of complemental cDNA. As primer thymidine oligonucleotides are used, which are able to bind to the polyadenylated tail of mRNA. 1 µg of mRNA was incubated for 5 min at 65 °C together with 100 pmol of oligo$(dT)_{18}$ primer in H_2O in a total volume of 12.5 µL. Afterwards the samples were chilled on ice and 4 µL of 5x reaction buffer, 2 µL of 10 mM dNTPs and 1 µL of reverse transcriptase were added. The samples were incubated for 60 min at 42 °C. The reaction was terminated by heating at 70°C for 10 min.

3.1.3 Polymerase chain reaction (PCR)

PCR is a method to specifically amplify DNA sequences. The thermostable *Taq*-DNA-polymerase of *Thermophilus aquaticus* is used for amplification. As startpoints for the reaction oligonucleotide primers are used that bind specifically before and after the target sequence. These primers can be used to introduce recognition sites for restriction enzymes into the sequence. The amplification is accomplished in three steps. In the first step the DNA is heated to 95 °C to separate the strands, which serve as matrix for the amplification. This is followed by cooling down to permit the hybridization of the

primers to their complementary sequence. Hereby the annealing temperature is specific for each primer. Starting at the primer the polymerase now elongates the new DNA in 5'-3' direction at 72 °C. After each amplification cycle the copy number of the target sequence is doubled. As the PCR is a very sensitive method a control sample without template is always included. The reaction mixture contains:

50-500 ng	Template DNA
2.5 µL	Forward primer (10 µM)
2.5 µL	Reverse primer (10 µM)
2.5 µL	10x *Taq* buffer
2.5 µL	$MgCl_2$ (25 mM)
2.5 µL	dNTP Mix (2 mM)
0.2 µL	*Taq*-DNA-Polymerase
H_2O ad 25 µL	

The PCR was accomplished in 0.2 mL reaction tubes. The following PCR program was adjusted to the primers by modification of annealing temperature.

Initial melting	95 °C	180 sec	
Melting	95 °C	30 sec	40 cycles
Annealing	50-70 °C	60 sec	
Elongation	72 °C	60 sec	
Completing of incomplete amplificates	72 °C	600 sec	
Cooling	4 °C	∞	

3.1.4 Quantitative realtime PCR (qPCR)

To quantify gene expression levels qPCR can be used to analyse the amounts of a specific mRNA in comparison to the mRNA of a housekeeping gene, which is expressed constantly and stabily (housekeeping gene). In this thesis two different approaches of qPCR were used to analyse the expression of IL-23p19 mRNA in comparison to expression of mRNA of RPS9 as a housekeeping gene. Therefore the mRNA was previously transcribed into cDNA using reverse transcription.

IL-23p19 mRNA expression in stimulated BMDC was analyzed using a Taqman gene expression assay (*Applied Biosystems*). Hereby a probe, localized to span between two

exons, is labeled with a fluorophor and a quencher in close proximity. It anneals to the DNA together with the primers and is degraded during elongation by exonuclease activity of the polymerase. Thereby fluorophor and quencher are separated and the signal of the fluorophor can be detected after every cycle. As there is exactly one fluorophor released for each amplified strand this method is very specific and sensitive. The following reaction mixture and qPCR program were used:

Reaction mix		**qPCR program**		
5 µl	2x Taqman Master Mix	50 °C	2 min	
0.5 µl	Gene Expression Assay	95 °C	10 min	
4.5 µL	cDNA (10 ng/mL in H_20)	95 °C	15 sec	40x
		60 °C	60 sec	

Expression of IL-23p19 mRNA in tissue of infected mice was analyzed using a Sybrgreen based qPCR. The fluorescent dye Sybrgreen is only detectable when intercalated into double stranded DNA. After every PCR cycle the increase of fluorescence intensity indicates the newly amplified strands. After the PCR the melting curve is analyzed, whereby the specificity of the PCR reaction is indicated by decrease of fluorescence at the specific melting temperature of the amplificate. The following reaction mixture and qPCR program were used:

Reaction mix		**qPCR program**		
5 µl	2x Sybr Green Master Mix	95 °C	10 min	
0.3 µl	Primer for (10 pmol/µl)	94 °C	30 sec	35x
0.3 µl	Primer rev (10 pmol/µl)	64 °C	40 sec	
50 ng	cDNA (1 µL)	Touchdown of 1 °C / cycle for 6 cycles		
4 µl	H_2O			
		72 °C	30 sec	
		95 °C	1 min	
		Melting curve analysis from67 °C to 95 °C		
		40 °C	20 sec	

Relative expression was determined using the ΔΔCt method.

3.1.5 Agarose gel electrophoresis

Agarose gel electrophoresis is a method to separate DNA molecules according to their size. Therefore they are exposed to an electric field and the distance they covered during exposure is in reverse proportion to the common logarithm of their size. In this thesis all gels have a concentration of 1 % agarose dissolved in TBE buffer. For detection of the DNA 0.2 µg/mL ethidiumbromide were added to the agarose gel, a fluorescent dye that can be detected under UV light after intercalating into DNA. As standard for fragment size the 100 bp DNA ladder plus and the 1 kb DNA ladder were used (*Fermentas*).

3.1.6 Purification of DNA from agarose gels

For Purification of DNA from agarose gels the desired fragment was cut out of the gel using a scalpel under UV light. Purification of the DNA was accomplished using the 'QIAquick' DNA gel extraction kit (*QIAGEN*).

3.1.7 Enzymatic DNA manipulation (restriction, ligation, cloning)

Type II endonucleases recognize specific palindromic DNA sequences, where they bind and cut the DNA. Some of them leave overhanging bases ("sticky ends"), which can be used to connect DNA Fragments. In this thesis 1 to 3 µg DNA were cut using 1 U 'Fast Digest' restriction enzyme (*Fermentas*) according to the manufacturer's protocol.

The ligase from the bacteriophage T4 uses ATP to bind a free 5' phosphate residue to a free 3' hydroxyl residue of a neighboring fragment. For cloning of PCR products the 'PCR Cloning Kit' (QIAGEN) was used. Inclosed is the cloning vector pDrive, which has a PCR cloning site flanked with overhanging uridine. The overhanging adenine, created by the terminal transferase activity of the Taq-Polymerase at the end of a PCR product, can anneal there. The successful cloning was analysed by restriction of the vector and subsequent agarose gel analysis as well as by sequencing of the insert using the primers 'M13 uni -21' and 'M13 rev -29'. Cloning of fragments into the expression vector was achieved using restriction sites that were introduced during PCR. The target sequence was excised from the cloning vector using specific restriction enzymes. The same enzymes were used to open the expression vector. Ligation was accomplished at 4 °C overnight, using following the mixture:

7 µL	fragment DNA
1 µL	vector DNA
1 µL	10x DNA ligase buffer
1 µL	T4 DNA ligase

3.1.8 Competent bacteria, transformation and selection

To obtain bacteria that can be transformed by heatshock a 10 mL culture was incubated overnight at 37 °C in a shaker. 1 mL of this culture was inoculated into 40 mL prewarmed LB medium and shaken at 37 °C until the OD at 550 nm of 0.3. 10 mL of the second culture were inoculated into 200 mL prewarmed LB medium and shaken for approx. 2 hours, until an OD at 550 nm of 0.45. The cells were collected and cooled on ice for 15 min. Afterwards they were centrifuged for 15 min at 2500 rpm and 4 °C. The cell pellet was resuspended in 2 mL TFB1 and the volume was afterwards adjusted to 20 mL with TFB2. After incubation of the suspension for 15 min on ice the cells were centrifuged for 15 min at 2500 rpm and 4 °C. The pellet was resuspended in 2 mL TFB2 and incubated on ice for 15 min. 200 µL aliquots were made and stored at -70 °C.

For transformation the bacteria were thawed on ice, and, after addition of DNA, incubated on ice for 30 to 45 min. Heatshock was accomplished at 42 °C for 90 sec, afterwards the cells were cooled on ice. 1 mL LB medium was added and the cells were incubated for 1 hour at 37 °C. The cells were plated on LB-Agar and incubated overnight at 37 °C.

Selection of bacteria, which were successfully transformed with the cloning vector, was accomplished by addition of ampicillin and X-Gal to the culture plates. Only transformed bacteria are able to grow on these plates due to an ampicillin resistance encoded on the vector. Successful insertion of the PCR product disrupts a *lacZ'*-gene encoded on the vector. Bacteria containing the vector with an insert were therefore detectable as white colonies, while bacteria containing a vector without insert were detectable as blue colonies. Bacteria, which were successfully transformed with the expression vector, were selected on plates containing zeocin, as the vector contains a zeocin resistance.

3.1.9 Purification of plasmids from bacterial culture

For small scale purification of plasmid DNA selection antibiotic was added to 5 mL of LB.

The liquid culture was inoculated with bacteria from a culture plate and shaken overnight at 37 °C and 2000 rpm. Plasmid purification was accomplished using 'Nucleo Spin Plasmid Kit' (*Macherey & Nagel*).

To purify higher amounts of plasmid DNA a liquid culture with antibiotic selection was performed for 8 h. It was added to 250 mL LB medium and the culture was shaken overnight at 37 °C and 2000 rpm. For purification the Plasmid Kit Plus 'Maxi' (*QIAGEN*) was used.

Concentrations of DNA and plasmids were determined photometrically using a nanodrop photometer.

3.1.10 Cryoconservation of bacteria

Bacteria containing the expression vector were cryoconserved for storage at –80 °C. Therefore 150 µL of glycerol were added to 850 µL of a 5 mL bacterial culture to prevent formation of ice crystals during freezing.

3.2 Biochemistry

3.2.1 Purification of IgG fusion molecules via affinity chromatography

The IL-35-Fc fusion molecule was purified from the supernatant of transfected cells by binding of the IgG part to recombinant Protein G coupled to Sepharose Matrix. The recombinant protein G binds IgG with high affinity, but in contrast to native protein G, it does not bind serum albumine. 2 mL protein G sepharose matrix were packed in a 10 mL plastic column and rinsed with 30 mL PBS. Cell culture supernatant containing IL-35-Fc was applied two times to the column with a flow rate of 50-200 mL/h. Afterwards the column was rinsed with 30 mL PBS. The protein was eluted by applying 10 mL elution buffer and taking 1 mL fractions. Neutralization of pH was accomplished by immediate addition of 100 µL Tris buffer, pH 9, to the eluate fractions. Fractions containing protein were identified by addition of 10 µL eluate to 150 µL Bradford test solution. Presence of protein was indicated by bright blue color of the solution.

The solvent of the obtained protein solution was changed to PBS by dialysis. Therefore two rounds of dialysis, corresponding in total to 10,000 times dilution of the solvent, were performed.

Concentration of the protein was determined photometrically using a nanodrop photometer.

3.2.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

During SDS PAGE proteins are separated according to their size in an electric field. The detergent SDS in matrix and loading dye facilitates separation of proteins independent from their charge and conformation. It denatures the protein and the heavily negative charged SDS molecules associate to the non-polar amino acid chains, thereby compensating the native charge of a protein. Therefore the migration rate of proteins in the gel matrix is solely proportional to their size. Addition of dithitreithol (DTT) to the loading dye leads to reduction and therefore breaking of sulfate bonds. Furthermore, during discontinuous SDS PAGE the proteins are first focused in the stacking gel (5 % acrylamide, pH 6.8), leading to distinct seperation of the proteins in the separation gel (10 % acrylamide, pH 8.8).

For SDS PAGE the samples were in a volume of 5 to 20 µL, whereby 1/5 of sample volume was loading dye. The samples where heated to 95 °C for 10 min. After loading into the gel, the protein samples where focused for 10 min at 60 V and afterwards separated for 90 min at 120 V. Electrophoresis was stopped when the bromophenol from the loading dye reaches the end of the gel. As standard for protein size the ‘Page Ruler unstained’ protein ladder (for subsequent silver stain) and the ‘Page Ruler prestained’ protein ladder (for subsequent western blot) were used (*Fermentas*).

3.2.3 Silver stain

Following SDS PAGE the separated proteins in a gel can be visualized by staining with silver nitrate. This is a very sensitive detection with a detection limit of 50 ng of protein. First the gel was incubated in fixation solution for at least 1 hour. Afterwards it was incubated for 20 min in washing solution. Following 20 sec of sensitizing in sensitizing solution and two times 20 sec of rinsing with distilled water, the gel was incubated for 20 min in silver nitrate solution. Again the gel was rinsed twice for 20 sec with distilled water and afterwards developed with developing solution for less than 10 min and until the desired intensity of the staining was reached. Development was stopped by rinsing with distilled water and subsequent incubation in fixation solution for at least 10 min. Afterwards the gel was stored in water for a short period or conserved by drying.

3.2.4 Western blot

Western blot is a method to specifically identify proteins after SDS PAGE. Therefore the proteins are transferred to a membrane by an electric field vertical to the gel surface (blot). On the membrane they are detected by specific antibodies.

The gel-sized membrane was preactivated by short incubation in methanol. Six gel sized whatman filters, gel and membrane were soaked in CAPS buffer and three filters, followed by membrane, gel and three more filters, were stacked between the electrodes. Transfer was carried out for 1 h at 1 mA/cm^2 of blot surface.

Specific antibodies were used for detection of the protein. Therefore the membrane was incubated overnight with blocking solution at 4 °C. The IL-35-FC fusion molecule was detected with 1:10,000 dilution of a horseradish-peroxidase (HRP) conjugated rabbit-anti-human IgG antibody (*DAKO*) in blocking solution, or with a 1:300 dilution of a primary rat-anti-mouse EBI3 antibody in PBS with 1 % BSA, followed by a 1:10,000 dilution of a secondary HRP conjugated goat-anti-rat IgG antibody in blocking solution. Following incubation with an antibody three washing cycles of 10 min in PBS were performed. After the last washing cycle the membrane was coated with ECL detection solution (*Amersham*). Chemiluminescence evoked by the reaction of HRP with ECL was detected using an x-ray film. The film was processed on an automatic film processor.

3.2.5 ELISA

The enzyme linked immunosorbent assay (ELISA) is a method to quantify low amounts of a protein in a sample with help of specific antibodies. In the here used sandwich ELISA a specific antibody (capture antibody) against the desired protein is immobilized on the polytstyrene surface of a 96 well ELISA plate. It binds proteins that themselves would only weakly bind to the polystyrene. The binding of the protein out of the sample is detected with another specific, biotin conjugated, antibody (detection antibody). Streptavidine conjugated to HRP (Strep-HRP) can bind to the biotin, a step which enhances the signal intensity. Reaction of the HRP with a substrate leads to a color change of the substrate solution, which can be quantified photometrically.

ELISA Kits for IFNγ and IL-17 were obtained from *R&D Systems* and conducted according to the enclosed protocol. The samples were measured in triplicates and without further dilution. Standards were measured in duplicates. For IL-12p40 ELISA the capture

antibody was diluted to a concentration of 2 µg/mL in PBS and 50 µL per well were incubated at 4 °C overnight. After every incubation step the plates were washed with PBS containing 0.05 % Tween. The wells were blocked with 100 µL blocking solution for 2 h at room temperature. 50 µL of 1:10 diluted samples were loaded in triplicates. Standard was serially diluted, starting with a concentration of 2 ng/mL, and was loaded in duplicates. The plate was incubated over night. Detection antibody was diluted to a concentration of 0.5 µg/mL and 50 mL per well were incubated for 1 h. Afterwards the wells were incubated for 30 min to 1 h with 50 µL of Strep-HRP. After washing 100 µL of substrate solution were added to the wells and incubated for less than 10 min. The reaction was stopped with 25 µL 2 M H_2SO_4 per well. Quantification was conducted using an ELISA reader photometer.

3.2.6 Cytometric bead array (CBA)

Concentration of cytokines in serum samples was quantified using CBA (*BD Bioscience*). Fluorescent beads, covered with specific antibodies against a cytokine, are incubated with the serum samples and a detection antibody conjugated to a fluorophor. The beads are detected via flow cytomtery, whereby a specific bead can be identified by its own fluorescence intensity whereas the concentration of cytokine in the sample is in proportion to the fluorescence intensity of the detection antibody. In this thesis the Mouse Inflammation CBA Kit was used according to the manufacturer's protocol. Serum samples were diluted 1:4.

3.3 Cell biology

3.3.1 Cell culture, cell counting and cryoconservation of eukaryotic cells

Cell culture was conducted under sterile conditions in a class II biological safety cabinet. Sterile disposable plastic equipment, as well as sterilized glassware and solutions were used. Solutions and glassware were sterilized with steam for 20 min at 135 °C and 2 to 2.2 bar. All cells were cultivated in RPMI-1640 medium, supplemented with FCS, glutamine and gentamycin. Culture plates were incubated in a CO_2 incubator at 37 °C and 5 % CO_2. Centrifugation of cells was performed for 5 min at 4 °C and 300 g. CHO cells were cultivated in 6-well cell culture plates and every 2 to 3 days they were removed using trypsine-EDTA and diluted 1:6 to 1:10 before they were plated in a new 6-well.

Counting of cells was performed in a Neubauer counting chamber. This chamber has

optically contrasted quadrants, whereby in one big quadrant encloses 0.1 µL cell suspension. The cell number determined in one big quadrant was multiplied with 10,000 to obtain the number of cells per mL. Before counting, cells were diluted 1:2 with trypane blue, which diffuses into dead cells. They are stained blue, while live cells remain bright. The dilution with trypane blue has to be regarded in the determination of cell number.

Eukaryotic cells can be conserved over a longer period of time at -196 °C in liquid nitrogen. DMSO added to the medium draws water out of the cells and prevents building of ice crystals. For cryoconservation cells were centrifuged and the pellet was resuspended in freezing solution and transferred into cryotubes. Afterwards they were stored for 24 h at -80 °C before they were transferred to liquid nitrogen. When used again the cells were thawed at 37 °C and, after three washing steps with prewarmed medium, taken back into culture. Hereby the medium was changed after 24 h.

