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The cytokine IL-22 modulates the immune response during *Plasmodium berghei* ANKA infection in mice (*mus musculus*)

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Declaration on oath
Eidestattliche 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.

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Abbreviations

°C	Degree Celsius
∞	Endless
α	Anti -
µg	Microgram
µL	Microliter
ACT	adenylate cyclase toxoid
APC	Antigen presenting cell
bp	Base pair
B cell	Bone - marrow derived lymphocyte
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
CM	Cerebral malaria
CSP	Circumsporozoite protein
Cy	Cyanine
d	day
DC	Dendritic cell
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
et al.	et alia
EDTA	Ethylendiamintetraacetate
ELISA	Enzyme linked immunosorbent assay
FACS	Flow activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein Isothiocyanate
fw	forward
g	Gram
GFP	Green fluorescent protein
h	Hour(s)
HEPES	2',4',2-Hydroxyethyl-1-piperazinyl-ethansulfonsäure
i.p.	Intraperitoneal
i.v.	Intravenous
IFNγ	Interferon γ
Ig	Immunglobuline
IL	Interleukine
iRBC	Infected red blood cell
JAK	Janus kinase
L	Litre
LPS	Lipopolysaccharide
M	Molar
MACS	Magnetic activated cell separation
MHC	Major histocompatibility complex
min	Minute(s)

mL	Milliliter
mRNA	Messenger ribonucleic acid
ng	Nanogram
NK cell	Natural Killer cell
NK T cell	Natural Killer T cell
OD	Optical density
OVA	Ovalbulin
p.i.	Post infection
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrine
PerCP	Peridinin chlorophyll protein
pH	negative decadal logarithm of the concentration of hydrogen-ions
pHepa	Primary hepatocyte
PMA	Phorbol-12-myristrate-13-acetate
PM	Perfusion medium buffer
pmol	Pico molar
PPML	Pre - perfusion medium buffer
PRR	Pattern recognition receptor
rcf	Relative centrifugal force
rev	Reverse
rpm	Rounds per minute
sec	Seconds
SEM	Standard error of mean
SOCS3	Suppressor of cytokine signalling 3
Tab.	Table
T cell	Thymus derived lymphocyte
TCR	T cell receptor
TGF-β	Transforming growth factor β
T_H cell	T helper cell
TNFα	Tumour necrosis factor α
TLR	Toll-like receptor
T_R cell	T regulatory cell
WHO	World health organization
V	Volt
v/v	Volume to Volume
w/v	weight to Volume

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Table of contents

List of Tables

List of Figures

1 Introduction

1.1 The immune system

The term “immune system” came to appear in the context of the development of defence at a cellular level against pathogen invasions in complex organisms, at first in the 1960s (1). The word immunity derives from the Latin word *immunitas* and refers to the defence of the identity (2). Regarding the human organism, immunity means the defence against invading pathogens like bacteria, viruses, fungi or parasites as well as malign tumours of endogenous cells. To preserve no harmful action of the immune response on the host, it has to be in a perfect equilibrium between self-tolerance and elimination against pathogens. In order to fulfil this purpose, a tightly controlled activation and regulation of the immune response is of crucial importance. In vertebrates the immune system is divided into two parts, the innate and the adaptive immune system.

1.1 The Innate immune system

The innate immune system is also described as non-specific immune system. Its roots lie in the early stadium of evolution (3, 4). Therefore it is found in various organisms like fungi, plants, insects and vertebrates (3). The innate immune system reacts directly on pathogens after they penetrated mechanic barriers like the skin or the mucosa. The first cells getting into contact with pathogens, which succeeded to break through mechanical barriers, are usually macrophages and neutrophilic granulocytes. These cells have the ability to distinguish between endogenous and exogenous structures with the help of pathogen-recognition receptors (PRRs). The PRRs bind to pathogen-associated molecular patterns (PAMPs) which are highly conserved structures of pathogens. PAMPs are essential for the survival and are therefore different to alter by pathogens. The binding of a PAMP to a PRR leads to further downstream pathways leading to the transcription of important pro - inflammatory cytokine genes which can in turn activate the adaptive immune system (5). An additional pathway to activate the adaptive immune system is the uptake of pathogens into the cell, where it is destructed into small protein fragments. Either theses small fragments are released into the periphery of the cell or the fragments are presented as antigens on the cell surface to induce cell activation of the adaptive immune system. The

destruction of the pathogen is performed in vesicles in the cell, called phagolysosomes. These phagolysosomes are the product of a merged pathogen containing phagosome and a lysosome. The phagolysosomes is characterized by a highly acidic pH level of the lysosome which leads to the destruction of the pathogen. Additionally reactive oxygen species support this mechanism (3). Furthermore macrophages secrete cytokines during phagocytosis which leads to an inflammatory reaction and additional immune responses of the organism. Besides the activation of the complement system, a further reaction is the accumulation of additional phagocytes, neutrophils, basophils, eosinophils, mast cells and natural killer cells (NK cells). Many of these cells are able to induce death of pathogens with the release of cytotoxic granules, called degranulation. An important type of phagocytic cells are dendritic cells (DCs). This cell type is able to phagocyte pathogens or exogenous molecules. Subsequently incorporated parts are processed intracellularly and fragments are presented as antigens on the major histocompatibility complex (MHC) molecules. With the help of this antigen presenting cells the adaptive immune system can be activated. Besides phagocytosis and antigen presentation, cells of the innate immune system secret pro-inflammatory chemokines and cytokines like the tumour necrosis factor (TNF), Interleukin- (IL-) 6 and IL-1 which additionally attract and activate the adaptive immune system (6).

1.1.1 Antigen processing

Any nucleus containing cell of vertebrates is able to present antigens on its surface. The presentation of antigens is an important step to activate the adaptive immune system and to allow a specific immune response against the invading pathogen or mal-functional cells including tumor cells. There are two main pathways of antigen processing, the endogenous and the exogenous pathway. The endogenous pathway includes the ubiquitination of proteins within the cell; this can be a virus particle of an infected cell or any other protein within the cell. The ubiquitin tagged proteins are then degraded by the proteasome into small peptide fragments for the presentation on MHC class I molecules. These peptides are then transported into the lumen of the rough endoplasmatic reticulum, where correctly folded and empty MHC class I molecules are located. Once the MHC class I molecule is loaded with the peptide, the complex is transported to the golgi apparatus and subsequently to the surface of the cell (Fig. 1.1). $CD8^+$ T cells of the adaptive immune system are able to recognize the peptide presented by a MHC class I molecules and can be further driven to

immune modulatory tasks depending on additional presented molecules of the antigen presenting cell (APC).

The exogenous pathway is restricted to antigen presenting cells, e.g. dendritic cells (DCs) as well as B cells. Within this pathway MHC class II molecules are loaded with peptides of endocytosed proteins. This means that proteins are endocytosed by the APC and subsequently degraded in a highly acidic endosome by lysosomal enzymes. Since the MHC class II molecule is folded in the rough endoplasmatic reticulum as the MHC class I molecule, it would be likely that peptides which are intended for MHC class I molecules accidentally bind to MHC class II molecules. For that reason the binding site of the MHC class II molecule is blocked with an invariant chain. This invariant chain additionally facilitates the transport of the MHC class II molecules into vesicles. These vesicles fuse with the late endosomes to allow the brake down of the invariant chain. The loss of the invariant chain does not confer the loading of the MHC class II molecule, since in the binding site is still blocked with a small protein called CLIP. After the removal of the CLIP the MHC class II is ready for the antigen binding and presentation and can be transported to the surface of the APC. $CD4^+$ T cells of the adaptive immune system are then capable to recognize the presented antigen (Fig. 1.1) (6-8).

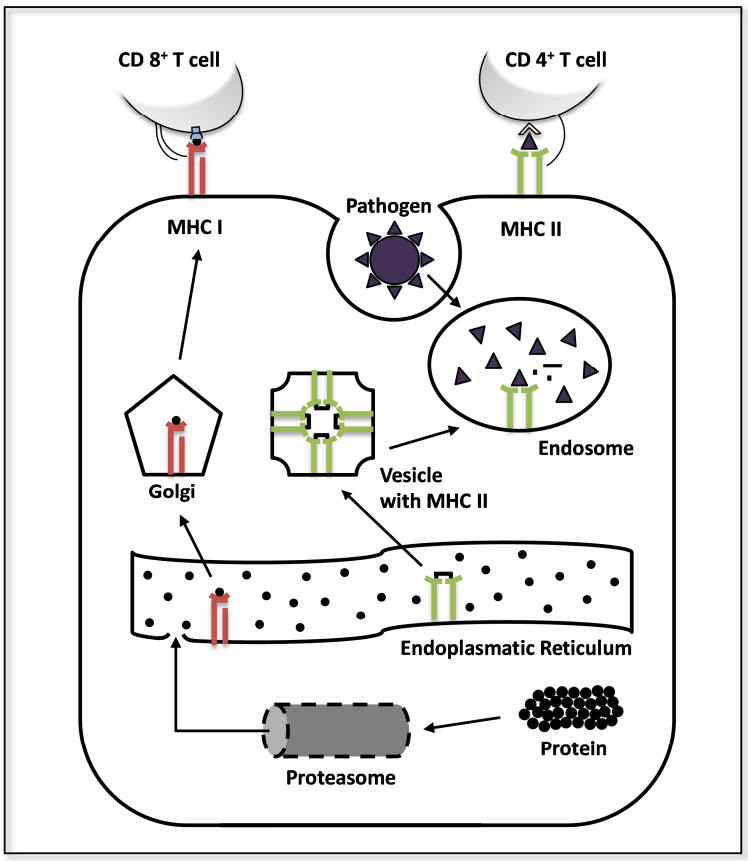

Fig. 1.1: Antigen processing of professional antigen presenting cells. Endogenous proteins are destructed by the proteasome and brought onto the rough endoplasmatic reticulum where MHC class I molecules are located. These are then loaded with the antigen and subsequently transported via the golgi apparatus to the surface of the cell. Peptides presented over MHC class I can be exclusively be recognised by $CD8^+$ T cells. MHC class II molecules are located in the rough endoplasmatic reticulum as well but they cannot be loaded with an antigen since their binding site is blocked. Vesicles which contain MHC class II molecules are subsequently formed which merge with phagosomes which in turn contain peptides of destructed pathogens. After the proteins which block the binding site of the MHC class II are removed the peptide can be loaded to the binding site and presented on the cell surface. Peptides presented over MHC class II can be exclusively be recognised by $CD4^+$ T cells. The graphic was created in accordance to following literature: Abbas, 2012 (6); Germain *et al.*, 1994 (7); Pfeifer *et al.*, 1993 (8); Villadangos *et al.*, 2007 (9).

Beside the endogenous and exogenous pathway of antigen presentation an additional way of presentation is possible which contains parts of both pathways, known as cross - presentation. Within this pathway the APC omits the endogenous proteasome pathway. Peptides of phagocytosed particles or pathogens, which usually would have been presented by MHC class II molecules of the DC, are presented by the APC via MHC class I. Hence, $CD8^+$ T cells are stimulated with this way of antigen presentation. Processing of antigens by cross presentation is of crucial importance for the development of tumour immunity and vaccination with protein antigens (9).

1.2 The Adaptive immune system

Lymphocytes are the cell population which induces adaptive immunity. They are able to induce a highly defined immunity against infections. Lymphocytes are antigen specific cells which are able to perform different effector functions during infection and to develop an immunological memory. They are mostly located in the blood and the lymphatic fluid as well as in lymphatic organs, like the thymus, the spleen or the bone marrow. To obtain an immunological response of the adaptive immune system T and B lymphocytes are of crucial importance. Both cell populations derive from the same pluripotent hematopoietic stem cell in the bone marrow. While T cells migrate to the thymus, B cells stay and mature in the bone marrow. There they pass through different stages, including the rearrangement and expression of the B cell receptor (BCR) genes. After the BCR development is completed, the mature but naive B cells migrate to adjacent peripheral tissues. T cells mature in the thymus where they get into contact with stroma cells and undergo their differentiation. Similar to B cells, the differentiation of T cells needs a rearrangement and the expression of the T cell receptor (TCR). The next step is the development of the TCR complex which consists of the TCR itself, the surface molecules CD3 and CD4 or CD8, respectively. Before mature but still naive T cells are released into the periphery, the functionality of the TCR-complex is tested. If the interaction with the antigen presenting MHC molecule is too strong or too low, T cells will undergo programmed cell death (negative selection), called apoptosis. The positive selection of immature T cells includes those cells which recognises only self MHC molecules and which express also the appropriate coreceptor (CD4 or CD8). Subsequently the selected T cells will recycle in secondary lymphatic organs until they get fully activated by an APC. A proper T cell activation needs three signals: the specific antigen recognition, the ligation of one of the costimulatory molecules CD80 or CD86 to the CD28 molecule of the T cell and furthermore distinctive cytokines. The cytokine milieu drives the T cell into differentiation into the particular T helper cell type which owns a specific task in immune modulation (6).

T and B cells represent the two most important mechanisms of the adaptive immune system, the cellular and humoral immune response. B cells are able to produce antigen specific antibodies. This mechanism belongs to the humoral immune response (Lat. [h]umor = humidity, liquid). Even though B cells are able to produce antigen specific antibodies without T cell, this ability is drastically improved in the presence of T cells (10). This phenomenon is based on the function of a subpopulation of T cells which are known as

T helper cells (T_H cells). This T cell population is able to express the CD4 coreceptor which binds to MHC class II molecules, expressed by DCs, macrophages and B cells. When the T cell recognizes a foreign antigen, the activation of the T cell occurs. The T cell response is divided into T_H1, T_H2, T_H17 or T_H22, depending on the cytokine milieu in which they are situated at the time point of activation. The different cell populations are characterized concerning their activated transcription factors and secreted cytokines (Fig. 1.2). The presence of IL-12 and IFNγ drives a naive T cell to differentiate into T_H1 which in turn secretes even more IFNγ but also TNFα and IL-2. Besides driving T cell differentiation into T_H1, IFNγ also activates macrophages (11). The secreted cytokines of T_H1 cells are known to have a regulatory effect on the differentiation of naive T cells into T_H17 cells (12). This population will appear if the naive T cell remains in a milieu dominated by IL-1β, TGF-β, IL-6 and IL-23. An additional cytokine which dampens the T_H17 differentiation is IL-4. The presence of IL-4 is highly important for the maturation of T cells into T_H2 cells. This cell population secrets IL-4, IL-5, IL-6, IL-13 and IL-2. Even though T_H17 and T_H22 cells secrete more or less the same cytokines, they are separated from each other because of their different transcription factors. Both cell populations can produce IL-17 and IL-22 but T_H17 cells are driven by the RORγτ transcription factor and T_H22 cells depend highly on the aryl hydrocarbon receptor (AHR).

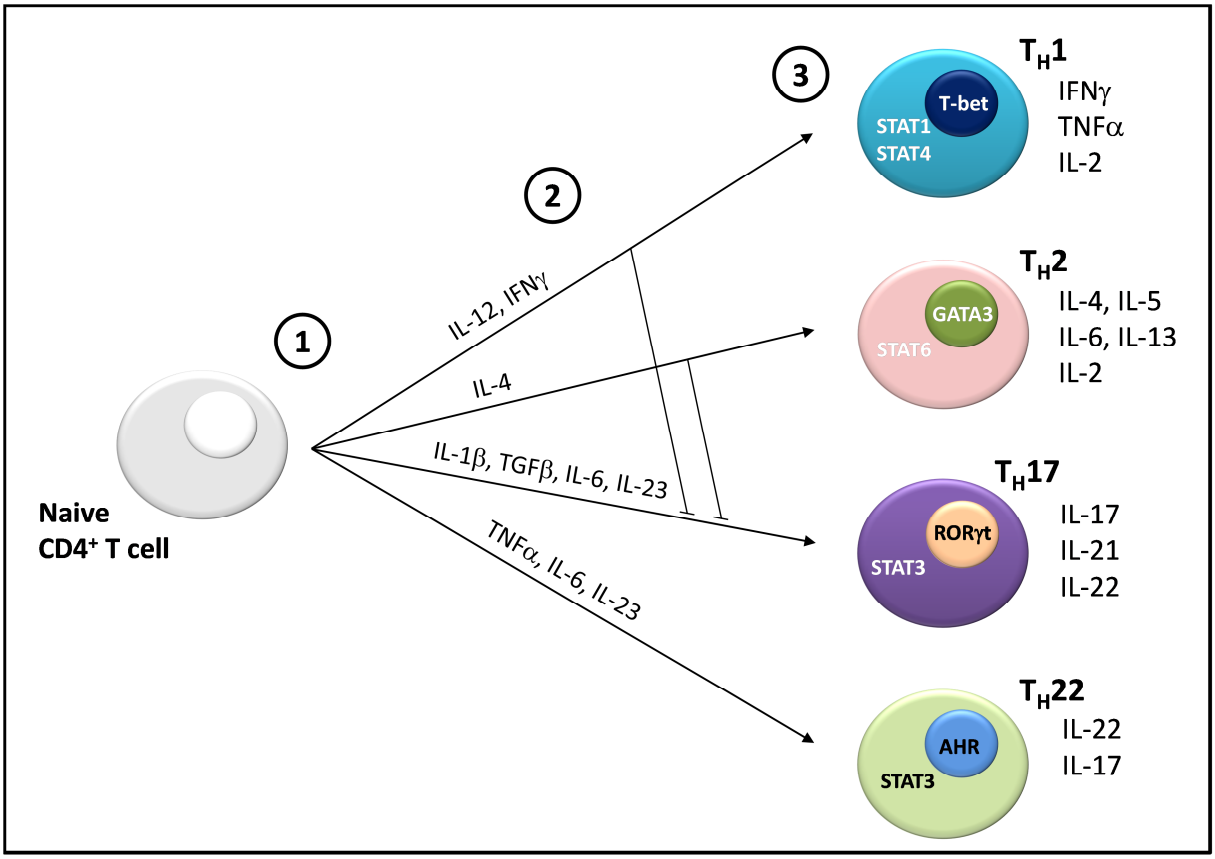

Fig. 1.2: T cell differentiation. Naive $CD4^+$ T cells (1) differentiate into a particular T cell subpopulation (3) in accordance to the prevalent cytokine milieu (2). The graphic was created in accordance to following literature: O'Shea *et al.*, 2010 (13), Jetten *et al.*, 2009 (14), Zhu *et al.*, 2010 (15)

In contrast to previously mentioned T cell subpopulations, which act mostly pro - inflammatory, regulatory T cells (T_R) act as strong immunosuppressors. Foxp3 is an important and characteristic transcription factor of this cell population. T_R cells express a highly affine IL-2 receptor chain, called CD25 and are able to secrete IL-10, a highly immunosuppressive cytokine. Their task is it to control the immune system or to calm down the immune system when the infection is finally defeated in order to preserve the organism from a destructive immune reaction (16). Furthermore these cells are able to maintain tolerance to self-antigens and to inhibit DC maturation and function (17). The mechanisms to prevent DC maturation and function are still not well understood (18).

$CD8^+$ T cells are able to recognize peptides presented by MHC class I molecules. Nearly each nucleus containing cell throughout the whole body and platelets expresses MHC class I molecules. Besides the cytotoxic activity of $CD8^+$ T cells, they are also highly capable of secreting proinflammatory cytokines, e.g. IFNγ and TNFα. This induces the migration of further lymphocytes to the side of infection and to cause inflammation.

Furthermore, the adaptive immune system is not only able to combat infections but also to develop an immunological memory. After a successful fight against infection a small number of differentiated T cells still circulate in the organism ready to expand as soon as they recognize their particular antigen. This fast and prompt mechanism is preferred over the usual classical pathway of differentiation, activation and proliferation of T cell activation.

1.2.1 The role of IL-10 family cytokines in the immune response

The IL-10 cytokine family consists of three subfamilies, divided accordingly to their biological function: The IL-10 subfamily, including IL-10 itself; the IL-20 subfamily represented by IL-19, IL-20, IL-22, IL-24 and IL-26 and the subfamily of the type III interferons (IL-28A, IL-28B and IL-29) (Tab. **1.1**) (19).

Tab. 1.1: IL-10 cytokine family

	IL-10 cytokine family		
Subfamily	IL-10	IL-20	Type III Interferon
Members	IL-10	IL-19, IL-20, IL-22, IL-24, IL-26	IL-28A, IL-28B, IL-29

Thus, nine different cytokines are members of the IL-10 family sharing the characteristic feature of facilitating tissue healing and maintaining the integrity of epithelial layers. IL-10 is the main immunosuppressive cytokine known so far. It is expressed by a variety of cell types of the adaptive immune system, including T_H1, T_H17, T_{reg}, $CD8^+$ T and B cells, as well as of cell types of the innate immune system like macrophages, dendritic cells and mast cells (20, 21). The signalling of IL-10 is driven by the formation of a receptor tetramer which consists of dimers of the IL-10Rα chain and the IL-10Rβ chain (Fig. 1.3). The intracellular signalling cascade of IL-10 leads to the expression of various IL-10 target genes, including the suppressor of cytokine signalling 3 (SOCS3). SOCS3 in turn inhibits the tyrosine-kinase activity by binding to the active centre of Janus kinase (JAK), an important enzyme for the phosphorylation of proteins of the signal transduction pathways of cytokines (22). Hence, the IL-10 signalling induces a decrease of further cytokine secretion, mediating an overall suppressive effect on the immune response.

