Effect of IL-22 on T cell-derived cytokine production

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1 Introduction

1.1 The immune system
The immune system is a network of various tissues, cells and organs that is in charge of protecting an organism from the aggression of exogenous agents like virus, bacteria and parasites. It is also responsible for recognizing internal non-pathogenic microorganisms and avoiding an attack against them (tolerance), which is why it is so important that the immune system maintains its homeostasis. The cells and molecules involved in this system are distributed through the entire organism waiting for a pathogen to enter the body. Depending on the speed of the reaction and its specificity, the immune system is divided into innate and adaptive immune responses. (Abbas, Lichtman et al. 2012).

1.1.1 Innate immune system
The innate immune system provides the first line of defence against an injury from pathogens. This system is present in all plants and animals; it acts unspecific and does not change over exposition to the same pathogen. The main actors of the innate immune response are barriers, cells derived from common myeloid progenitor cells like neutrophils and macrophages, dendritic cells and natural killer cells; the latter recognize and kill target cells like tumour or infected cells. When a pathogen enters a host, macrophages, neutrophils and dendritic cells migrate to the site of infection, where they recognize the pathogen-associated molecular patterns (PAMPs) through their pattern recognition receptors (PRRs) like the scavenger and toll-like receptors (TLRs). Even though these immune cells can only recognize a limited number of molecules; the antigens they recognize are crucial for the microbes survival. Therefore, the pathogens cannot discard these molecules and thus are not able to evade host defence. Once the antigens are bound to their receptors, antimicrobial and pro-inflammatory functions are activated on the immune cells. In order for immune cells to migrate to the infection tissues, they need to get activated. (Abbas, Lichtman et al. 2012).

1.1.1.1 Antigen Presenting Cells
Antigen presenting cells (APCs) are specialized in capturing antigens, processing and displaying them to lymphocytes and starting the adaptive immune response. They also express co-stimulators and produce cytokines that enhance the differentiation as well as the proliferation of lymphocytes. There are different cell populations that can act as APCs but normally have other main functions, such as B cells and macrophages. On the other hand, the dendritic cells are called professional APCs because their main task is to present antigens to lymphocytes. The APCs make a link between the innate and acquired immune responses and thus are considered to belong to both of these systems. (Abbas, Lichtman et al. 2012).

1.1.1.2 Dendritic Cells
Dendritic cells (DCs) are specialized is displaying antigens to naïve T cells and activating them. They can be found in parenchyma, mucosal epithelium and in other lymphoid tissues. They arise from a
monocyte precursor, and need a growth factor called fms-related tyrosine kinase 3 ligand (Flt3 ligand) to maturate, as well as IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF). (Abbas, Lichtman et al. 2012).

DCs can be divided into immature and mature dendritic cells, depending on their ability to activate T cells. Immature DCs may be found in homeostatic non-lymphoid tissues like epithelia. On this organ they recognize antigens but do not express co-stimulators and thus are not able to activate T cells. Immature DCs might be important in preventing autoimmunity, since they can present self-antigens to self-reactive lymphocytes and induce the lymphocytes anergy. Mature DCs on the other hand, have already encountered an antigen through PAMPs or DAMPS and migrate to the lymph nodes to display the antigen and activate T cells. (Abbas, Lichtman et al. 2012).

Dendritic cells possess long membranous projections that ease capturing antigens. This task is simplified by the numerous TLRs and cytoplasmic pattern recognition receptors that DCs express on their surface, making them capable of recognizing many diverse molecules. Once an antigen is captured, the dendritic cell processes the ingested protein into peptides which bind to proteins called major histocompatibility complex (MHC). There are two types of major histocompatibility complexes, MHC I and MHC II, which are selected depending on the source of the antigen. When a peptide is produced by the proteolytic degradation of cytosolic proteins inside the APC, this peptide is transported to the endoplasmic reticulum and it is bound to a MHC I molecule. All of the nucleated cells possess MHC I and are therefore able to present antigens to CD8$^+$ T cells. On the contrary, when an antigen is captured outside of the APC, it is internalized via endosomes where it is degraded enzymatically generating peptides that are able to bind MHC II. Once bound to MHC II, the antigen can be presented to CD4$^+$ T cells. (Abbas, Lichtman et al. 2012).