3.3.2 Transfection of eukaryotic cells

During transfection vector DNA containing a transgene is transported into the nucleus of an eukaryotic cell, where the transgene will be transcribed. For transient transfection the vector only has to be located in the nucleus. For stable transfection the vector has to be included in the genome, in order to be duplicated during cell division. The non-lipid transfection reagent Turbofect (*Fermentas*) forms kationic polymers with DNA, which are taken up by cells by endocytosis. High charge of the polymers leads to breaking of endosomes, so the polymers can migrate into the nucleus.

For transfection cells were cultivated to 80 % confluence. The medium was discarded and 0.4 mL medium per cm^2 were applied. Per cm^2 were first 0.4 µg DNA and then 0.4 µL Turbofect added to 400 µL medium. The suspension was incubated for 20 min at room temperature and added dropwise to the cells afterwards. Success of the transformation could be determined afterwards by intracellular staining for human IgG1 and flow cytometric analysis. For stable transfection single cells were sorted using the FACS Aria. The cells were cultivated in medium, containing 400 µg/mL Zeocin.

3.3.3 Lymphocyte isolation from organs

Lymphocytes from blood were isolated by lysis of erythrocytes. Therefore up to 500 µL of blood were diluted in 15 mL RBC lysis buffer for about 10 min. RBC lysis was

complete when the suspension had turned from turbid to clear. Afterwards the cells were centrifuged and washed twice with medium.

Lymphocytes from spleen were isolated by flushing the organ with medium using syringe and needle. Afterwards the cells were centrifuged and the pellet was suspended in RBC lysis buffer. Lysis was stopped after 2 min by addition of medium followed by centrifugation. The cells were washed twice with medium and resuspended in 5 mL medium.

Lymphocytes from liver were separated from hepatocytes using a Nycoprep gradient. Whole liver was mechanically disrupted by pressing through a cell strainer. The filter was rinsed with 10 mL medium. The cells were centrifuged and the pellet was resuspended in 1 mL medium. This was mixed with 5.5 mL of 30 % Nycoprep. 2 mL of medium in a 15 mL centrifugation tube were underlayed with the Nycoprep suspension. Gradient centrifugation was performed at 2500 rpm, 4 °C without brakes. Afterwards the lymphocytes in the middle layer were carefully removed and suspended in 5 mL medium. Centrifugation was perfomed at 1800 rpm and 4 °C. The cells were washed one additional time and resuspended in 300 µL medium.

3.3.4 Restimulation of lymphocytes for intracellular cytokine analysis

Preceding intracellular cytokine staining 1x 10^6 lymphocytes per well were plated into a 96-well plate and restimulated in 200 µL medium with 100 ng/mL PMA and 500 ng/mL Ionomycin in presence of 0.75 µL Golgi-Stop (*BD Bioscience*) per well. Incubation was performed for 4 h at 37 °C.

3.3.5 Flow cytometry and cell sorting

During flow cytometry single cells of a cell suspension can be analysed for light scatter and fluorescent signals. Therefore the cells are separated in the laminar flow of the cytometer and guided through the beam of a laser. Reflection and scatter of the beam are dependent on size and granularity of the cells, respectively. Furthermore the absorption of light at different wavelengths can be analysed, which detects fluorescent labeling of the cells with specific antibodies. The flow cytometers FACS Calibur and Accuri C6 (*BD Bioscience*) used in this thesis are able to detect forward scatter (size) and sideward scatter (granularity), as well as fluorescence in four different channels.

All staining procedures were performed in wells of 96-well plates. Staining of surface markers with fluorescent labeled antibodies was conducted in 15 µL FACS buffer for 1 h at 4 °C. Afterwards the cells were washed two times with 150 µL FACS buffer. For staining of intracellular molecules the cells were fixed and permeabilized for 10 min in 100 µL Cytofix/Cytoperm (*BD Bioscience*). The cells were washed two times with Perm/Wash and were incubated with the fluorescent labeled antibody in 15 µL Perm/Wash. Afterwards the cells were washed two times with 150 µL Perm/Wash and resuspended in 150 µL Perm/Wash for flow cytometric analysis. Specificity of antibody staining was verified by comparison with cells incubated with antibodies of the same isotype but irrelevant specificity (isotype control).

For sorting via flow cytometry spleen cells were stained extracellularly with fluorescent labeled antibodies in 1 mL medium. After two washing steps with medium the cells were resuspended in medium to a concentration of 2x 10^7/mL to 5x 10^7/mL for sorting.

All flow cytometric analyses were performed using FlowJo software (*Tree Star*).

3.3.6 Stimulation and *in vitro* differentiation of T cells

The antibody used in this thesis against the CD3 ε chain of the TCR complex leads to strong cross-linking of TCRs on the T cell surface and therefore to an antigen independent activation of T cells. To stimulate T cells, 2x 10^5 spleen cells per well were cultivated in 96-well plates in 200 µL medium with 1 µg/mL anti-CD3 antibody. The T cells of C57Bl/6 mice differentiate thereby mostly into a T_H1 like phenotype producing high amounts of IFNγ. To obtain cells producing IL-17, 20 ng/mL IL-6 and 2 ng/mL TGFβ were added to the culture medium.

3.3.7 Generation of bone marrow-derived dendritic cells (BMDC)

In presence of the cytokine GM-CSF, monocyte precursors from bone marrow can be differentiated into dendritic cells *in vitro*. Therefore bone marrow was flushed from tibia and femur of mice with medium, using syringe and needle. A single cell suspension was generated with help of a cell strainer. The suspension was incubated for 10 min on ice, until remains of bone have sedimented. The supernatant was collected, centrifuged and resuspended in RBC lysis buffer. Lysis was stopped after 2 min by addition of medium followed by centrifugation. The cells were washed with medium and resuspended in 5 mL medium. Cells were counted and 4x 10^6 cells were plated in 10 mL medium in a

petri dish. 1 mL GM-CSF containing cell supernatant was added and the cells were incubated at 37 °C and 5 % CO_2. After 3 days, 10 mL medium and 1 mL GM-CSF containing cell supernatant were added. Additional 3 days later, 10 mL supernatant were discarded and 10 mL medium and 1 mL GM-CSF containing cell supernatant were added. On day 7 the cells were used and stimulated for assays or cryoconservated for later use. Thawed cells were incubated with medium containing 10 % GMCSF containing cell supernatant for 24 h prior to stimulation.

3.3.8 γδ T cell stimulation in the presence of APC

For stimulation of γδ T cells 2x 10^4 BMDC per well were plated into a 96-well plate. Lymphocytes from spleen were taken and T cells were enriched magnetically using MACS Pan T cell purification Kit (*Miltenyi*) according to the manufacturer's protocol. γδ T cells from spleen were sorted for γδ TCR and CD27 expression and 1x10^3 γδ T cells per well were added. Stimulation was performed with 100 ng/mL PamCys for 48 hours at 37 °C and 5 % CO_2.

3.4 Infection model

3.4.1 Parasites

P. berghei ANKA parasites were maintained at the mosquito colony (*BNI*). This was achieved by alternating passage of the parasites in *Anopheles stephensi* mosquitoes and BALB/c mice. For i.v. infection with sporozoites the parasites were prepared out of the salivary glands of mosquitoes. Stabilate for i.p. infection was generated by collecting blood from highly parasitemic mice using a heparinized syringe. Aliquots of 2x 10^7 pRBC were resuspended in a solution containing 0.9% NaCl, 4.6% sorbitol, and 35% glycerol and stored in liquid nitrogen.

3.4.2 Infection model

Infection with sporozoites was accomplished by i.v. injection of 1000 sporozoites in 100 µL PBS into the tail vein. When infected with stabilate mice received an i.p. injection of 2x 10^6 pRBC in PBS. Parasitaemia was determined on day 6 p.i., unless stated otherwise. Between days 7 and 10 p.i. C57BL/6 started to develop neurological symptoms, including ataxia, convulsions, and coma, and ultimately leads to death of the mice. During this phase mice were closely monitored. To avoid unnecessary suffering

mice were scored upon observation of cerebral malaria according to the severity of the symptoms and subsequently euthanized.

3.4.3 Determination of parasitaemia

Glass slides with blood smears of blood from the tail vein of infected mice were used for the determination of parasitaemia. They were fixed for 10 min in methanol and stained for at least 45 min in 10 % Giemsa. Afterwards the slides were rinsed with water and dried at room temperature. At least 1000 erythrocytes in three fields of view were counted and parasitaemia was indicated as ratio of pRBC/(RBC+pRBC) in percent.

3.5 Statistical Analyses

All statistical analyses were performed with the software ‘GraphPad Prism’ using the students t test.

4 Results

4.1 Regulation of IL-17 by IL-35 during experimental cerebral malaria

The first part of the thesis deals with the influence of the regulatory IL-12 family cytokine IL-35 on the pathologic proinflammatory immune response during experimental cerebral malaria (ECM). IL-35 is produced by regulatory T cells rather than by antigen-presenting cells like other IL-12 family members (Collison, L.W. et al. 2007). It is described to regulate IL-17 production of T_H17 cells as well as to activate and induce regulatory T cells (Niedbala, W. et al. 2007). While investigating the influence of IL-35 it has to be considered that the pathology of ECM is believed to be mediated by proinflammatory cytokines like IFNγ and TNFα, which are hallmarks of a T_H1 polarisation of the immune response rather than for a T_H17 polarisation that would most likely be influenced by IL-35.

4.1.1 Cloning of the IL-35-Fc construction vector

To conduct studies on IL-35 *in vitro* and *in vivo* the first aim was to construct a fusion molecule of murine single chain IL-35 linked to the Fc domain of human IgG1, in which the two subunits p35 and EBI3 are connected by a flexible linker. This molecule has the advantage of being easily detectable by an antibody against the human IgG1 domain and easy to purify with the help of protein G coupled to Sepharose.

The chosen expression vector was the pFuse-hIgG1-Fc2 vector. This vector already contains a slightly modified Fc-part of the human IgG1. Additionally, it contains the signal sequence of human IL-2, which will ensure secretion of the expressed molecule into the supernatant by transfected cells. Between these two elements of the vector the multiple cloning site is located with the restriction sites of the enzymes *Eco*RI, *Nco*I and *Bgl*II. The molecule was constructed with the sequence of the p35 subunit at the 3' end of the IL-2 sequence, flanked by restriction sites for *Nco*I and *Bgl*II. This is followed by a glycine-serine linker containing four repeats of GlyGlyGlySer and the EBI3 sequence connected to the 5' end of the IgG1 sequence by a restriction site for *Eco*RI.

To obtain the sequences of the murine p35 and EBI3 subunits BMDC of mice were generated and stimulated for 24 hours with 100 ng/mL LPS to induce upregulation of

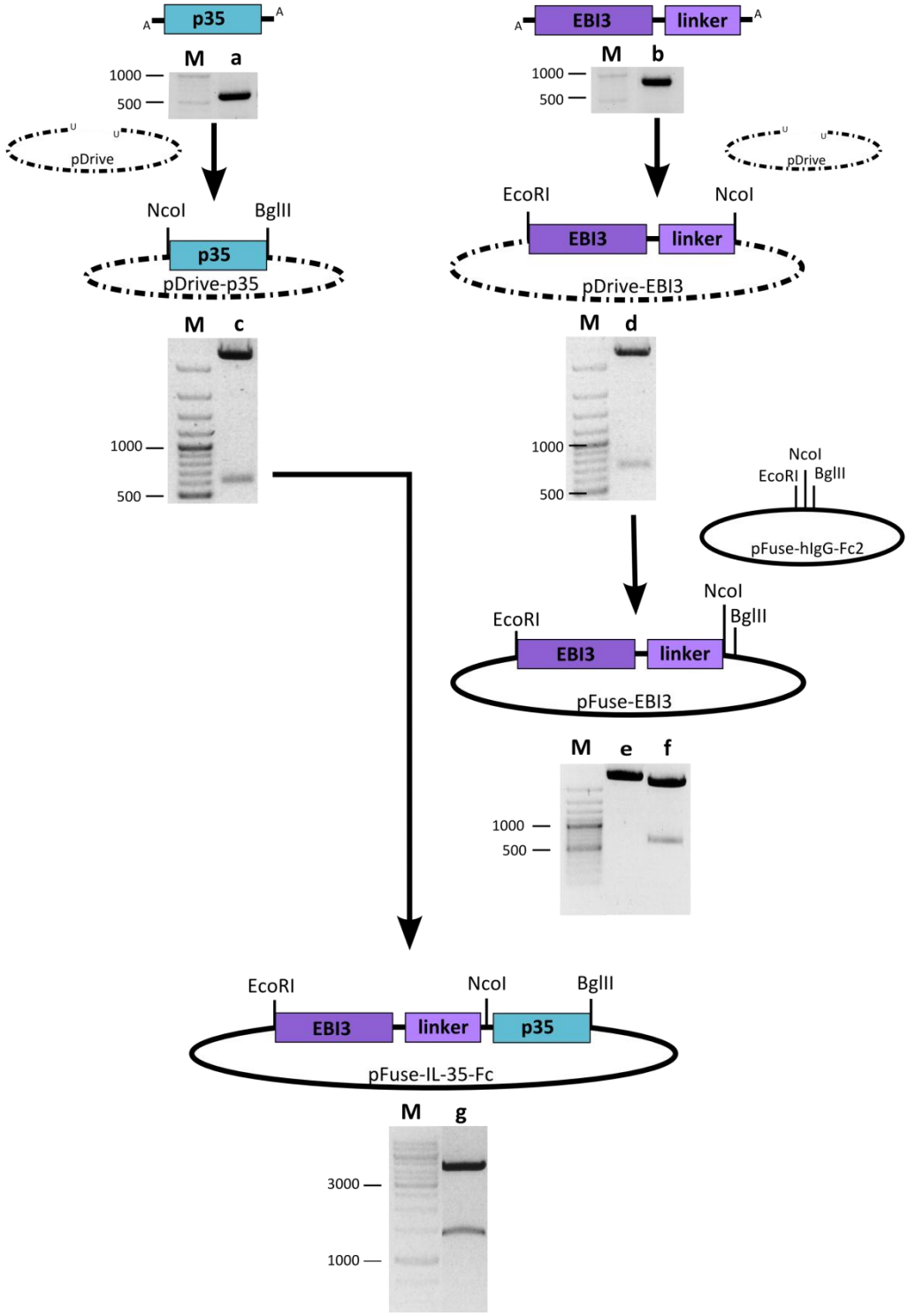

Figure 4-1: **Construction of the IL-35-Fc expression vector**

Murine p35 and murine EBI3 fused to a linker sequence were amplified via PCR and ligated into the pDrive cloning vector. The linker and EBI3 were excised from the cloning vector using *Nco*I and *Eco*RI and cloned into the pFuse expression vector. p35 was excised from the cloning vector using *Bgl*II and *Nco*I and cloned into the expression vector already containing the linker and EBI3. **M:** Marker. **a:** p35 PCR Fragment (expected size: 610 bp) **b:** linker + EBI3 PCR Fragment (expected size: 712 bp) **c:** p35 Fragment excised from pDrive using *Bgl*II and *Nco*I (expected size: 602 bp) **d:** linker + EBI3 Fragment excised from pDrive using *Nco*I and *Eco*RI (expected size: 705 bp) **e:** pFuse containing linker and EBI3, linearised using *Bgl*II and *Nco*I **f:** linker + EBI3 Fragment excised from pFuse using *Nco*I and *Eco*RI (expected size: 705 bp) **g:** Fragment containing p35, linker and Ebi3, excised from expression vector using *Eco*RI and *Bgl*II (expected size: 1307 bp)

the mRNA of the IL-12 family cytokine subunits. The cells were harvested and mRNA was obtained via phenol-chloform extraction using Trizol reagent. An oligo(dT)$_{18}$ primer was used to generate cDNA, which served as template for the PCR. The amplified sequences were analyzed via agarose gel elektrophoresis. The analysis of the PCR for p35 showed the correct fragment size of 610 bp (**Figure 4-1 a**). The forward primer for the EBI3 sequence already contained the linker sequence, so a fragment of the correct size of 710 bp was detected in the analysis (**Figure 4-1 b**). Each fragment was purified from the gel and ligated into the pDrive cloning vector. With the cloning vectors containing the respective subunits *E coli* DH5α were transformed and positive clones were selected by Ampicillin resistance encoded on the vector and blue/white screening, taking advantage of the insertion of the insert within a ß-Galactosidase gene on the cloning vector. Vector DNA from 5 mL cultures of selected clones was purified and tested by restriction analysis followed by agarose gel elektrophoresis. In the analysis of the vector containing p35 the restriction with the enzymes *Bgl*II and *Nco*I yielded a fragment of the expected size of 602 bp (**Figure 4-1 c**). The vector containing the linker and the EBI3 was digested with the enzymes *Nco*I and *Eco*RI the fragment of the expected size of 705 bp could be shown in the analysis (**Figure 4-1 d**). Additionally, both subunits were sequenced within the cloning vector using M13 primers. They matched the NCBI reference sequences. The EBI3 fragment was purified from the agarose gel and ligated into the expression vector, which had been digested with the same enzymes before. The resulting vector was used to transform *E coli* Top Ten, which were selected by a Zeocin resistance encoded on the vector. Selected clones were analyzed by restriction with *Eco*RI and *Nco*I and subsequent agarose gel elektrophoresis. The fragment with the correct size of 705 bp could be detected (**Figure 4-1 f**). The resulting vector containing the linker and EBI3 sequences was linearised using *Bgl*II and *Nco*I (**Figure 4-1 e**) and used for ligation with the gel purified fragment of p35, which was restricted from the cloning vector using the same enzymes (**Figure 4-1 c**). The resulting vector was used to transform *E coli* Top Ten, which were selected by a Zeocin resistance encoded on the vector. The selected clone was analyzed by restriction with *Eco*RI and and *Bgl*II and subsequent agarose gel elektrophoresis. The fragment with the correct size of 1307 bp could be detected (**Figure 4-1 g**). The obtained expression vector was propagated in *E. coli* Top Ten and used to transfect CHO cells.