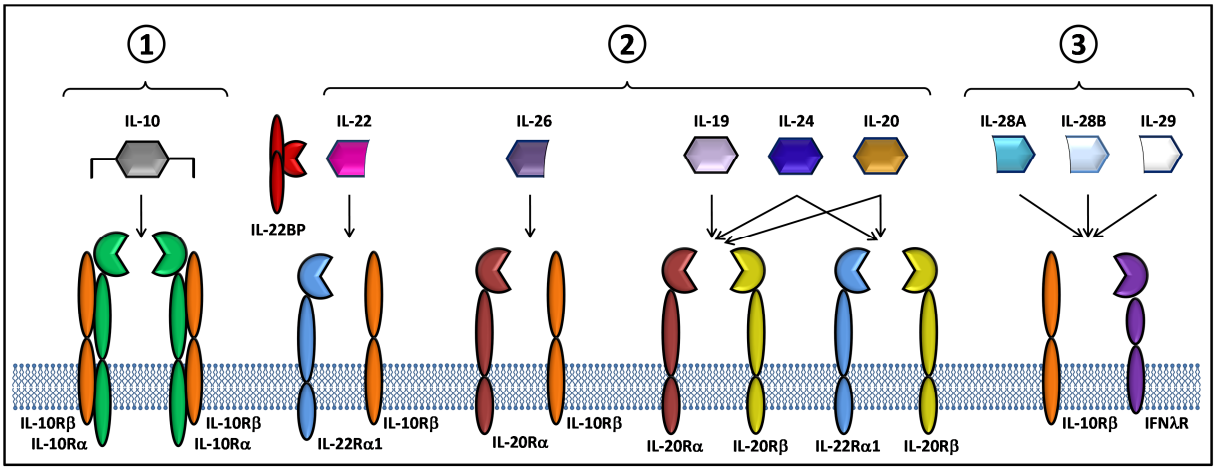

Fig. 1.3: IL-10 cytokine family and their receptors. The IL-10 cytokine family is divided into three subfamilies. The first subfamily is only represented by IL-10 itself (1). The second subfamily, named as IL-20 subfamily, is the largest one and consists of IL-22, IL-26, IL-19, IL-24 and IL-20 (2). The last family is named type III interferon subfamily and includes IL-28A, IL-28B and IL-29 (3). The graphic was created in accordance to following literature: Sziksz *et al.*, 2015 (19).

The characteristic feature of the IL-20 cytokine subfamily is the ability to link the immune system with epithelial tissues. The IL-20 cytokines (IL-19, IL-20, IL-22, IL-24 and IL-26) are mainly produced by cells of the immune system, including monocytes, lymphocytes, NK cells and macrophages. By contrast, the expression of the receptors was so far particularly shown on non-hematopoietic cells (23, 24) and the expression can vary between different organ structures, leading to a distinctive modulation of the responding tissue. Nevertheless recent studies were able to show an IL-22 receptor alpha 1 (IL-22Rα1) expression on haematopoietic cells, especially in arthritis (25) and during a lymphoma disease (26). Apart of IL-10 the remaining cytokines all bind to heterodimeric receptors. IL-22 binds to the IL-22Rα1 chain and subsequently to the IL-10Rβ chain which is also important for a proper signalling of IL-26 and the third subgroup of the IL-10 family. Besides the binding of IL-26 to the IL-10Rβ chain, a further connection of IL-26 to the IL-20Rα chain is necessary to gain a proper response of receptor expressing cells. While IL-24 and IL-20 can bind to heterodimeric receptors composed of IL-20Rα/IL-20Rβ and IL-22Rα1/IL-20Rβ, IL-19 is only capable to induce signalling through the IL-20Rα/IL-20Rβ heterodimer. Besides the binding to the IL-10Rβ, the members of the type three interferons need a further binding to the IFNλR chain for a successful signalling of the receptor expressing cells (Fig. 1.3).

1.2.2 IL-22 - IL-22Rα1 - IL-22Rα2 (IL-22BP)

A member of the IL-10 cytokine family which came more into focus over the last years concerning its immune modulatory effect is IL-22 which is mainly expressed by homeostatic cells. The discovery and the molecular characterization of IL-22 was made by Xie et la., 2000 (27). The amino acid sequence of the cDNA of human IL-22 is to 23 % similar to IL-10 and to 78 % similar to the mouse IL-22 which was firstly named as IL-10 related T cell inducible factor (IL-TIF) (27, 28). It took seven years to create a murine IL-22 knock out model which was further characterized in an acute liver inflammation model (29). IL-22 signalling is provided by a receptor complex of the IL-10Rβ and the IL-22Rα1 which was identified by Kotnenko *et al.*, 2001 (30). The expression of the IL-22Rα1 receptor is so far only shown on non-hematopoietic cells (23, 24) but nevertheless recent studies were able to show an IL-22Rα1 expression on haematopoietic cells. The receptor seems to play a role on $CD4^+$ T cells in arthritis (25) and has recently been detected on PBMC in patients suffering from Sjörgen syndrome, a lymphoma disease (26). The binding of IL-22 to its receptor induces a phosphorylation of the kinases Jak1 and Tyk2 and the transcription factors STAT1, STAT3 and STAT5 (27, 31-33) and leads to the maintenance of the homeostasis of epithelial cells at barrier surfaces (34, 35). The binding of IL-22 to its receptor can be prevented by a soluble IL-22 binding protein (IL-22BP or IL-22Rα2) which was discovered by Dumoutier *et al.* in 2000 (31). IL-22BP is mainly expressed in the mucosal tissues and the spleen (36). The exact cell type is not ultimately defined even though first findings show that immature $CD103^+$ $CD11b^+$ dendritic cells in the intestinal lamina propria produce IL-22BP under homeostatic conditions in response to retinoic acid (36, 37). Hence, the mode of action of IL-22 on IL-22Rα1 - expressing cells is controlled by the soluble IL-22BP which has a higher affinity to IL-22 than the IL-22Rα1 chain (38). IL-22 is produced by different cells of the immune system including T, B, NK, γδ T cells and ILC 3 cells (39, 40). IL-6, IL-21 and IL-23 are STAT3 activating cytokines which are able to induce IL-22 production by T cells and ILC 3 cells (41-44). Other important pathways known to induce IL-22 in T cells are the Notch and aryl hydrocarbon receptor (AHR) signalling (45, 46). The function of IL-22 in immunity is controversially discussed. In psoriasis IL-22 is increased, promotes inflammation, epidermal hyperplasia and additionally induces chemokines and pro-inflammatory cytokines (47). In inflammatory bowel disease IL-22 is highly expressed as well but has the opposite effect. It was shown that IL-22 has protective effects on the inflamed intestine (48-50). In the liver where the IL-22Rα1

is highly expressed on hepatocytes, IL-22 has protective effects in hepatitis (29). Additionally IL-22 is known to play a role concerning the composition of the microbiota (51), especially in infection (52). Moreover, IL-22 is involved in antimicrobial host defence by the induction of antimicrobial peptides, including the S100 family (53, 54).

1.3 Malaria

Malaria is still a major health problem. More than 3.3 billion people living in malaria endemic regions and around 130 million infections were registered worldwide in 2013 (Fig. 1.4). About 584 000 deaths occurred caused by the parasitic protozoa of the *Plasmodium* genus (55). The devastating fact that 78 % of these dead patients were children under the age of 5 drives malaria deeply into the concern of further investigations with the aim to eradicate this

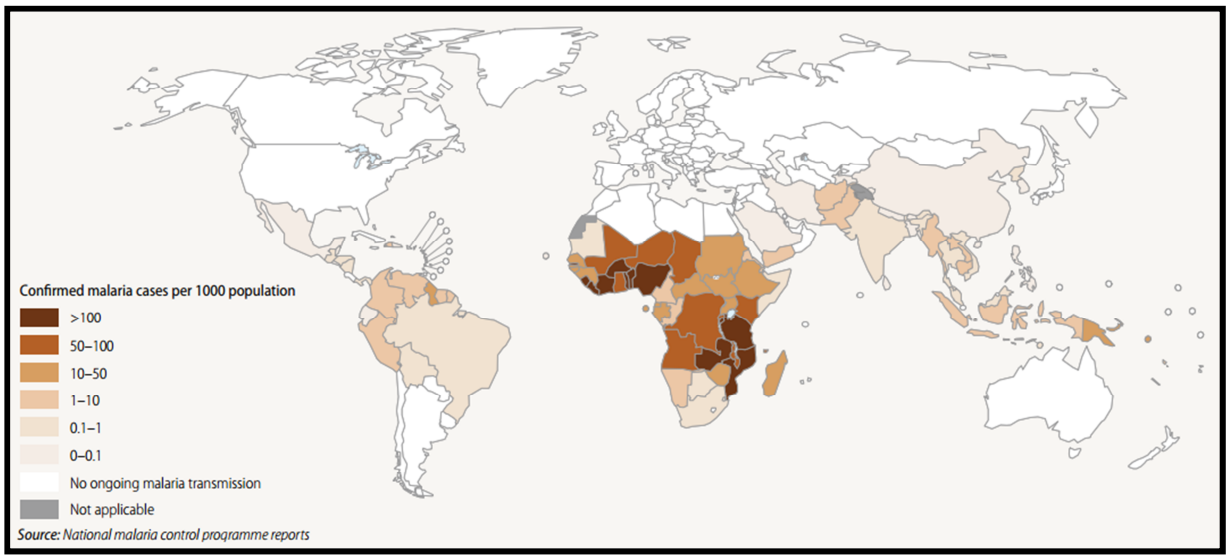

Fig. 1.4: Countries with ongoing transmission of malaria, 2013 (WHO) (55)

infectious disease. The worldwide implementation of prevention programs leads to a decline in malaria case incidence of 30 % and a decline of mortality by 47 % since the year 2000 (55). These prevention programs address mostly the eradication of the vector. There are more than 400 *Anopheles* mosquitos' species known, whereas only 30 of them are able to transmit the plasmodial parasite. Nevertheless *Plasmodium falciparum* (*P. falciparum*) is the most virulent and lethal species and the most prevalent plasmodial species on the African continent. Beside *P. falciparum* there are four more human pathogenic *Plasmodium* species (ssp.), including *Plasmodium vivax* (*P. vivax*), *Plasmodium* ovale (*P. ovale*), *Plasmodium*

malariae (*P. malariae*) and *Plasmodium knowlesi* (*P. knowlesi*). *P. vivax* affects around 75 % of all people living at risk for malaria (56, 57), caused by the unique property of the *P. vivax* carrying vector to develop at cooler temperatures. Even though there are more people living at risk for a *P. vivax* infection, the African population is mostly protected against this infection, since humans living in African malaria endemic regions mostly do not express the Duffy gene which is indispensable for *P. vivax* to invade red blood cells in order to complete its life cycle (58).

1.4 The *Plasmodium* life cycle

Malaria is a disease caused by the transmission of the *Plasmodium* spp. from the vector (female *Anopheles* mosquitos) to its host (vertebrates). With the bite of an infected female *Anopheles* mosquito sporozoites are released into its host body. This infectious stadium of the *Plasmodium* finds its way with the blood stream to the liver. There it crosses the sinusoidal wall and migrates through several hepatocytes till it finds its host cell were it starts do differentiate into merozoites in a parasitophorous vacuole (Fig. 1.5, (1)). Each sporozoite will develop into thousands of merozoites, the infectious stadium for erythrocytes. This part of the life cycle is named as the liver phase. The characteristic feature of the liver phase is that it stays nearly asymptomatic. The sporozoite exploits the elusive property of the immune regulatory environment in the liver which makes it even harder for the immune system to detect and to eradicate infected hepatocytes. Additionally the time in which the sporozoite develops into merozoites conducts only 2 -16 days (depending on the *Plasmodium* spp.) which makes it difficult for the adaptive immune system to develop a proper immune response in this short time window. To fulfil the life cycle, merozoites are packed into merosomes which crosses the sinusoidal wall and release the merozoites into the blood stream (59), thus leading to the initiation of the blood stage. There they infect circulating erythrocytes (Fig. 1.5, (2)) in which they start to develop from the ring stage (Fig. 1.5, (3)) into trophozoites (Fig. 1.5, (4)) and subsequently into schizonts (Fig. 1.5, (5)). Finally the schizonts which are packed with fully differentiated merozoites burst and thousands of new merozoites are released into the blood stream and re-infect erythrocytes (Fig. 1.5, (6)). During the blood phase the merozoites can also develop into the sexual state of the *Plasmodium* spp., called gametocytes (Fig. 1.5, (7)). These gametocytes can then be taken up by another bite of a female *Anopheles* mosquito. The infected erythrocytes will then burst in

the gut of the mosquito, releasing the gametocytes which in turn will further develop into mature gametes. Male and female gametes will then fuse to form a diploid zygote (Fig. 1.5, 8). These zygotes will further differentiate into actively moving ookinetes (Fig. 1.5, 9) which fuses with the mosquito midgut wall and form oocytes (Fig. 1.5, 10). Many infectious sporozoites are then released with the burst of one oocyte after about 8 - 15 days (Fig. 1.5, 11). The last step of the whole life cycle of the *Plasmodium* spp. is the invasion of the sporozoites into the salivary gland of the mosquito. With the injection of these sporozoites into the body of a vertebrate by a blood meal of the female *Anopheles* mosquito the life cycle will be reproduced (60).

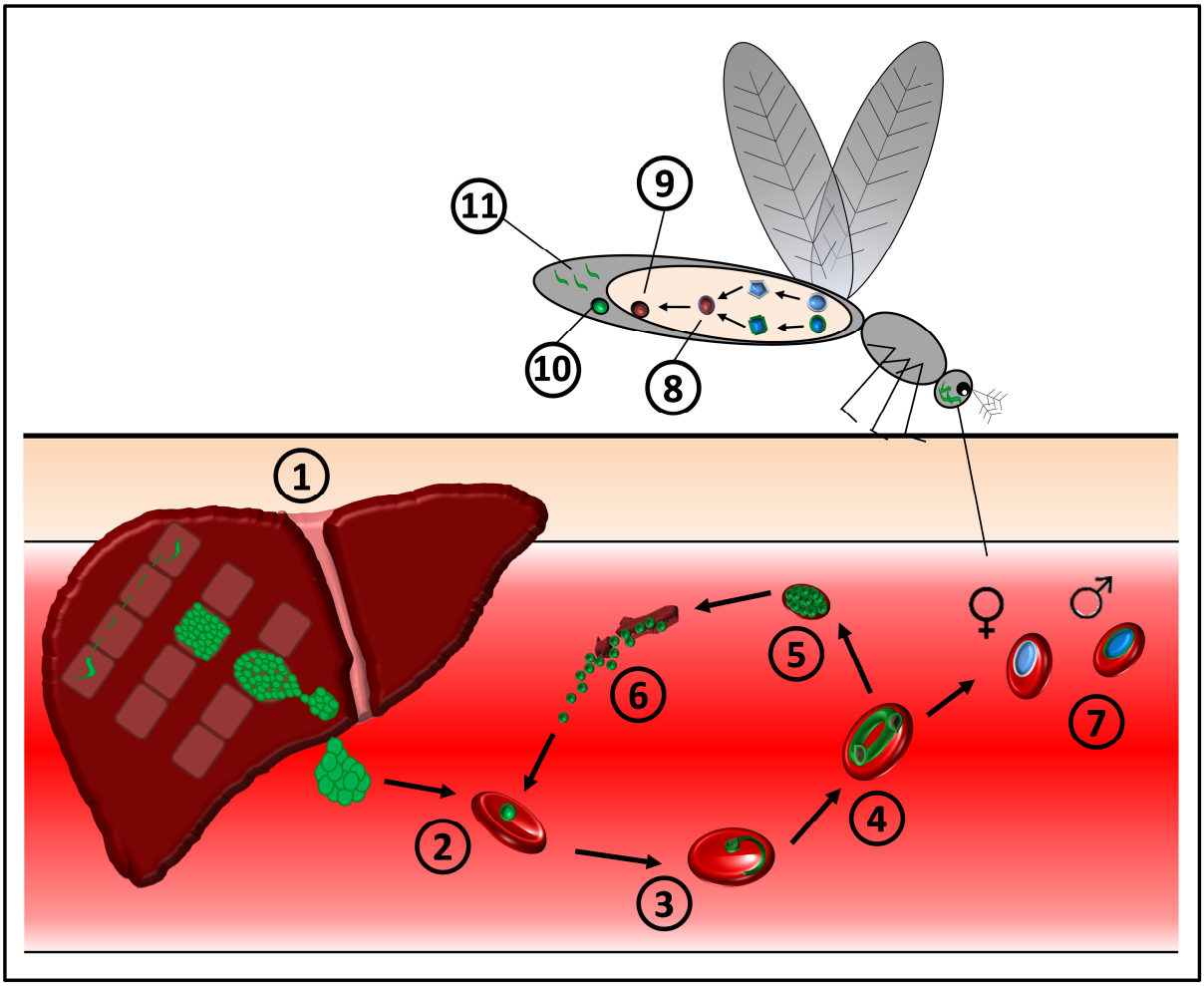

Fig. 1.5: The plasmodial life cycle. 1) liver stage; 2) merozoites infection of the erythrocyte; 3) ring stage; 4) trophozoite; 5) schizonts; 6) gametozoite; 7) raptured schizonts and released merozoites; 8) zygote; 9) ookinete; 10) oocyte; 11) sporozoites. The graphic was created in accordance to following literature: CDC, 2012 (61).

1.4.1 Experimental malaria models

The limited host range of the human infecting *Plasmodium* strains causes one of the major challenges in studying human malaria. Only large apes, including gorillas and chimpanzees and some smaller non-human primate species, are susceptible for *P. falciparum* and *P. vivax* (the two most prevalent malaria *Plasmodium* strains) infection. The most valuable and closest experimental malaria model to human malaria is the *Plasmodium* infection of chimpanzees. Nevertheless, the ethical concern that arises with the use of primates is to respect. Furthermore the high costs and scarcity of primates leads to small cohort sizes in experiments. Additionally the reproducibility of research data is limited due to the inter - individual variability of outbred species.

To bridge this ambiguous problem, scientists established a murine model to elucidate the pathology induced by the infection of plasmodial species. The advantages of a small animal model, including the fairly easy amenability of genetic manipulation, made the murine model the chosen system to investigate malaria. There are four *Plasmodium* species available, including *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei*. All those species where isolated from thicket rats and were firstly described in the middle of the 20th century. Since the infection of mice with one of those species will not mirror all human aspects of malaria pathology, the murine infection with one of these species for the investigation of a defined tasks concerning *Plasmodium* infection has to be chosen precisely. For example the infection with *P. yoelii* NL (a non - lethal strain) will lead to a high parasitemia with a high immune response able to clear infection without harming the host and to create an immunological memory (62). A widely established plasmodial strain to investigate cerebral malarial symptoms in C57BL/6 mice is P. *berghei* ANKA (PbA) in which the incidence of the lethal pathology is high and reproducible. This model highlighted the importance of the immune system in the modulation of cerebral malaria and made it possible to elucidate key factors in the development of cerebral malaria, e.g. IFNγ (63-66). The pathologic inflammation in malaria is highly promoted by IFNγ - producing $CD8^+$ T cells that adhere to the brain endothelium accompanied by a T_H1 response of $CD4^+$ T cells (67). Cerebral symptoms can be prevented with the depletion of $CD8^+$ as well as $CD4^+$ T cells (68). This effect shows how pathology is closely related to the immunological response in *Plasmodium* infection.

1.4.2 Immune response and malaria pathology

Because of the short time window of sporozoite development and the elusive property of the liver to create an immune regulatory environment it is nearly impossible to establish a proper adaptive immune response against sporozoites. Hence, only few is known about this pre-erythrocytic stage, which causes no symptoms in the host. Nevertheless IgG antibodies directed against proteins of the sporozoite are detectable, furthermore memory B cells have been discovered (69, 70). Even though little is known about the $CD4^+$ T cell response in this stage of plasmodial development, $CD4^+$ T cells which were specific for antigens of this stage have been documented (71). Protective $CD8^+$ T cells against a specific peptide of the circumsporozoite protein (CSP), a protein highly specific for the sporozoite stage of plasmodial development, have been reported as well. This CSP specific $CD8^+$ T cell group was shown to be primed by DCs in the lymph node after infection with sporozoites in an experimental mouse model (72). Nevertheless, this immune response is not effective enough to combat sporozoite infection. So in the end of the liver phase merozoites are formed which will subsequently infect erythrocytes in the blood phase of malaria. With the infection of erythrocytes the parasite evades the immune system one more time, since these cells do not express MHC molecules. The rupture of the iRBCs and release of the merozoites leads to the induction of the immune response which is associated with fever in the host. The most severe form of malaria has been associated with the infection of *P. falciparum* in human. These severe forms are known as complicated malaria and have similar symptoms to sepsis (73). The symptoms of complicated malaria can affect many organs and occur mainly in children under the age of five. Especially these young children are affected because their immune system has not been fully developed (6). The characteristic indications are high fever, coma, cramps, shock and anaemia resulting in difficulties in breathing. The recognition of the *Plasmodium* in the blood phase through the innate immune system through the recognition of the parasites by toll-like receptors (TLRs) is controversially discussed. It was postulated that the parasitic glycosylphosphatidylinositol binds to TLR 2 (74, 75) and that the haemozoin, which is formed by the degradation of haemoglobin, can be detected by the TLR 9 (76). The binding of the TLRs with parasitic molecules subsequently is meant to lead to the production of inflammatory cytokines like TNFα, IFNγ and nitric oxide as well of the TLR expressing cell. Even though the deficiency of TLR 2/4/9 in PbA - infected mice showed no significant differences in the occurrence of cerebral malaria symptoms (77). Nevertheless

cytokines released of innate immune cells attract cells of the adaptive immune response to the site of inflammation. Since the blood stage of plasmodial infection affects the whole body in the host, the immune response can lead to tremendous side effects and subsequently to pathology. An important characteristic feature which contributes to severe pathology of *P. falciparum* - induced malaria is the ability of infected red blood cells (iRBCs) to adhere to endothelial cells, a process known as sequestration. The ability of iRBC to bind to the endothelial is mediated by the formation of knob like structures on the surface of the iRBC on which the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is expressed. This molecule can bind to CD36 (a molecule highly expressed in fatty tissue), intracellular adhesion molecule 1 (ICAM1) and to the platelet endothelial cell adhesion molecule 1 (PECAM1). With the sequestration to the endothelial of small vessels and in fatty tissue, the *Plasmodium* parasite avoids the clearance during the passage through the spleen (78). Especially the sequestration of iRBCs to the endothelium of capillaries in the brain leads to cerebral pathology. This includes the activation of endothelial cells and subsequently the release of pro-inflammatory cytokines. Theses cytokines attract $CD4^+$ and $CD8^+$ T cells which in turn secrete cytokines as well. This so - called cytokine storm leads to an impairment of the blood brain barrier (79). One cytokine which is necessary for the development of cerebral malaria symptoms is IFNγ. It was shown by several groups that the deficiency of its receptor leads to the absence of cerebral malaria (80, 81). During the blood phase the high IFNγ level is due to the released by a variety of different T cell subsets as well as of NK cells. This is mainly mediated by IL-12 produced by macrophages and DCs and drives the inflammation into a T_H1 immune response. The classical T_H2 response does not play an elusive role in plasmodial infection. The presence of its characteristic IL-4 was thought to be indispensable for B cell differentiation into antibody - secreting plasma cells in malaria but this was rejected by the group of von der Weid, *et al.* (82). Furthermore a subset of IL-21 - releasing T cells (T follicular helper cells, T_{fH} cells) came recently more into focus concerning the differentiation of B cells into plasma cells (83). The impairment of specific antibody release of plasma cells in the absence of IL-21 was also recently described in malaria (84). Nevertheless the role of IL-21 in B cell differentiation, especially in malaria has to be further investigated.