To start a T cell response against a specific antigen, the first signal needed is recognition of the antigen bound to a MHC molecule. When the APC displays the antigen, it also releases cytokines and co-stimulators to further activate lymphocytes, such as IL-12, CD80 and CD86, respectively. Shortly after being activated, T cells express high levels of IL-2 and IL-2 receptors to enhance their own proliferation. (Abbas, Lichtman et al. 2012).

1.1.1.3 Plasmacytoid dendritic cells

Plasmacytoid dendritic cells (PDCs) are morphologically similar to antibody-producing cells; they also originate from the bone marrow and are found in the blood, spleen and lymph nodes. PDCs are specialized in generating antiviral proteins like type I interferons in response to viral infections. This is possible due to the high expression of TLRs 3, 7, 8, 9 they possess, which capture nucleic acid of intracellular viruses. (Abbas, Lichtman et al. 2012).
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1.1.1.4 Follicular dendritic cells
Follicular dendritic cells (FDCs) are usually found coalescent with B cells in the lymph nodes, mucosal epithelium and spleen. They capture antigens that are bound to antibodies or complement proteins and display the antigens to B cells. FDCs are not derived from the bone marrow like other DCs but are of mesenchymal origin. (Abbas, Lichtman et al. 2012).

1.1.2 Adaptive immune system
Both the innate and acquired immune responses do not work by themselves but are closely related to each other. When a pathogen enters an organism, the host tries to eliminate it with the unspecific innate immune response. If this response is not able to get rid of the pathogen, the innate immune system stimulates the adaptive response, which takes several days to develop, but is highly specific. (Abbas, Lichtman et al. 2012).

In contrast to the innate immunity, the adaptive immune system is exclusive of vertebrates and has memory (the immune response gets faster and more vigorous with repeated exposures to the same pathogen). The main components of the acquired immune response are the lymphocytes and their produced antibodies. (Abbas, Lichtman et al. 2012).

Lymphocytes are distributed through the whole organism, they can be found in blood, bone marrow and lymphoid tissues. When a lymphocyte has encountered an antigen, there are clones made from this cell which express the same receptor for that specific antigen. Once this so-called clonal expansion happens, millions of lymphocytes clones are distributed in the body and wait to recognize their antigen. (Abbas, Lichtman et al. 2012).

There are two main types of lymphocytes; the bone marrow-derived B cells and the thymus matured T cells.

B lymphocytes bind epitopes through their membrane-bound immunoglobulin receptors (B cell receptors) and consequently turn into plasma cells. Plasma cells are in charge of producing antibodies that tag their specific epitope for the elimination of the antigen. This type of immunity is called humoral immune response and is best suited against extracellular microbes and their toxins, since the antibodies do not have the capacity to enter any cell. When an antibody is secreted, it can be transported into the lumen of mucosal organs, where it triggers the release of inflammatory mediators and also activates phagocytosis of the pathogen. (Abbas, Lichtman et al. 2012).

While B cells are in charge of the humoral immune response, the T lymphocytes elicit the cellular immune response. T cells do not produce antibodies but clear antigens by direct contact with the infected cells or by enhancing the activation of other immune cells. T lymphocytes are produced in the bone-marrow and fully mature in the thymus. They express a receptor called TCR receptor which recognizes antigens bound to MHC molecules. The TCR receptor consists of two chains, either αβ or γδ. Approximately 90% of T lymphocytes express the αβ TCR, which gives rise to CD4+ and
CD8$^+$ T cells. CD4 and CD8 are co-receptors which will be committed to the cells depending on their ability to recognize either MHC II or MHC I, respectively. Lymphocytes expressing the TCR $\gamma\delta$-chains develop into $\gamma\delta$ T cells, which will be described later. (Abbas, Lichtman et al. 2012).