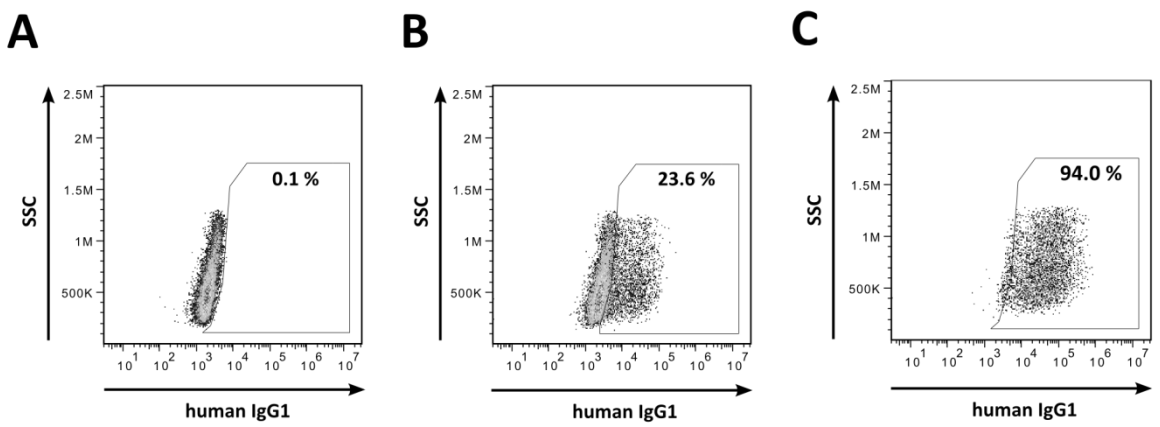

Figure 4-2: Expression of the IL-35-Fc construct in CHO cells

CHO cells were transfected with the IL-35-Fc expression vector using Turbofect transfection reagent. Efficiency of the transfection was analyzed in transiently transfected cells and in stabily transfected cells that were subcloned and cultivated in presence of the selective agent Zeocin. The cells were permeabilised and intracellularly stained using PE labeled antibody against the Fc part of the human IgG1 and analyzed via flow cytometry. **A**: Untransfected CHO cells. **B:** Transiently transfected CHO cells 48 hours after transfection. **C:** Stabily transfected CHO cells, clone A2. Plots are representative of 6 to 8 analyzes of expression of the IL-35-Fc.

4.1.2 Expression and purification of the IL-35-Fc fusion molecule

The recombinant IL-35-Fc fusion molecule was produced in CHO cells. The production in eukaryotic cells has certain advantages. On the one hand these cells are able to perform posttranslational modifications that bacteria are not capable of, such as glycosylation. On the other hand, the chance of endotoxin contamination is lowered, which is crucial to avoid artificial activation of immune cells by pyrogenicity.

The introduction of the vector into CHO cells was first accomplished by transient transfection with the Turbofect transfection reagent. To analyze the transfection efficiency the cells were trypsinised, collected and permeabilised 48 hours after transfection. Afterwards, they were stained with an antibody against human IgG1 and analyzed via flow cytometry (**Figure 4-2 B**). CHO cells treated with the same amount of transfection reagent but without vector served as a control for the transfection efficiency (**Figure 4-2 A**). This method yielded only low transfection efficiencies of 20 % to 25 % of the cells producing the IL-35-Fc fusion molecule.

To get higher percentages of cells producing the fusion molecule, stable transfectants of CHO cells producing the desired molecule were created in the context of the master

thesis of Aline Taenzer (Taenzer, A. 2010). The cells were transfected using the same transfection protocol as for the transient transfection and were cultivated under selection with the selective agent Zeocin for two weeks. Afterwards, single cell sorting into 96 well plates was conducted using the FACS Aria cell sorter. The clones were further cultivated under selective pressure and screened for production of the IL-35-Fc fusion molecule by intracellular staining with an antibody against human IgG1 and analysis via flow cytometry (**Figure 4-2 C**). Using this method more than 90 % of the cells were shown to produce the fusion molecule, which was maintained stabily under selective pressure of Zeocin containing medium and was as well maintained over the period of three weeks without addition of Zeocin. During this time the cells were expanded and supernatants containing IL-35-Fc were collected.

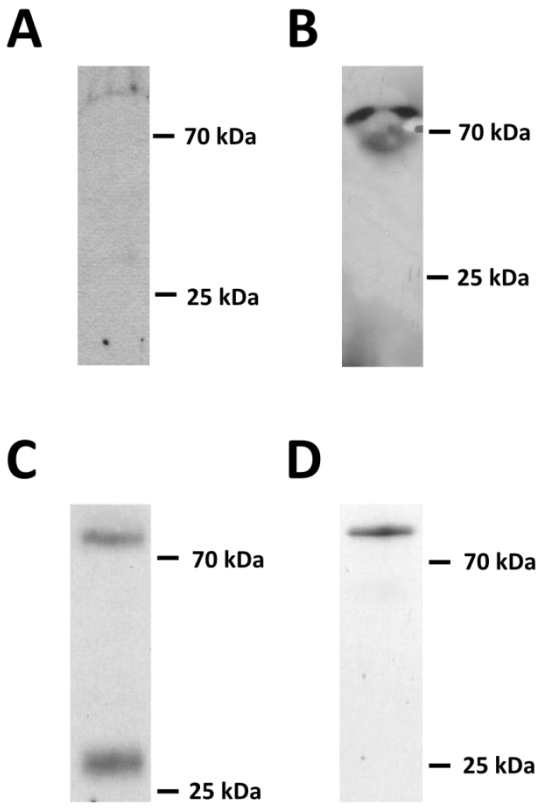

Figure 4-3: Purification of IL-35-Fc from supernatants of transfected CHO cells

CHO cells were transfected with the IL-35-FC expression vector using Turbofect transfection reagent. After five days the supernatant was collected and tested for the presence of the recombinant protein using Western Blot. The calculated size of the protein is 74 kDa. **A:** Western Blot of supernatants of transfected CHO cells using a specific antibody for human IgG1 **B:** Western Blot of supernatant of transfected CHO cells using a specific antibody for murine EBI3.
IL-35-Fc was purified from CHO cell supernatant using a G-Protein coupled sepharose column. **C:** Western Blot of purified IL-35-Fc using a specific antibody for human IgG1. **D:** Silver stain of purified IL-35-Fc.

The next requirement was to verify if the fully transcribed fusion molecule was secreted into the supernatant. For this purpose transfected CHO cells were cultivated for five days in the same medium. Afterwards, the supernatant was collected and used for SDS PAGE and subsequent western blot. The size of the single chain molecule was estimated at 71 kDa. First the molecule was detected with a directly HRP-labeled antibody against the human IgG1 (**Figure 4-3 A**). This detection showed only a weak and irregularly curved signal shortly above the 70 kDa marker

signal. Additionally, the western blot was conducted using a monoclonal rat antibody against the murine EBI3. This was detected using a secondary HRP-labeld anti-rat antibody (**Figure 4-3 B**). This western blot showed a stronger, but still uneven and curved signal of the same size as in the blot with the detection against human IgG.

For the production of larger amounts of the molecule the cells were expanded. The medium used had been depleted from bovine IgG using a Protein-G coupled Sepharose column beforehand. About 1 L of supernatant was collected after five days of culture without change of medium. Since the IgG1 part of the fusion molecule binds to Protein-G, another Protein-G coupled sepharose column was used to purify the protein. A Bradford test showed protein content in 5 of the 10 eluate fractions, which were pooled. Afterwards the solvent was changed to PBS by dialysis. The specificity and purity of the recombinant fusion protein was analyzed via western blot and silver staining. The western blot was conducted using a HRP-labeled antibody against human IgG1 (**Figure 4-3 C**). It shows two signals, one at the expected size of 71 kDa and one with a lower molecular weight between 25 and 30 kDa, which would correspond to the size of only the modified human IgG1 Fc-part. In the silver staining the second signal between 25 and 30 kDa is not visible, there is only one at the expected size of 71 kDa (**Figure 4-3 D**). The absence of any other proteins in detectable concentration on the silver staining shows the purity of the protein.

4.1.3 *In vitro* test of the IL-35-Fc

IL-35 is a regulatory cytokine that has been shown to be produced by regulatory T cells (Niedbala, W. et al. 2007). As such, it has been shown to induce regulatory T cells and on the other hand it displays a capability to specifically regulate IL-17 production in T_H17 cells. This has been demonstrated by experiments, where addition of IL-35 was sufficient to suppress IL-17 production in a T_H17 polarizing stimulation of $CD4^+$ T cells *in vitro* (Niedbala, W. et al. 2007). To verify, if the produced single chain IL-35-Fc fusion molecule displays the functionality of endogenous murine IL-35 newly synthesized IL-35-Fc was tested in an *in vitro* stimulation of T cells under T_H17 polarizing conditions.

For the experiments MACS-purified T cells from spleenocytes of C57Bl/6 mice were taken and stimulated in 96 well plates under different conditions. Stimulated with a crosslinking antibody against CD3 most of the T cells of C57Bl/6 mice obtain a T_H1 phenotype characterized by high production of IFNγ. To generate T cells with a T_H17

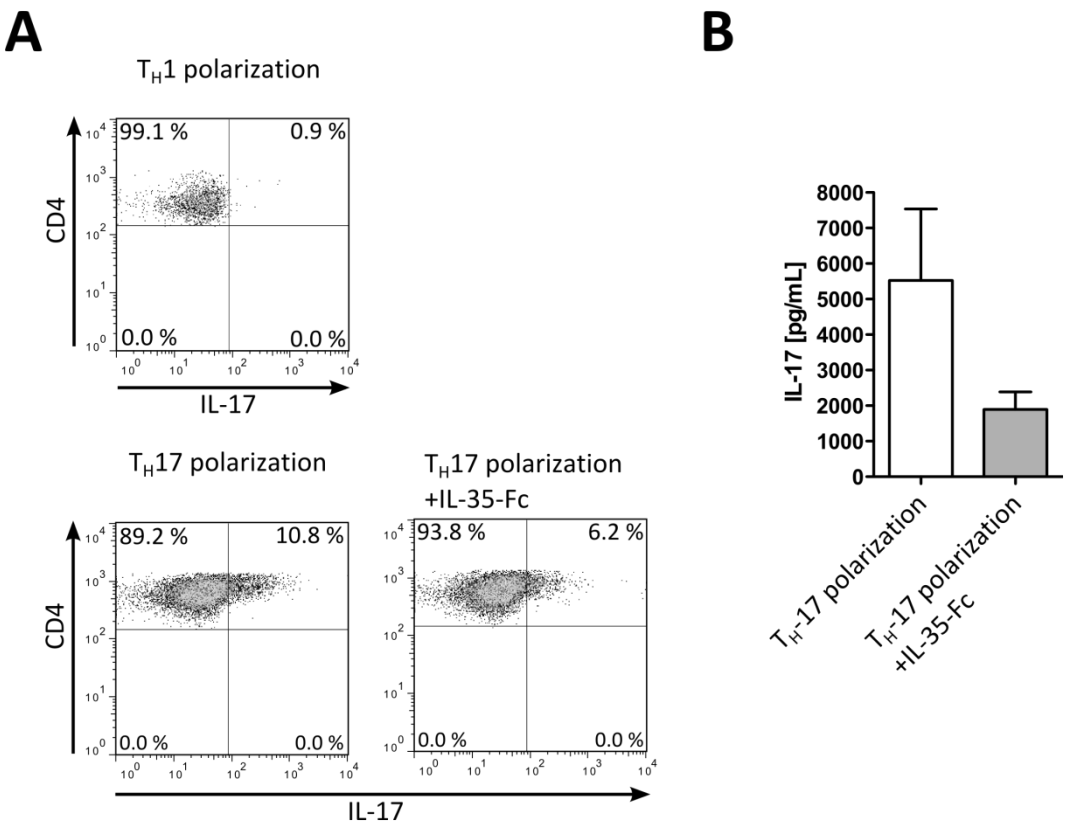

Figure 4-4: IL-35-Fc suppresses *in vitro* T_H17-polarisation of $CD4^+$ T cells

T cells from spleen were cultured for 72 h under T_H1 polarizing conditions, stimulated with an anti-CD3 antibody without additional cytokines, or under T_H17 polarizing conditions, stimulated with an anti-CD3 antibody in the presence of IL-6 and TGFβ in presence or absence of 100 µg/mL IL-35-Fc. Afterwards the supernatant was harvested after 68 hours and the cells were restimulated with PMA and Ionomycin in presence of Golgi-Stop for 4 hours. **A:** The cells were extracellularly stained with a PE labeled antibody against CD4 followed by fixation, permeabilization and staining with an APC labeled antibody against IL-17. Afterwards the cells were analyzed via flow-cytometry. Plots are representative for three independent experiments **B:** Supernatants were tested for IL-17 via ELISA.

phenotype, in addition to the anti-CD3-antibody the cytokines IL-6 and TGFβ were added and the cells were cultivated in presence or absence of IL-35. The cells were stimulated with PMA and Ionomycin for 4 hours; afterwards three wells per stimulating condition were pooled. They were stained extracellularly for CD4, fixed and permeabilised, followed by intracellular staining of IL-17. Analysis was conducted via flow cytometry (**Figure 4-4 A**). In the mainly T_H1 polarized stimulation only very few IL-17 expressing $CD4^+$ T cells would be expected, therefore this stimulation served as the negative control. After stimulation under T_H17 polarizing conditions 10 % of the $CD4^+$ T cells expressed IL-17. Under T_H17 polarising conditions in presence of IL-35 only 6 % of the $CD4^+$ T cells remained positive for IL-17. Additonally, the supernatants of the stimulation were tested for IL-17 via ELISA (**Figure 4-4 B**). In the supernatant of the cells

lower concentration of IL-17 can be noticed. Hence the flow cytometric analysis shows the reduction of IL-17 producing cells and the ELISA indicates an overall reduction of the secreted cytokine in the supernatant.

4.1.4 *In vivo* application of IL-35-Fc

In humans an infection with *Plasmodium falciparum* may lead to the severe complication of cerebral malaria. The appearance of an inflammation in the otherwise immunopriviliged tissue of the brain causes severe neurological defects and eventually death (Idro, R. et al.). The murine model for cerebral malaria is the infection of C57Bl/6 mice with *Plasmodium berghei* ANKA, which leads to neurologic defects after seven to ten days, followed by coma and death within less than 24 hours (Li, C. et al. 2001). In mice the severe course of infection is dependent on a strong activation of proinflammatory immune responses and a sequestration of $CD8^+$ T cells to the endothelium of the brain (Hermsen, C. et al. 1997). The survival in the early phase of infection is therefore the readout for the severity of the immunopathology. As IL-35 is an

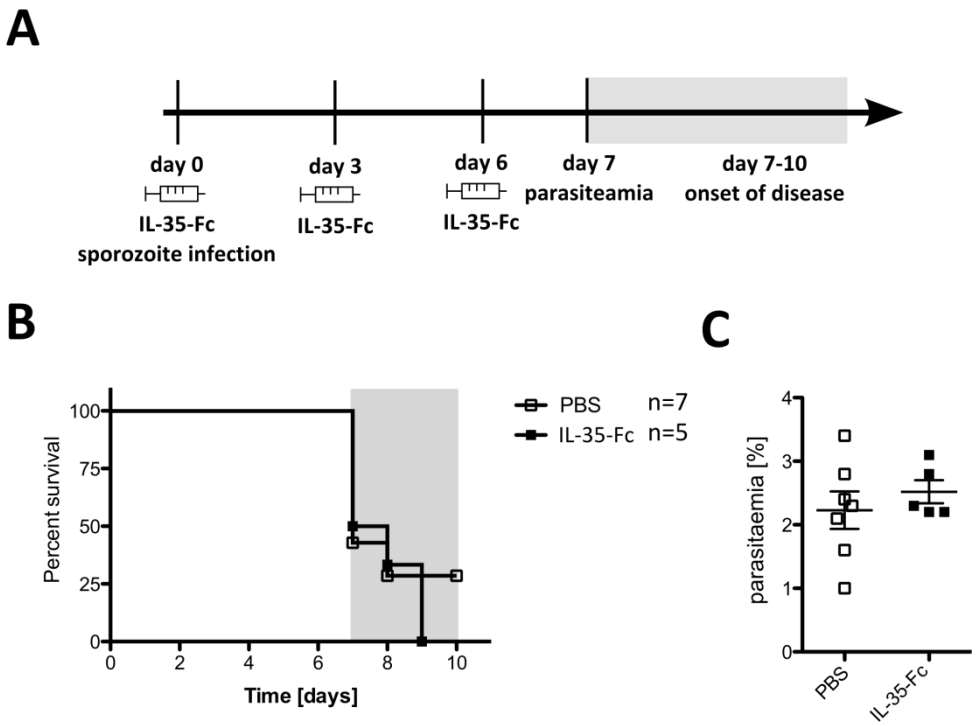

Figure 4-5: *In vivo* application of IL-35-Fc has no influence on the course of *P. berghei* ANKA infection in mice

Mice receiving 30 µg IL-35-Fc i.p. on day 0, 3 and 6 of infection and mice receiving PBS i.p. on day 0, 3 and 6 of infection were infected i.v. with 1000 *Plasmodium berghei* ANKA sporozoites. **A:** Experimental setup **B:** Onset of cerebral malaria was monitored until day 10. **C:** Parasitaemia was determined on day 6.

immunoregulatory effector the question arouse, if it might be able to dampen the immunopathology that leads to the severe symptoms. During the early phase of infection the increasing parasitaemia cannot be controlled sufficiently by adaptive immunity. Therefore an early influence on the parasitaemia would be conducted by innate mechanisms.