1.4.3 The role of IL-22 in malaria

Since IL-22 plays an elusive role concerning its involvement in different immune diseases and infection models it is of special interest to elucidate its role in malaria. An important scientific contribution concerning IL-22 in malaria was done by Koch *et al.* (85). The group showed that the *IL22* gene is located in proximity to the highly important IFNG gene for malaria. Subsequently the *IL22* gene was taken into consideration for further investigations regarding the severeness of malaria symptoms in a large case-control study. Two haplotypes of the *IL22* gene were found to induce either resistance or susceptibility to *P. falciparum* infection. This study made IL-22 interesting concerning its function in plasmodial infection. Furthermore, the role of IL-22 concerning its ability to maintain the homeostasis of epithelial cells at barrier surfaces (34, 35) brought this cytokine into light for additional investigation for malaria, since the disruption of the brain epithelium leads to lethal symptoms. This fact goes in line with the decreased survival of *P. chabaudi* - infected IL-22$^{-/-}$ mice (86). Additionally IL-22 was shown to modulate the IFNγ response by suppressing the upregulation of MHC class I and II expression on epithelial cell during inflammation (87).

1.5 Aim

Severe malaria pathology is induced by a misbalance of pro - inflammatory and anti - inflammatory immune responses. IL-22 is closely related concerning the sequence homology to IL-10, a strong immune regulatory cytokine. It was shown that IL-22 - deficient mice had a decreased survival rate in *P. chabaudi* infection (86). To further investigate the impact of IL-22 during malaria a murine model was applied which includes the infection of C57BL/6 mice with *P. berghei* ANKA (PbA). This model is widely accepted to reflect the severe malaria symptoms of *P. falciparum* - infected human in a small animal model (67). IL-22 is known to maintain the homeostasis of epithelial cells at barrier surfaces (34, 35) and additionally it was shown that IL-22 can have regulatory effects on the IFNγ secretion (87). These two effects are essential for the immune response during plasmodial infection and the development of malaria pathology. Taken together, the hypothesis was created that IL-22 prevents pathology during malaria. To confirm this hypothesis, IL-22 - deficient mice were infected with PbA and monitored concerning the occurrence of CM and development of parasitemia. Furthermore the immune profile of different lymphocyte populations,

regarding their ability to produce IFNγ and IL-17 was analysed. Moreover it was of special interest to determine the link between the immune system and IL-22, keeping in mind that hematopoietic cells are postulated to express no IL-22 receptor (88).

2 Material

2.1 Laboratory equipment

Tab. 2.1: Laboratory equipment

EQUIPMENT	COMPANY
Agarose gel electrophoresis	*PeqLab Biotechnology, Erlangen*
Analytical scales	*Sartorius AG, Göttingen*
Benchtop rotator "Duomax 2030"	*Heidolph Instruments, Schwalbach*
Benchtop microcentrifuge	*Eppendorf, Hamburg*
Centrifuge "5415 R"	*Eppendorf, Hamburg*
Centrifuge "5810 R"	*Eppendorf, Hamburg*
Centrifuge "Megafuge 1.0 R"	*Heraeus Instruments, Hanau*
CO_2 incubator	*ThermoFisher Scientific, Waltham (USA)*
Class II biological safety cabinet „Biowizard"	*Kojair, Finnland*
ELISA reader "MRX II"	*Dynex Technologies, Berlin*
Flow cytometer "Accuri C6"	*BD Bioscience, Heidelberg*
Flow cytometer "FACSariaIII"	*BD Bioscience, Heidelberg*
Flow cytometer "LSR 2"	*BD Bioscience, Heidelberg*
Geldocumentation	*Vilber Lourmat, France*
Inverse microscope 'Nikon TMS'	*Nikon Instruments, Badhoeverdorp, Netherlands*
IVC 'Blue Line IVC Sealsafe'	*Tecniplast, Valencia, Italy*
Microscope 'Olympus CK2'	*Olympus, Hamburg*
Microscope 'Axiostar plus'	*Zeiss, Oberkochen*

EQUIPMENT	COMPANY
Microwave	*Panasonic, Wiesbaden*
Nano-drop photometer 2000 c	*ThermoFisher Scientific, Waltham (USA)*
PCR Machine "Primus 96^{plus}"	*MWG AG Biotech, Ebersberg*
pH meter HI 221	*Hanna Instruments, Kehl am Rhein*
Pipettes	*Gilson, Middleton (USA)*
Pipette controller „Swiftpet+"	*Abimed, Langenfeld*
Power supply for agarose gel electrophoresis	*BioRad, Munich*
Quantitative real time PCR Cycler "Corbett RG6000"	*Qiagen, Hilden*
Thermomixer „5436"	*Eppendorf, Hamburg*
Vortex 'MS1 Minishaker'	*IKA Labortechnik, Staufen*
Peristaltic Pump P1	*GE Healthcare, Freiburg*

2.2 Glass and plastic ware

Tab. 2.2: Glass and plastic ware

EQUIPMENT	COMPANY
10 mL plastic column	*Mo Bi Tec, Göttingen*
Cell culture plates (6-, 24- and 96-well)	*Greiner bio-one, Frickenhausen*
Cell strainer 'Falcon' (50 µm & 100 µm)	*BD Biosciences, Bedford (USA)*
Centrifuge tubes (15 and 50 mL)	*Greiner bio-one, Frickenhausen*
Connectors, Barbed for Pump P1	*GE healthcare, Freiburg*
Disposable chirugical scalpel	*Braun, Melsungen*
Disposable pipette (5, 10 and 25 mL)	*BD Bioscience, Heidelberg*

EQUIPMENT	COMPANY
DuoSet ELISA for IFNγ	*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*
Tubing for Pump P1	*GE Healthcare, Freiburg*

2.3 Chemicals

All chemicals derive from Merck (Darmstadt), Roth (Karlsruhe) or Sigma-Aldrich (Taufkirchen), if not otherwise declared.

2.4 Material for molecular biology

2.4.1 Oligonucleotide Primer

Oligonucleotide primers used for PCR and qPCR were obtained from MWG Biotech (Ebersberg).

Tab. 2.3: Oligonucleotide primer

PRIMER	SEQUENCE
Pb 18S rRNA forward	5‘-GGATGTATTCGCTTTATTTAATGCTT-3‘
Pb 18S rRNA reverse	5‘-CACGCGTGCAGCCTAGTAT-3‘
GAPDH forward	5‘-GGGTGTGAACCACGAGAAAT-3‘
GAPDH reverse	5‘-CCTTCCACAATGCCAAAGTT-3‘
IL-22Rα1 forward	5’-CTACGTGTGCCGAGTGAAGA-3’
IL-22Rα1 reverse	5‘-AAGCGTAGGGGTTGAAAGGT-3‘
GAPDH forward (for IL-22Rα1-PCR)	5’- GTCGGTGTGAACGGATTTGG-3‘
GAPDH reverse (for IL-22Rα1-PCR)	5‘-TTCCCATTCTCGGCCTTGAC-3‘

2.4.2 Reagents for molecular biology

Tab. 2.4: Reagents for molecular biology

REAGENT	COMPANY
Agarose	*Biomol, Hamburg*
Ampuwa H_2O	*Fresenius, Graz (Austria)*
dNTPs	*ThermoFisher Scientific, Waltham (USA)*
DNA standard “100 bp DNA Ladder plus”	*ThermoFisher Scientific, Waltham (USA)*
Ethidium bromide	*Sigma, Deisendorf*
Maxima SYBR Green qPCR Master Mix	*ThermoFisher Scientific, Waltham (USA)*
stainless steel beads, 5mm	*Qiagen, Hilden*
TRIzol	*Life Technologie, Carlsbad (USA)*

2.4.3 Kits for molecular biology

Tab. 2.5: Kits for molecular biology

KIT	COMPANY
Maxima First Strand cDNA Kit with dsDNase	*ThermoFisher Scientific, Waltham (USA)*
Maxima First Strand cDNA Kit	*ThermoFisher Scientific, Waltham (USA)*

2.5 Material for cell biology

2.5.1 Reagents for cell biology

Tab. 2.6: Reagents for cell biology

REAGENT	COMPANY
DMEM (without L-Glutmain)	*PAN-Biotech, Aidenbach*
EDTA reaction tubes	*Kabe, Nümbrecht-Elsenroth*
Fetal calf serum (FCS)	*PAN-Biotech, Aidenbach*
Fc-Bock	*BNITM,Hamburg*
Gentamycin solution	*PAA, Pasching (Austria)*
Incidin Liquid	*Ecolab, Düsseldorf*
L-Glutamine	*PAA, Pasching (Austria)*
Monensin (1000x)	*Biolegend, San Diego (USA)*
Peptide Pb1 SQLLNAKYL	*Jerini Biotools, Berlin*
Peptide Pb2 IITDFENL	*Jerini Biotools, Berlin*
Peptide Pb3 EIYIFTNI	*Jerini Biotools, Berlin*
Percoll	*GE Healthcare, Uppsala (Schweden)*
RPMI 1640 (without L-Glutamine)	*PAN-Biotech, Aidenbach*
William's Medium + GlutaMAX	*Gibco life technologie, Carlsbad (USA)*

2.5.2 Kits for cell biology

Tab. 2.7: Kits for cell biology

KIT	COMPANY
Foxp3 / Transcription factor staining buffer set	*eBioscience, San Diego (USA)*
MACS CD8a$^+$ T cell isolation kit, mouse	*Miltenyi Biotec GmbH, Bergisch Gladbach*
MACS CD146 (LSEC) Microbeads, mouse	*Miltenyi Biotec GmbH, Bergisch Gladbach*

2.6 Antibodies and fluorescent dyes

Tab. 2.8: Antibodies and fluorescent dyes

ANTIBODY	CLONE	COMPANY
αD3 PerCP Cy5.5	145-2C11	*Biolegend, San Diego (USA)*
αCD8 eFluor 450	53-6.7	*eBioscience, San Diego (USA)*
αCD11b V450	M1/70	*BD Pharmingen, New Jersey (USA)*
αCD11c Allophycocyanin	N418	*Biolegend, San Diego (USA)*
αCD11c PE-Cy7	N418	*Biolegend, San Diego (USA)*
αCD44 PE-C7	IM7	*Biolegend, San Diego (USA)*
αCD80 FITC	16-10A1	*BD Pharmingen, New Jersey (USA)*
αCD86 Allophycocyanin	GL-1	*Biolegend, San Diego (USA)*
αγδT PE-Cy7	GL3	*Biolegend, San Diego (USA)*
αIFNγ PE	XMG 1.2	*eBioscience, San Diego (USA)*
αIL-17A Allophycocyanin	17B7	*eBioscience, San Diego (USA)*
αIL-22 PE	1H8PWSR	*eBioscience, San Diego (USA)*
αNK1.1 PE-Cy7	PK 136	*BD Pharmingen, New Jersey (USA)*

ANTIBODY	CLONE	COMPANY
αIL-22	polyclonal	*R&D Systems, Wiesbaden*
Normal Goat IgG Control		*R&D Systems, Wiesbaden*
CFSE		*Biolegend, San Diego (USA)*

2.7 Buffer, solutions and culture media

Tab. 2.9: Buffers, solutions and culture media

BUFFER	INGREDIENTS
Block buffer (ELISA)	1 % BSA in PBS
Compete DMEM medium	500 mL DMEM High Glucose (4,5 g/L) with 25 mM HEPES 50 mL FCS 5 mL Glutamin (200 mM) 2.5 mL Gentamicin (10 mg/mL)
Complete RPMI 1640 medium	500 mL RPMI 1640 50 mL FCS 5 mL Glutamin (200 mM) 2.5 mL Penicillin/Streptavidin (100x)
Ethidium bromide solution	10 mg/mL
Erythrocyte lysis buffer	0.1 M Tris-HCl (pH 7,5) 0.16 M ammonium chloride (NH_4Cl)
FACS-Buffer	1 % FCS 0.1 % sodium azide (NaN_3) in PBS
LB-Medium	20 g LB-Broth ad 1 L with ddH_2O,
Liberase solution	1mg / ml in PM
MACS-Buffer	2 nM EDTA 0.5 % BSA in PBS
PBS (20x) pH 7,4	160 g sodium chloride (NaCl) 4 g potassium chloride (KCl) 28,8 g disodium phosphate (NaH_2PO_4) 4.8 g monopotassium phosphate (KH_2PO_4) ad 1 L ddH_2O; pH 7,4

BUFFER	INGREDIENTS
PM	400 mg KCL 190 mg $MgSO_4$ x 7 H_2O 190 mg $MgCl_2$ x 6 H_2O 60 mg Na_2HPO_4 x 2 H_2O 2.38 mg Hepes 8 g NaCl 60 mg KH_2PO_4 2 g glucose 220 mg $CaCl_2$ 2 g BSA ad 1 L ddH_2O, pH 7,4
PPML	400 mg KCL 58 mg KH_2PO_4 350 mg $NaHCO_3$ 8.06 g NaCl 68 mg Na_2HPO_4 x 2 H_2O 1 g glucose 190 mg EGTA 11.91 g Hepes ad 1 L H_2O, pH 7,35
Stop solution (ELISA)	2 M sulfuric acid (H_2SO_4)
Substrate buffer (ELISA) **pH 5,5**	100 mM Sodiumhydrogenphosphate (NaH_2PO_4)
Substrate solution (ELISA)	12 mL ELISA substrate buffer 200 µL TMB stock solution 1.2 µL hydrogen peroxide (H_2O_2, 30 %)
TMB stock solution	30 mg Tetramethylbenzidine in 5 mL DMSO
TBE-Puffer (10x) **pH 8,0**	0.89 M Tris base 0.89 M boric acid (H_3BO_3) 20 mM EDTA

2.8 Enzymes

Tab. 2.10: Enzymes

ENZYME	COMPANY
Liberase TH Reasearch Grade	*Roche, Rotkreuz (Switzerland)*

2.9 Parasites

Tab. 2.11: Parasites

STRAIN	ORIGIN	ABBREVIATION USED IN THE THESIS
***Plasmodium* berghei ANKA**	BNI, Hamburg	PbA
***Plasmodium* berghei ANKA OVAtg**	BNI, Hamburg	PbA-OVAtg
***Plasmodium* berghei ANKA GFP**	BNI, Hamburg	PbA-GFP
***Plasmodium* yoelii non-lethal**	BNI, Hamburg	PyNL

2.10 Mice

Tab. 2.12: Mice

STRAIN	ORIGIN	ABBREVIATION USED IN THE THESIS
C57BL/6	BNI, Hamburg	Wt
C57BL/6 IL-22-/-	BNI, Hamburg	IL-22$^{-/-}$
C57BL/6 IL-22BP-/-	UKE, Hamburg	IL-22BP$^{-/-}$
C57BL/6 IL-22RA_1 KO	UKE, Hamburg	IL-22Rα1$^{-/-}$
C57BL/6 OT1	BNI, Hamburg	OT1

2.11 Material for animal experiments

Tab. 2.13: Material for animal experiments

SUBSTANCE	COMPANY
Rompun 2 % (Xylanzin)	*Bayer, Leverkusen*
Ketavet 100 mg/mL (Ketamin)	*Pfizer, Berlin*
Wright's stain	*Sigma Chemical, St. Louis (USA)*

2.12 Software and data bases

Tab. 2.14: Software and data bases

PROGRAM	PURPOSE
Adobe Reader X	Reading PDF data
FlowJo 10	Analysis of flow cytometry data
GraphPad Prism 5	Statistical analysis
Microsoft Office 2010	Word and graphic processing, spreadsheets
PDF24 Editor	Creating PDF data
PubMed	Literature database

3 Methods

3.1 Molecular biology

3.1.1 Isolation of RNA

Cells or tissues were collected and stored at - 70 °C in 500 µL or 1000 µL TRIzol reagent (Life Technologies), depending on the amount of tissue. For the further procedure the samples were thawed at room temperature (RT). Tissue samples were additionally homogenized with the TissueLyzer LT (Qiagen) in the presence of a 5 mm stainless steel bead per tube at 50 Hz for 4 min. In the next step the chloroform was added in a 1 to 6 ratio (v/v) and the samples were vortexed and incubated at RT for three minutes. Subsequently they were centrifuged at 4 °C and 12 000 rcf for 15 min. The aqueous RNA containing solution was transferred into a new tube and 500 µL of isopropanol were added, followed by vortexing and a 10 min incubation time at RT. The samples were then centrifuged at 4 °C and 12 000 rcf for 10 min and subsequently the RNA pellet was suspended in 500 µL 75 % ethanol and further centrifuged at 4 °C and 12 000 rcf for 5 min. The supernatant was discarded and the RNA pellet dried under the laminar flow cabinet for 20 min. By incubating the sample at 60 °C for 10 min the RNA was solved in 20 - 100 µL RNAse free Ampuwa depending on the amount of used cells or tissue, respectively. The RNA concentration was measured at the NanoDrop 2000c (ThermoFisher Scientific) and the samples were stored at - 70 °C.

3.1.2 Reverse transcription of mRNA into cDNA

Depending on the RNA content of the previous mRNA isolation, 60 ng - 200 ng mRNA were used for the reversed transcription into cDNA. The reverse transcription was performed accordingly to the Maxima First Strand cDNA Kit with dsDNase (ThermoFisher Scientific) protocol for cells and splenic tissue or accordingly to the Maxima First Strand cDNA Kit (ThermoFisher Scientific) protocol for the reversed transcription of mRNA of PBS perfused liver tissue. The obtained cDNA was stored at - 20 °C.

3.1.3 Polymerase chain reaction (PCR)

The polymerase chain reaction is a method to amplify exponentially a defined nucleic acid sequence *in vitro*. The specificity is determined with the accurate choice of the primer oligonucleotide sequences.

The DNA strand serves as matrix, obtained as part from the DNA-RNA-hybrid from the previous reversed transcription. In the beginning it is necessary to denature the target DNA at 95 °C, allowing the specific primers to anneal before and after the target DNA sequence at 60 °C. The annealing temperature is specific defined for the chosen primers pair. The DNA synthesis is performed by a Taq-polymerase, which is stable even at high temperatures. The elongation is performed in the 5' - 3' direction at 72 °C. With each cycle of denaturation, annealing and elongation the target sequence is doubled. To exclude a false positive control through DNA contamination, an additional control sample without template cDNA was carried out. To control if the applied amount of cDNA is the same in each sample a control PCR is performed on a ubiquitous expressed housekeeping gene.

The reaction mixture contains:

Template cDNA	10 ng
dNTP (2 mM)	2 µL
Forward primer (2 pmol)	0.2 µL
Reverse primer (2 pmol)	0.2 µL
DreamTaq buffer (10 x)	2 µL
DreamTaq 5U/µL	0.2 µL
ddH_2O	ad to 20 µL

The following PCR program was established for the here chosen target DNA. The PCR was performed in 0.2 mL reaction tubes.

		T [°C]	Time [sec]
	Initial melting	95	180
40 x	Denaturation	95	30
	Primer Annealing	60	20
	Elongation	72	15
	Final elongation	72	300
	Storage	8	∞

3.1.4 Quantitative polymerase chain reaction (qPCR)

For the exact quantification of the expression of a DNA product, a PCR of the target gene and an additional reference gene, known as housekeeping gene, are carried out. Thus the expression of the RNA is determined in relation to an ubiquitous expressed housekeeping gene. An intercalating fluorescent dye is utilized to determine the exact amount of the DNA products. The fluorescence intensity stands in direct relation to the DNA product.

In this study the amount of the *Plasmodium* 18S rRNA was quantified in relation to the mouse - GAPDH housekeeping gene. For this purpose the samples were analysed in duplicates. The following reaction mixture was chosen:

Template cDNA	50 ng
Forward primer (5 pmol)	2 µL
Reverse primer (5 pmol)	2 µL
Sybr Green (2 x)	10 µL
ddH_2O	ad to 20 µL

The following PCR program was chosen for the performance of the 18S specific qPCR:

		T [°C]	Time [sec]
	Initial melting	95	900
35 x	Denaturation	95	15
	Primer Annealing	50	20
	Elongation	68	20
	Final Denaturation	95	30
	Melting curve analysis from 67 °C to 95 °C		

The relative expression of the target sequence was analysed using the $\Delta\Delta c_t$ method.