During maturation in the thymus, T cells go through positive selection. This process consists of promoting survival of T lymphocytes that weakly react to the self MHC complexes. Afterwards, lymphocytes that recognize self-antigens with high affinity are negative selected and undergo apoptosis or turn into regulatory T cells. Lymphocytes that were not negatively selected go into the periphery as naïve T cells and wait for an antigen to be presented to them. Since this deletion of self-reactive lymphocytes is not a perfect system, some self-reactive cells survive and may lead to autoimmune diseases like type 1 diabetes. (Baldwin, Sandau et al. 2005)

As described before, T cells are in charge of the cellular immunity and depending on their phenotype, T cells can directly kill the pathogen (CD8$^+$ cytotoxic T cells), recruit more immune cells or enhance the production of specific antibodies against the antigens (CD4$^+$ helper T cells). In the blood and lymphoid tissues approximately 65% of all T cells express CD4 and 35% CD8. Distinct to B cells, T cells cannot recognize antigens by themselves but always need an APC to perform this task for them. (Abbas, Lichtman et al. 2012).

When an antigen is recognized for the second or subsequent time, the immune system can start an immune response without having to wait for naïve T cells to become activated. This is possible due to the ability that B and T cells have in becoming long-lived antigen specific memory cells, which are more numerous than the effector T cells specific for an antigen. Memory B cells produce antibodies that bind antigens with a much higher affinity than the antibodies produced the first time the antigen was recognized. Furthermore, memory T cells are able to act faster and more effectively than effector T cells do. (Abbas, Lichtman et al. 2012).

As soon as the infection is abolished, the immune system needs to go back to its basal state or homeostasis. When an antigen is presented and triggered, the immune response works to eliminate it and therefore many cytokines and co-stimulators are secreted. Once the antigen is eradicated, no more cytokines and co-stimulators are produced and therefore most cells die by apoptosis, with the exception of memory cells. (Abbas, Lichtman et al. 2012)

1.1.3 $\gamma\delta$ T Lymphocytes
These cells are mainly found in epithelia. Around 5% of all T lymphocytes are $\gamma\delta$ T lymphocytes, which do not only recognize antigens associated to the MHC molecules, but are also able to bind small phosphorylated molecules, alkyl amines and lipids. Some $\gamma\delta$ T cells have the ability to directly recognize antigens without the help from an APC. (Abbas, Lichtman et al. 2012).
1.1.3.1 **CD8**+ T cells

CD8**+** T cells are specialized in killing abnormal cells like virus-infected or tumour cells. They possess two different mechanisms to function; the first one involves the granzyme and perforin proteins. Once a virus-antigen is encountered, processed and presented on a MHC I molecule to a CD8**+** T cell, clonal expansion takes place and the clones made go into the tissues in search of their specific antigen. When CTLs find a cell infected with the antigen, they take direct contact with the infected cell and release perforin into its membrane, which forms a pore. Through this pore enzymes like granzyme B enter the anomalous cell and activate the programmed cell death through pro-caspases. (Abbas, Lichtman et al. 2012).

The second mechanism takes place when the infected or abnormal cell expresses the Fas molecule, which interacts with the membrane protein Fas-ligand (FasL) that resides on the surface of T cells. When the Fas molecule binds to the FasL, apoptosis of the infected cell is induced. Since T cells are not affected by this procedure, they move on in search of more pathologic cells expressing the specific antigen. To further enhance the elimination of pathogens, CTLs produce great amounts of IFNγ. IFNγ up-regulates the expression of MHC molecules, promotes macrophages activation and increases the release of reactive oxygen species by inducible nitric-oxide synthase (Tsuji, Shiraki et al.), which leads to apoptosis of abnormal cells. (Street, Trapani et al. 2002)

1.1.3.2 **CD4**+ T cells

The main function of CD4**+** helper T cells is to activate other immune cells. CD4**+** T lymphocytes regulate CD8**+** T cell responses and maintain macrophages function by releasing different cytokines. CD4**+** T cells can also activate B cells to produce antibodies and once the antigen is eradicated, they stop the immune response. (Zhu, Yamane et al. 2010).

When CD4**+** T cells encounter their specific antigen, they differentiate either into Th1, Th2 Th17 or Th22 subsets; each one of these populations produces different cytokines and therefore has diverse functions. Depending on the source of the pathogen threatening the organism, the cells gain a specific phenotype in order to induce an appropriate immune response and get rid of the pathogen. (Abbas, Lichtman et al. 2012).