After the *in vitro* experiments suggested that the IL-35-Fc fusion molecule was fully functional, it was tested in the model of experimental cerebral malaria. Therefore mice were infected with 1000 sporozoites, each. One group was treated with 30 µg IL-35-Fc in PBS and a control group was treated with PBS on days 0, 3 and 6 of malaria infection. Blood from the tail vein was taken on day 7 to analyze the parasitaemia and the mice were monitored until day 10 for neurological symptoms (**Figure 4-5 A**). Both groups showed comparable survival rates (**Figure 4-5 B**). Thus, the administration of IL-35-Fc does thus not influence the immunopathology during the malaria infection. Neither did the parasitaemia on day 7 differ between the two groups (**Figure 4-5 C**), so the immunoregulatory function of IL-35-Fc did not dampen anti-parasite-immunity during the infection.

4.2 Induction of IL-17 by IL-23 during experimental cerebral malaria

After the first part of the thesis has dealt with the regulation of the proinflammatory, especially the T_H17 polarized, immune response by the IL-12 family member IL-35, the second part focuses on the activation of this immune response and its consequences. The T_H17 polarized immune response, that would have been counterregulated by IL-35 is induced by the IL-12 family cytokine IL-23 and leads to secretion of IL-17 (Aggarwal, S. et al. 2003). IL-12 itself activates in contrast a T_H1 polarized immune response (Hsieh, C.S. et al. 1993).

Still the model chosen for the investigations is the ECM. However, while during the studies on IL-35 the infection was evoked by injection of sporozoites directly into the bloodstream, in the second part of the thesis mice were infected with 2x 10^6 pRBC i.p.. Hereby the asymptomatic liver stage, where the high multiplication rates can be the source of high variability in the number of parasites that enter the blood stage, is skipped. The stability of the infection course is improved and it begins directly with the blood stage, where the immune responses and the immune pathology take place.

4.2.1 Deficiency of IL-12 protects from ECM whereas deficiency of IL-17 leads to an increased parasitaemia.

During an infection with *P. berghei* ANKA different parameters can be analyzed, that respond on different aspects of the immune status. The development of neurological defects and death before day 10 of infection shows the severity of pathological immune responses. Which direction the polarization of the immune response has taken can be concluded from cytokines detected in serum samples. The parasitaemia on the contrary shows, how a protective immune response is able to cope with the infection. In these three parameters mice lacking T_H17 polarized immune responses and mice lacking T_H1 polarized immune responses were analyzed.

To analyze the infection in absence of T_H1 polarisation mice deficient for IL-12p35, and thus lacking functional IL-12, and WT mice were infected with parasitized red blood cells and monitored for neurological defects until day ten (**Figure 4-6 A**). More than 50 % of the WT mice developed cerebral malaria, in contrast to one out of ten in the IL-12p35$^{-/-}$. Additionally, serum cytokines were analyzed on day six p.i. (**Figure 4-6 B**). IFNγ, which is viewed as a typical T_H1 type cytokine, is strongly reduced in the

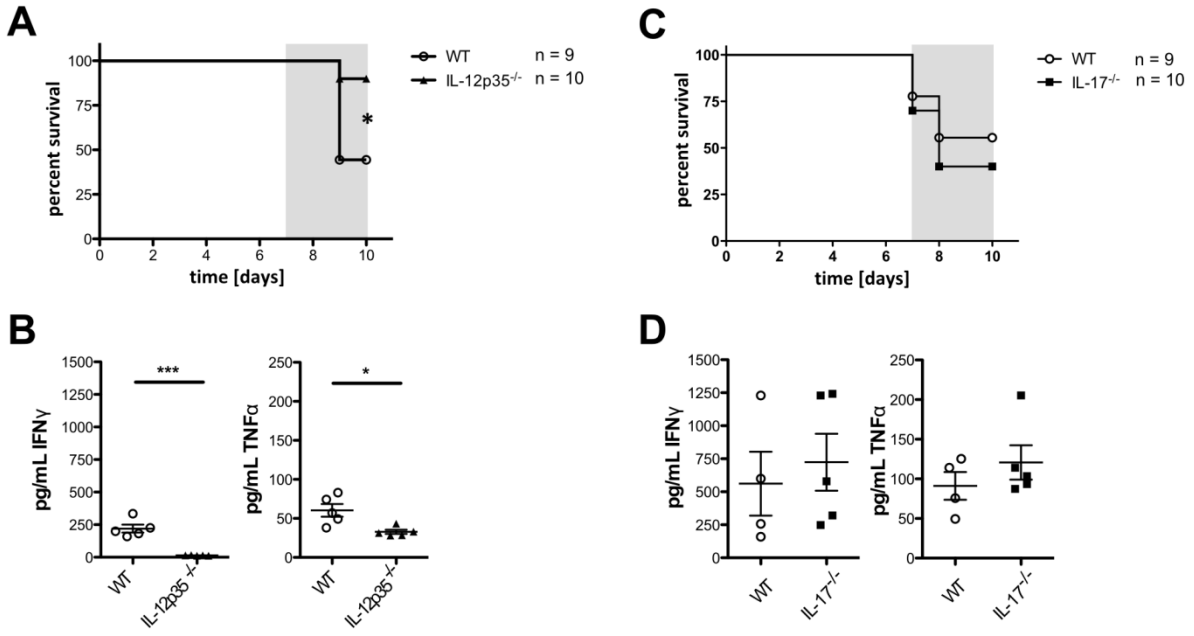

Figure 4-6: Deficiency of IL-12 protects against cerebral Malaria

Mice were infected with 2x 10^6 *Plasmodium berghei* ANKA parasitized red blood cells. The onset of cerebral malaria was monitored until day 10 and serum cytokine concentrations were determined on day 6. **A:** Onset of cerebral malaria in IL-12p35$^{-/-}$ vs. WT control mice. **B:** Serum cytokine concentration of IL-12p35$^{-/-}$ vs. WT control mice. **C:** Onset of cerebral malaria in IL-17$^{-/-}$ vs. WT control mice. **D:** Serum cytokine concentration of IL-17$^{-/-}$ vs. WT control mice **A,B:** Pooled results of two independent experiments. **C,D:** Representative results of two independent experiments. * $p<0.05$, *** $p<0.005$

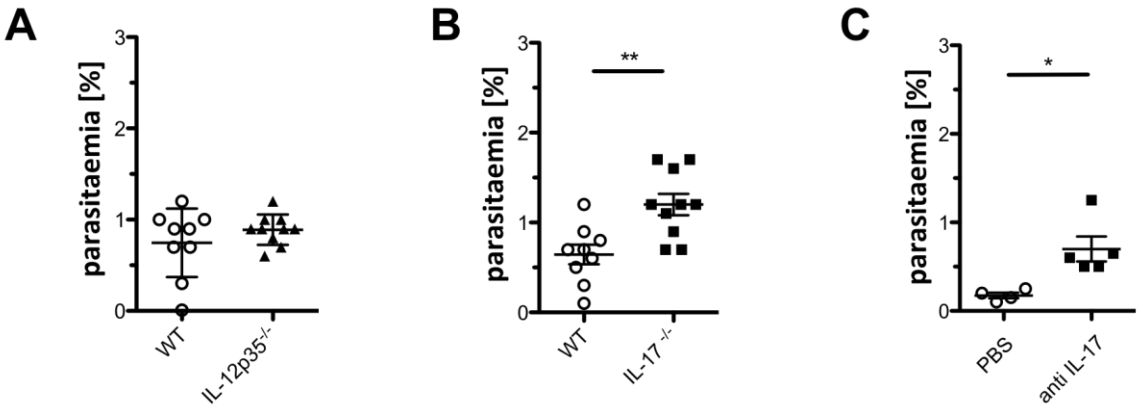

Figure 4-7: Deficiency of IL-17 leads to an increased parasitaemia.

Mice were infected with 2x 10^6 *Plasmodium berghei* ANKA parasitized red blood cells. Parasitaemia was determined on day 6. **A:** Parasiteamia of IL-12p35$^{-/-}$ vs. WT control mice. **B:** Parasitaemia of IL-17$^{-/-}$ vs. WT control mice **C:** Parasitaemia of mice receiving a neutralising antibody against IL-17 vs. mice receiving control IgG. **A+B:** Pooled results of two independent experiments. **C:** Representative results of three independent experiments. * $p<0.05$, ** $p<0.01$

IL-12p35$^{-/-}$ mice. Similarly, the proinflammatory cytokine TNFα is reduced. Absence of IL-12, and, in consequence, absence of a T_H1 polarized immune response, thus leads to a decrease in pathology. The absence of IL-17 was analyzed in mice deficient for IL-17. These mice were as well infected with pRBC and monitored for neurological defects until day ten, with WT mice serving as a control (**Figure 4-6 C**). Mice in both groups developed cerebral malaria to a similar extent. Serum cytokines were analyzed on day 6 p.i. as well (**Figure 4-6 D**), and in both groups the same levels of proinflammatory cytokines were present. In contrast to the absence of the T_H1 polarisation, absence of the T_H17 polarized immune response has no impact on the pathology.

Additionally to monitoring of neurological symptoms and analysis of serum cytokines, parasitaemia was determined on day 6 p.i. for IL-12p35$^{-/-}$ mice (**Figure 4-7 A**) and IL-17$^{-/-}$ mice (**Figure 4-7 B**), each in comparison to WT mice. In the IL-12p35$^{-/-}$ mice there was no difference in the parasitaemia in comparison to the WT mice. The lack of T_H1 responses has therefore no influence on the protective immunity against the parasite. Contrarily, the IL-17$^{-/-}$ mice showed an increased parasitaemia compared to WT mice. Thus, the physiological antiparasitic immune response is weakened by the absence of a T_H17 polarized immune response. In the IL-17$^{-/-}$ mice the cytokine is absent during ontogeny. To ensure, that potential compensatory mechanism formed during the development of the immune system may not interfere with the course of infection, the same experiment was conducted with mice that received a neutralizing antibody against IL-17. This procedure creates a transient absence of IL-17 mediated effects during the infection course. Control mice received unspecific rat IgG, and the parasitaemia was determined on day 6 p.i. (**Figure 4-7 C**). In mice where IL-17 was neutralized the parasitaemia was increased as well, verifying the result revealed in the infection of IL-17$^{-/-}$ mice.

4.2.2 The main population of IL-17 producing cells are γδ T cells

IL-17$^{-/-}$ mice displayed no difference in the pathology of cerebral malaria compared to WT mice, but the parasitaemia during infection with *P. berghei* ANKA was increased, revealing a role of IL-17 in the anti-parasite immune response. The difference in parasitaemia occurs on day 6 p.i.. Neither CD4$^+$ nor CD8$^+$ T cells are likely to be responsible for the difference in parasiteamia, as it has been shown that the parasiteamia is not significantly altered after depletion of either subset during *P. berghei* ANKA infection (Hermsen, C. et al. 1997). But since IL-17 is a T cell derived cytokine,

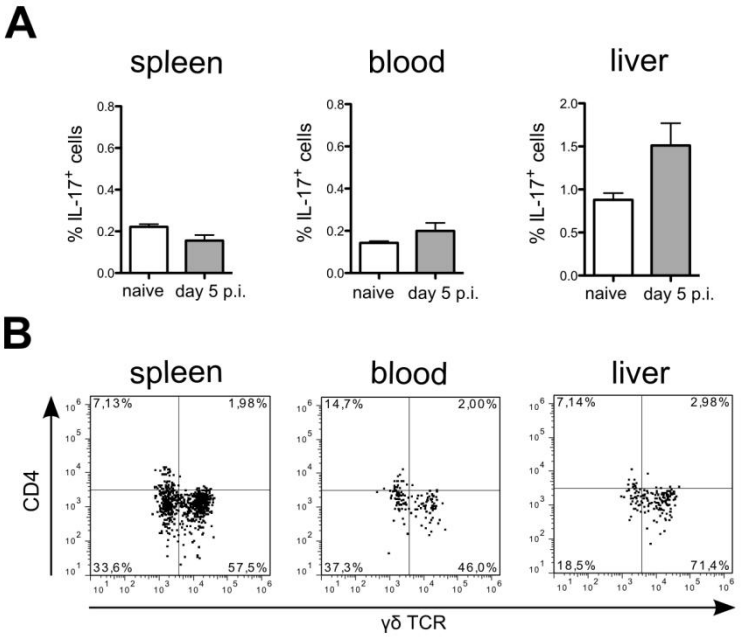

Figure 4-8: γδ T cells produce IL-17 during infection with P. berghei ANKA

Mice were infected with 2x 10^6 *P. berghei* ANKA parasitized red blood cells. On day 5 p.i. leukocytes from spleen, blood and liver were isolated. Cells from naïve mice served as a control. The cells were restimulated with PMA and Ionomycin in presence of Golgi-Stop for 4 hours. Afterwards the cells were stained with a PE labeled antibody against CD3, PerCp labeled antibodies against CD4 and CD8 and a FITC labeled antibody against the γδ TCR, followed by permeabilisation and intracellular staining with an APC labeled antibody against IL-17. Via flow cytometry the cells were analyzed first for IL-17 production and then for lineage affiliation of the IL-17 producing T cells. **A:** Percentage of IL-17 producing cells in Lymphocytes. **B:** Representative plot of the staining of γδ TCR and CD4 in IL-17 producing lymphocytes on day 5 post infection.

thus the most likely source besides conventional $CD4^+$ αβ T cells are non-conventional innate-like T cells like γδ T cells or NK T cells.

In order to identify of the source of IL-17 early in malaria infection the amount of IL-17 producing cells and their lineage affiliation was determined. Therefore leukocytes of uninfected mice and mice on day five p.i. were isolated from spleen, blood and liver and the percentage of IL-17 producing T cells in whole lymphocytes was compared (**Figure 4-8 A**). In the spleen the amount of IL-17 producing cells did not increase during infection and in blood only a marginal increase was found. Only in the liver the population of IL-17 producing cells expanded. Additionally to a staining for IL-17 the cells were stained for CD3, CD4, CD8 and the γδ TCR. $CD3^-$ IL-17 producing cells were not observed. The IL-17 producing T cells on day 5 p.i. were then analyzed for their lineage affiliation by plotting CD4 against the γδ TCR in the $CD3^+$ IL-17 producing population for spleen, blood and liver (**Figure 4-8 B**). Only a very low proportion of 7 % to 15 % of the IL-

17^{+} cells were conventional CD4^{+} T cells. IL-17 producing CD8^{+} T cells were not detectable. The largest fraction of 46 % to 72 % of the IL-17 producing cells were γδ T cells. The cells that were neither positive for CD4 nor for the γδ T cell receptor added up to 18 % to 37 % of the IL-17 producing T cells and were assumed to be NK T cells, as all other T cell lineages have been excluded by their specific markers. As expected, IL-17 production was assigned to the unconventional T cells and within these mainly to the γδ T cells.

Now that the γδ T cells were identified as mainly responsible for the IL-17 production the phenotype of increased parasitaemia in the IL-17$^{-/-}$ mice should be reproducible by removal of γδ T cells in WT mice. As there is no antibody available that reliably depletes γδ T cells mice were treated with a monoclonal antibody against the γδ TCR, the clone GL-3. This antibody strongly crosslinks the γδ T cell receptor, leading to hyperactivation followed by downregulation of the γδ TCR. This state is maintained for more than two weeks in which the γδ T cells are thought to be insensitive for further stimulation (Koenecke, C. et al. 2009). This was used as substitute for depletion of γδ T cells and mice receiving PBS were used as control. Efficacy of the antibody treatment was controlled by staining of the γδ TCR and subsequent flow cytometric analysis, revealing 0.39 % (±0.09 %) γδ TCR^{+} cells in blood lymphocytes of PBS treated mice and 0.07 % (± 0.03 %) γδ TCR^{+} cells in blood lymphocytes of antibody treated mice. One day after antibody treatment the mice were infected with *P. berghei* ANKA and the parasitaemia was determined on day 7 p.i. (**Figure 4-9**). Parasitaemia is significantly increased in mice receiving the anti γδ TCR antibody compared to mice receiving PBS, thus supporting the conclusion, that the IL-17 production of γδ T cells is playing a pivotal role in early anti-parasite defense.

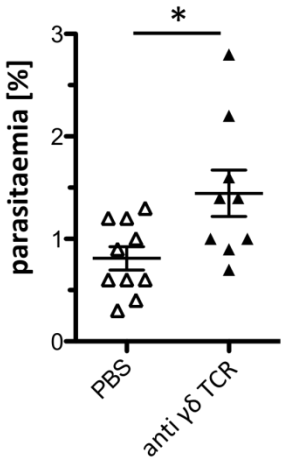

Figure 4-9: Deficiency of γδ T cells leads to increased parasitaemia

Mice receiving a depleting antibody against the γδ TCR (GL-3) and mice receiving PBS were infected i.p. with 2x 10^{6} *Plasmodium berghei* ANKA parasitized red blood cells. Parasitaemia was determined on day 7. Pooled results from two indepent experiments are shown. * $p<0.05$

4.2.3 IL-17 is preferentially produced by CD27$^-$ γδ T cells

Besides being the main producer of IL-17 the total number of γδ T cells increases upon infection. To specify this increase, mice were infected and on day 5 p.i. lymphocytes from spleen, blood and liver were stained for the γδ T cell receptor and analyzed via flow cytometry. The percentage of γδ T cells in total lymphocytes in the organs were compared to uninfected mice (**Figure 4-10 A**). In the spleen the induction of γδ T cells was almost undetectable, whereas in blood it was pronounced and the strongest increase was found in the liver.