3.1.5 Agarose gel electrophoresis

The DNA is charged negatively and moves in an electric field toward the anode. The agarose forms a net in which DNA fragments are separated according to their size. Taller DNA fragments are hindered and move slower to the anode than smaller DNA fragments. The distance they move in the electric field stands in reverse proportion to the logarithm of their size. In this study gels had an agarose concentration of 1.5 % (w/v) in TBE buffer. The preparation of the gel includes the bowling of the appropriate amount of agarose in TBE buffer and the addition of ethidium bromide, a DNA intercalating dye which allows the detection under UV - light of the separated DNA fragments after gel electrophoresis. The gel

was poured into a gel chamber and the samples were loaded directly, since the used DreamTaq PCR buffer already includes a loading dye. The fragment sizes were determined with the addition of a 100 kb DNA ladder plus (GeneRuler 100 bp plus; ThermoFisher Scientific). The agarose gel electrophoresis was carried out at 60 V.

3.2 Biochemistry

3.2.1 Enzyme - linked immunosorbent assay (ELISA)

For a quantitative determination of the IL-22 concentration in the blood plasma or of the IFNγ concentration in supernatants via ELISA, special coated 96 well plates were utilized. The ELISA allows the determination of protein concentration in low amount with the usage of specific antibodies. To start, the plate was incubated over night at 4 °C with the capture antibody diluted in PBS. After this incubation the antibody solution was discarded and the plate was washed four times in 0.05 % Tween 20 in PBS (v/v). To avoid an unspecific binding of the coating antibody the wells were incubated with 150 µL/well 1 % BSA in PBS (v/v) at RT for 2 h. After discarding the blocking buffer, the samples were loaded in 50 µL/well in a 1 to 4 dilution in PBS as triplicates. A standard was loaded on the plate in duplicate as reference to determine the exact concentration of the searched protein. The samples were incubated over night at 4 °C. In this step the antigens bind to the pre-coated capture antibody. The next day the samples were removed and the plate was washed four times in 0.05 % Tween 20 in PBS (v/v) to discard remaining unbound particles. The biotinylated detection antibody was diluted in 0.1 % BSA in PBS (v/v) and added in 50 µL/well. The binding of the detection antibody to the selected protein took place at RT. After one hour of incubation time the detection antibody solution was discarded and the plate was washed four times in 0.05 % Tween 20 in PBS (v/v). During the incubation time of 30 minutes with 50 µL/well streptavidin horseradish peroxidase (1:200 in 0.1 % BSA in PBS (v/v)), the streptavidin linked enzyme binds to the biotinylated detection antibody. After repeating the washing procedure the plate was incubated with the 100 µL/well substrate solution (for one plate: 12 mL substrate solution; 200 µL TMB solution; 1.2 µL H_2O_2) for 15 to 30 minutes. Within this step the peroxidase reduces the substrate toward a blue product. The reaction was stopped with 25 µL of 2 M sulphoric acid. This induces a colour change from blue to yellow. The intensity of the colour is related to the concentration of the specific chosen

protein and will be detected with an ELISA reader at a wavelength of 450 nm. The exact quantification is determined in accordance to the standard and the negative control.

3.3 Eukaryotic cell biology

3.3.1 Determination of the cell concentration

Single cell suspensions were diluted 1 to 5 in trypan blue. The determination of the cell concentration was performed with the help of a Neubauer cell counting chamber. The cells from four counting grids were counted within each of the four large squares. Hence, 16 counting grids were counted in total. The total number of cells per millilitre was determined by multiplying the dilution factor with 10^4 and with the counted number of cells:

$$Cell\ number/ml = Counted\ cells \times dilution\ factor \times 10^4$$

If the cell concentration of many samples had to be calculated at the same time, the application of the flow cytometer Accuri C6 was preferred. The single cell suspensions were diluted 1 to 20 in a total volume of 200 µL in PBS and subsequently the flow cytometer determined the cell number in a volume of 50 µL per sample. The Accuri C6 software allows an automatized determination of the cell concentration of several samples at a time. Afterwards the single cell suspensions were adjusted to the right concentration and further procedures were carried out.

3.3.2 Generation of bone marrow derived dendritic cells (BMDCs)

For the isolation of BMDC the hind leg was taken from a mouse and subsequently the bone was separated from the flesh. In the next step the bone was disinfected in 70 % isopropanol and the ends of the tibia were cut with sterile scissors. The bone marrow was flushed with cell culture media into a petri dish. The gained bone marrow was suspended in cell culture media and centrifuged at 1200 rpm for 5 min at 4 °C. The supernatant was discarded and the cells were treated with 5 mL erythrocyte lysis buffer for 5 min at RT. The lysis was stopped with the addition of 5 mL PBS. Afterwards the cells were again centrifuged at 1200 rpm for 5 min at 4 °C. Monocyte precursors from the bone marrow can differentiate into dendritic cells (DCs) in the presence of the cytokine GM-CSF. Hence, the cells were suspended in GM-CSF containing cell culture medium (DMEM containing 10 % FCS, 1 % L-Glutamine, 0.5 % Gentamycin and 10 % GM-CSF) and the cell concentration was determined. 3×10^6 cells were cultured in 8 mL GM-CSF containing cell culture medium in a petri dish at 37 °C and 9 %

CO_2. At day three and six after bone marrow isolation 8 mL of the GM-CSF containing cell culture medium were added additionally to the culture. At day 7 the BMDCs were fully differentiated and used for further experiments.

3.3.3 Lymphocyte isolation from the spleen

For the isolation of murine splenic cells mice were euthanized and the spleens were dissected and transported from the mouse facility in 5 mL sterile PBS on ice to the laboratory. The spleens were mashed through a cell strainer (Falcon) with the stamp of a sterile 5 mL syringe and rinsed with 10 mL sterile PBS into a 50 mL Falcon and subsequently centrifuged at 1200 rpm for 5 min at 4 °C. The supernatant was discarded and the cells were suspended in 5 mL erythrocyte lysis buffer for 5 min at RT. The lysis was stopped with the addition of 5 mL PBS. Afterwards the cells were centrifuged at 1200 rpm for 5 min at 4 °C. After discarding the supernatant the cells were suspended in 10 mL sterile cell culture medium (RPMI containing 10 % FCS, 1 % L-Glutamine and 0.5 % Penicillin/Streptomycin). The cell number was determined and the exact cell concentration was adjusted.

3.3.4 Isolation of primary hepatocytes

In order to isolate primary hepatocytes out of a mouse the appropriate buffers (30 mL PPML, 25 mL PM, 50 mL PM) have to be warmed up in a water bath at 42 °C. During this time the tube of the pump was prepared and the right flow velocity was adjusted with 20 mL ddH_2O. The flow through had to be between a slow continuous flow and shortly before a drop-like flow. The mouse was anesthetized with an overdose of 1 to 6.67 diluted Ketaminhydrochloride (Stock: 100 mg/mL) and a 1 to 200 dilution of Xylanzin (Stock: 20 mg/mL) in PBS. During the mouse falls asleep the tube of the pump was rinsed with 10 mL of the PPML buffer and 1 mL of the Liberase enzyme (Stock: 1 mg/mL) was diluted in 25 mL of the PM buffer by inverting the falcon. The fully anesthetized mouse was fixed, a median incision was performed and the peritoneum and thorax were opened to obtain a clear view on the beating heart. The gut, spleen, stomach were thrust aside and fixed. A threat was used to pinch off the vena carva superior. With this step the portal vein swells because the blood accumulates there. This is the time point were the cannula, which is connected to the pump and to the reservoir of the enzyme containing PM buffer, is inserted into the portal vein. When the liver is completely swollen the aorta is cut to release the blood and the pass through of the buffer. The total volume of 25 mL is slowly and

contentiously inserted into the portal vein. It is important to mention that the whole procedure has to be carried out without any air in the system. After the flow through of the enzyme the gallbladder is removed and the yellowish liver is transferred to a 10 cm petri dish which already contains 20 mL of PM buffer. The freed hepatocytes inside the liver and all other cells are shacked out into the dish containing the PM buffer. Subsequently this solution is poured through a cell strainer and the volume was adjusted to 50 mL. Then the hepatocyte containing solution was incubated for 20 min at RT, allowing them to sink to the bottom. This step was carried out without closing the lid, since the hepatocytes need the surrounding air oxygen. Subsequently the upper 24 mL were replaced with a 100 % Percoll solution, the cell suspension was carefully inverted several times and centrifuged at 550 rpm, 10 min at 4 °C. The supernatant was removed and the remaining hepatocytes were washed once with 20 mL complete primary hepatocytes culture medium (Williams E Glutamax containing 10 % FCS, 1 % L-Glutamine, 1 % Penicillin/Streptomycin). After centrifugation at 550 rpm, 10 min and 4 °C the hepatocyte pellet was diluted in 10 mL complete primary hepatocytes culture medium and the cell concentration was determined using only big pipet tips to avoid the disruption of hepatocytes through shear forces. 5×10^5 hepatocytes were transferred in a RNAse free reaction tube and stored in TRIzol at - 70 °C for further RNA isolation.

3.3.5 Isolation of liver sinusoidal endothelial cells (LSECs)

To obtain LSECs the same procedure as in 3.3.4 was carried out. Nevertheless the whole supernatant was collected after the hepatocytes were settled down. This supernatant contains many different cell types including the LSECs. In order to get these cells purified, two washing steps were carried out. The cells were centrifuged at 1200 rpm, 5 min, 4 °C and resuspended in 10 mL PBS. LSECs express the surface marker CD146 in a high extend. Hence these cells were enriched using the Magnetic activated cell separation (MACS technique) as well as a FACS sort. The obtained cells were then stored in TRIzol in RNAse free reaction tubes at - 70 °C.

3.3.6 Magnetic activated cell separation (MACS)

With the MACS technique it is possible to purify cell types due to their surface marker expression. Herein cells are tagged with specific antibodies for selected markers. These antibodies were in turn labelled with magnetic beads. After this procedure of labelling, the

single cell suspension was poured through a column which was located in a magnetic field. The antibody tagged cells remained in the column (positive selection). Cells which were collected in the flow through were negatively selected. The purity of the selected cell population was checked with flow cytometry. The purity of the positively selected LSECs reached 65 - 70 %. The purity of the negatively selected $CD8^+$ T cells reached 95 - 98 %.

3.3.7 Peptide specific restimulation of $CD8^+$ T cells

BMDCs were utilized in order to present PbA specific peptides on the MHC class I receptor. For that reason 3000 BMDC were seeded in a well of a 96 U-shaped plate and incubated with 1 µg/mL PbA specific peptides (Pb1: SQLLNAKYL; Pb2: IITDFENL; Pb3: EIYIFTNI) of the parasitic blood phase (89, 90) for 5 h in complete cell culture medium (DMEM containing 10 % FCS, 1 % L-glutamine and 0.5 % gentamycin). During this time splenic lymphocytes were isolated from PbA - infected mice on d6 p.i. in accordance to 3.3.3. Subsequently the lymphocytes were purified with the MACS technique in order to obtain $CD8^+$ T cells (3.3.6). In the next step the PbA peptide pulsed BMDCs were centrifuged (1500 rpm, 5 min, 4 °C), washed with 200 µL PBS per well and centrifuged again. The supernatant was discarded and 5×10^4 $CD8^+$ T cells were added per well in 200 µL complete cell culture medium. The assay was incubated for 24 h at 37 °C and 9 % CO_2. The supernatant was collected and stored at - 20 °C for further procedures.

3.3.8 Restimulation of splenocytes and intracellular cytokine staining

For the detection of cytokine producing cell types, the cells are stimulated in order to show their capability to produce cytokines at the time point of stimulation. For this purpose 2×10^6 splenic lymphocytes were stimulated with 50 ng/mL phorbol-12-myristrate-13-acetate (PMA) and 500 ng/mL ionomycin for at 37 °C and 5 % CO_2 for four hours. PMA activates the protein kinase C, since it has a similar structure to the natural ligand diacylglycerol. Ionomycin is an ionophor and allows calcium to influx into the cell. The combination of PMA and Ionomycin serves to simulate the activating effect of a stimulated T cell receptor. In the last three hours of stimulation, 2 µM Monensin were added to the stimulation cell culture medium (RPMI containing 10 % FCS, 1 % L-glutamine and 0.5 % penicillin/streptomycin) in order to inhibit the protein transport in the cell. This results in a concentration of the cytokines in the golgi-apparatus and leads to a highly improved detection of the produced cytokines during the intracellular staining for flow cytometry.

In order to stain the cells, the 96 U-shaped plate, containing 2 x 10^6 cells per well, had to be centrifuged at 1500 rpm, 5 min and 4 °C and the supernatant was discarded. 150 µL per well FACS buffer were added and the plate was centrifuged again (1500 rpm, 5 min and 4 °C). After this step the cells were stained with surface antibodies in a volume of 15 µL of Fc-block buffer. This buffer contains antibodies directed against the Fc-receptors expressed by some lymphocyte subpopulations. Hence the specificity of the chosen antibodies is ameliorated. After an incubation time of 25 min at 4 °C the cells were washed with FACS buffer and centrifuged (1500 rpm, 5 min and 4 °C). In the next step the cells were fixed and permeabilized in accordance to the manufacturer's protocol of the Foxp3 / Transcription factor staining buffer set (eBioscience).

3.3.9 CFSE proliferation assay

Splenic cells were isolated as in 3.3.3 and washed twice with PBS. Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent dye which integrates into the cell membrane. The lymphocyte concentration was adjusted to 1 x 10^7 cells/mL in 1 µM CFSE in PBS and incubated 2 min and 40 sec at RT. The CFSE labelling of splenic cells was stopped with 1 to 1 (v/v) complete cell culture medium and centrifuged (1200 rpm, 5 min and 4 °C). The washing step was repeated twice and the CFES labelling of the cells was checked by flow cytometry. The cell concentration was adjusted to 1 x 10^8 cells / mL in PBS and 2 x 10^7 were transferred intravenous (i.v.) into recipient mice. The proliferation of the CFSE labelled lymphocytes can be observed by flow cytometry. Hence each cell division is related to a decrease of CFSE contend in the membrane of the descendant cell population which leads to a decrease of fluorescence intensity of the proliferating cells.

3.3.10 Flow cytometry

With the help of the fluorescent activated cell sorting (FACS), structures, size, granularity as well as extra- and intra-protein expression can be detected. Cells are labelled with antibodies which are coupled to a fluorescent dye. Cells passes in a laminar flow a monochromatic laser which stimulates the fluorescent dye. The characterization of the cell composition is possible with the application of different fluorochromes which have unique emission wavelengths. With the forward scatter (FCS) and the sideward scatter (SSC) the relative size and granularity can be analysed. For all shown plots the LSR 2 was utilized.
In Tab. 3.1 the utilized fluorochromes are listed.

Tab. 3.1: Chosen fluorochromes for flow cytometry

FLUOROCHROME	LASER	max. ABSORPTION	max. EMISSION
eFluor 450	405 nm	404 nm	448 nm
AF 488	488 nm	495 nm	515 nm
Fluorescein Isothiocyanat (FITC)	488 nm	495 nm	519 nm
PE-Cy7	488 nm	496 - 565 nm	780 nm
PerCP Cy5.5	488 nm	465 nm	695 nm
Phycoerythrin (PE)	448 nm	565 nm	578 nm
Allophycocyanin	633 nm	650 nm	660 nm

Despite the capacity for analysing the composition of cell populations, the method of cell sorting can be applied based on the FACS procedure to enrich chosen cell populations. The method includes the labelling of a chosen cell type which is detected by flow cytometry and which is furthermore isolated in a new tube. These purified cell populations were subsequently reanalysed for purity which was between 92 - 98 % and stored in TRIzol at - 70 °C for further investigations.

3.4 Infection model

3.4.1 Mice

Mice were taken into experiment at an age between 7 and 10 weeks. The euthanasia was performed with CO_2 and a subsequent neck dislocation.

3.4.2 *Plasmodium* parasites and infections.

PbA and PyNL strains were stored in liquid nitrogen in a solution containing 0.9 % NaCl, 4.6 % sorbitol and 35 % glycerol. To start an experiment, mice were injected with the respective *Plasmodium* strain intraperitoneal (i.p.) and the blood was collected after 5 to 6 days post infection (p.i.). Infected red blood cells (iRBCs) were subsequently used to infect the experimental mice with either 1 x 10^5 iRBC PbA or 2 x10^4 iRBC PyNL i.p.. To maintain the life cycle of plasmodia, NMRI mice with a high parasitemia were anesthetized and used for a

blood meal of *Anopheles stephensi* mosquito at the mosquito colony of the Bernhard Nocht Institute for Tropical Medicine. After 18 to 25 days the sporozoites were isolated by manual dissection of the salivary glands of mosquitos. For further experiments 1000 sporozoites were injected i.v. into each mouse.

PbA - infected C57BL/6 mice develop cerebral malaria symptoms which includes the occurrence of neurological symptoms such as ataxia, convulsion and coma. These symptoms usually appear between d6 and 8 p.i. and ultimately lead to dead. Mice developing cerebral symptoms were scored daily (score 0: no symptoms; score 1: deceleration and weight loss < 10 %; score 2: ruffled fur; score 3: weight loss < 20 %, strong deceleration, paralysis of one leg; score 4: coma; score 5: dead). To avoid unnecessary suffering, mice were euthanized when reaching score 3 and scored as dead, since mice developing score 3 never cleared symptoms in our model and would progress to score 5 within several hours.

The parasitemia was analysed in a thin blood smear of *Plasmodium* - infected mice. This blood smear was then stained with WRIGHT stain. The percentage of infected erythrocytes was calculated on a total count of erythrocytes between 700 and 1000.

All experiments were in accordance with the local Animal Ethics Committee Regulations.

3.5 Neutralisation of IL-22 *in vivo* by antibody treatment

For the *in vivo* neutralization mice were treated with 20 µg αIL-22. The antibody was injected i.p. in 100 µL per mouse at the same time point of plasmodial infection. The control group was treated with the corresponding IgG.

3.6 Statistical Analysis

The GraphPad Prism 5 software was used to perform statistical analysis. Statistical differences in survival were analysed using Kaplan-Meier survival curve statistics. Additional statistical differences were identified using an unpaired t-test. For parasitemia testing the Mann-Whitney test was performed, assuming no Gaussian distribution for tested experiments. A regular two way ANOVA was performed for pooled data. p-Values were indicated as *,$p<0.05$; **, $p<0.01$; ***, $p<0.001$. All experiments were performed at least twice and indicated error bars are based on SEM values.

4 Results

4.1 Increased IL-22 plasma level in human and murine malaria

Two haplotypes of the *IL22* gene were shown to have an impact on the pathology of malaria. To determine whether the IL-22 protein is induced in malaria, its concentration was analysed in the blood plasma during *Plasmodium* species (spp.) infection with an IL-22 specific ELISA. The plasma of *P. falciparum* - infected human individuals (Fig. 4.1, A) as well as the plasma of *Plasmodium berghei* ANKA (PbA) - infected mice on d6 post infection (p.i.) (Fig. 4.1, B) was analysed concerning their IL-22 concentration and compared to healthy participants of the study or naive mice, respectively. In human as well as in mice the IL-22 level was significantly elevated in the blood plasma during plasmodial infection.

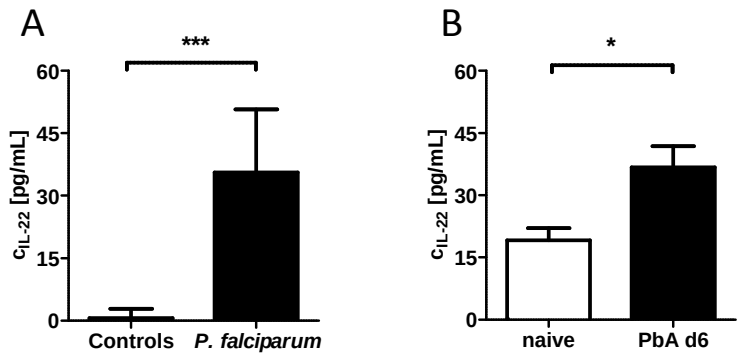

Fig. 4.1: Increased IL-22 plasma levels during malaria. IL-22 plasma levels of patients with *P. falciparum* infection were compared to healthy controls ($n_{control}$ = 9; $n_{P. falciparum}$ = 30) [A]. IL-22 plasma levels of PbA blood stage - infected wt mice were compared to PBS - treated naive controls on d6 p.i. ($n_{control}$ = 43; $n_{PbA\ d6}$ = 36). The concentration of IL-22 was analysed with an ELISA [B]. Indicated are the means with SEM. The statistical significance was analysed with the student's t-test (unpaired, two-tailed); $p<0.05^{*}$; $p<0.01^{**}$; $p<0.001^{***}$.

4.2 IL-22 - producing cell types in PbA infection

To elucidate which cell types are responsible for this increased IL-22 production in malaria, mice were infected with 1×10^5 PbA - infected red blood cells (iRBC) intra peritoneal (i.p.) and sacrificed on d6 p.i.. Subsequently splenocytes were isolated, stimulated with PMA/Ionomycin and intracellularly stained with an IL-22 specific antibody. The result was examined by FACS analysis. The $\gamma\delta^+$ T cell population showed the strongest ability to produce IL-22 upon PbA infection. Nevertheless other lymphocyte populations of PbA - infected mice showed also an increased IL-22 production on d6 p.i. compared to naive mice (Fig. 4.2).