1.1.3.2.1 **Th1 cells**

The Th1 subtype of effector cells is induced by IFNγ and IL-12, the latter cytokine is produced when cells like macrophages or dendritic cells bind an antigen through a TLR. IL-12 also activates the transcription factor T-bet which in turn induces IFNγ production and as a result macrophages become activated. (Szabo, Kim et al. 2000). Other cytokines produced by these helper T cells are IL-2, which induces Th1 cell proliferation, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF), which together stimulate the bone marrow to produce more phagocytes. Th1 cells also help B cells in the antibody production and stimulate CD8**+** T cells proliferation. The immune response set by this effector cell subset is specialized against intracellular bacteria like *Listeria* and *Mycobacterium*,
and also against parasites like *Plasmodium*. Th1 cells can also play a regulatory role by eliminating defective cells through apoptosis, like active macrophages who fail to kill an abnormal cell. (Abbas, Lichtman et al. 2012).

1.1.3.2.2 Th2 cells
Th2 cells main function is to lead an immune response against extracellular bacteria and parasites. They can also activate B cells to produce antibodies, especially IgE which is necessary to clear a parasitic infection. The Th2 subset is induced in the presence of IL-4, which is produced by basophils or by Th2 cells themselves. IL-4 is also in charge of inhibiting another helper T pathway; Th1 response. The transcription factor induced by these helper T cells is the trans-acting T-cell-specific transcription factor GATA-3. GATA-3 acts as a master regulator from the Th2 subtype, since it blocks the Th1 differentiation and also potentiates Th2 development through a positive feed-back loop. Some other cytokines produced by these CD4+ T cells are IL-5, IL-9, IL-10 and IL-13. IL-5 activates eosinophils to attack the parasites while IL-10 suppresses Th1 cell differentiation. (Abbas, Lichtman et al. 2012).

1.1.3.2.3 Th17 cells
These CD4+ T cells mediate the immune response against extracellular bacteria and fungi by recruiting neutrophils to the site of infection. The Th17 subset is triggered by IL-6 and TGF-β and as main cytokines it produces IL-17A, IL-17F, IL-21 and IL-22. TGF-β acts more as a suppressor of Th1 and Th2 and thus removes the inhibitory effects these two populations can have upon Th17 cells. The main transcription factors involved in the development of Th17 cells are the signal transducer and activator of transcription (STAT3) and retinoid-related orphan receptor T (ROR γt), the latter controls the differentiation of this cell population. (Abbas, Lichtman et al. 2012). Th17 cells can influence other immune cells by eliciting the expression of pro-inflammatory cytokines and chemokines like IL-6, G-CSF and monocyte chemoattractant protein-1 (MCP-1), which recruit neutrophils and activate T cells (Aggarwal 2003). When there is an uncontrolled up-regulation of the Th17 cytokines, several autoimmune diseases like rheumatoid arthritis and type 1 diabetes can develop. (Gaffen 2009).

1.1.3.2.4 Th22 cells
Th22 cells are the most recently identified T helper subset; they mainly express IL-22, as well as IL-26 and IL-13 (Eyerich, Eyerich et al. 2009), but no IL-17 or IFNγ. (Duhen, Geiger et al. 2009). Th22 cells seem to play a protective role in mucosal antimicrobial defence (Basu, O’Quinn et al. 2012) and can be induced in the presence of IL-6 and TNF-α. The expansion of IL-22 producing cells is regulated by the aryl hydrocarbon receptor (AhR) transcription factor. The cytokine IL-22 affects mainly epithelial and stromal cells since its main receptor sub-unit seems to be lacking on hematopoietic cells. The Th22 cell population might be involved in diseases like type 1 diabetes, atopic eczema and rheumatic arthritis when not regulated. (Kirkham, Lassere et al. 2006, Honkanen, Nieminen et al. 2010).
A summary of the above mentioned helper T cell populations is presented in order to have a better understanding at their functions and main inducers.