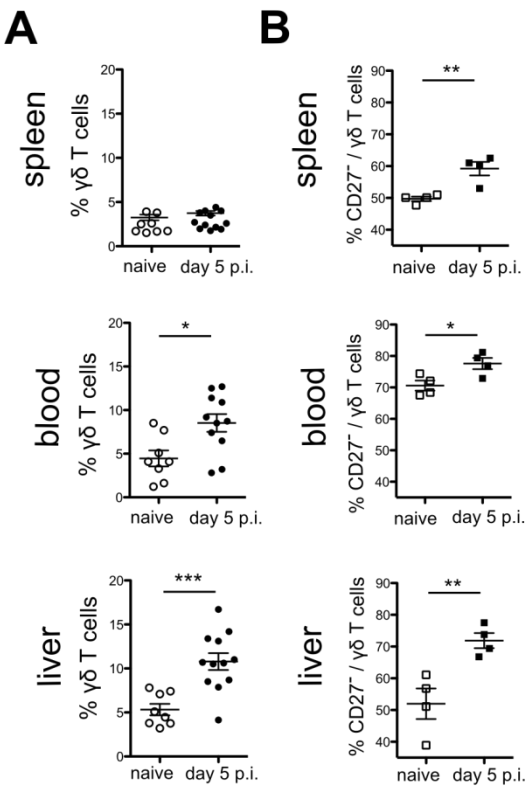

Figure 4-10: CD27$^-$ γδ T cells expand during infection with PbA

Mice were infected with *Plasmodium berghei* ANKA. On day 5 post infection leukocytes from spleen, blood and liver were isolated. Cells from uninfected mice served as a control. **A:** The cells were stained with a FITC labeled antibody against the γδ TCR and analyzed via flow cytometry for the percentage of γδ T cells in the lymphocyte population. Pooled results from three independent experiments are shown. **B:** The cells were stained with a FITC labeled antibody against the γδ TCR and a PE labeled antibody against CD27. They were analyzed via flow cytometry for the percentage of CD27$^-$ cells in the γδ T cell pool. * p<0.05; ** p<0.01; *** p<0.005

Several studies have shown that it is possible to discriminate between γδ T cells that produce IL-17 and those that produce IFNγ by certain surface markers. Hence, CD27$^+$ CCR6$^-$ γδ T cells rather produce IFNγ whereas CD27$^-$ CCR6$^+$ γδ T cells produce IL-17 (Haas, J.D. et al. 2009; Ribot, J.C. et al. 2009). If the IL-17 producing γδ T cells expand in the infection a simultaneous expansion of CD27$^-$ cells within the γδ T cells could be expected. To analyze this, leukocytes from uninfected mice and from mice on day 5 p.i. with *P. berghei* ANKA were taken from spleen, blood and liver and were stained for the γδ T cell receptor and CD27. They were analyzed via flow cytometry and compared for the percentage of CD27$^-$ cells in the γδ T cell pool (**Figure 4-10 B**). The expansion of CD27$^-$ γδ T cells was clearly detectable in all analyzed organs, and again the difference was most pronounced in the liver.

For a closer observation of the mechanisms that lead to the activation of CD27$^-$ γδ T cells an *in vitro* stimulation system was established. γδ T cells were sorted based on their expression of CD27 using a FACS Aria Cell sorter (**Figure 4-11 A**). CD27$^+$ and CD27$^-$ γδ T cells were separated to a purity of more than 90 % each. Mainly two TLRs are ligated during Malaria infection, TLR2 by the typical GPI anchor of *Plasmodium* parasites, and TLR9 by hemozoin, the metabolite originating from the digestion of hemoglobin (Coban, C. et al. 2005; Krishnegowda, G. et al. 2005). PamCys was chosen as the stimulating agent in this *in vitro* model system to mimic the activation of TLR2 by malaria GPI. For the assay BMDC were used as APC, cocultured with either CD27$^-$ or CD27$^+$ γδ T cells and stimulated with PamCys (**Figure 4-11 B**). After 48 hours the supernatants were tested for IL-17 (**Figure 4-11 C**) or IFNγ (**Figure 4-11 D**) via ELISA.

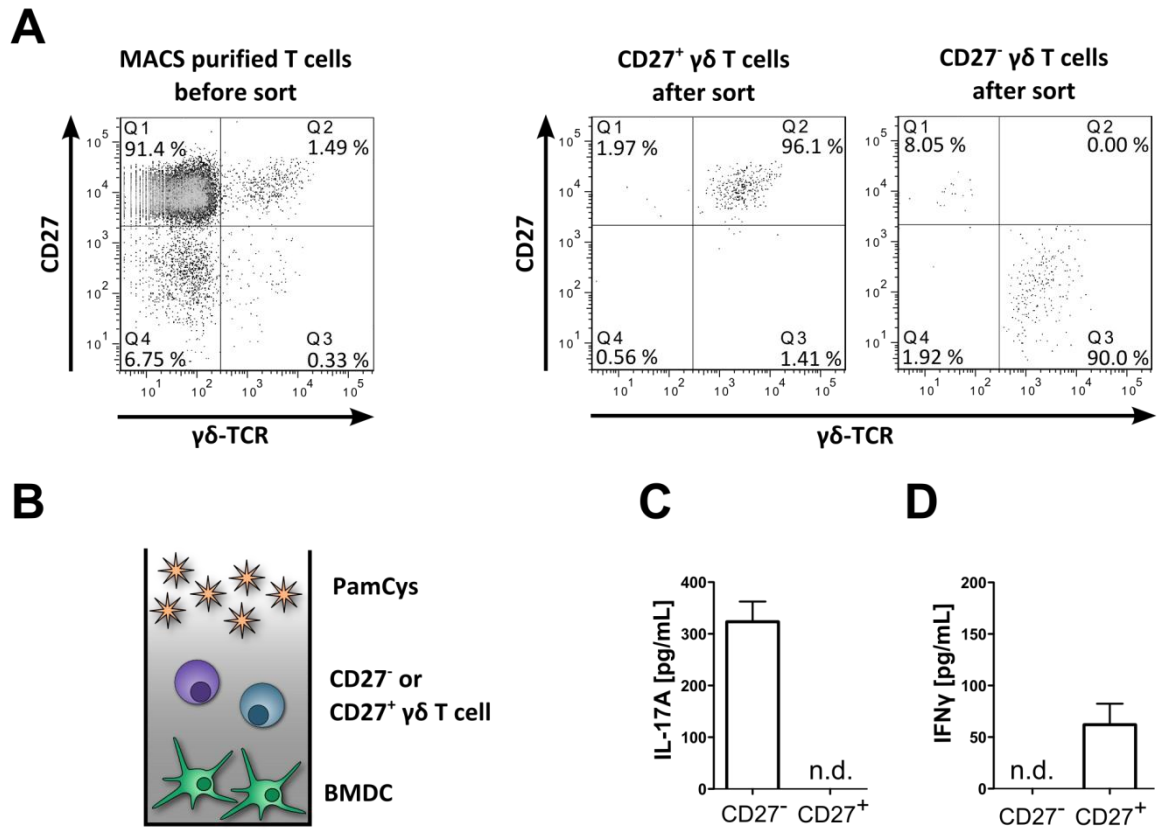

Figure 4-11: CD27$^-$ γδ T cells produce IL-17 in presence of DCs and the TLR2 stimulant PamCys

A: MACS purified T cells from spleen were stained with a FITC labeled antibody against the γδ TCR and a PE labeled antibody against CD27 and sorted for γδ T cells positive or negative for CD27 using the FACS Aria cell sorter. **B:** Experimental setup: CD27$^+$ or CD27$^-$ γδ T cells were cocultured with BMDCs and stimulated with PamCys. **C:** Supernatant of CD27$^-$ or CD27$^+$ γδ T cells, cocultured with WT DCs and stimulated with PamCys for 48 hours, was tested for IL-17 via ELISA. **D:** Supernatant of CD27$^-$ or CD27$^+$ γδ T cells, cocultured with WT DCs and stimulated with PamCys for 48 hours, was tested for IFNγ via ELISA.

Exclusively the CD27$^-$ γδ T cells produced IL-17, whereas only the CD27$^+$ γδ T cells produced IFNγ. This *in vitro* assay was then used to further analyze the activation of especially CD27$^-$ γδ T cells in the presence of APC by a TLR2 agonist, and their subsequent production of IL-17.

4.2.4 IL-17 production in CD27$^-$ γδ T cells is induced by IL-23 from TLR2-activated APC

It has been demonstrated by quantitative real time PCR that γδ T cells are able to express TLR2 (Mokuno, Y. et al. 2000). Therefore, the first question addressed using the *in vitro* assay was if TLR2 on the γδ T cell or on the antigen presenting cell is necessary for the induction of IL-17 in the γδ T cell. Either TLR2$^{-/-}$ BMDC were cocultured with WT CD27$^-$ γδ T cells or TLR2$^{-/-}$ γδ T cells were cocultured with WT BMDC (**Figure 4-12 A**).

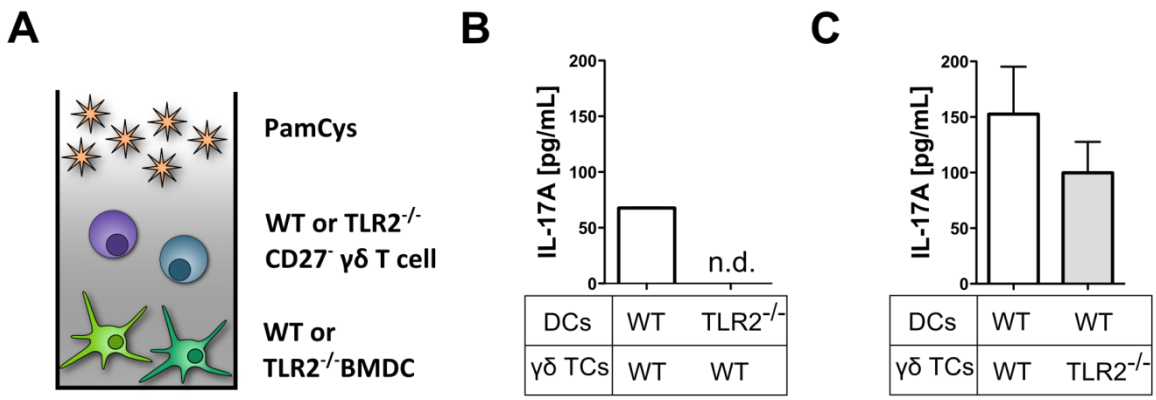

Figure 4-12: TLR2 on BMDCs but not on γδ T cells is necessary for IL-17 production of CD27$^-$ γδ T cells

A: WT or TLR2$^{-/-}$ CD27$^-$ γδ T cells were cocultured with TLR2$^{-/-}$ or WT DCs and stimulated wih PamCys. **B** Supernatant of CD27$^-$ γδ T cells, cocultured with WT or TLR2$^{-/-}$ DCs and stimulated with PamCys, was tested for IL-17 via ELISA. **C:** Supernatant of WT or TLR2$^{-/-}$ γδ T cells, cocultured with WT DCs and stimulated with PamCys, was tested for IL-17 via ELISA.

In the first experiment WT CD27$^-$ γδ T cells were cocultured either with WT or TLR2$^{-/-}$ BMDC, stimulated with PamCys and the concentration of IL-17 in the supernatants were quantified (**Figure 4-12 B**). After coculture of CD27$^-$ γδ T cells with WT BMDC IL-17 was detectable in the supernatant, whereas it was absent in the supernatant after coculture with TLR2$^{-/-}$ BMDCs. In the second experiment WT BMDC were cocultured either with WT or TLR2$^{-/-}$ γδ T cells, stimulated with PamCys and the concentration of IL-17 in the

supernatant was determined (**Figure 4-12 C**). In this setting WT as well as TLR2$^{-/-}$ γδ T cells did produce IL-17. The amount produced by TLR2$^{-/-}$ γδ T cells was slightly reduced compared to WT γδ T cells, which might suggest, that TLR2 on the CD27^{-} γδ T cells contributes to the IL-17 production but is not sufficient for the induction without additional signals from activated APC.

After it was shown that the IL-17 production is induced by a factor produced by the BMDC, IL-23 was the most likely candidate to mediate this effect. It was already identified to play a role in inducing IL-17 in γδ T cells even without ligation of the γδ T cell receptor (Sutton, C.E. et al. 2009). Therefore quantitative real time PCR was performed to analyze, if an expression of IL-23 can be observed in the *in vitro* assay as well as in the malaria infection.

To analyze the in vitro situation BMDC were stimulated with PamCys for six hours. Control cells were cultured in the same medium without stimulation. The cells were harvested, mRNA was isolated, transcribed into cDNA and the upregulation of IL-23p19 mRNA was analyzed (**Figure 4-13 A**). Stimulation of TLR2 by PamCys led to a pronounced upregulation of the IL-23p19 gene already after 6 hours.

For *in vivo* analysis the IL-23p19 mRNA in spleen and liver was determined. Mice were infected with *P. berghei* ANKA pRBC. Mice that received PBS injection instead of pRBC served as control. 12 and 24 hours p.i. spleen and liver were taken and mRNA from whole organs was isolated. The mRNA was transcribed into cDNA and upregulation of IL-23p19 gene expression was analyzed.

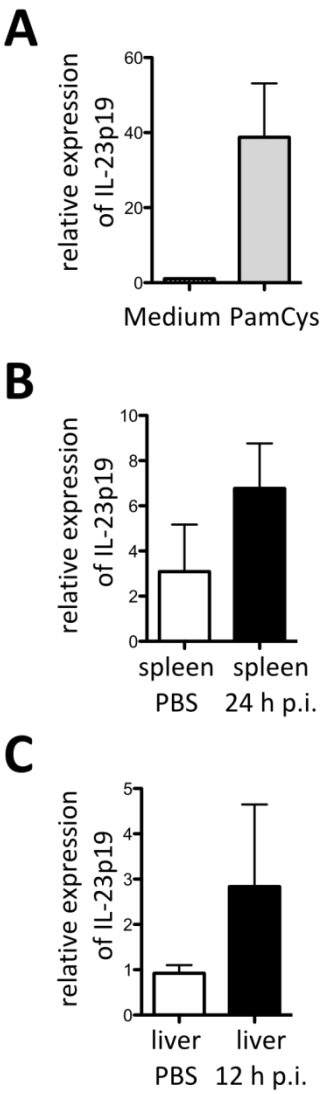

Figure 4-13: IL-23 is produced by APC *in vitro* and *in vivo*

A: WT DCs were stimulated with PamCys for six hours. Afterwards the mRNA was isolated from these cells and transcribed into cDNA. **B,C:** Mice were infected with 2x 10^6 *P. berghei* ANKA parasitized red blood cells. Mice that received PBS injection instead of pRBCS served as control. **B:** 24 hours post infection mRNA from whole spleen was purified and translated into cDNA. **C:** 12 hours post infection mRNA from liver was purified and translated into cDNA.
A-C: Upregulation of IL-23p19 gene expression was analyzed via quantitative real-time PCR.

For spleen the upregulation of the IL-23p19 transcript was detectable 24 hours p.i. (**Figure 4-13 B**). In the liver already 12 hours p.i. an upregulation of the IL-12p19 mRNA was detectable (**Figure 4-13 C**). The detected IL-23 mRNA expression was only small as it was determined in whole organs and not on a cellular level. Furthermore, the difference between infected and PBS injected mice was slightly more pronounced in the liver than in the spleen.