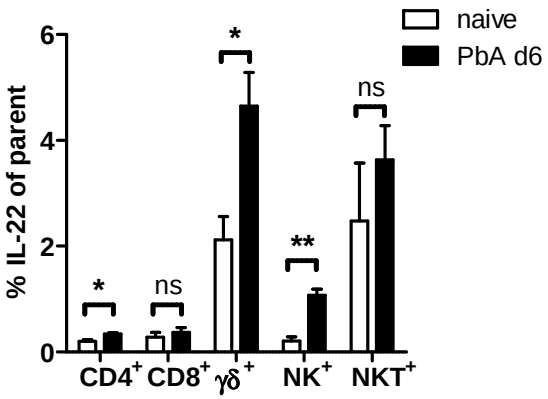

Fig. 4.2: Induction of IL-22 production in PbA infection of wt mice in different cell types. Wt mice were infected with 1 x 10^5 PbA - iRBC i.p. or treated with PBS as naive control. At d6 p.i. mice were sacrificed and 2 x 10^6 splenocytes were stimulated for 6 h with PMA/Ionomycin including the last 5 h with Monensin. The percentages in relation to the parent cell population of IL-22 secreting cells were determined by FACS analysis and are depicted in relation to the parent cell populations (n_{naive} = 3; $n_{PbA\ d6}$ = 5). Indicated are the means with SEM. The statistical significance was analysed with the student's t-test (unpaired, two-tailed); $p<0.05^{*}$; $p<0.01^{**}$; $p<0.001^{***}$.

4.3 Impact of IL-22 on malaria pathology

Since the IL-22 level was elevated in plasmodial infection it was of interest to elucidate which role IL-22 plays during murine malaria. Hence the effect of IL-22 on the pathology and parasitemia of *Plasmodium* - infection in mice was investigated (Fig. 4.3). For this purpose wt and IL-$22^{-/-}$ mice were infected with 1000 PbA sporozoites intravenous (i.v.) and the progression of disease was monitored (Fig. 4.3, A). No differences were observed between wt and IL-$22^{-/-}$ mice concerning the experimental cerebral malaria (CM) score at d7 p.i. (Fig. 4.3, B) and the weight loss (Fig. 4.3, C) at d7 p.i.. This was confirmed by a similar survival in IL-22 - deficient mice compared to wt mice. Thus the infection with PbA sporozoites, which includes the liver and the blood stage of the parasite life cycle, is not influenced by the lack of IL-22. Even though the survival was not affected, the blood stage parasitemia is significantly lower in IL-$22^{-/-}$ mice compared to wt mice on d6 p.i. (Fig. 4.3, D).

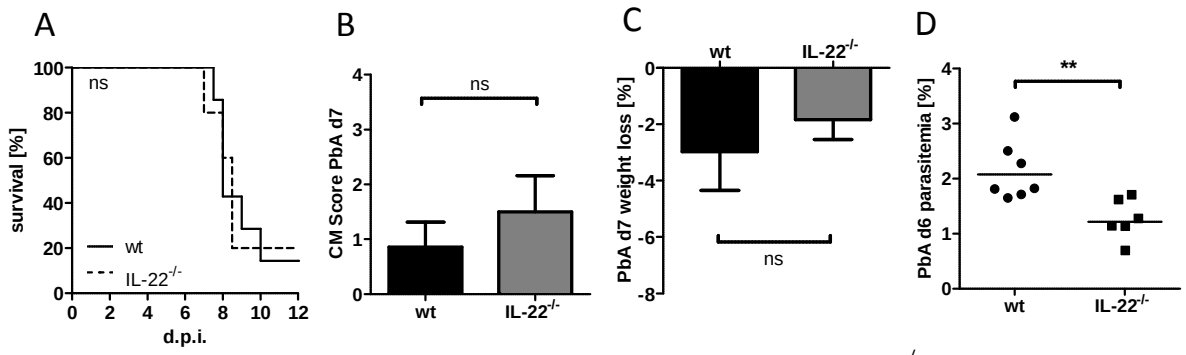

Fig. 4.3: Impact of IL-22 on the course of PbA sporozoite infection. Wt and IL-22$^{-/-}$ mice were infected with 1000 PbA sporozoites i.v. and the course of disease was monitored [A]. The CM score [B] and the weight loss [C] were plotted at the onset of disease (d7 p.i.). The parasitemia was analysed on d6 p.i. [D] (n_{wt} = 7; $n_{IL\text{-}22\text{-}/\text{-}}$ = 6). Indicated are the means +/- SEM or only the mean. Statistical differences in the survival were analysed using the Kaplan-Meier survival curve statistic. Figure B and C were analysed with the student's t-test (unpaired, two-tailed). The statistical differences of the parasitemia is calculated with the Mann-Whitney test; $p<0.05^{*}$; $p<0.01^{**}$; $p<0.001^{***}$.

To elucidate if the lower parasitemia is due to a decreased burden of the sporozoite in the liver, IL-22$^{-/-}$ mice were infected with 1000 sporozoites i.v. and sacrificed 24 h p.i.. The relative amount of sporozoites in the liver was analysed with a *Plasmodium* spp. - specific 18S qPCR (91). No differences were found between wt and IL-22$^{-/-}$ mice concerning the sporozoite burden in the liver 24 h p.i. (Fig. 4.4).

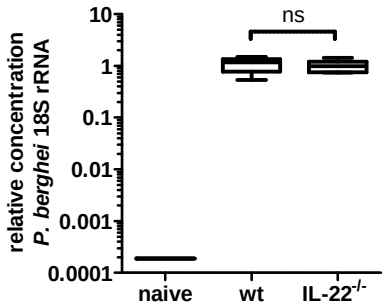

Fig. 4.4: PbA sporozoite burden in the liver. Wt and IL-22$^{-/-}$ mice were infected with 1000 sporozoites i.v.. The parasite burden in the liver was determined 24 h after sporozoite infection by qPCR (n_{wt} = 5; $n_{IL\text{-}22\text{-}/\text{-}}$ = 6). Indicated are the means +/- SEM. Statistical differences were calculated with the Mann Whitney test; p<0.05*; p<0.01**; p<0.001***.

The lack of IL-22 showed an effect on the parasitemia, indicating that IL-22 has an influence on the blood stage. Thus the survival of mice in murine malaria was monitored in the absence of the liver stage. For this purpose mice were infected i.p. with PbA - iRBCs. Subsequently the pathology of PbA blood stage - infected wt and IL-22$^{-/-}$ mice was monitored (Fig. 4.5). The lack of IL-22 led in a PbA blood stage infection to an earlier onset of malaria - specific symptoms (e.g. coma, ruffled fur and weight loss) (Fig. 4.5, A). This was also reflected by a higher incidence of CM in PbA - infected IL-22$^{-/-}$ mice compared to wt mice on

d6 p.i. (Fig. 4.5, B). Furthermore the parasitemia of blood stage - infected IL-22$^{-/-}$ mice was significantly decreased compared to wt mice (Fig. 4.5, D). Nevertheless the weight loss was not different between both groups (Fig. 4.5, C),

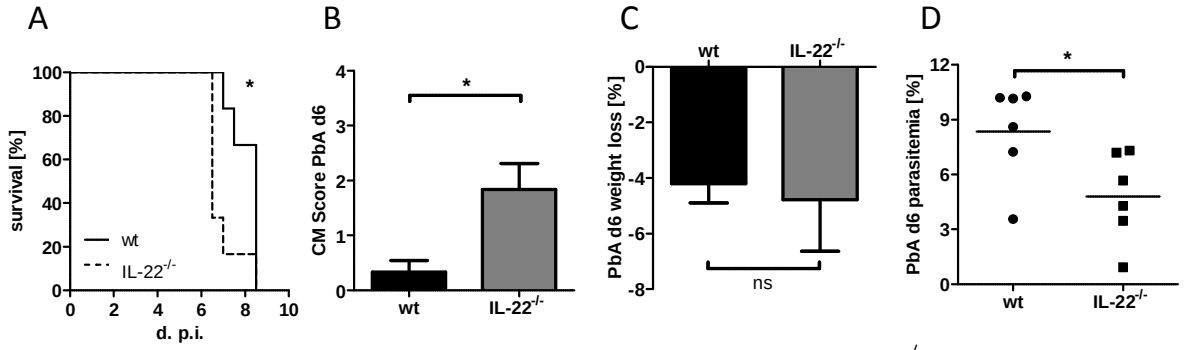

Fig. 4.5: Impact of IL-22 on the course of PbA blood stage infection. Wt and IL-22$^{-/-}$ mice were infected with 1 x 10^5 PbA iRBC i.p. and the course of disease was monitored [A]. The CM score [B] and the weight loss [C] were plotted at the onset of disease (d6 p.i.),. The parasitemia was analysed on d6 p.i. [D] (n_{wt} = 6; $n_{IL\text{-}22\text{-}/\text{-}}$ = 6). Indicated are the means with/without SEM. Statistical differences in the survival were analysed using the Kaplan-Meier survival curve statistic. Figure B and C were analysed with the student's t-test (unpaired, two-tailed). The statistical differences of the parasitemia is calculated with the Mann-Whitney test; p<0.05*; p<0.01**; p<0.001***.

To confirm that the absence of IL-22 has no significant impact on the liver during the course of a PbA blood stage infection, transaminase levels in the blood plasma of PbA blood stage - infected wt and IL-22$^{-/-}$ mice were analysed as indicators of liver damage on d6 p.i.. The level of alanine transaminase (ALT) and aspartate transaminase (AST) did not differ between PbA - infected wt and IL-22$^{-/-}$ mice on d6 p.i. (Fig. 4.6). Even though the ALT level increased significantly in PbA - infected wt mice compared to naive wt mice, the induction of ALT and AST in PbA - infected IL-22$^{-/-}$ mice was not significantly enhanced compared to naive IL-22$^{-/-}$ mice. More important is the fact, that the ALT and AST levels were comparable between PbA blood stage - infected wt and IL-22$^{-/-}$ mice at d6 p.i..

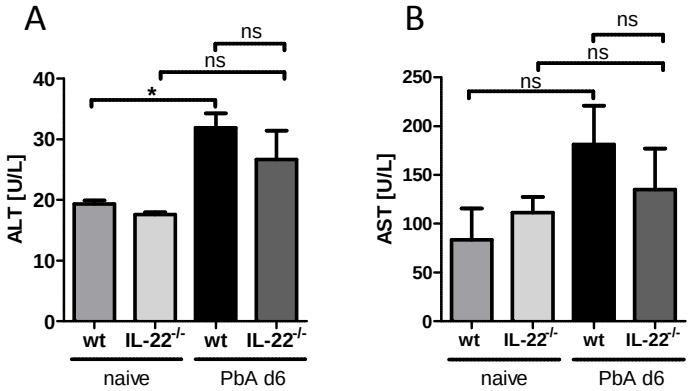

Fig. 4.6: Liver damage in PbA blood stage infection in mice. ALT [A] and AST [B] levels in the plasma are used as liver damage markers and were analysed in naive and in wt and IL-22$^{-/-}$ mice at d6 p.i. ($n_{wt\ naive}$ = 2; $n_{IL\text{-}22\text{-}/\text{-}\ naive}$ = 2; $n_{wt\ PbA\ d6}$ = 5; $n_{IL\text{-}22\text{-}/\text{-}\ PbA\ d6}$ = 5). Indicated are the means with SEM. Statistical differences were calculated with the student's t-test (unpaired, two-tailed); p<0.05*; p<0.01**; p<0.001***.

To elucidate if the effects of the IL-22 knock-out might be due to pleiotropic mechanisms or if the knock-out leads to secondary effects during ontogeny of the immune system, the course of PbA blood stage infection was monitored in wt mice treated with an IL-22 specific antibody (αIL-22). This leads only to a transient loss of the IL-22 protein function without influencing possible effects of the *il22* mRNA. Additional compensatory effects of the immune system which might be induced in IL-22 - deficient mice might be not so strong in transiently IL-22 - blocked wt mice. This transient blockade of IL-22 in the beginning of PbA blood stage infection of wt mice resulted in a strong increase of CM symptoms compared to control IgG treaded mice (Fig. 4.7, A). Furthermore the administration of an IL-22 specific antibody in PbA infection of mice led to a slightly higher CM score (Fig. 4.7, B) and to a significant increased weight loss on d6 p.i. (Fig. 4.7, C). Nevertheless the parasitemia on d6 p.i. did not differ in both groups (Fig. 4.7, D). These results show that the IL-22$^{-/-}$ mice and the αIL-22 treated mice respond similar concerning the advanced onset of malaria pathology compared to wt mice.

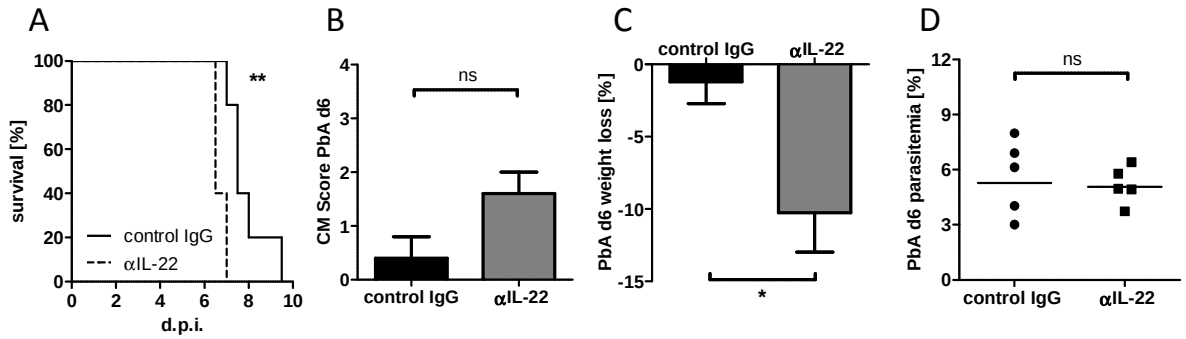

Fig. 4.7: Impact of αIL-22 treatment of wt mice on the course of PbA blood stage infection. Wt mice were infected with 1 x 10^5 PbA iRBC i.p. and simultaneously treated with 20 µg αIL-22 or control IgG, subsequently the survival was observed [A]. The CM score [B] and the weight loss [C] were plotted on d6 p.i., at the onset of disease. The parasitemia was analysed on d6 p.i. [D]. (n_{wt} = 5; $n_{\alpha IL\text{-}22}$ = 5). Indicated are the means +/- SEM or only the mean. Statistical differences in the survival were analysed using the Kaplan Meier survival curve statistic. Figure B and C were analysed with the student's t-test (unpaired, two tailed). The statistical differences of the parasitemia is calculated with the Mann Whitney test; p<0.05*; p<0.01**; p<0.001***.

Taken together the phenotype of a significant decreased survival in PbA blood stage infection of IL-22$^{-/-}$ mice compared to wt mice was abolished when the liver stage of the disease was included. Nevertheless no stronger liver damage or even inflammation could have been observed in the absence of IL-22 during PbA blood stage infection, even though the IL-22 receptor alpha 1 (IL-22Rα1) chain is highly expressed on hepatocytes. This leads to the conclusion that the absence of IL-22 has no impact on the liver function during PbA

infection. Furthermore the absence of IL-22 leads to the effect of a lower of parasitemia which was not due to an altered sporozoite liver burden.

4.4 The bioavailability of IL-22 modulates parasitemia

The lack of IL-22 in PbA blood stage infection resulted in an increased severity of pathology and a decreased parasitemia. To elucidate if the lower parasitemia is not only restricted to PbA infection, IL-22$^{-/-}$ mice and wt mice were infected with 2 x 10^4 *Plasmodium* yoelii non - lethal (PyNL) iRBC i.p.. The infection with PyNL does not induce CM symptoms and leads to a transient parasitemia which is cleared within three weeks. As in the PbA model, PyNL infected IL-22$^{-/-}$ mice developed a lower parasitemia at d7 p.i. compared to wt mice (Fig. 4.8, A). To elucidate if the differences in parasitemia is due to effects of the knock-out of IL-22, like an incorrect development of the mouse strain during ontogenesis or pleiotropic mechanisms due to the knock-out, wt mice were treated with an IL-22 specific antibody in the beginning of PyNL blood stage infection. So for this purpose wt mice were treated with the αIL-22 antibody or with the carrier and the parasitemia was analysed at d2, d4, d7, d9, d11, d14, d16, d18 and d21 until mice cleared the infection. The transient blockade of IL-22 in the beginning of infection led to a significant decrease of blood stage infection during the whole period of PyNL infection (Fig. 4.8 B).

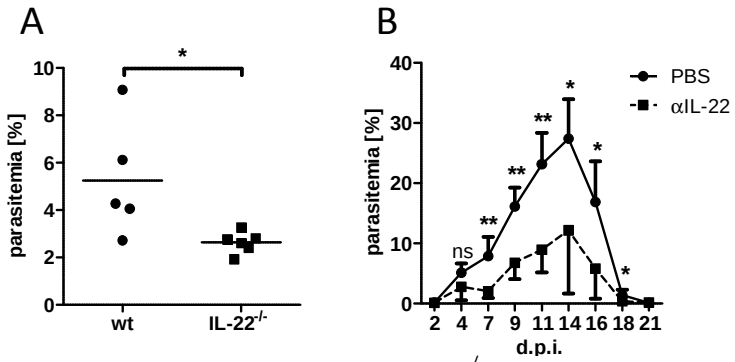

Fig. 4.8: PyNL parasitemia in the absence of IL-22. Wt and IL-22$^{-/-}$ mice were infected with 2 x 10^4 PyNL - iRBC i.p. The parasitemia was analysed on d7 p.i. (n_{wt} = 5; $n_{IL\text{-}22\text{-}/\text{-}}$ = 6) [A]. Wt mice were infected with 2 x 10^4 *P. yoelii* NL iRBC i.p. and simultaneously treated with 20 µg of αIL-22 or PBS as a control ($n_{control}$ = 5; $n_{\alpha IL\text{-}22}$ = 5). The parasitemia curve was analysed over a time period of three weeks [B]. Indicated are the means +/- SEM or only the mean. The statistical differences of the parasitemia are calculated with the Mann Whitney test; p<0.05*; p<0.01**; p<0.001***.

The IL-22 level *in vivo* is controlled by a soluble IL-22 binding protein (IL-22BP). The IL-22BP is secreted by immature dendritic cells and can be induced by retinoic acid (36). Thus, the maturation of dendritic cells leads to a reduction of IL-22BP and hence to an increase of free

IL-22. To confirm this mechanism during *Plasmodium* spp. blood stage infection the IL-22 level was analysed in wt and IL-22BP$^{-/-}$ mice which were infected with PbA iRBCs. The blood plasma was collected at d6 p.i. and the IL-22 concentration was determined by ELISA. A significant increase of IL-22 was observed in IL-22BP$^{-/-}$ mice compared to wt mice in PbA blood stage infection on d6 p.i (Fig. 4.9, A). Using the IL-22BP$^{-/-}$ mice it is possible to elucidate the effect of an increased level of bioactive IL-22 during infection. One might expect an increased parasitemia with an increase of bioavailability of IL-22. As expected, a significantly increased parasitemia was observed on d9 and d11 p.i. (Fig. 4.9, B) in PyNL - infected IL-22BP$^{-/-}$ mice compared to wt mice.

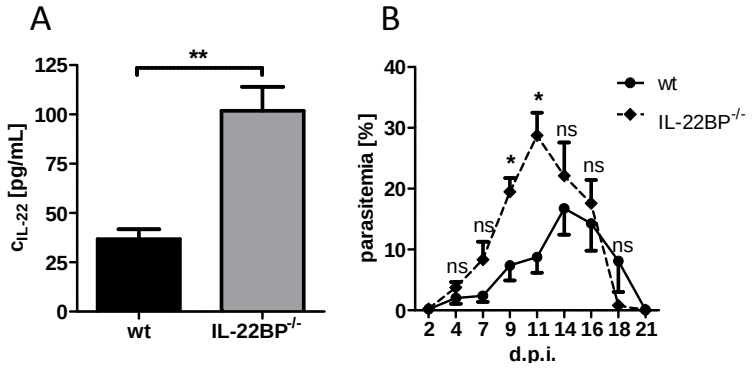

Fig. 4.9: Impact of the IL-22BP deficiency on the parasitemia in murine plasmodial infection. Wt and IL-22BP$^{-/-}$ mice were infected with 1 x 10^5 PbA iRBC i.p.. IL-22 plasma level in PbA - infected IL-22BP$^{-/-}$ mice on d6 p.i. ($n_{wt\ PbA\ d6}$ =36; $n_{IL-22BP-/-\ PbA\ d6}$ =5) [A]. Wt and IL-22BP$^{-/-}$ mice were infected with 2 x 10^4 *P. yoelii* NL iRBC i.p. (n_{wt} = 5; $n_{IL-22BP-/-}$ = 3). The parasitemia was analysed over a time period of 3 weeks [B]. Indicated are the means +/- SEM. The statistical significance of the ELISA was analysed with the student's t-test (unpaired, two-tailed). The statistical differences of the parasitemia is calculated with the Mann Whitney test; p<0.05*; p<0.01**; p<0.001***.

In summery the parasitemia of *Plasmodium* spp. - infected mice is influenced by the bioavailability of IL-22. The lack of IL-22 induces a decrease of parasitemia whereas high level of IL-22 led to an increase of parasitemia of *Plasmodium* spp. - infected mice.