### Table 1: CD4+ helper T cell subsets

<table>
<thead>
<tr>
<th>Type</th>
<th>Cytokine stimulus</th>
<th>Transcription factors</th>
<th>Effector cytokines</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>IL-12, IFNγ</td>
<td>T-bet, STAT1, STAT4</td>
<td>IFNγ, TNFα, GM-CSF</td>
<td>Macrophage and CTL activation against intracellular pathogens</td>
</tr>
<tr>
<td>Th2</td>
<td>IL-4</td>
<td>GATA-3, STAT6</td>
<td>IL-4, IL-5, IL-9, IL-10, IL-13</td>
<td>Allergic reaction, antibody production by B cells</td>
</tr>
<tr>
<td>Th17</td>
<td>IL-6, TGF-β, IL-23</td>
<td>RORγt, STAT3, AhR</td>
<td>IL-6, IL-17A, IL-17F, IL-21, IL-22</td>
<td>Granulocyte activation, eradication of pathogens not handled by Th1/Th2, autoimmune diseases</td>
</tr>
<tr>
<td>Th22</td>
<td>IL-6, TNFα</td>
<td>STAT3, AhR</td>
<td>IL-22, IL-26, IL-13</td>
<td>Mucosal antimicrobial defense, skin autoimmune diseases</td>
</tr>
</tbody>
</table>

#### 1.1.4 Cytokines

Cytokines are a group of polypeptides secreted by cells from both the innate and the acquired immune systems that are in charge of mediating the cellular and humoral immune responses. Monocytes, lymphocytes, tissue cells and some epithelial cells have the ability to produce cytokines, which can only act when their receptor is expressed on their target cells. Cytokines cannot be stored as molecules, but must perform their function as soon as they are secreted. Once the cytokines are in the bloodstream, they are able to act not only on one cell but on many different ones (pleiotropism) and they can influence their target cells in either an agonist or antagonist manner. The function of the cytokines is very redundant; this means that different cytokines may have the same effect on cells, which makes it difficult to antagonize one cytokine since other proteins might compensate its function. Cytokines are in charge of triggering proliferation and differentiation of lymphocytes, activating effector cells to kill pathogens or stimulating the development of hematopoietic cells. (Abbas, Lichtman et al. 2012).

#### 1.1.4.1 IL-10 family

Interleukin 10, also known as cytokine synthesis inhibitory factor, is considered an anti-inflammatory and immunosuppressive cytokine since it has the ability to block the production of IL-2, IL-6, IL-12, IFNγ and TNFα. IL-10 plays an important role in proliferation and differentiation of T and
B lymphocytes as well as mast cells. (Pestka, Krause et al. 2004). This cytokine is a dimer consisting of two six-helix bundle domains. In order to have an effect on immune cells, both domains of the IL-10 molecule need to bind to the IL-10 receptor, which consists of two chains; denominated IL-10R1 and IL-10R2 (Kotenko, Izotova et al. 2001). These chains have extracellular, transmembrane and intracellular domains and both chains belong to the cytokine receptor family class 2 (CRF2) (Pestka, Krause et al. 2004).

Several IL-10 homologs have been identified and are currently denominated as IL-10 family members. These homologous structures display between 20-30% amino acid identity with IL-10, they possess a similar structure and location of their encoding genes and share some receptor subunits (Fickenscher, Hör et al. 2002). The IL-10 family includes IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B and IL-29. These IL-10 related cytokines have very different functions but are overall essential for maintaining the homeostasis of epithelial layers, as they activate the innate immune response to limit the damage caused by an infection. The IL-10 family of cytokines is also able to repress pro-inflammatory responses and limit tissue disruption caused by inflammation. (Rutz, Wang et al. 2014).

1.1.4.2 Interleukin 22

IL-22 was discovered in 2000 by the Renauld group during the search of expressed genes in IL-9 stimulated murine thymocytes. At first, this cytokine was denominated IL-10-related T cell-derived inducible factor (IL-TIF) due to the 22% sequence homology murine IL-22 shares with IL-10. Human IL-22 was discovered shortly after the murine counterpart was found. Murine and human IL-22 share around 79% homology. The gene responsible for IL-22 is located on the longer arm of chromosome 12, on 12q15q in humans, near the IFNγ and IL-26 loci. Whereas in mice, the IL-22 gene is located on chromosome 10 also close to the IFNγ locus. (Dumoutier, Louahed et al. 2000). Identical to all of the other members of the IL-10 cytokine family; IL-22 is formed by six α-helices, which are arranged in antiparallel conformation and form a monomeric, bundle-like protein (Nagem, Colau et al. 2002).