Now that it has been shown that IL-23 is expressed by BMDC after stimulation of TLR2 the question arouse, if TLR2 deficiency would alter the IL-23 production by BMDC upon stimulation with *P. berghei* ANKA pRBC. To investigate this, pRBC were purified fromblood of infected mice, taking advantage of the paramagnetic properties of the accumulated hemozoin. The disadvantage of this method is that only in late stages of the

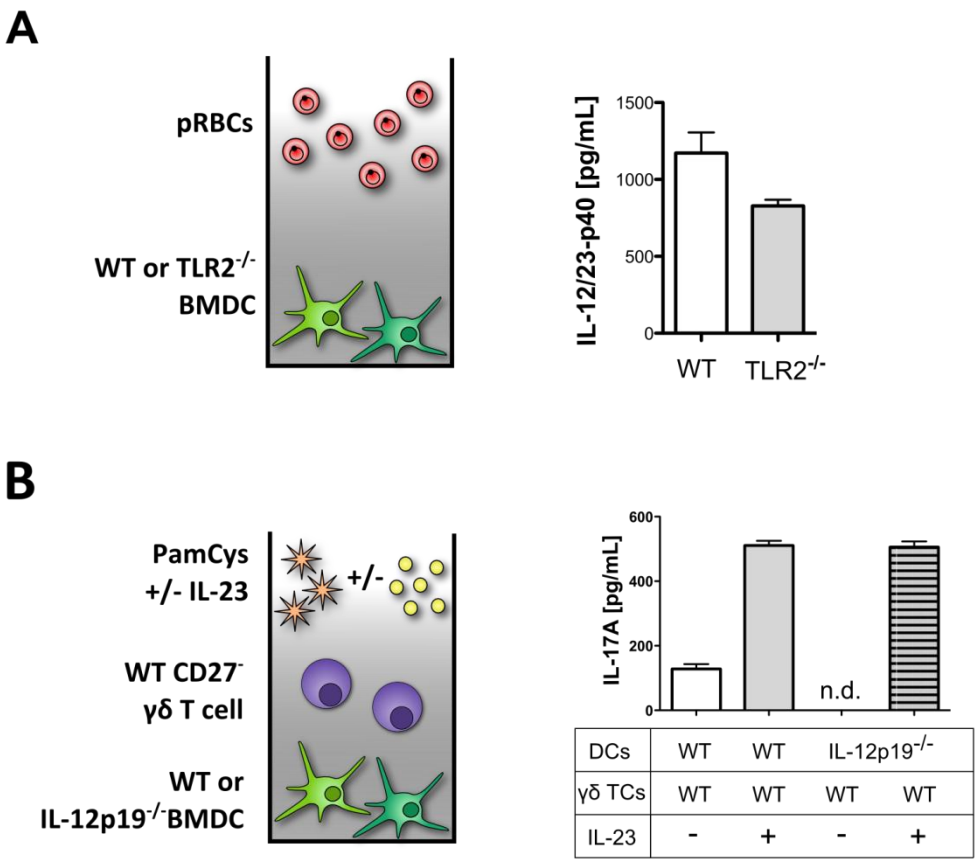

Figure 4-14: CD27$^-$ γδ T cells produce IL-17 upon stimulation with IL-23

B: Parastised red blood cells were purified from whole blood of infected mice using MACS magnetic separation. WT or TLR2$^{-/-}$ DCs were stimulated with pRBCs for 48 hours. The supernatant was tested for IL-12/23-p40 via ELISA.
A: CD27$^-$ γδ T cells were cocultured with WT or IL-23p19 BMDC in presence or absence of recombinant IL-23 and stimulated with PamCys. After 48 hours the supernatant of the cultures tested for IL-17 via ELISA.

erythrocytic life cycle of the parasite enough hemeozoin is accumulated to render them paramagnetic. Earlier stages, especially young ring stages are lost during purification. WT and TLR2$^{-/-}$ BMDC were coincubated with the pRBC and the supernatants were tested for IL-12/23p40 via ELISA (**Figure 4-14 A**). Both cell types produce the protein, whereby the TLR2$^{-/-}$ BMDC produce less. Probably only a part of the detected IL-12/23p40 is bound in IL-23 though, as it is the large subunit for IL-12 as well, and both cytokines might be produced by the BMDC on encounter with th pRBC. Additionally, pRBC are able to stimulate other receptors beyond TLR2 in the APC, but absence of TLR2 already reduces the induction of IL-12/23p40 slightly.

It remains to be clarified, if the production of IL-23 induced by ligation of TLR2 is responsible for the induction of IL-17 in γδ T cells. To further verify this, CD27^{-} γδ T cells were cocultured with either WT or IL-23p19 deficient BMDC and stimulated with PamCys in the presence or absence of recombinant IL-23. The supernatants of the cultures were tested for IL-17 via ELISA (**Figure 4-14 B**). In coculture with the WT BMDC the γδ T cells produce IL-17, which is even strongly enhanced if recombinant IL-23 is added to the culture. In coculture with the IL-23p19$^{-/-}$ BMDC no IL-17 production by the γδ T cells is detectable. This can be rescued by addition of recombinant IL-23 leading to an IL-17 concentration comparable to the coculture of γδ T cells with WT BMDC and additional IL-23. Thus, the IL-17 production by γδ T cells is induced by IL-23 that originates from antigen presenting cells. The ligation of TLR2 by PAMPs of *Plasmodium spp.* is an important stimulus of APC leading to their production of IL-23 during early infection with *P. berghei* ANKA.

4.2.5 The role of IL-17F in IL-17A deficiency during ECM

When IL-17 was mentioned up to this point, it was always in reference to IL-17A. The IL-17 family includes besides IL-17A, which was the first identified member, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. IL-17A binds to the IL-17RA and has been widely investigated for its role in inflammation. IL-17F is the closest relative to IL-17A and binds with high affinity to IL-17RC, but additionally with weak affinity to IL-17RA. It is even able to build a heterodimer with IL-17A (Liang, S.C. et al. 2007). Some studies now claim a possible compensatory or counteractive role of IL-17F in IL-17A deficient mice in different models of disease (Ishigame, H. et al. 2009).

To investigate a possible influence of IL-17F in the infection with *P. berghei* ANKA, IL 17A deficient mice and WT control mice were infected with pRBC. On day 5 p.i. cells from spleen, liver and blood were restimulated with PMA and Ionomycin, stained extracellularly for the γδ TCR and intracellularly for IL-17F. The percentage of IL-17F producing cells in the γδ T cell pool was compared (**Figure 4-15 A**). The amount of γδ T

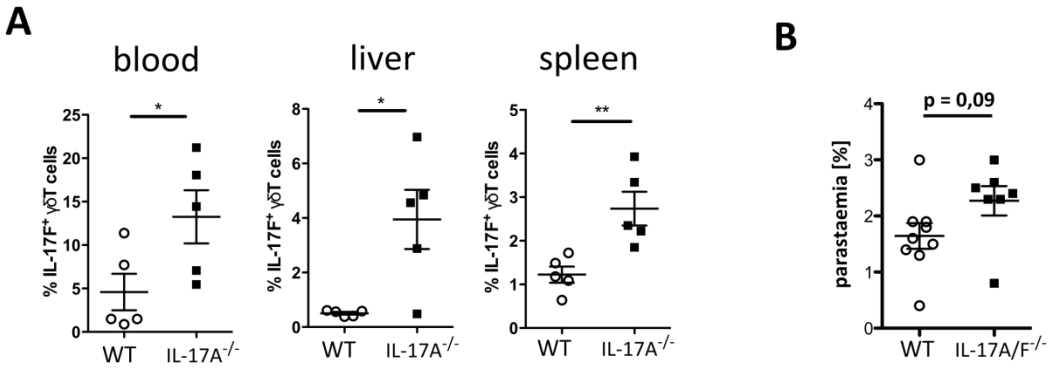

Figure 4-15: γδ T cells of IL-17 deficient mice produce higher amounts of IL-17F during infection with PbA

A: WT and IL-17A$^{-/-}$mice were infected i.p. with 2x 10^6 *P. berghei* ANKA parasitized red blood cells. On day 5 p.i. leukocytes from spleen, blood and liver were isolated. The cells were restimulated with PMA and Ionomycin for 4 hours. Afterwards they were first stained with a FITC labeled antibody against the γδ TCR and then permeabilised and stained intracellularly with a PE labeled antibody against IL-17F. The cells were analyzed for their IL-17F expression via flow cytomtery. **B:** WT and IL-17A$^{-/-}$IL-17F$^{-/-}$mice were infected i.p. with 2x 10^6 *P. berghei* ANKA parasitized red blood cells. Parasitaemia was determined on day 8. Results are pooled of two independent experiments. * $p<0.05$; ** $p<0.01$

cells producing IL-17F in the IL-17A$^{-/-}$ mice was increased in all analyzed organs. This strong compensatory upregulation of IL-17F upon absence of IL-17A might lead to a ligation of IL-17RA despite the weaker affinity of IL-17F compared to IL-17A. Therefore, in addition to mice deficient for IL-17A, mice double deficient for IL-17A and IL-17F were investigated. These, and WT mice as a control, were infected with *P. berghei* ANKA and the parasitaemia was determined on day 8 p.i. (**Figure 4-15 B**). The parasitaemia was higher in the IL-17A$^{-/-}$IL-17F$^{-/-}$ mice without reaching statistical significance. It can be concluded that the compensatory upregulation of IL-17F has no effect on the parasitaemia, because if a ligation of IL-17RA by compensatory upregulated IL-17F in the IL-17 A$^{-/-}$ mice would occur, the elevated parasitaemia, as an effect of IL-17 deficiency, should be more pronounced in mice deficient for both cytokines.

4.2.6 Neutrophils are not the mediators of IL-17 induced effects on parasitaemia during ECM

The pathway leading to IL-17 production during ECM is started by the production of IL-23 by APC. But IL-17 itself as a cytokine cannot directly act against the parasite. Neutrophils were demonstrated to be attracted and activated by IL-17 (Miyamoto, M. et al. 2003). Furthermore, they can produce effector molecules like reactive oxygen species that might be able to harm the parasite even in the erythrocyte. Therefore neutrophils as possible effector cells were investigated.

First the kinetic of neutrophil number early in infection was analyzed before and after the timepoint that correlates to the observed effect of increased parasitaemia in the absence of IL-17. For this, mice were infected with *P. berghei* ANKA pRBC and uninfected mice served as control. On day 5 p.i. and on day 8 p.i. leukocytes from blood, liver and spleen were taken. The cells were stained with antibodies against CD11b and GR-1, analyzed via flow cytometry and the number of neutrophils, identified as $CD11b^{+}$ and $GR\text{-}1^{high}$, was determined (**Figure 4-16**). In blood about 2.5 % of the leukocytes are neutrophils, which expand to up to 7 % to 12 % on day 5 p.i. and contract afterwards to around 5 % on day 8 p.i.. In the liver the percentages of neutrophils are around 2.5 % in the naïve mouse, up to 10 % on day 5 and 2 % to 2.5 % on day 8 p.i.. In spleen the numbers of neutrophils are smaller, only 0.5 % of the leukocytes are neutrophils in the

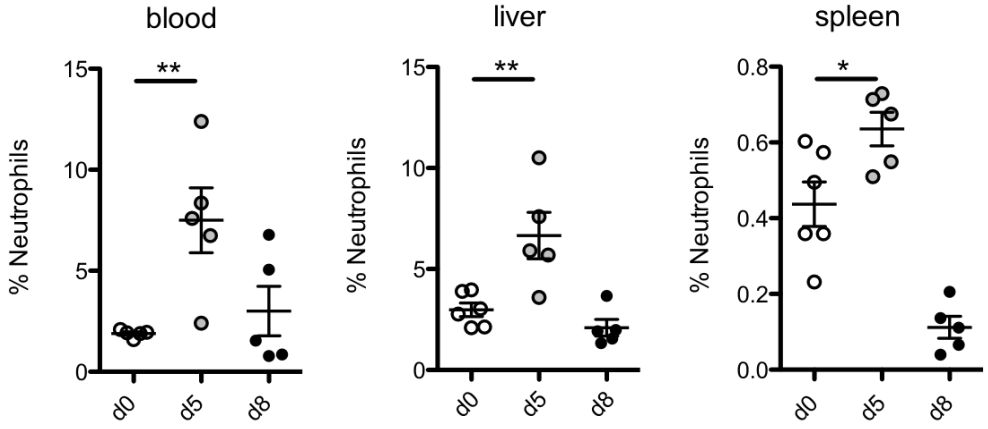

Figure 4-16: Neutrophils expand transiently after infection with *Plasmodium berghei* ANKA

Mice were infected with *Plasmodium berghei* ANKA. From uninfected mice and on day 5 and 8 post infection leukocytes from blood, spleen and liver were isolated and stained with an FITC labeled antibody against CD11b and a PE labeled antibody against GR-1. The percentage of neutrophils in leukocytes of blood, liver and spleen was analyzed via flow cytometry. * $p<0.05$; ** $p<0.01$

uninfected mouse. But they expand as well to 0.7 % to 0.8 % on day five p.i. and contract even more to 0.1 % on day 8 p.i.. In all organs a transient increase on day 5 p. i. was detected, which concurs with the increase of IL-17 producing γδ T cells on day 5. This result can be due to an induction and activation of neutrophils by IL-17 originating from γδ T cells early in *P. berghei* ANKA infection.

If neutrophils are mediating the anti-parasitic effect induced by IL-17, these effect should be lost in the absence of neutrophils. The parasitaemia would be increased, comparable to the effect seen in IL-17 deficient mice. To deplete neutrophils mice were injected with a depleting antibody against GR-1. This antibody does not only deplete neutrophils but all cells with a high expression of GR-1, which is true also for some monocyte subsets. Mice that received PBS injection served as a control. Efficacy of the antibody treatment was controlled by staining of CD11 and GR-1 and subsequent flow cytometric analysis, revealing 65.1 % (±13.8 %) Neutrophils in blood granulocytes of PBS treated mice and 0.08 % (± 011 %) Neutrophils in blood granulocytes of antibody treated mice. The mice were infected with *P. berghei* ANKA pRBC and the parasitaemia was determined on day seven p. i. (**Figure 4-17 A**). There was no difference in the

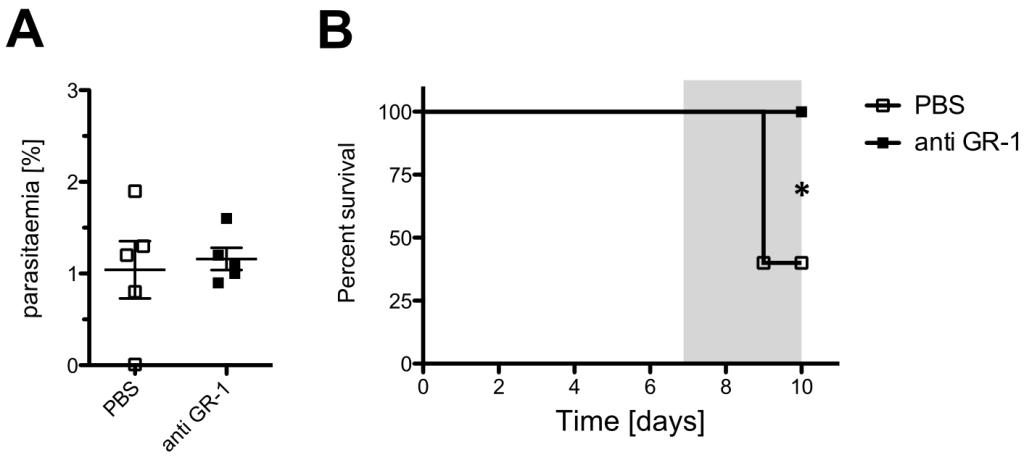

Figure 4-17: Neutrophils are not the source of IL-17 induced effects in the infection with P. berghei ANKA

Mice receiving a depleting antibody against GR-1 and mice receiving PBS one day before infection were infected with *Plasmodium berghei* ANKA. **B:** Parasitaemia was determined on day 7 **C:** Onset of cerebral malaria was monitored until day 10.* $p<0.05$.

parasitaemia detectable, thus neither neutrophils nor one of the other GR-1 high expressing cell subsets that were depleted by the antibody mediate the IL-17 induced effect on the parasitaemia. Additionally to the determination of the parasitaemia the mice were monitored for neurological symptoms until day ten (**Figure 4-17 B**). As has been demonstrated before, using a different antibody (Chen, L. et al. 2000), no neurological impairment in the mice that received the depleting antibody against GR-1 was observed. Thus it can be concluded that the GR-1 expressing cells do not contribute to the protection but to immune pathology.

Although it could be shown, that IL-17 from γδ T cells, induced by IL-23 from APC, plays a role in the protective immune response against *P. berghei* ANKA. Neutrophils are not the mediators of this effect.

5 Discussion

This thesis aims for further investigation of the interplay of the IL-12 family cytokines IL-35 and IL-23 during $CD4^+$ T cell polarization and their influence on the mainly IL-12 driven proinflammatory immune response during ECM.

Therefore a fusion molecule of the recently discovered regulatory cytokine IL-35 with the Fc domain of human IgG1 was produced to investigate regulation of T_H17 responses and activation of regulatory T cells during ECM. Proinflammatory responses were investigated regarding IL-17 production, to study the effect of IL-23 and its downstream mechanisms. Hereby the effect of IL-23 on IL-17 production by non-conventional T cells was included. The role of T cells activated by IL-23 was compared to the activation of T_H1 cells by IL-12.

5.1 Regulation of IL-17 by IL-35 during experimental cerebral malaria

To investigate the role of a protein under certain circumstances, either loss or excess of this protein is created and compared to the physiological situation. In the ECM model loss of a protein could be created by a gene deficiency of the mice. This is not applicable on IL-35, as the subunits of this cytokine are shared with other cytokines of the IL-12 family (Devergne, O. et al. 1997). Knockout of either subunit would result in deficiency for at least two cytokines. No specific antibody for IL-35 was commercially available, which could have been used to neutralize the cytokine. Therefore the role of IL-35 was investigated by creating an excess of the cytokine. Previous studies have used two approaches to achieve an excess of IL-35. One was a recombinant single chain protein that can be purified and used *in vitro* and *in vivo* (Niedbala, W. et al. 2007). The second was a retroviral vector which was used to transfect cells to produce single chain IL-35 (Collison, L.W. et al. 2007). This retroviral transduction was used for cell culture as well as for gene therapeutic approaches in mice (Collison, L.W. et al. 2010). None of the tools were commercially available, so the generation of a recombinant IL-35 protein was required. Here the approach of a purified single chain IL-35 linked to the Fc domain of human IgG1 protein was established. The advantage of a fusion molecule with the Fc domain of IgG is the easy detection by an antibody against the human IgG1 and the easy purification via protein G Sepharose. Furthermore it can be used at a defined

concentration *in vitro* and *in vivo*, where the IL-35 overexpression induced by viral transduction would be more variable. *In vivo* the Fc part stabilizes the fusion molecule and reduces degradation (Jazayeri, J.A. and Carroll, G.J. 2008).

The fusion molecule was cloned into the expression vector pFuse-hIgG1-Fc2. This vector already contains a slightly modified Fc domain of the human IgG1 and the signal sequence of human IL-2, which ensures that the expressed molecule is secreted into the supernatant by transfected cells. The successful construction of the vector was verified via restriction and subsequent agarose gel analysis.