4.5 IFNγ and IL-17 production is modulated in murine PbA infection in the absence of IL-22

The lack of IL-22 induced a more severe course of murine malaria. The severe pathology of malaria is caused by an overwhelming pro - inflammatory immune response of the adaptive immune response accompanied by a strong release of cytokines. Therefore, it was of interest to analyse if the T cell response is altered in the absence of IL-22. In order to investigate the cytokine release by T cells in PbA blood stage infection, the IL-17 and IFNγ production of different lymphocyte subsets in wt and IL-22$^{-/-}$ mice were determined. Mice

were infected with 1 x 10^5 PbA - iRBC i.p. and the cytokine production was analysed on d3 and d6 p.i.. IFNγ is of crucial importance concerning the development of CM. Thus, its expression was analysed after stimulation of single cell suspensions of splenocytes with PMA/Ionomycin and an intracellular staining by FACS. At d3 p.i. the IFNγ release was significantly higher in the absence of IL-22. This strong release of IFNγ was observed in different lymphocyte subsets of IL-$22^{-/-}$ mice including $CD3^+$ (Fig. 4.10 A), $CD8^+$ (Fig. 4.10 B), $CD3^+$ $CD8^-$ (representing $CD4^+$ T cells, since the CD4 receptor is internalized and degraded upon stimulation) (Fig. 4.10 C) and $\gamma\delta^+$ T cells (Fig. 4.10 D). Regarding the later time point of PbA blood stage infection (d6 p.i.) the IFNγ expression was alike in wt and IL-$22^{-/-}$ mice.

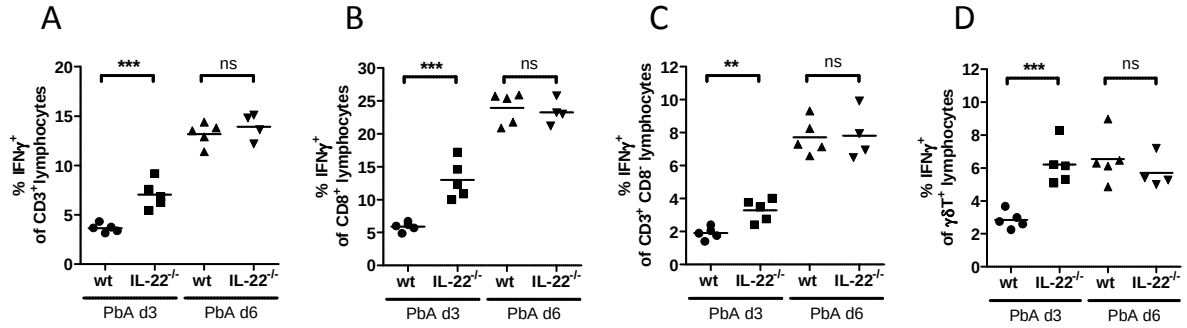

Fig. 4.10: IFNγ production in the absence of IL-22 in murine PbA infection. Splenic cells were isolated from PbA - infected wt and IL-$22^{-/-}$ mice at d3 (n_{wt}= 5; $n_{IL\text{-}22\text{-}/\text{-}}$= 5) and d6 ($n_{wt}$= 5; $n_{IL\text{-}22\text{-}/\text{-}}$= 4) p.i. and stimulated for 4 h with PMA/Ionomycin including the last 3 h with Monensin. $CD3^+$ [A], $CD3^+$ $CD8^-$ (representing CD4+ T cells, since the CD4 receptor is internalized and degraded upon stimulation) [B], $CD8^+$ [C] and $\gamma\delta^+$ [D] T cells were analysed for IFNγ expression by FACS analysis. Indicated are the means. The statistical significance was analysed with the student's t-test (unpaired, two tailed); p<0.05*; p<0.01**; p<0.001***.

To confirm that the early induction of IFNγ is not due to pleiotropic mechanisms or an incorrect development of the IL-22 knock-out mouse strain during ontogeny, wt mice were treated with an IL-22 specific antibody in the beginning of PbA blood stage infection. The results obtained in the IL-22 knock-out mouse were confirmed with a transient blockade of IL-22 in wt mice. A higher production of IFNγ in $CD3^+$ T cells was observed in PbA blood stage - infected αIL-22 - treated mice at d3 p.i. compared to control IgG - treated mice (Fig. 4.11).

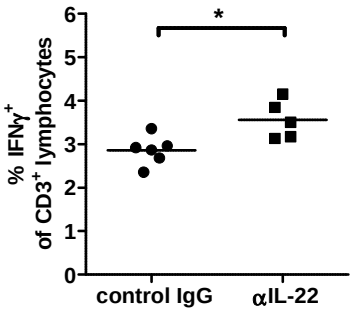

Fig. 4.11: IFNγ production of αIL-22 treated wt mice in murine PbA infection. Mice were treated with αIL-22 or the corresponding control IgG and subsequently infected with 1 x 10^5 PbA - iRBC i.p.. The IFNγ expression of $CD3^+$ T cells was analysed by FACS analysis after PMA/Ionomycin stimulation for 4 h including the last 3 h with Monensin ($n_{control\ IgG}$= 6; $n_{\alpha IL\text{-}22}$= 5). Indicated are the means. The statistical significance was analysed with the student's t-test (unpaired, two tailed); p<0.05*; p<0.01**; p<0.001***.

Because it is known that IFNγ has an immune modulatory effect on T_H17 differentiation (12), it was of interest to elucidate if the expression of IL-17 is altered as an effect of higher IFNγ level in IL-$22^{-/-}$ mice during murine malaria. Hence mice were infected with PbA - iRBCs and the expression of IL-17 was analysed by FACS analysis on d3 and d6 p.i. after PMA/Ionomycin stimulation and intracellular staining. Regardless of the time point or T cell population, the IL-17 expression was significantly lower in IL-$22^{-/-}$ mice compared to wt mice during PbA blood stage infection (Fig. 4.12, A, B).

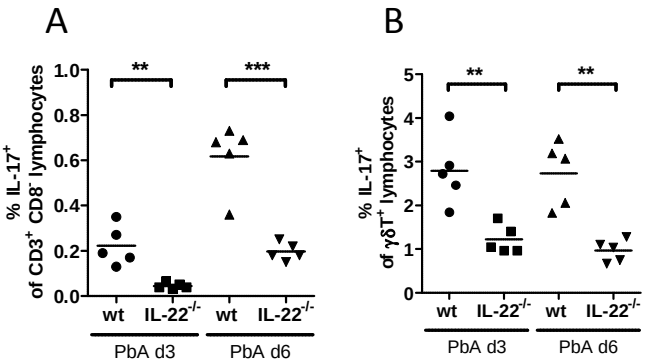

Fig. 4.12: IL-17 production in the absence of IL-22 in murine PbA infection. Splenic cells were isolated from PbA - infected wt and IL-$22^{-/-}$ mice at d3 (n_{wt}= 5; $n_{IL\text{-}22\text{-}/\text{-}}$= 5) and d6 ($n_{wt}$= 5; $n_{IL\text{-}22\text{-}/\text{-}}$= 5) p.i. and stimulated for 4 h with PMA/Ionomycin including the last 3 h with Monensin. $CD3^+$ $CD8^-$ (representing $CD4^+$ T cells, since the CD4 receptor is internalized and degraded upon stimulation) [A] and $\gamma\delta^+$ [B] T cells were analysed for IL-17 secretion by FACS analysis. Indicated are the means. The statistical significance was analysed with the student's t-test (unpaired, two tailed); p<0.05*; p<0.01**; p<0.001***.

Finally the absence of IL-22 modulated the immune response to PbA blood stage infection. The IFNγ production was higher at an early time point of infection in a variety of splenic lymphocyte populations of IL-$22^{-/-}$ mice compared to wt mice. The low IL-17 production from $CD3^+$ $CD8^-$ T cells (representing $CD4^+$ T cells, since the CD4 receptor is internalized and

degraded upon stimulation) and $\gamma\delta^+$ T cells might be regarded as an effect of the early IFNγ secretion in the absence of IL-22.

4.6 Lipopolysaccharide stimulated bone marrow - derived dendritic cells express the IL-22Rα1 chain

IL-22 acts on cells which express the IL-22Rα1 chain, especially non - hematopoietic cells. Here, it was shown that immune cells, which were described to have no IL-22Rα1 receptor, are influenced by the absence of IL-22. To elucidate which cell type could play as a connection point between the IL-22 and the modulatory effect on the immune system, several cell populations were chosen and analysed concerning their expression of the *il22ra1* chain on mRNA level (Fig. 4.13). For this purpose different cell populations were enriched by Magnetic activated cell separation (MACS technology) or stained for markers which define certain cell populations for a subsequent sort by FACS. The cDNA of these samples was purified and used for an IL-22Rα1 specific PCR. The samples were loaded on a gel and analysed for an *il22ra1* - specific DNA product of a size of 189 bp. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. Splenic $CD3^+$ T cells of a naive wt mouse were used as negative and primary hepatocytes (pHepa) as positive control. $CD11c^+$ cells of a spleen from a naive mouse were analysed concerning their *il22ra1* chain mRNA expression. Additionally bone marrow - derived dendritic cells (BMDCs) were stimulated with LPS for 24 h and compared to unstimulated control BMDCs. Another cell population which potentially could express the IL-22Rα1 chain were liver endothelial sinusoidal cells (LSECs). To ensure that the applied MACS technology for the LSEC enrichment lead to a good purity of the cell population an additional cell sort for CD146 was performed. CD146 is a characteristic marker for LSECs. The only cell population which showed an *il22ra1* chain expression on mRNA level were LPS - stimulated BMDCs. The specificity of this signal was confirmed by a correct positive control (pHepa) and negative control (naive $CD3^+$ T cells). All other cell populations showed no characteristic band for the *il22ra1* chain. This new finding leads to further questions concerning the ability of dendritic cells (DCs) to express the IL-22Rα1 chain upon *in vivo* stimulation, including *Plasmodium* spp. infection.

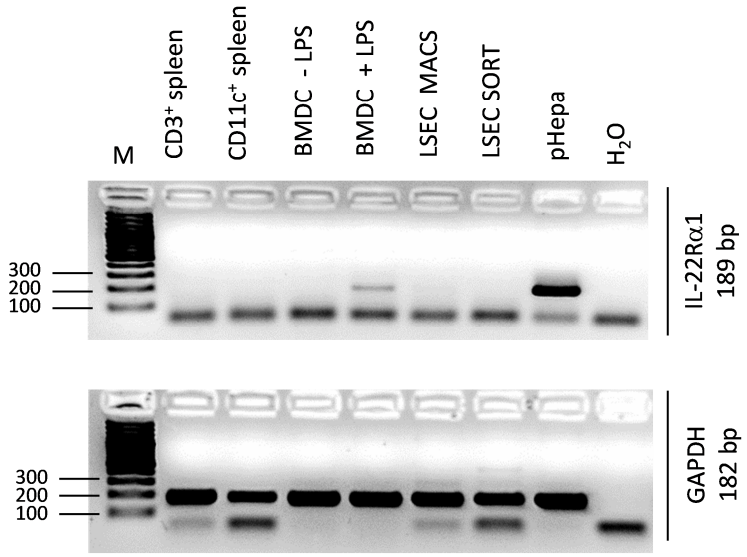

Fig. 4.13: IL-22Rα1 chain - specific PCR on cDNA samples of different cell populations. Different cell populations of a wt mouse were stained and either sorted for $CD3^+$ or $CD11c^+$ cells. Additionally freshly generated BMDCs were stimulated in the absence/presence of LPS. LSECs were either enriched by MACS technology or sorted for CD146. Primary hepatocytes were isolated and used as a positive control. mRNA isolation and cDNA transcription was performed subsequently. 10 ng of cDNA of each sample was analysed for IL-22Rα1 transcription levels. GAPDH was used as loading control.

To confirm that the performed IL-22Rα1 PCR is highly specific for the IL-22Rα1 chain, IL-22Rα1$^{-/-}$ mice were used as a control (Fig. 4.14). Therefore, wt mice and IL-22Rα1$^{-/-}$ mice were infected with 1 x 10^5 PbA iRBC i.p. and sacrificed at d6 p.i.. Splenic tissue was collected and the mRNA was subsequently isolated and transcribed into cDNA. A visible induction of the amount of the PCR *il22ra1* DNA product was observed in the splenic tissue samples of infected wt mice compared to the splenic tissue sample from the naive mouse. By using the PCR for the IL-22Rα1 chain on naive and PbA - infected IL-22Rα1$^{-/-}$ mice the specificity was confirmed, since there was no band for the IL-22Rα1 chain visible.

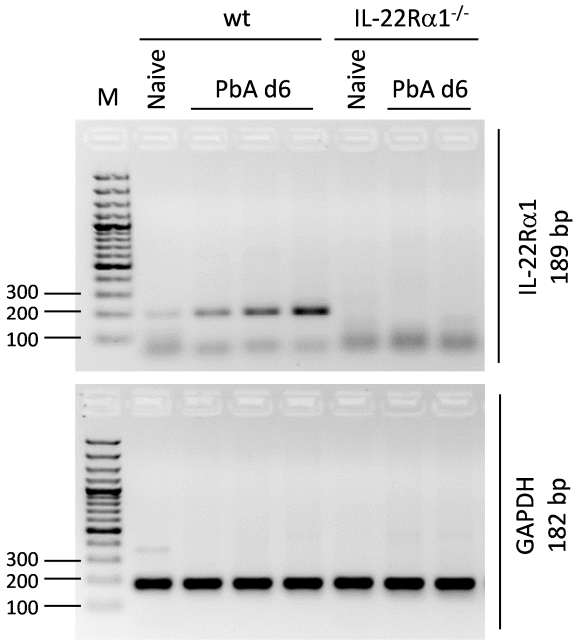

Fig. 4.14: IL-22Rα1 chain control PCR on splenic tissue of wt and IL-22Rα1$^{-/-}$ mice. Wt and IL-22Rα1$^{-/-}$ mice were infected with 1 x 10^5 PbA iRBCs i.p. and sacrificed on d6 p.i.. Splenic tissue was collected and mRNA was isolated and subsequently transcribed into cDNA. 10 ng of cDNA of each sample was analysed for IL-22Rα1 transcription levels. GAPDH was used as loading control.

The IL-22Rα1 chain specific PCR on cDNA samples of different cell populations and the IL-22Rα1 chain control PCR show that splenic cells are able to express the *il22ra1* chain on mRNA level upon stimulation.

4.7 Impact of IL-22 on antigen presenting cells

To elucidate if the IL-22Rα1 chain is expressed on DCs during murine *Plasmodium* spp. infection, mice were infected with 1 x 10^5 PbA - iRBC i.p. and sacrificed at d6 p.i.. The expression of the *il22ra1* chain on DCs of naive and PbA - infected wt mice was determined using the IL-22Rα1 chain specific PCR. Therefore, splenic CD11c$^+$ cells were sorted by FACS and the mRNA was subsequently purified and transcribed into cDNA. A specific *il22ra1* chain DNA band in CD11c$^+$ cells was observed for several PbA - infected mice (Fig. 4.15). CD4$^+$ T cells from a naive and a PbA blood stage - infected mouse were used as control. Surprisingly CD4$^+$ T cells of the PbA blood stage - infected mouse showed a specific band for the IL-22Rα1 chain in PbA infection.

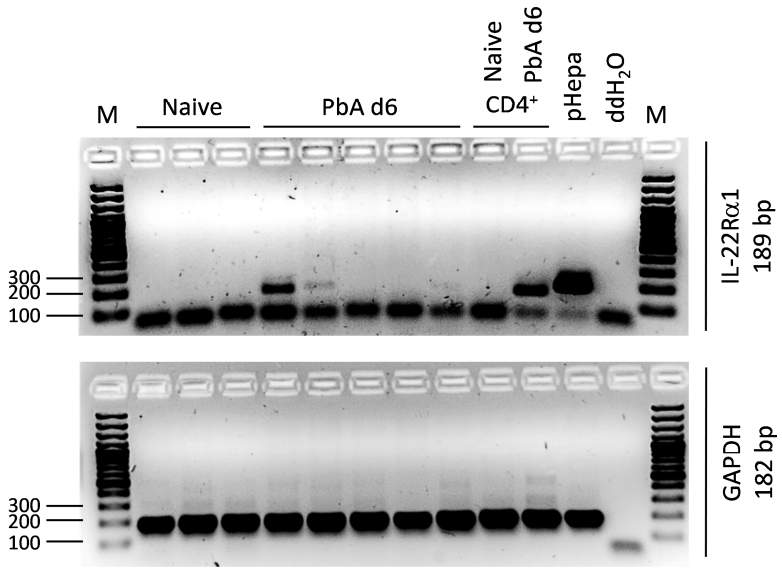

Fig. 4.15: IL-22Rα1 chain PCR on splenic DCs of PbA - infected mice. Wt mice were infected with 1 x 10^5 iRBC PbA i.p. or treated with PBS as control. On d6 p.i. mice were sacrificed and analysed for IL-22Rα1 expression on splenic DCs via IL-22Rα1 specific PCR (n_{naive}= 3; $n_{PbA\ d6}$= 5). cDNA samples were analysed for IL-22Rα1 expression of $CD11c^+$ sorted cells (n_{naive}= 3; $n_{PbA\ d6}$= 5). The expected IL-22Rα1 band has a size of 189 bp. GAPDH was used as loading control.

The next step was to investigate if DCs in IL-$22^{-/-}$ mice show a different phenotype compared to wt mice during murine malaria. Since DCs are important for the priming of T cells in the beginning of infection, PbA - infected mice were sacrificed at d3 p.i.. Splenocytes were stained with specific antibodies for CD11c and CD11b (Fig. 4.16, A) and the different subsets of antigen presenting cells (APCs) were analysed concerning their ability to express CD86, an important costimulatory receptor for immune activation. IL-$22^{-/-}$ mice showed an increased expression of CD86 on $CD11c^{++}$ $CD11b^-$ splenocytes (Fig. 4.16, B), concluding that professional antigen presenting cells in IL-$22^{-/-}$ mice possess a higher ability to activate T cells at an early time point of infection.

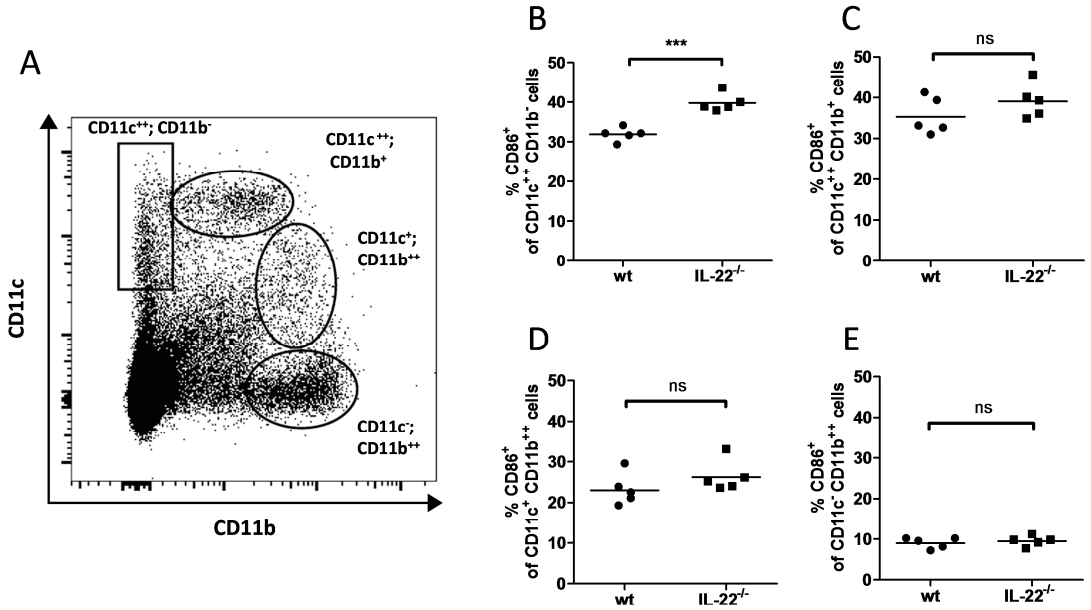

Fig. 4.16: CD86 expression on different subsets of antigen presenting cells in PbA - infected wt and IL-22$^{-/-}$ mice. PbA - infected wt and IL-22$^{-/-}$ mice were sacrificed at d3 p.i. and splenic cells were isolated. The exemplary dotplot shows the subsets of APCs, characterized by a different expression pattern of CD11c and CD11b on splenocytes of a PbA infected wt mouse on d3 p.i. [A]. These subsets were analysed regarding their CD86 expression [B; C; D; E] (n_{wt}= 5; $n_{IL\text{-}22\text{-}/\text{-}}$= 5). Indicated are the means. The statistical significance was analysed with the student's t-test (unpaired, two tailed); p<0.05*; p<0.01**; p<0.001***.

Concerning the higher the CD86 expression on splenic DCs of PbA - infected IL-22$^{-/-}$ mice on d3 p.i., it was of further interest to investigate if this finding can be confirmed *in vitro*. For that reason bone BMDCs of wt and IL-22$^{-/-}$ mice were stimulated for 24 h with LPS or PBS as control and their capacity for CD80 and CD86 expression was determined by FACS analysis. Here, the LPS stimulation induced an increase of CD80 and CD86 expression, both costimulatory receptors, on BMDC, regardless of the IL-22 level. Moreover, the expression of these receptors was significantly higher on IL-22$^{-/-}$ derived BMDCs compared to wt BMDCs after LPS stimulation (Fig. 4.17).

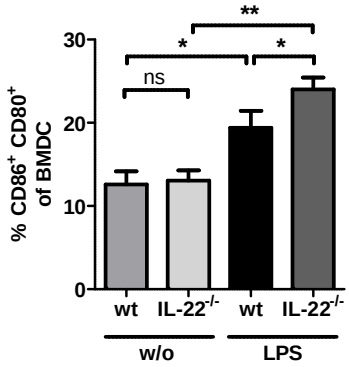

Fig. 4.17: Induction of CD86 and CD80 expression of BMDCs after LPS stimulation. Bone marrow of wt and IL-22$^{-/-}$ mice were isolated and cultured for 7 days in the presence of GM-CSF. On d7 BMDCs were transferred into a 96 well plate and stimulated with 100 ng/mL LPS or equal volume of PBS for 24 h. The induction of CD86 and CD80 expression was determined by comparing the untreated samples with the LPS stimulated samples. The pool of four independent experiments is shown. Indicated are the means with SEM. The statistical significance was calculated with a regular ANOVA test; <0.05*; p<0.01**; p<0.001***.