The main producers of IL-22 in the adaptive immune system are γδ T cells, Th1, Th17 and Th22 cells as well as natural killer T (NKT) cells. In the innate immune system mast cells and innate lymphoid cells (ILCs) are the main sources of IL-22. (Sonnenberg, Fouser et al. 2011). Even though IL-22 is expressed by immune cells, this cytokine can only act non-hematopoietic cells like hepatocytes, pancreatic α and β cells, keratinocytes, lung and intestinal epithelial cells. The ability of IL-22 to act exclusively on these cells lies on the IL-22 receptor expression, which is thought to be absent on immune cells (Whittington, Armstrong et al. 2004, Sabat, Ouyang et al. 2014).
Since IL-22 cannot act directly on immune cells, it is supposed that this cytokine must mediate a cross-talk between cells from the immune system and cells expressing its receptor (Sonnenberg, Fouser et al. 2011). The IL-22 receptor complex consists of two chains; IL-22R1α and IL-10Rβ2, both of them belong to CRF2. IL-22R1α has the longer chain (325aa) and it contains four putative STAT recruitment sites (Kotenko, Izotova et al. 2001). The second subunit (IL-10Rβ2) has only 79aa and in contrast to IL-22R1α, has no affinity for the IL-22 molecule. IL-10Rβ2 also functions as an accessory receptor chain for IL-10, IL-26, IL-28 and IL-29. (Langer, Cutrone et al. 2004). While IL-10Rβ2 is ubiquitously expressed, IL-22R1α expression is found in very few tissue cells, with the highest amounts being expressed in cells from pancreas and kidney (Kotenko, Izotova et al. 2001). To induce down-stream signalling on a cell, IL-22 must first bind to the unique IL-22R1α chain; this interaction leads to a conformational change on the IL-22 molecule and makes it suitable to bind the second subunit, IL-10Rβ2 (Pestka, Krause et al. 2004). Binding to the second chain is vital for IL-22 to elicit a down-stream signalling on a cell, since IL-10Rβ2 absence turns cells non-responsive to IL-22. Once IL-22 is bound to its receptor, the JAK/STAT pathway is triggered. First, the tyrosine kinases associated to the intracellular domain of each receptor sub-unit become activated and phosphorylate the receptor’s tyrosine residues. These residues serve as docking sites for src homology 2 SH2-domains of the STAT molecule. Subsequently, the STAT molecules dimerize and translocate to their target genes in the cell nucleus and regulate the function of the cell. STAT3 induces expression of suppressor of cytokine signaling-3 (SOCS3), which negatively regulates the STAT3 signalling. (Kotenko, Izotova et al. 2001). STAT3 is also capable of inducing genes that encode anti-apoptotic...
proteins like B-cell CLL/lymphoma 2 (Bcl-2), B-cell lymphoma extra-large (Bcl-xL), and myeloid cell leukaemia sequence 1 (Mcl-1), among others (Dauer, Ferraro et al. 2005).

In addition to the IL-22 receptor complex, there is a soluble receptor denominated IL-22 binding protein (IL-22BP) that can bind to IL-22. Like the two IL-22 receptor sub-units, IL-22BP is a CRF2 member, and it mainly differs from IL-22R1α and IL-10Rβ2 by lacking the intracellular and transmembrane domains. This soluble single-chain protein has a much higher affinity (up to 1000-fold) in binding to IL-22 than IL-22R1α does (Huber, Gagliani et al. 2012) and it shares 33% sequence homology with the extracellular domain IL-22R1α (Dumoutier, Louahed et al. 2000). IL-22BP is in charge of regulating the IL-22 bioavailability, since it blocks IL-22 binding to IL-22R1α and thus antagonizes the effect of IL-22 (Xu, Presnell et al. 2001). This IL-22 antagonist is expressed in several tissues like the skin, pancreas, intestine, thymus, spleen and lungs (Dumoutier, Louahed et al. 2000). It has also been recently discovered on immature DCs (Martin, Bériou et al. 2014).

There are several cytokines that can induce IL-22 such as IL-6, TGF-β and IL-23, the latter cytokine is said to be the main inducer of IL-22 (Zheng, Danilenko et al. 2007). When IL-22 expression takes place through the IL-23 pathway; induction of STAT3 is needed (Dumoutier, Louahed et al. 2000). IL-22 can also be induced by the aryl hydrocarbon receptor transcription factor on Th17 cells. However, the exact mechanism by which the AhR transcription factor induces IL-22 expression is still unknown (Veldhoen, Hirota et al. 2009).