The generated expression vector was used to transfect CHO cells. These eukaryotic cells are able to perform most of the posttranslational modifications that bacteria are not capable of, such as glycosylation. Additionally, the level of endotoxin contamination is lowered and therefore artificial activation of immune cells by pyrogenicity is avoided. The transient transfection of CHO cells led only to low transfection efficiencies of 20 % – 25 % of the cells producing the IL-35-Fc fusion molecule as shown by intracellular staining of the lgG part of the molecule and subsequent flow cytometric analysis. Therefore stable transfectants of CHO cells producing the desired molecule were created in the context of the master thesis of Aline Taenzer (Taenzer, A. 2010). Intracellular staining of the human lgG and flow cytometric analysis showed that in the generated cell line more than 90% of the cells produced the fusion molecule. As this was stable even without selective pressure, this cell line was used to produce the fusion molecule.

In the next step it was verified that the fully transcribed fusion molecule was secreted into the supernatant, from where it should be purified. Therefore, the supernatant of the transfectants was applied in western blot analysis and the IgG1 as well as the EBI3 part of the molecule were specifically stained. As the medium contains FCS the signal on both blots is located in a very protein rich region of the gel in which many other abundant serum proteins, for example the 67 kDa sized BSA, would be expected. The high protein density could lead to an irregular distribution of the protein in this part of the gel, what might explain the unevenness and curved appearance of the signals on both blots. Additionally, the signal is only very weak after detection with the antibody against the IgG1, what might be due to different detection methods. The antibody against IgG1 is directly labeled with HRP. The monoclonal antibody against EBl3 is not labeled and has therefore to be detected with a HRP labeled secondary antibody. This two step detection

leads to an enhancement of the signal provided by the avidity of the secondary antibody.

The purification of the fusion molecule was accomplished with one liter cell culture supernatant. The purification yielded about 1 mg protein. The purified protein was analyzed via SDS page and subsequent western blot or silver staining. The western blot showed an additional band at a size of about 30 kDa, which would correspond to the size of the Fc domain and might be a degradation product.

After it was demonstrated that the newly synthesized IL-35-Fc fusion molecule was expressed and could be purified, it needed to be verified that it displays the functionality of endogenous murine IL-35. It has been shown that IL-35 has the capability to specifically regulate IL-17 production by T_H17 cells during *in vitro* differentiation (Niedbala, W. et al. 2007). Therefore the recombinant IL-35-Fc was tested in an *in vitro* T_H17 differentiation assay. Addition of IL-35 reduced the number IL-17 producing cells among the $CD4^+$ T cells by 40 %. The concentration of IL-17 in the supernatants of the cells was reduced from about 5500 pg/mL to about 2000 pg/mL after addition of IL-35-Fc. This reduction was comparable to the reduction of T_H17 cells demonstrated in other studies (Niedbala, W. et al. 2007). This indicates that the recombinant IL-35-Fc indeed reduces the number of IL-17 producing cells during *in vitro* T_H17 differentiation, which in turn leads to less IL-17 in the supernatant.

Use of a recombinant IL-35-Fc fusion molecule has been shown to attenuate collagen induced arthritis through induction of regulatory T cells that produce IL-10 (Kochetkova, I. et al. 2010; Niedbala, W. et al. 2007). These T cells suppress IL-17 production and can be induced *in vitro*, exerting their regulatory function in different models of severe immunopatholgy in $FoxP3^{-/-}$ mice, EAE and IBD (Collison, L.W. et al. 2010). The models described have in common that a contribution of T_H17 cells has been demonstrated (Komiyama, Y. et al. 2006; Yen, D. et al. 2006). This led to the question, if the regulatory effect of IL-35 is able to affect the pathological proinflammatory immune response that causes the severe pathology of cerebral malaria. To this end the mouse model of ECM was employed. There was no difference in either pathology or parasitaemia between mice receiving recombinant IL-35-Fc and mice receiving PBS injections. It has been demonstrated, in this thesis as well as in other studies, that IL-17 does not contribute to ECM (Ishida, H. et al.). The role of regulatory T cells has been investigated during ECM as well, showing that depletion of them has little influence on

the course of disease (Steeg, C. et al. 2009). Other studies have shown that blockade of CTLA-4 exacerbates ECM and *in vivo* expanded regulatory T cells mediate protection via CTLA4 and not IL-10 (Haque, A. et al.; Jacobs, T. et al. 2002). These studies might explain the lack of influence of IL-35 on the pathology of ECM, as it acts through induction of IL-10 and suppression of IL-17.

In many infectious diseases the activation of proinflammatory immune responses is inevitable for recovery as well as source of bystander tissue injury. Besides for severe malaria this has been shown for many viral infections as well as for tuberculosis and chagas disease (Dorhoi, A. et al.; DosReis, G.A. et al. 2005; Wack, A. et al.). Up to now IL-35 has been mostly investigated in models of autoimmune pathology. It might be worthwhile to study the role of IL-35 in models of T cell mediated pathology, which could benefit from IL-10 producing regulatory T cells and regulation of IL-17. IL-35, which has been demonstrated in this thesis to have no influence on the proinflammatory immune response leading to ECM, might regulate pathologic, IL-17 mediated, immune responses without inhibiting protection.

5.2 Induction of IL-17 by IL-23 during experimental cerebral malaria

To further unravel the role of differential T cell activation the second part of the thesis focused on the function of the proinflammatory cytokine IL-23. As the other IL-12 family members it consists of two subunits, of which it shares one with the cytokine IL-12. They both use the large subunit p40 but differ in their small subunit, which is p35 in IL-12 and p19 in IL-23 (Oppmann, B. et al. 2000). Although both are proinflammatory cytokines they have different effects on $CD4^+$ T cell polarization. IL-12 has been shown to be inevitable for the mounting of an effective T_H1 response (Macatonia, S.E. et al. 1995). The role of IL-23 has been thoroughly investigated in different models of autoimmunity and was identified to induce the pathology exerting T_H17 cells in EAE (Chen, Y. et al. 2006; Cua, D.J. et al. 2003). Despite this role in pathology T_H17 cells and the cytokine IL-17 play an important physiological part in the control of some bacterial infections like in infections with *M tuberculosis* (Khader, S.A. et al. 2005).

In the first experiment mice lacking T_H17 polarized immune responses and mice lacking T_H1 polarized immune responses were analyzed and compared to wildtype C57Bl/6 mice in the model of ECM. However, while during the studies on IL-35 the infection was

evoked by injection of sporozoites directly into the bloodstream, in the second part of the thesis mice were infected with pRBC intraperitoneally. Hereby the asymptomatic liver stage, where the high multiplication rates can be the source of high variability in the number of parasites that enter the blood stage, is skipped. The stability of the infection course is improved and it begins directly with the blood stage, where the immune responses and the immune pathology take place (Hafalla, J.C. et al. 2011). To study the immune response without T_H1 polarization IL-12p35 deficient mice were infected. IL-17 deficient mice were used for the observations of the infection course in absence of T_H17 polarization. Three main parameters were analyzed in the infection: the development of neurological defects, the concentration of cytokines in serum of the mice and the parasitaemia at an early timepoint.

The development of neurological defects and death before day 10 of infection show the severity of pathological immune responses. The incidence of neurological complications in both knock-out mice strains was compared to wildtype mice. Only 10 % of the IL-12p35$^{-/-}$ mice developed symptoms whereas in the wildtype mice more than 50 % of the mice died from cerebral malaria. In contrast the IL-17$^{-/-}$ mice developed ECM to a similar extent as the wildtype mice. This clearly shows, that a T_H1 polarized immune response contributes strongly to the pathology, whereas lack of the T_H17 polarized immune response does not improve the course of disease. This result is reflected in the amount of cytokines detected in the serum. In the IL-12p35$^{-/-}$ mice the proinflammatory cytokines IFNγ and TNFα are virtually absent, showing that the absence of IL-12 leads to lack of their activation. This is in line with results, which demonstrated that absence of IFNγ signaling leads to protection against pathology (Amani, V. et al. 2000). Furthermore, it has been demonstrated that resistant BALB/c mice succumb to ECM after strong induction of IL-12, accompanied by high amounts of IFNγ (Schmidt, K.E. et al. 2011). However, contradictory results have been shown with mice that were as well deficient for IL-12p35, but created using a different method. In these IL-12p35 deficient mice susceptibility for ECM was sustained. This cannot yet be explained, as mice deficient for the IL-12Rβ2 were protected (Fauconnier, M. et al. 2012). In IL-17$^{-/-}$ mice the proinflammatory cytokine production is as pronounced as in the C57Bl/6 wildtype mice. The incidence of cerebral malaria is as high as in the control mice and they show a comparable amount of proinflammatory cytokines in serum. Thus, it can be concluded, that the T_H17 polarized helper T cells do not influence the immune response that leads

to pathology. Susceptibility of IL-17$^{-/-}$ mice to ECM was reported by others, as well (Ishida, H. et al. 2010).

The parasitaemia, on the other hand, is not connected to the pathology but might show if a protective immune response is mounted against the infection. The analysis of the parasitaemia led to results divergent to the analysis of neurological impairment. There was no difference in parasitaemia between wildtype mice and mice deficient for IL-12p35. Thus, in spite of being responsible for the pathology, T cells activated by IL-12 have no influence on anti-parasite immunity during the early phase of infection. But IL-17$^{-/-}$ mice showed an increase in parasitaemia compared to C57Bl/6 wildtype mice. Therefore the conclusion could be drawn, that a release of IL-17 early during infection, which is absent in the IL-17$^{-/-}$ mice, contributes to anti-malarial immunity. This experiment was repeated with mice receiving a neutralizing antibody against IL-17 instead of IL-17 deficient mice. In these mice the potential formation of a compensatory mechanism during the development of the immune system could not occur, as IL-17 mediated effects are only absent during the infection course. In mice where IL-17 was neutralized the parasitaemia was increased as well. This verified the result revealed during the infection of IL-17$^{-/-}$ mice.

The cytokine IL-17 has been associated to release of antimicrobial substances from tissues and to neutrophil activation (Iwakura, Y. et al. 2008; Miyamoto, M. et al. 2003). *Plasmodium spp*, blood borne obligate intracellular parasites, would be expected to be controlled by NK cells and macrophages, activated by a cellular immune response, not by effectors induced by IL-17 (Hafalla, J.C. et al. 2011). Therefore the result of a contribution of IL-17 to anti-malarial immunity was unexpected. The induction of IL-17 was then investigated for triggers and downstream effectors.

The difference in parasitaemia, caused by IL-17 deficiency, occurred as early as on day 6 after infection. IL-17 has been investigated thoroughly as a cytokine produced by T_H17 cells. The differentiated CD4$^+$ T cells are effectors of the adaptive immune system, which do not contribute to defense in *P. berghei* infection, as their absence does not significantly alter the parasitaemia (Hermsen, C. et al. 1997). Nevertheless, IL-17 is still a cytokine produced mainly by T cells (Gaffen, S.L. 2008). Thus, likely sources of IL-17 besides conventional CD4$^+$ $\alpha\beta$ T cells are non-conventional T cells like $\gamma\delta$ T cells or NK T cells, as both have been demonstrated to be capable of IL-17 production (Caccamo,

N. et al. 2011; Lockhart, E. et al. 2006; Rachitskaya, A.V. et al. 2008). To analyse IL-17 producing cells in early *P. berghei* ANKA infection their amount and their lineage affiliation was determined in spleen, blood and liver. The percentage of IL-17 producing cells in spleen and blood expanded only marginally, but a significant increase was measurable in the liver. To determine the lineage affiliation the cells were stained for CD3, CD8, CD4 and the γδ TCR. IL-17 producing $CD8^+$ T cells were not observed. The IL-17^+ cells were mostly γδ T cells. Conventional $CD4^+$ T_H17 cells, were only very few. The cells that were neither positive for CD4 nor for the γδ T cell receptor were assumed to be NK T cells, as all other T cell lineages have been excluded by their specific markers.

When the lack of IL-17 production by γδ T cells is responsible for the increased parasitaemia in the IL-$17^{-/-}$ mice the removal of γδ T cells in C57Bl/6 mice should lead to increased parasitaemia as well. Unfortunately there is no antibody available that reliably deplets γδ T cells in mice. Instead the mice were treated with a monoclonal antibody against the γδ TCR. This antibody induces strong crosslinking of the γδ TCR, leading to hyperactivation, followed by downregulation of the γδ TCR. This state is maintained for more than two weeks in which the γδ T cells are thought to be insensitive for further stimulation (Koenecke, C. et al. 2009). Therefore this method was used as a substitute for depletion of γδ T cells. After treatment with the antibody, *P. berghei* ANKA infected mice developed significantly increased parasitaemia compared to mice that received PBS. This leads to the conclusion that γδ T cells are the most important source of protective IL-17 during infection with *P. berghei* ANKA.

On day 5 p.i. the percentage of γδ T cells increases in blood, liver and less pronounced in spleen. Since it was demonstrated that the IL-17 producing γδ T cells are $CD27^-$ and $CCR6^+$ and the IFNγ producing γδ T cells are $CD27^+$ and $CCR6^-$ these surface markers can be used to distinguish between the two populations (Ribot, J.C. et al. 2009). In the next experiment CD27 was used to resolve the question if and how much the numbers of IL-17 producing γδ T cells increase within the γδ T cell pool. It could be shown, that the percentage of $CD27^-$ γδ T cells was higher on day 5 p.i. than in the naïve state. This was again most pronounced in the liver. Together the two experiments show that not only the overall number of γδ T cells expands but that within them the proportion of IL-17 producing cells increases. Notably the expansion of IL-17 producing cells and especially γδ T cells was in all experiments most pronounced in the liver. Research on malaria very

seldomly concentrates on the liver beyond analysis of the liver stage. But some studies demonstrated already, that the liver is important for the immune response against malaria blood stage. Liver specific macrophages play an important role in the uptake of pRBC in defense against *P. yoelii* infection (Lee, S.H. et al. 1986). Adoptive transfer of cells isolated from liver can even mediate protection against *P. chabaudi* blood stage infection (Balmer, P. et al. 2000). In other infections the important role of liver derived non conventional T cells was demonstrated, like the role of γδ T cells in defense against *T. cruzi*, and especially IL-17 producing γδ T cells in defense against *L. monocytogenes* (Emoto, M. et al.; Hamada, S. et al. 2008; Sardinha, L.R. et al. 2006). During infection with *P. yoelii* the expanding γδ T cells in the liver of DBA/2 mice have even been demonstrated to be responsible for their rapid recovery compared to less resistant C57Bl/6 mice (Bakir, H.Y. et al. 2006). Combined, these studies and the results of the analysis of γδ T cell distribution during early *P. berghei* infection lead to the conclusion, that expanding IL-17 producing γδ T cells in the liver activate effector mechanisms against *P. berghei* ANKA blood stage parasites.

Further analysis of the pathway leading to IL-17production by γδ T cells in malaria was conducted in an *in vitro* stimulation system. In this system $CD27^-$ and $CD27^+$ γδ T cells, but also the role of APC, could be analyzed separately. γδ T cells were sorted for their expression of CD27 and stimulated in presence of BMDC, which served as APC. For stimulation the TLR2 ligand PamCys was chosen, because TLR2 ligation occurs during malaria infection by the typical GPI anchor of the *Plasmodium* parasite (Krishnegowda, G. et al. 2005). The supernatants of the cultures of either $CD27^-$ or $CD27^+$ γδ T cells in coculture with BMDC and PamCys were tested for IFNγ or IL-17. IL-17 was detected exclusively in the supernatant of $CD27^-$ γδ T cells, whereas only the supernatant of $CD27^+$ γδ T cells contained IFNγ. This is in line with the literature that claims that only the $CD27^-$ $CCR6^+$ γδ T cells produce IL-17 (Ribot, J.C. et al. 2009). This result further supports the conclusion, that the expanding $CD27^-$ γδ T cells are the source of protective IL-17 during *P. berghei* ANKA infection.

The *in vitro* assay was then used to analyze, if the activation of γδ T cells occurs directly by stimulation of PRR on their surface or if a stimulated APC has to be present. It has been demonstrated by quantitative real time PCR that γδ T cells are able to express TLR2 (Mokuno, Y. et al. 2000). To test, if the stimulation of TLR2 on γδ T cell is sufficient for

activation the assay was conducted with either the APC or the γδ T cell being deficient for TLR2. In a setting where the APC were deficient for TLR2 no IL-17 was detectable in the supernatant, whereas in the control, with APC and γδ T cells both possessing TLR2, IL-17 was detectable. When only the γδ T cell was deficient for IL-17 the amount of IL-17 was slightly reduced, compared to a setting where both cells were from wildtype origin, but still present. This leads to the conclusion that TLR2 activation on the γδ T cells might contribute to the IL-17 production but is alone not sufficient for their induction. The APC is therefore responsible for the activation of IL-17 production in the γδ T cell. The activation is not necessarily dependent on the γδ TCR, as a TLR2 ligand alone is sufficient to activate these cells. Activation of IL-17 production independent of TCR ligation has been demonstrated before in an environment of activated APC, which produce the IL-12 family cytokine IL-23 (Sutton, C.E. et al. 2009).

The following experiments concentrated on IL-23 gene expression, as this should be detectable both in the *in vitro* experiment and in the mouse model, when IL-23 is responsible for γδ T cell activation. *In vitro* the stimulation of TLR2 led to an almost 40 fold upregulation of the IL-23p19 gene in BMDC already after 6 hours. *In vivo*, the expression of IL-23p19 mRNA was analyzed in spleen and liver early after infection with *P. berghei* ANKA. In the spleen an upregulation of the IL-23p19 gene was detectable 24 h p.i.. In the liver already 12 h p.i. an upregulation of the IL-12p19 mRNA was detectable. As IL-23 mRNA was detected in RNA of whole organ tissue and the fraction of IL-23 expressing cells per organ would be very low, the level of detected IL-23 expression was expected to be very small. This was the case, nevertheless the upregulation was clearly detectable and the increase of expression in the liver was more pronounced and earlier than in spleen. This would fit to the stronger increase of IL-17 producing γδ T cells in the liver that was described earlier.