The next experiment served to answer the questions if T cells are better activated in IL-22$^{-/-}$ mice during PbA infection compared to wt mice and if IL-22$^{-/-}$ - derived BMDCs are better APCs than wt BMDCs. To investigate these questions BMDCs were generated out of wt and IL-22$^{-/-}$ mice and pulsed with PbA specific peptides. After a washing step, to ensure peptide presentation only by BMDCs, purified CD8^{+} T cells of PbA infected wt and IL-22$^{-/-}$ mice were added. The supernatant was analysed for IFNγ secretion after 24 h by ELISA as an indicator of T cell activation. This approach showed that IL-22$^{-/-}$ CD8^{+} T cells secrete more IFNγ after *ex vivo* antigen specific stimulation compared to wt CD8^{+} T cells regardless of the origin of peptide presenting BMDCs. Furthermore IL-22$^{-/-}$ BMDCs showed an stronger stimulatory effect on wt and IL-22$^{-/-}$ CD8^{+} T cells compared to wt BMDCs regarding their ability to secrete IFNγ (Fig. 4.18).

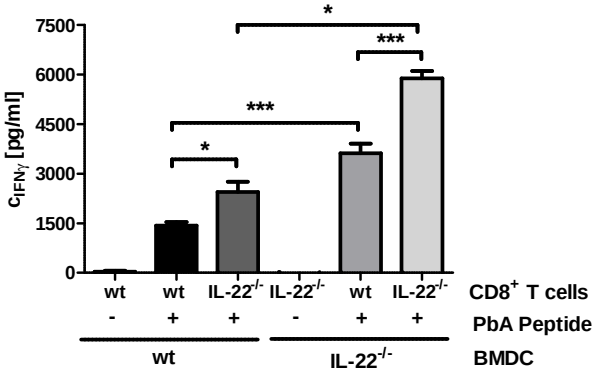

Fig. 4.18: IFNγ secretion of CD8^{+} T cells of PbA - infected mice, stimulated on BMDCs with PbA peptide. Wt and IL-22$^{-/-}$ mice were infected with 1 x 10^5 PbA iRBC. On d6 p.i. mice were sacrificed and splenic CD8^{+} T cells were purified. CD8^{+} T cells of wt or IL-22$^{-/-}$ mice were subsequently incubated on previously PbA peptide pulsed wt or IL-22$^{-/-}$ BMDCs. After an incubation time of 24 h the IFNγ concentration in the supernatant was analysed with an ELISA ($n_{wt\ CD8+;\ w/o\ PbA\ Peptide;\ wt\ BMDC}$= 8; $n_{wt\ CD8+;\ PbA\ Peptide;\ wt\ BMDC}$= 6; $n_{IL\text{-}22\text{-}/\text{-}\ CD8+;\ PbA\ Peptide;\ wt\text{-}BMDC}$= 6; $n_{IL\text{-}22\text{-}/\text{-}\ CD8+;\ w/o\ PbA\ Peptide;\ IL\text{-}22\text{-}/\text{-}\ BMDC}$= 8; $n_{wt\ CD8+;\ PbA\ Peptide;\ IL\text{-}22\text{-}/\text{-}\ BMDC}$= 8; $n_{IL\text{-}22\text{-}/\text{-}\ CD8+;\ PbA\ Peptide;\ IL\text{-}22\text{-}/\text{-}\ BMDC}$= 8). Indicated are the means with SEM. The statistical significance of the ELISA was analysed with the student's t-test (unpaired, two tailed); p<0.05*; p<0.01**; p<0.001***.

The IL-22 deficiency leads to an upregulation of costimulatory molecules on DCs *in vitro* upon LPS stimulation as well as during malaria *in vivo*. The function of these upregulated costimulatory molecules on IL-22$^{-/-}$ BMDCs was conferred by their improved stimulation of CD8^{+} T cells.

4.8 Improved recovery of antigen specific T cells in IL-22$^{-/-}$ mice

The different T cell response including the enhanced expression of costimulatory receptors on DCs led to the question if the proliferation of T cells in PbA infection is modulated in the absence of IL-22. To elucidate the proliferation *in vivo*, OT1 splenic cells were labelled with CFSE dye and transferred into recipient mice. OT1 mice express an Ovalbumin - specific transgenic T cell receptor. The recipient mice were subsequently infected with an OVA peptide - expressing PbA strain (PbA-OVAtg) or a green fluorescent protein (GFP) - expressing PbA strain (PbA-GFP) as control. The proliferation of antigen specific T cells was the determined by the CFSE dilution using FACS analysis. Nevertheless the proliferation steps were not different comparing OT1 CD8$^+$ T cells in PbA-OVAtg - infected wt or IL-22$^{-/-}$ recipient mice on d5 p.i. (Fig. 4.19, A). Even though the proliferation was alike, the recovery of OT1 CD8$^+$ T cells was significantly higher in PbA-OVAtg - infected IL-22$^{-/-}$ recipient mice on d5 p.i. compared to wt recipient mice (Fig. 4.19, B).

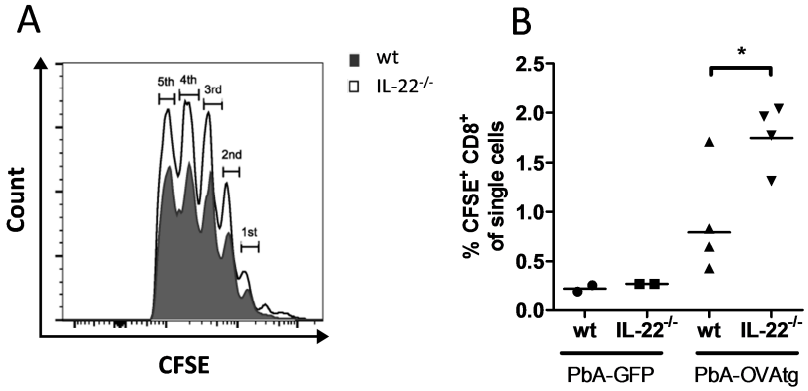

Fig. 4.19: Proliferation of CFSE - labelled OT1 splenocytes in PbA-OVAtg - infected wt and IL-22$^{-/-}$ mice. Splenocytes were isolated from OT1 mice, labelled with CFSE and subsequently transferred in wt and IL-22$^{-/-}$ recipient mice. After 3 hours these mice were either infected with PbA-GFP as a control or PbA-OVAtg. Mice were sacrificed on d5 p.i. and subsequently the cell proliferation steps of CFSE$^+$ CD8$^+$ lymphocytes (CFSE dilution steps 1 to 5) [A] and the relative amount of CFSE$^+$ CD8$^+$ lymphocytes was analysed on d5 p.i. [B] ($n_{\text{wt PbA-GFP}}$= 2; $n_{\text{IL-22-/- PbA-GFP}}$= 2; $n_{\text{wt PbA-OVAtg}}$= 4; $n_{\text{IL-22-/- PbA-OVAtg}}$= 4). Indicated are the means. The statistical significance of the ELISA was analysed with the student's t-test (unpaired, two tailed); p<0.05*; p<0.01**; p<0.001***.

5 Discussion

The role of T_H1 cytokines in malaria have been mostly clarified (66, 92-94), but there are only few studies available regarding the role of T_H17 cytokines like IL-17 or IL-22. The previously assumed protective role of IL-17 in a *Plasmodium chabaudi* (*P. chabaudi*) infection in mice was recently rejected and instead IL-22 came more into focus. Mastelic *et al.*, 2012 observed increased level of IL-17 as well as of IL-22 during a *P. chabaudi* infection in the blood plasma of mice (86). Although the IL-17 concentration was high upon plasmodial infection, there was no effect concerning the parasitemia or survival in *P. chabaudi* - infected IL-$17^{-/-}$ mice compared to wt mice. Furthermore IL-22 seems to play a more essential role in malaria. The survival of *P. chabaudi* - infected IL-$22^{-/-}$ mice was 50 % lower compared to wt mice, even though the parasitemia was not altered (86). This observation made it interesting for the present study to elucidate the role of IL-22 in murine malaria. For this purpose mice were infected with *P. berghei* ANKA (PbA), a murine *Plasmodium* strain highly similar to *P. falciparum* in human regarding nucleotide (70.3 %) and protein (62.9 %) identity (95). Additionally this murine strain allows the observation of cerebral malaria (CM) symptoms in C57BL6J mice similar to CM occurrence in *P. falciparum* - infected human (67). Hence, to investigate the role of IL-22 in murine malaria, mice were infected with PbA and examined regarding malaria pathology and immune response.

5.1 Elevated IL-22 level in the blood plasma of PbA - infected mice

The plasmodial infection can be separated into two stages: an asymptomatic liver stage and the blood stage which induces the severe form malaria disease which includes kidney and liver dysfunction, anaemia and CM (67). Because the liver stage is almost undetected by the immune system and does not induce pathology (96), this study focuses mainly on the blood stage. As a first step the concentration of IL-22 was analysed during the blood stage of PbA - infected mice and *P. falciparum* - infected human individuals. In both, human and mice, the IL-22 concentration in the plasma was significantly higher compared to healthy participants of the study and naive mice, respectively, indicating that the PbA infection mouse model is similar to *P. falciparum* infection concerning the role of IL-22 in plasmodial

infection (Fig. 4.1). The increased concentration of IL-22 in the blood plasma fits to the overall activated immune system during malaria which includes the induction of several pro - inflammatory cytokines, especially TNFα and IFNγ. This increased release of pro - inflammatory cytokines leads to a counter regulation including the production of immunosuppressive cytokines including IL-10 and TGF-β (97). The high concentration of IL-22 shows that this cytokine plays a role during the immune response to plasmodial infection. Nevertheless it is not clear if IL-22 acts pro - or anti - inflammatory.

To determine which cell type is responsible for the high level of IL-22 in malaria, splenocytes of naive and PbA - infected mice were stimulated with PMA/Ionomycin and the IL-22 production was analysed by FACS analysis. It is already published for other immune system - related models, e.g. arthritis, that the majority of IL-22 - secreting cells are activated lymphocytes and innate lymphocytes (42, 98, 99). In *P. chabaudi* infection of mice it was shown that $CD4^+$ T cells are mainly responsible for IL-22 production during infection (86). Here the chosen model of PbA infection in C57BL/6 mice shows that $\gamma\delta^+$ and NK T cells are besides $CD4^+$ T cells additionally responsible for a significant induction of IL-22 concentration during plasmodial infection (Fig. 4.2). Nevertheless $\gamma\delta^+$ T cells are the most likely cell population to produce IL-22 during PbA infection. This cell population is known to be of crucial importance during plasmodial infection in mice. The group of McKenna showed that the deficiency of $\gamma\delta^+$ T cells leads to an increase of sporozoite burden in the liver (100). Additionally $\gamma\delta^+$ T cells play an important role during the development of CM as well, since the depletion of $\gamma\delta^+$ T cells with an antibody leads to resistance to CM (101). In humans Goodier *et al.*, 1995 showed that $\gamma\delta^+$ T cells were the major producer of IFNγ upon *in vitro* stimulation with schizonts antigens (102).

Taken together $\gamma\delta^+$ T cells show to be of importance in the combat against plasmodial infection. Consequently IL-22 is released by cells which are known to play important roles for the development of a proper immune response against plasmodial infection. Hence it is likely that this cytokine owns a crucial function in *Plasmodium* spp. infection.

5.2 Lack of IL-22 induces pathology in PbA blood stage infection

IL-10 has already been described as an important mediator in controlling inflammation in *P. chabaudi* induced malaria (103). IL-22 is a member of the IL-10 family and is known to act

bi-functionally, pro - or anti - inflammatory depending on the environmental context (25, 104). It was shown that IL-22 can promote psoriasis. During this disease, IL-22 acts on keratinocytes which in turn upregulate the production of pro-inflammatory calcium binding proteins (88). An additional pro-inflammatory function of IL-22 was observed in patients with multiple sclerosis. It is assumed that the increased level of IL-22 in MS, which goes along with increased level of IL-17, leads to a permeabilisation of the blood-brain barrier resulting in an influx of inflammatory molecules to the central nervous system (105). In hepatitis Radaeva *et al.*, 2004 and Zenewicz *et al.*, 2007 could show a protective role of IL-22 (29, 106). The high susceptibility of IL-22 - deficient mice to hepatitis can be ameliorated with the transfer of IL-22 - expressing T_H17 cells.

Since the IL-22 concentration is increased in human and in murine malaria, the question arises which role IL-22 plays in *Plasmodium* spp. infection. To elucidate the impact of IL-22 on the progression of malaria, IL-$22^{-/-}$ mice were infected with freshly isolated sporozoites out of salivary glands of PbA - infected *Anopheles stephensi* mosquitos. The development of disease in IL-$22^{-/-}$ was compared to wt mice. By the use of the sporozoite infection route, the natural course of disease, including the liver and the blood stage, is reproduced. No differences were observed regarding the incidence of cerebral malaria symptoms in both groups (Fig. 4.3, A). Despite the same occurrence of cerebral symptoms the blood stage parasitemia was significantly lower in IL-$22^{-/-}$ mice (Fig. 4.3, D). This gave the hint that IL-22 has apparently an effect during the blood stage of plasmodian infection, but not during the liver stage, even though the IL-22Rα1 chain is highly expressed on hepatocytes (88). Furthermore the lower parasitemia in the absence of IL-22 was not due to reduced sporozoite load in the liver of IL-$22^{-/-}$ mice, since the sporozoite burden in the liver was comparable to wt mice (Fig. 4.4). Hence the lower parasitemia in IL-$22^{-/-}$ mice during the blood stage was not due to an effect of sporozoite infectivity in the absence of IL-22, concluding that the hepatoprotective function of IL-22 plays no role for sporozoite infection of hepatocytes (29, 106, 107). To proof the hypothesis that IL-22 does not play a role during liver stage but during the blood stage, the liver stage was evaded by infecting mice with PbA - infected red blood cells of the blood stage (iRBCs). In this experiment, the lack of IL-22 induced an earlier abundance of cerebral malaria symptoms when the liver stage was omitted (Fig. 4.5, A). This indicates that the sporozoite infection of the liver can induce suppressive immune modulatory effects, regardless of presence or absence of IL-22, which

are strong enough to eradicate the earlier CM occurrence triggered by a blood stage infection of IL-22$^{-/-}$ mice compared to wt mice. If the liver stage was evaded, the parasitemia was still lower in IL-22$^{-/-}$ mice compared to wt mice, indicating that the immune response against PbA in the blood stage was improved in the absence of IL-22. In order to clarify that the liver damage and inflammation in PbA blood stage infection in the absence of IL-22 shows no differences compared to wt mice, even though hepatocytes highly express the IL-22Rα1 chain (88), alanine (ALT) and aspartate (AST) transaminase level were measured in the plasma of PbA blood stage - infected mice (Fig. 4.6). The ALT level increases significantly in PbA iRBC infection compared to naive wt mice, but this elevation was not observed for AST. In PbA iRBC - infected IL-22$^{-/-}$ mice there were no differences observed regardless to any compared group. This observation led to the assumption that IL-22 has hardly any influence on the liver physiology during the blood stage of PbA infection.

It is likely that the obtained results concerning the increased susceptibility to PbA blood stage infection and lower parasitemia are due to a direct effect of lacking IL-22. To rule out any indirect effects of the IL-22 knock-out e.g. during ontogenesis of the immune system a method was chosen to transiently block IL-22. This also has the advantage to elucidate if the knock-out of IL-22 has pleiotropic effects. For this purpose, wt mice were treated with an IL-22 specific antibody at d0 of PbA blood stage infection and the course of the disease was monitored. Even though there was no difference in parasitemia (Fig. 4.7, D) the occurrence of cerebral symptoms was significantly earlier (Fig. 4.7, A) and the weight loss (Fig. 4.7, C) was increased in IL-22 neutralized mice, indicating that no abnormalities in ontogenesis or pleiotropic mechanisms occur in IL-22$^{-/-}$ mice. Even though the incidence of cerebral symptoms was increased there were no differences concerning the parasitemia. This leads to the assumption that the antibody treatment was not efficient enough to imitate the whole phenotype in the IL-22 knock-out. To confirm this it may be necessary to introduce an increased amount of the IL-22 - neutralizing antibody or even to prolong the treatment to an additional day. An alternative explanation might be that the earlier onset of CM and the lower parasitemia in PbA blood stage - infected IL-22$^{-/-}$ mice compared to wt mice are of different immunological origin. Hence the treatment with the IL-22 antibody shows the same effect considering the induced susceptibility to CM but has no influence on the unknown pathway concerning the strong reduction of PbA parasitemia as seen in IL-22$^{-/-}$ mice.

5.3 The bioavailability of IL-22 modulates parasitemia

To elucidate if the finding of the increased parasite clearance in the absence of IL-22 is due to altered mechanism of the host to the parasite or due to altered mechanisms of the parasite acting differentially on the immune system of the host, an additional *Plasmodium* strain was chosen. In contrast to PbA, *Plasmodium yoelii* non-lethal (PyNL) infections are cleared within 3 weeks by C57BL/6 mice without developing CM. Already in the late 1970s it was proven that this clearance is due to humoral factors including antibodies. The transfer of serum collected from PyNL immune donors to naive mice induce a protection in the recipient mice to PyNL infection (108). The antimalarial response in PbA infection is especially driven by the hallmark T_H1 cytokine IFNγ by limiting the parasitemia through the activation of macrophages (109). IFNγ additionally plays an important role for the development of CM in C57BL/6 mice, the fatal outcome of PbA infection. The treatment of mice with an IFNγ specific antibody which resulted in a reduced outcome of CM, gave the first hint that this cytokine is of crucial importance concerning the development of CM (63). Further publication confirmed this finding in IFNγ$^{-/-}$ mice (64, 65, 110) as well as in IFNγR$^{-/-}$ mice (80, 81).

PyNL - infected IL-22$^{-/-}$ mice developed a significant lower of parasitemia at d7 p.i. compared to PyNL - infected wt mice (Fig. 4.8, A). In this experimental setup as well, wt mice were treated in the beginning of infection with an IL-22 specific antibody to rule out any effect of an incorrect development during ontogenesis or any pleiotropic effects of the knock-out. Because this model does not induce cerebral symptoms it is assumed that the antibody treatment should have an effect on the parasitemia. Indeed the development of the parasitemia of PyNL infection was drastically decreased in αIL-22 treated mice. Taken together, both plasmodial strains, PbA as well as PyNL, showed a lower parasitemia in IL-22$^{-/-}$ mice compared to wt mice. Even though the treatment of wt mice with αIL-22 led to no differences in PbA parasitemia, a decreased parasitemia was observed in αIL-22 treated and PyNL - infected mice compared to the IgG control. Furthermore, the increased pathology after αIL-22 treatment in the beginning of PbA blood stage infection and the significant lower parasitemia in PyNL infection in αIL-22 treated mice, led to the assumption that IL-22 has an influence in the beginning of disease development which includes the activation of the adaptive immune response.

However, if the concentration of IL-22 is not controlled by the binding of the soluble IL-22BP protein (111), like in IL-22BP$^{-/-}$ mice, a significant high level of IL-22 is observed in PbA blood stage infection at d6 p.i. compared to wt mice (Fig. 4.9, A). Already Huber *et al.*, 2012 showed in the model of a dextran-sulphate sodium - induced colitis a decrease of the *il22bp* mRNA. During the steady-state the amount of *il22bp* mRNA was expressed at high levels as well as during the phase of recovery. In correspondence to this the *il22* mRNA levels were the highest when *il22bp* mRNA was reduced (37).

Keeping the high level of IL-22 in IL-22BP - deficient mice in *Plasmodium* spp. infection in mind, it is likely to assume a reversed phenotype concerning the parasitemia in PyNL infection (Fig. 4.9, B). Indeed, the absence of IL-22BP leads to an increase of parasitemia during PyNL infection.

Comparing the PyNL curves of the αIL-22 treated mice compared to carrier treated mice and the parasitemia curve of PyNL blood stage - infected IL-22BP$^{-/-}$ mice compared to wt mice, it seems that the wt mice develop a higher parasitemia in Fig. 4.8 B than in Fig. 4.9 B. This can be explained by the gender differences. In Fig 4.8 B only female mice were infected, whereas in Fig 4.9 B only male mice were used which are known to be more obese then female mice at the same age. Keeping that in mind and knowing that *Plasmodium* spp. parasites adhere to CD36, a molecule highly expressed in fatty tissue, in mice as well as in human (112, 113) lead to the assumption that a different parasite load in the blood are determined even though male and female mice have the same age. Combining these facts, the lower PyNL parasitemia curve from Fig. 4.9 B compared to the wt curve from Fig. 4.8 B might be explained by the increased sequestration of plasmodial parasites in the fatty tissue via CD36 in male mice, hence less parasites are detectable in the blood.

Despite this gender difference it may be of interest to perform further experiments in IL-22BP$^{-/-}$ mice regarding the influence of high level of IL-22 during plasmodial infection. It is especially of interest if the absence of IL-22BP could lead to a protection against pathology in malaria. For that reason it would be necessary to perform further survival experiments with PbA blood stage - infected IL-22BP$^{-/-}$ mice.

5.4 IL-22 modulated the cytokine release

The phenotype of IL-22$^{-/-}$ mice in PbA blood stage infection is characterized by the aggravated pathology accompanied by a lower parasitemia. To elucidate how the immune system is involved into malaria pathology in IL-22 - deficient mice it is important to know how and when the immune system is acting in plasmodial infection. Herein, different cell types are involved in defending the host against the pathogen, including cells of the innate and adaptive immune system. Since IL-22 is mainly involved in the pathology and clearance of *Plasmodium* spp. infection it was of interest to investigate how the absence of IL-22 influences the adaptive immune system in a plasmodial infection. One of the main key players in the immune response in malaria is IFNγ which is the hallmark cytokine of a T_H1 dominated immune response. IFNγ is important to further push T_H1/T_H2 differentiation forward and to activate macrophages (109). But still it is not really clear if IFNγ induces pathology (63-66, 80, 81, 110) or confer protection (92-94) in malaria. Nevertheless in the absence of IL-22 in PbA infection, IFNγ is significantly increased in different splenic cell types, including CD3$^+$ (Fig. 4.10, A), CD8$^+$ (Fig. 4.10, B), CD3$^+$ CD8$^-$ (representing CD4$^+$ T cells, since the CD4 receptor is internalized and degraded upon stimulation) (Fig. 4.10, C) and γδ$^+$ (Fig. 4.10, D) T cells at d3 p.i.. This high level of IFNγ in the beginning of PbA blood stage infection gives the cytokine a more pro-inflammatory function during plasmodial infection, resulting in an earlier onset of cerebral malaria symptoms in IL-22$^{-/-}$ mice compared to wt mice. This result is in accordance to the finding that IFNγ$^{-/-}$ mice (64, 65, 110) as well as IFNγR$^{-/-}$ (80, 81) mice develop significantly less cerebral malaria symptoms. It was shown that IFNγ promotes the trafficking of leucocyte into the brain in PbA infection (110, 114). IFNγ signalling leads to an induction of ICAM-1 expression on the brain endothelium which in turn attract lymphocytes and lead subsequently to a sequestration of lymphocytes and parasite - infected erythrocytes (81). The sequestration of these cells induces an influx of inflammatory molecules in the brain and subsequently to a disruption of the blood - brain barrier resulting to the induction of cerebral malaria symptoms. However, at a later time point of PbA infection in mice (d6 p.i.) the IFNγ secretion in CD3$^+$ (Fig. 4.10, A), CD8$^+$ (Fig. 4.10, B), CD3$^+$ CD8$^-$ (representing CD4$^+$ T cells, since the CD4 receptor is internalized and degraded upon stimulation) (Fig. 4.10, C) and γδ$^+$ (Fig. 4.10, D) T cells was alike in IL-22$^{-/-}$ and wt mice. Therefore, a different kinetic of IFNγ secretion in IL-22$^{-/-}$ compared to wt mice can be suggested in PbA infection.

The enhanced IFNγ secretion at d3 p.i. of splenic $CD3^+$ T cells was confirmed in αIL-22 treated mice, indicating that this effect is specific to IL-22 and not due to incorrect development of the immune system during ontogenesis or any other secondary effects of the knock-out (Fig. 4.11).

A cytokine which recently comes more into focus concerning its pro-inflammatory action is IL-17 which is highly secreted by T_H17 and $\gamma\delta^+$ T cells upon stimulation. As previously described, T_H17 differentiation is driven by IL-1β IL-23, TGF-β and IL-6, by inhibiting T-bet (T_H1 transcription factor) and Foxp3 (Treg transcription factor) induction in $CD4^+$ T cells (42, 115). Additionally IL-23 is necessary for the IL-22 release of T cells (19, 44, 116). Furthermore it is essential for the development of IL-22 producing ILCs (117-119). So IL-17 and IL-22 shares IL-23 as an inducer cytokine. Even though IL-23 can induce both, T_H17 and T_H22 differentiation, further inducer cytokines of IL-17, like TGF-β, are inhibiting the differentiation of T_H22 T cells. Vice versa, ligands of the Aryl hydrocarbon receptor, the key transcription factor of T_H22, are known to inhibit the production of IL-17 (120). To shed light into this diverse interaction pathway of IL-17 and IL-22 secretion in *Plasmodium* spp. infection, the IL-17 secretion of $CD3^+$ $CD8^-$ and $\gamma\delta^+$ T cells was analysed on d3 and d6 p.i. of PbA blood stage - infected IL $22^{-/-}$ and wt mice. At both time points and in both cell types the IL-17 production was significantly reduced (Fig. 4.12, A, B). Since the IFNγ secretion is induced at the early time point of PbA infection in IL-$22^{-/-}$ mice it can be assumed that T_H1 differentiation is more favoured in the absence of IL-22. Additionally it is known that IFNγ can suppress T_H17 differentiation, so it can be concluded that the increased amount of IFNγ in IL-$22^{-/-}$ mice during PbA infection hinders the IL-17 production of $CD3^+$ $CD8^-$ and $\gamma\delta^+$ T cells (121, 122). In conclusion, the low amount of IL-17 in IL-$22^{-/-}$ mice during PbA infection compared to wt mice is a consequence of the increased IFNγ secretion in the beginning of infection.

5.5 Dendritic cells express the IL-22Rα1 chain upon stimulation

Considering the modulated T cell response in PbA - infected IL-$22^{-/-}$ mice one can assume a link between IL-22 and the adaptive immune system. So far it is known that the IL-22Rα1 receptor is expressed on non-hematopoietic cells, especially on primary hepatocytes (pHepa) upon rIL-6 stimulation for 6 h. Wolk *et al.*, 2004 showed that different T cell

populations do not express an IL-22Rα1 chain upon αCD3 and αCD28 stimulation for 6, 18, 42 and 66 h. Additionally the same publication showed that macrophages as well as dendritic cell do not express IL-22Rα1 chain upon LPS stimulation for 6 h (88).

Hence in the present study different cell types were analysed concerning their *il22ra1* chain mRNA expression. For this purpose FACS sorted splenic $CD3^+$ T cells of a naive mouse were used as negative control (23) and primary hepatocytes as positive control (88) for an IL-22Rα1 chain specific PCR (Fig. 4.13). Since the liver can adopt the splenic capacity of trapping *Plasmodium* spp. - infected red blood cells if the spleen is already exhausted by infection (123, 124), and since the liver has the ability to modulate immune response (125), it was of special interest to analyse if another cell type in the liver than hepatocytes can be sensible to IL-22. For that reason liver sinusoidal endothelial cells (LSECs) came into focus. LSECs are located in close proximity to the surrounding blood flow and are consequently in direct contact with the pathogen and circulating cells of the immune system. Hence it is likely to assume that these cells can be influenced by the bioavailability of IL-22 and subsequently modulate the immune response. Additionally it is reported that LSECs are able to interact with T cells (126) and furthermore they are highly capable to induce regulatory T cell responses (127). Nevertheless LSECs did not show a specific signal for the IL-22Rα1 chain and are therefore not the connecting cell type between IL-22 and the IL-22 responsive immune response to PbA infection in mice. Another cell type which is commonly known to be the classical connection point between the innate and adaptive immune system are dendritic cells (DCs) (6). Indeed bone marrow derived DCs (BMDCs) are able to express the *il22ra1* chain mRNA after 24 h LPS stimulation (Fig. 4.13). To confirm that the mRNA expression of the *il22ra1* chain mRNA is induced due to PbA iRBC infection, wt mice were infected with PbA iRBC and sacrificed at d6 p.i. and the cDNA of splenic tissue was generated subsequently. Additionally IL-22Rα$1^{-/-}$ mice were analysed as negative control to proof the sensitivity and specificity of the chosen IL-22Rα1 primers for this PCR (Fig. 4.14). The result of this PCR showed that there is an induction of the expression of the *il22ra1* chain on mRNA level in the spleen and the use of the IL-22Rα$1^{-/-}$ mice proved that this PCR is highly sensitive and specific for the IL-22Rα1 chain. In the next step, further experiments with this PCR can be performed as quantitative PCR, since the sensitivity and specificity is proven.

To elucidate if plasmodial infection induces IL-22Rα1 chain mRNA expression in DCs *in vivo*, mice were infected with PbA iRBC and sacrificed at d6 p.i.. Sorted $CD11c^+$ cells of naive and

PbA blood stage - infected mice were subsequently analysed concerning their *il22ra1* chain mRNA expression by the IL-22Rα1 chain PCR (Fig. 4.15). The different expression of the *il22ra1* chain in DCs of the PCR result might be explained by a different disease progression in each mouse, caused e.g. by hierarchical stress in between the group of mice. Surprisingly the sorted $CD4^+$ T cell sample of a PbA blood stage - infected mouse, which was intended as negative control, showed an IL-22Rα1 chain PCR product. So far it was assumed that hematopoietic cells do not express the IL-22Rα1 chain and consequently are not susceptible for IL-22 signalling (23, 88). But a recent publication showed in an autoimmunity model an increased expression of the IL-22Rα1 chain on $CD4^+$ T cells (25). Additionally patients with the Sjögren syndrome lymphoma showed the expression of a functional IL-22Rα1 chain on peripheral blood mononuclear cells (PBMCs) (26). Hence $CD4^+$ T cells cannot be used negative control for the expression of IL-22Rα1 chain in PbA - infected mice. Therefore, the idea that cells of the immune system are completely absent of an IL-22Rα1 chain expression has to be reconsidered. It can be assumed that the expression of the IL-22Rα1 chain on hematopoietic cells is depending on the stimulatory environment.

5.6 IL-22$^{-/-}$ dendritic cells own an increased ability to stimulate T cells in PbA infection

The deficiency of IL-22 in PbA blood stage infection in mice leads to an early increase of IFNγ production in different lymphocyte subsets. Furthermore an early treatment with an IL-22 antibody *in vivo* leads to the same effect and additionally to a significant decreased parasitemia in PyNL infection. Hence these findings lead to the assumption that IL-22 plays a regulatory role during the early phase of immune response which includes the priming of T cells. Since DCs are highly important for this step they were taken into consideration regarding the IL-22Rα1 chain expression. Indeed an IL-22Rα1 chain expression was observed in PbA blood stage infection via PCR (Fig. 4.15). The expression of CD86, an important costimulatory molecule, was increased on $CD11c^{++}$ $CD11b^-$ DCs of PbA blood stage - infected IL-$22^{-/-}$ mice compared to wt mice on d3 p.i. (Fig. 4.16). The presence of increased CD86 expression on the surface of DCs of IL-$22^{-/-}$ mice in the early phase of infection may lead to a better antigenic T cell priming via DCs *in vivo*. This may explain the enhanced induction of IFNγ release by splenic lymphocytes and the decreased parasitemia during PbA and PyNL

infection of IL-22 - deficient mice. To confirm the induced expression of costimulatory molecules by DCs in the absence of IL-22 *in vitro*, BMDCs of IL-22$^{-/-}$ and wt mice were stimulated for 24 h with LPS and subsequently analysed concerning their CD80 and CD86 expression by FACS analysis. The result showed that the CD86 and CD80 expression of IL-22$^{-/-}$ BMDCs is significantly induced after LPS stimulation for 24 h (Fig. 4.17). Hence the expression of costimulatory factors is increased in DCs derived from IL-22$^{-/-}$ mice after stimulation *in vitro* as well as *in vivo.*

Bringing all together the assumption can be created that DCs of IL-22$^{-/-}$ mice own an increased ability to stimulate T cells in PbA blood stage infection. Hence the immune response to PbA blood stage infection in IL-22$^{-/-}$ mice is induced considering a regulatory modulation of DCs by IL-22. To confirm this assumption BMDC of wt and IL-22$^{-/-}$ mice were generated, pulsed with PbA specific peptides (89, 90) and subsequently incubated for 24 h with CD8^{+} T cells of PbA blood stage - infected wt and IL-22$^{-/-}$ mice (Fig. 4.18). The result showed that the IFNγ release of wt infected mice is lower than of PbA blood stage - infected IL-22$^{-/-}$ mice if they are either stimulated on wt or IL-22$^{-/-}$ BMDCs. The increased IFNγ release of CD8^{+} T cells from IL-22$^{-/-}$ mice, regardless of the origin of the stimulating BMDCs, leads to the conclusion that the absence of IL-22 induces already a higher antigen specific T cell activation *in vivo*. Even more important was the observation that IL-22$^{-/-}$ BMDCs showed a highly significant induction of IFNγ release by CD8^{+} T cells regardless if they derived from PbA - infected wt or IL-22$^{-/-}$ mice. So it is evident that BMDCs of IL-22$^{-/-}$ mice own a greater ability to induce pro-inflammatory immune responses.

Since T cells are shown to be better activated in the absence of IL-22, it was of interest if this is also reflected concerning their proliferation. For this reason CFSE labelled splenic OT1 T cells, which constitutively express a OVA specific T cell receptor on their surface, were injected into recipient IL-22$^{-/-}$ and wt mice which were subsequently infected with an OVA transgenic expressing PbA strain. With this experimental setup it is possible to track antigen specific proliferating T cells *in vivo*. Nevertheless the proliferation of CFSE^{+} CD8^{+} T cells was alike in IL-22$^{-/-}$ and wt mice at d5 p.i.(Fig. 4.19). However the lack of IL-22 results in a greater magnitude of proliferation, concluding that the absence of IL-22 during PbA infection leads to a significantly increased recovery of antigen specific CD8^{+} T cells. Hence it might be suggested that the lack of IL-22 leads to a more pronounced antigen specific activation of T cells and to a better survival of antigen recognizing T cells. Thus the increased abundance

of antigen specific T cells and the increased IFNγ release of these cells will explain the reduced parasitemia in PbA - infected IL-22$^{-/-}$ mice during the blood stage and the induced CM due to the increased amount of IFNγ.

Taken together this present study shows that die deficiency of IL-22 is modulating the immune response to plasmodial infection. On the one hand the parasitemia is dependent on the bioavailability of IL-22 and on the other hand the absence of IL-22 leads to an earlier onset of malaria symptoms. The increased susceptibility to CM in IL-22 - deficient mice can be explained due to the increased early release of IFNγ which and a diminished IL-17 production in PbA - infected IL-22$^{-/-}$ mice compared to wt mice. The different cytokine release in IL-22 - deficient mice compared to wt mice during malaria might be due to the enhanced expression of costimulatory receptors on dendritic cells in the absence of IL-22 upon plasmodial infection.

5.7 Outlook

The reported results in this study further characterize the pathophysiological mechanisms of *Plasmodium* spp. infection concerning the role of IL-22 during murine malaria. Nevertheless the exact mechanism how IL-22 is acting on the immune system in plasmodial infection is not fully understood. The decreased parasitemia in IL-22 - deficient mice might be due to an induced antigen specific immune activation by the increased ability of dendritic cells to express costimulatory receptors. Furthermore it was shown that the parasitemia is dependent on the bioavailability of IL-22, regarding PyNL - infection in IL-22$^{-/-}$ mice and in IL-22BP$^{-/-}$ mice. Despite the ability of IL-22BP to block the interaction between IL-22 and the IL-22Rα1 chain, its role is not well understood. For that reason the implementation of further tools for the characterization of the IL-22BP as well as the IL-22 - IL-22BP complex are of crucial importance, e.g. specific antibodies. Moreover the identification of a potential receptor for the IL-22 - IL-22BP complex would bring more light how IL-22 and its complex with IL-22BP might act on the immune response. Hence the results of this study cannot be attributed only to the lack of IL-22 alone since the function of the IL-22 - IL-22BP complex is not clarified. An additional question which seems to be not completely solved is the link between IL-22 and the immune system. Two recent publications showed that the IL-22Rα1 chain can be expressed on lymphocytes during arthritis and a lymphoma, respectively.

Hence it might be of interest to analyse further T cell subtypes concerning their ability to express the IL-22Rα1 chain during murine malaria. For this purpose tissue specific IL-22Rα1$^{-/-}$ mice can lead to further results concerning the identification of how IL-22 acts on the immune response during plasmodial infection. The new finding, which has to be taken into consideration, is the ability of IL-22$^{-/-}$ dendritic cells to express more costimulatory molecules upon stimulation than wt dendritic cells. For this reason it would be of special interest if the lack of IL-22 induces an improved T cell response against the liver stage of plasmodial infection upon vaccination. A well - established vaccine model against the liver stage in murine malaria is the application of a recombinant detoxified adenylate cyclase toxoid - circumsporozoite protein (ACT - CSP). ACT - CSP is capable to cargo the CSP peptide into the cytosol of CD11b expressing antigen presenting cells which in turn present the CSP by MHC class I molecules to CD8^{+} T cells in Balb/c mice. The expected result would be an improved immune response against the sporozoite load in the liver upon ACT-CSP vaccination in IL-22 - deficient mice.

6 Literature

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7 Appendix

7.1 Abstract

During plasmodial infection, it is of crucial importance to maintain the balance between pro - and anti - inflammatory immune response, since the disruption leads to severe malaria pathology. Members of the IL-10 cytokine family have a modulatory effect on the immune response and control the immune system during infection. Especially IL-10 itself acts highly immunosuppressive and its absence leads to a fatal outcome of disease. One member of the IL-10 cytokine family is IL-22. This cytokine is closely related to IL-10 concerning the sequence homology and shares a receptor chain with IL-10. The receptor complex of IL-22, which consists of the IL-10Rβ chain and the IL-22Rα1 chain, is thought to be expressed by non - hematopoietic cell, e.g. hepatocytes, even though IL-22 itself is mainly produced by cells of the immune system. In this present study, an increased level of IL-22 was determined in the blood plasma of *Plasmodium falciparum* (*P. falciparum*) infected humans compared to healthy participants of the study. The same result was obtained in a comparable murine malaria model which includes the infection of C57BL/6 mice with *Plasmodium berghei* ANKA (PbA). Furthermore IL-22 - deficient mice and wt mice were infected with PbA to elucidate the role of IL-22 during plasmodial infection concerning the pathology and the development of parasitemia. The occurrence of cerebral malaria symptoms was higher in the absence of IL-22 accompanied by an early induction of IFNγ and a decreased level of parasitemia. Since the PbA sporozoite burden in the liver of IL-22$^{-/-}$ mice is similar to wild type mice, the lower parasitemia is not due to a different infectivity of sporozoites to hepatocytes in the liver, even though hepatocytes express the IL 22 receptor complex. Hence the blood stage of malaria was further characterized and a higher secretion of IFNγ by different T cell subtypes was found at an early time point of PbA infection in IL-22$^{-/-}$ mice, indicating that the activation of the adaptive immune system is modulated in the absence of IL-22. This was confirmed with the observation that dendritic cells, the classical connection point between the innate and the adaptive immune system, of IL-22$^{-/-}$ mice show an enhanced expression of costimulatory receptors upon stimulation, *in vitro* by LPS as well as *in vivo* during PbA infection. Therefore, it can be assumed that dendritic cells are influenced by IL-22. This

assumption can be reinforced by the observation that the IL-22Rα1 chain is detectable upon stimulation of dendritic cells by PCR.

7.2 Zusammenfassung

Während der Malaria ist es von besonderer Bedeutung das Gleichgewicht zwischen einer pro - inflammatorischen und einer anti - inflammatorischen Immunantwort zu bewahren, da eine Beeinträchtigung des Systems zu einer schweren Pathologie der plasmodialen Infektion führen kann. Mitglieder der Interleukin (IL)-10 Zytokin - Familie besitzen die Fähigkeit die Immunantwort bezüglich einer Infektion zu beeinflussen. Besonders IL-10 selbst kennzeichnet sich durch immunregulatorische Funktionen aus. Dies konnte bereits durch die fatale Auswirkung der Abwesenheit von IL-10 während einer *Plasmodium* Infektion im Mausmodel gezeigt werden. Ein Mitglied der IL-10 Zytokin - Familie ist IL-22. Dieses Zytokin ist eng mit IL-10, bezüglich der Sequenzhomologie und der Eigenschaft sich eine Rezeptorkette mit IL-10 zu teilen, verwand. Die Expression des IL-22 Rezeptorkomplexes, bestehend aus der IL-10Rβ Kette und der IL-22Rα1 Kette, wurde bisher nicht - hämatopoetischen Zellen, z.B. Hepatozyten, zugeschrieben. Im Gegensatz dazu wird IL-22 von Zellen des Immunsystems produziert. In dieser Studie konnte gezeigt werden, dass die IL-22 Konzentration im Plasma *Plasmodium falciparum* infizierter Menschen im Vergleich zu gesunden Menschen erhöht ist. Das gleiche Ergebnis konnte auch in einem vergleichbaren Mausmodell dargestellt werden, welches die Infektion von Mäusen der Art C57BL/6 mit dem *Plasmodium berghei* ANKA (PbA) beinhaltet. Des Weiteren wurden IL-22 defiziente Mäuse und wild - typ Mäuse mit PbA infiziert, um die Rolle von IL-22 im Verlauf der Malaria bezüglich der Pathologie und der Parasitämie zu analysieren. Das Auftreten von zerebralen Symptomen war verstärkt in der Abwesenheit von IL-22, begleitet von einer frühen Induktion von IFNγ als auch einer niedrigeren Parasitämie. Da sich die PbA - Sporozoitenlast in der Leber nicht unterscheidet, kann die verringerte Parasitämie nicht durch eine unterschiedliche Infektiosität der PbA - Sporozoiten gegenüber der IL-22Rα1 Kette exprimierenden Hepatozyten erklärt werden. In der Blutphase konnte eine verstärkte Produktion von IFNγ durch verschiedene T - Zellpopulationen an einem frühen Zeitpunkt der PbA Infektion gezeigt werden. Dies führte zu der Annahme, dass die Aktivierung des adaptiven Immunsystems in der Abwesenheit von IL-22 modifiziert ist. Die klassische Verbindung zwischen dem angeborenen und dem adaptiven Immunsystem obliegt den dendritischen Zellen, welche in IL-22$^{-/-}$ Mäusen eine verstärkte Expression von kostimulatorischen Rezeptoren aufweisen. Dies konnte sowohl *in vitro*, durch LPS

Stimulation, als auch *in vivo*, in der PbA Infektion, gezeigt werden. Somit kann davon ausgegangen werden, dass dendritische Zellen durch IL-22 beeinflusst werden können. Diese Behauptung kann durch die per PCR nachgewiesene Expression der IL-22Rα1 Kette auf stimulierten dendritischer Zellen bestätigt werden.