If ligation of TLR2 on BMDC by *Plasmodium* derived molecules is responsible for IL-23 induction, less IL-23 should be produced by TLR2 deficient BMDC upon encounter of *P. berghei* ANKA parasites. This was tested in a setting where wildtype C57Bl/6 or TLR2$^{-/-}$ BMDC were stimulated with *P. berghei* ANKA pRBC. The stimulation was conducted with magnetically separated pRBC, the disadvantage of this method being, that only in late stages of the erythrocytic life cycle of the parasite enough hemozoin is accumulated to render them paramagnetic (Trang, D.T. et al. 2004). Earlier stages,

especially young ring stages are lost during purification. The supernatants of the stimulation were analyzed for concentration of IL-12/23p40, which is the common subunit of IL-12 and of IL-23 (Oppmann, B. et al. 2000). The observed production of the molecule shows that stimulation of APC by pRBC results in IL-12/23p40 secretion. In the supernatant of TLR2$^{-/-}$ BMDC a reduction of IL-12/23p40 concentration is observed. These results show, that the presence of pRBC leads to a ligation of TLR2 and a subsequent production of either IL-12 or IL-23, or both. It is not the only ligated PRR, as a reduced IL-12/23p40 production is maintained without TLR2. One additional ligated PRR that might account for residual IL-12/23p40 production could be TLR9, which binds the *Plasmodium* metabolite hemozoin (Coban, C. et al. 2005).

The preceding experiment showed that TLR2 activation induces production of IL-12/23p40. If this subunit is part of IL-23, the activator of CD27^{-} γδ T cells had to be verified in an additional experiment. Therefore CD27^{-} γδ T cells were stimulated in presence of WT or IL-23p19$^{-/-}$ BMDC and PamCys. In coculture with the WT BMDC γδ T cells produce IL-17, but in coculture with the IL-23p19$^{-/-}$ BMDC no IL-17 production was detectable. Addition of recombinant IL-23 to the culture strongly enhanced the production of IL-17 in the coculture with WT BMDC. In the coculture of γδ T cells with IL-23p19$^{-/-}$ BMDC the IL-17 production was not only rescued by the addition of recombinant IL-23 but was even enhanced to concentrations observed in the culture containing WT BMDC and recombinant IL-23. This experiment finally verifies that the IL-17 production by γδ T cells is induced by IL-23, which originates from APC.

Throughout the thesis mentioning of IL-17 always refers to IL-17A, which was the first identified member of the IL-17 family. This cytokine family further includes IL-17B, IL-17C, IL-17D, IL-17E and IL-17F (Gaffen, S.L. 2008). IL-17A has been widely investigated for its role in inflammation and binds to the receptor IL-17RA (Harrington, L.E. et al. 2005). IL-17F, the closest relative to IL-17A, can bind to the IL-17RA with weak affinity and is even able to build a heterodimer with IL-17A. The high affinity receptor for IL-17F is IL-17RC (Liang, S.C. et al. 2007). It has been demonstrated in some studies that IL-17F might have a possible compensatory or counteractive role in IL-17A deficient mice in different models of disease (Ishigame, H. et al. 2009). Also in this study the possibility of an influence of IL-17F, especially in the absence of IL-17A, was considered. So the expression of IL-17F in WT and IL-17A deficient mice was compared

during infection. This showed an increased amount of IL-17F production in γδ T cells in spleen, blood and liver of IL- 17A$^{-/-}$ mice in comparison to C57Bl/6 wildtype mice. The deficiency of IL-17A thus leads to a compensatory upregulation of IL-17F, which could indeed lead to an effect upon binding to IL-17RA, despite its weaker affinity. The effect of IL-17A without compensatory effects of IL-17 F can be investigated in mice double deficient for IL-17A and IL-17F. In these mice the parasitaemia was increased on day 8 p.i. compared to WT mice. Since the double deficient mice, like the mice deficient only for IL-17A, have an increased parasitaemia compared to WT mice, it can be concluded that IL-17F has no influence on the parasitaemia, even though it is upregulated in IL-17A deficient mice.

In IL-17$^{-/-}$ mice the absence of IL-17A is responsible for the increased parasitaemia. In reverse this implies that the IL-17 leads to activation of an effector mechanism, which is able to mediate anti-parasitic effects. One possible effector cell that was analyzed here was the neutrophil as it was demonstrated to be attracted and activated by IL-17 (Fossiez, F. et al. 1996; Laan, M. et al. 1999). These cells are part of the first wave of defense in many infectious diseases and are activated early and rapidly (Borregaard, N.). Additionally, effector molecules of neutrophils have been shown to at least partly confer protection to *Plasmodium* blood stage in rats (Pierrot, C. et al. 2007). The first step in the investigation of the role of neutrophils was to determine the course of their activation. Therefore the number of neutrophils early in infection was analyzed in spleen, blood and liver, before and after the timepoint of observation of increased parasitaemia in the absence of IL-17. In all organs the neutrophil numbers increased transiently on day 5 p.i., which concurs with the increase of IL-17 producing γδ T cells. On day 8 p.i. the number of neutrophils already declined. Mature neutrophils are stored as a reserve in the bone marrow and can be released upon infection by cytokines, especially IL-17 and G-CSF (Furze, R.C. and Rankin, S.M. 2008). Neutrophils survive only over short periods in blood and tissues and are cleared by other phagocytic cells, mainly macrophages (Mantovani, A. et al. 2011). Therefore, the transient expansion of the neutrophils on day 5 p.i. with *P. berghei* ANKA might result from an induction and activation by IL-17 produced by γδ T cells.

To analyze the importance of neutrophils, they were depleted one day prior to infection of mice by injection of a monoclonal antibody against GR-1. This antibody does not only

target neutrophils but also other cells with a high expression of GR-1 (Daley, J.M. et al. 2008). This might involve monocytes, plasmacytoid DC and some subsets of CD8+ T cells (Geissmann, F. et al. 2003; Matsuzaki, J. et al. 2003; Nakano, H. et al. 2001). On day 7 p.i. no difference in the parasitaemia was observed between mice that received the antibody and mice that received PBS as a control. Therefore it can be concluded, that neutrophils increase in number upon infection with *P. berghei* ANKA; but they are not mediating the anti-parasitic response. But, as has been demonstrated before using a different antibody, there was no incidence of cerebral malaria in the mice that received the depleting antibody against GR-1 (Chen, L. et al. 2000). In control group that received PBS more than 50 % of the animals developed neurological impairments and died from cerebral malaria. This implies that one or more of the subsets, which are sensitive to depletion with anti-GR-1 antibody, contributes to the immune pathology. Neutrophils or especially inflammatory monocytes might contribute by secretion of IL-12 and other inflammatory cytokines (Nakano, H. et al. 2009). Depletion of $CD8^+$ T cells might directly reduce the number cells contributing to the pathology (Matsuzaki, J. et al. 2003).

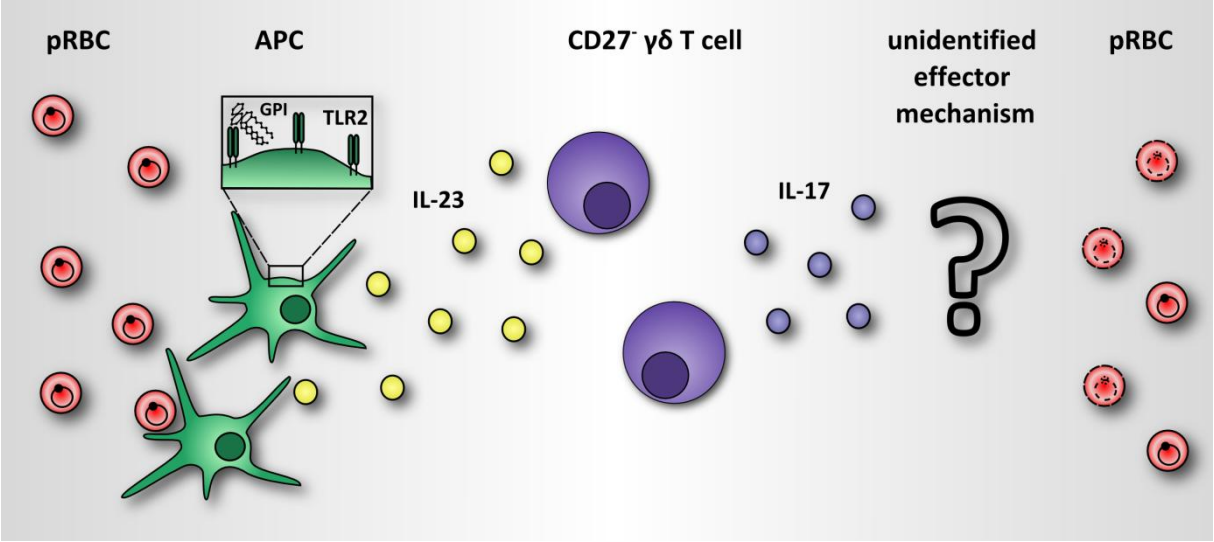

Figure 5-1: Activation of IL-17 secretion during ECM

pRBC induce production of IL-23 in APC by ligation of PRR like TLR2. IL-23 activates $CD27^-$ γδ T cells to produce IL-17. This mediates anti-parasitic effects by a still unidentified mediator.

Even though the final mechanism could not be identified, it was demonstrated in this thesis, that APC are activated by TLR2 to produce IL-23 during ECM. IL-23, in turn, induces IL-17 secretion by $CD27^-$ γδ T cells, which activates early anti-parasitic effects (**Figure 5-1**).

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7 Appendix

7.1 Sequence of the IL-35-Fc fusion molecule

Cloned Sequence of the IL-35, excluding signal sequence and IgG domain, recognition sites for the restricition enzymes *Eco*RI, *Nco*I ang *Bgl*II are underlined.

GAATTCCACTGAAACAGCTCTCGTGGCTCTAAGCCAGCCCAGAGTGCAATGCCATGCTTCTCG
GTATCCCGTGGCCGTGGACTGCTCCTGGACTCCTCTCCAGGCTCCCAACTCCACCAGATCCACG
TCCTTCATTGCCACTTACAGGCTCGGTGTGGCCACCCAGCAGCAGAGCCAGCCCTGCCTACAA
CGGAGCCCCCAGGCCTCCCGATGCACCATCCCCGACGTGCACCTGTTCTCCACGGTGCCCTACA
TGCTAAATGTCACTGCAGTGCACCCAGGCGGCGCCAGCAGCAGCCTCCTAGCCTTTGTGGCTG
AGCGAATCATCAAGCCGGACCCTCCGGAAGGCGTGCGCCTGCGCACAGCGGGACAGCGCCTGC
AGGTGCTCTGGCATCCCCCTGCTTCCTGGCCCTTCCCGGACATCTTCTCTCTCAAGTACCGACT
CCGCTACCGGCGCCGAGGAGCCTCTCACTTCCGCCAGGTGGGACCCATTGAAGCCACGACTTT
CACCCTCAGGAACTCGAAACCCCATGCCAAGTATTGCATCCAGGTGTCAGCTCAGGACCTCAC
AGATTATGGGAAACCAAGTGACTGGAGCCTCCCTGGGCAAGTAGAAAGTGCACCCCATAAGG
GTGGTGGTGGTTCTGGTGGTGGTGGTTCTGGTGGTGGTGGTTCTCCATGGATTCCAGTCTCTG
GACCTGCCAGGTGTCTTAGCCAGTCCCGAAACCTGCTGAAGACCACAGATGACATGGTGAAGA
CGGCCAGAGAAAAACTGAAACATTATTCCTGCACTGCTGAAGACATCGATCATGAAGACATC
ACACGGGACCAAACCAGCACATTGAAGACCTGTTTACCACTGGAACTACACAAGAACGAGAGT
TGCCTGGCTACTAGAGAGACTTCTTCCACAACAAGAGGGAGCTGCCTGCCCCCACAGAAGACG
TCTTTGATGATGACCCTGTGCCTTGGTAGCATCTATGAGGACTTGAAGATGTACCAGACAGAG
TTCCAGGCCATCAACGCAGCACTTCAGAATCACAACCATCAGCAGATCATTCTAGACAAGGGC
ATGCTGGTGGCCATCGATGAGCTGATGCAGTCTCTGAATCATAATGGCGAGACTCTGCGCCAG
AAACCTCCTGTGGGAGAAGCAGACCCTTACAGAGTGAAAATGAAGCTCTGCATCCTGCTTCAC
GCCTTCAGCACCCGCGTCGTGACCATCAACAGGGTGATGGGCTATCTGAGATCT

7.2 Abstract

During the infection of humans with the malaria parasite *Plasmodium falciparum* an overactivation of the immune system can lead to break-down of the blood-brain-barrier and an inflammation in the otherwise immuneprivilged tissue of the brain. In the development of the adaptive immune response during infection $CD4^+$ T helper cells, and their differentiation into different subsets, play an important role. In this thesis the role of selected IL-12 family cytokines and their influence on $CD4^+$ T cell differentiation during experimental cerebral malaria (ECM) were to be investigated. The first part of the thesis analyzes the regulatory function of the recently discovered cytokine IL-35. In the second part, IL-23 and its downstream mechanisms a proinflammatory immune response were investigated. Both cytokines have been reported to target the T_H17 response, IL-23 as its main activator and IL-35 as a direct suppressor in addition to its role as activator of regulatory T cells.

In the first part of the thesis an IL-35-Fc fusion molecule was successfully generated, expressed in CHO cells and purified via affinity chromatography. The purified molecule is active and able to suppress IL-17 production in $CD4^+$ T cells stimulated under T_H17 polarizing conditions. In the *in vivo* application of the molecule in a model of ECM, no effect of the IL-35-Fc on the pathology could be detected. This was in line with other studies, which also showed only little influence of IL-17 production by T_H17 cells or IL-10 production of regulatory T cells on the pathology.

In the second part of the thesis the T cell differentiaton was investigated in the model of ECM with focus on the proinflammatory cytokine IL-23 and activation of IL-17 release. Here it was demonstrated, that lack of IL-17 does no t influence the pathology but leads to higher parasite loads early in infection. The IL-17 producing cells are $CD27^-$ γδ T cells, which are activated by IL-23. Activation of IL-23 release by APC is activated upon ligation of PRRs, like TLR2, by microbial molecules. As IL-17 alone is not able to mediate the anti-parasitic effect, neutrophils were investigated as a possible downstream effector cell. Depletion of GR-1 expressing cells did not alter parasitaemia though, arguing against their role in immunity against the parasite.

7.3 Zusammenfassung

Die Infektion des Menschen mit dem Malaria-Parasiten *Plasmodium falciparum* kann zu einer Überaktivierung der Immunantwort führen, durch die eine Schädigung der Blut-Hirn-Schranke und eine Entzündung des normalerweise immunprivilegierten Gewebes im Gehirn auftritt. $CD4^+$ T-Zellen und deren Differenzierung in unterschiedliche Subtypen spielen beim Aufbau der adaptiven Immunantwort während einer Infektion eine wichtige Rolle. In dieser Arbeit wurden bestimmte IL-12-Familien-Zytokine auf ihren Einfluss auf die Differenzierung von $CD4^+$ T-Zellen im Modell der experimentellen zerebralen Malaria (EZM) untersucht. Der erste Teil beschäftigt sich mit dem unlängst entdeckten regulatorischen Zytokin IL-35. Im zweiten Teil lag der Fokus auf der proinflammatorischen Immunantwort, die durch IL-23, und durch von IL-23 aktivierten Effektor-T-Zellen, ausgelöst wird. Beide Zytokine beeinflussen T_H17 Zellen, IL-23 als ihr Hauptaktivator und IL-35, neben der Aktivierung regulatorischer T-Zellen, als Regulator.

Im ersten Teil der Arbeit wurde ein IL-35-Fc-Fusionsmolekül hergestellt, in CHO-Zellen exprimiert und mittels Affinitätschromatografie aufgereinigt. Das aufgereinigte Molekül ist biologisch aktiv und konnte die IL-17-Produktion von $CD4^+$ T-Zellen, die unter T_H17-polarisierenden Bediungungen kultiviert worden waren, supprimieren. Die Anwendung des Moleküls *in vivo* im Modell der EZM zeigte keinen Einfluss von IL-35 auf die Pathologie. Das Ergebnis ist im Einklang mit anderen Studien, die gezeigt haben, dass sowohl IL-17-Produktion von T_H17-Zellen als auch IL-10-Produktion von regulatorischen T-Zellen nur einen geringen Einfluss auf die Pathologie haben.

Im zweiten Teil der Arbeit wurde die T-Zell-Differenzierung im Modell der EZM analysiert, mit besonderer Beachtung von IL-23 und der Aktivierung von IL-17. Hier zeigte sich, dass ein Fehlen von IL-17 keinen Einfluss auf die Pathologie hat, aber zu einer erhöhten Parasitämie führt. IL-17 wird durch $CD27^-$ γδ-T-Zellen produziert, welche durch IL-23 aktiviert werden. Die IL-23-Produktion von antigenpräsentierenden Zellen (APZ) wurde zuvor durch Bindung von mikrobiellen Molekülen an bestimmte Rezeptoren, wie TLR2, aktiviert. Da IL-17 den anti-parasitären Effekt nicht direkt vermitteln kann, wurden Neutrophile als mögliche Effektor-Zellen untersucht. Depletion von GR-1-exprimierenden Zellen führte jedoch nicht zu einer Änderung in der Parasitämie, was gegen die Rolle dieser Zellen in der Bekämpfung des Parasiten spricht.