The functions of IL-22 are upregulating the innate immunity on tissue cells, protecting tissues from damage and increasing their regeneration capacity (Dudakov, Hanash et al. 2012). It has been demonstrated that IL-22 possesses antimicrobial abilities, especially at barrier surfaces like the intestine, skin and respiratory tract where it acts against extracellular bacteria like Klebsiella pneumonia and Citrobacter rodentium (Zheng, Valdez et al. 2008). It has been proved that this cytokine induces the production of anti-microbial substances like β-defensins, flagellin and S100 calcium binding protein A7 (S100A7) (Wolk, Kunz et al. 2004). In the liver, IL-22 can also induce acute-phase proteins like serum amyloid A (SAA), α1-antichimiotrypsin and haptoglobin. IL-22 plays a protective role in liver injury during concavalin-A induced hepatitis, where the absence of the cytokine worsens the disease. (Radaeva, Sun et al. 2004). In the intestine, IL-22 induces restitution of mucus-producing goblet cells in an STAT3 dependent mechanism, which leads to quick amelioration of intestinal inflammation (Sugimoto, Ogawa et al. 2008).

On the other hand, IL-22 can act as an inflammatory inducer by increasing the expression of G-CSF, IL-6, IL-1α and LPS-binding protein in models with Crohn’s disease (Wolk, Witte et al. 2007). IL-22 can also contribute to several autoimmune diseases when overexpressed. This is the case of rheumatoid arthritis, where high levels of IL-22 can be found in the synovial tissues, which is associated with osteoclastogenesis. (Ikeuchi, Kuroiwa et al. 2005).
Introduction

IL-22 can have differential and paradoxical functions in many tissues. This cytokine can enforce antimicrobial host defence, promote inflammation or induce regeneration. (Dudakov, Hanash et al. 2012) Therefore, in order to have a better understanding at the function of IL-22, it is essential to evaluate the tissue where IL-22 is found and the cytokines that are co-expressed.

1.2 Malaria disease
Malaria is a life-threatening illness that can occur in humans as well as in other animals. It is caused by protozoans that belong to the genus Plasmodium. There are five known species that can cause malaria in humans; P. malaria, P. ovale, P. vivax, P. falciparum and P. knowlesi. The first two subtypes produce asymptomatic diseases, while P. vivax causes a febrile illness that is rarely fatal. P. falciparum provokes the most severe form of malaria disease and counts with a high mortality rate. On the other hand, P. knowlesi is responsible for only a few cases of human malaria, and the symptoms elicited by this species are mild. (WHO 2013).

Malaria is typical of tropical and subtropical regions like Sub-Saharan Africa, Asia, Central and South-America, since in these areas the Plasmodium transmitter has the best conditions to survive. The malaria disease is caused by the bite of an infected female Anopheles mosquito, which transmits the malaria parasite. The diffusion of this disease depends on the vector, the immune system of the host and the environment. Anopheles mosquitoes breed in water like puddles, rice fields and swamps. When their lifespan is long like in Sub-Saharan regions in Africa, the parasite has the opportunity to finish its development inside the mosquito and consequently the transmission of the disease becomes more intense. This explains why 90% of the deaths caused by malaria occur in Africa. (WHO 2013).

Human immunity plays an important role in the severeness of the malaria infection. Adults that were repeatedly infected early in their lives develop certain mechanisms to limit the inflammatory response caused by the parasite (clinical immunity), while some acquire mechanisms to kill the parasite or inhibit parasite replication (anti-parasite immunity). (Artavanis-Tsakonas, Tongren et al. 2003).

According to the latest estimates, there were around 198 million malaria cases in 2013 from which 584 000 were fatal. Most deaths occurred to children under five years of age from regions in the Sub-Saharan Africa. The symptoms of malaria that a human host can suffer are present between 10-15 days after infection. The first symptoms developed are headaches, myalgia, nausea, vomiting and fever. If left untreated, malaria patients can manifest seizures, coma or even death. (WHO 2013).

1.2.1 Plasmodium life cycle
Once an infected mosquito takes a blood meal, the complex Plasmodium life cycle begins. This parasite has both intra- and extra-cellular forms; in humans it develops its asexual division inside hepatocytes and erythrocytes while in mosquitoes it is able to complete its sexual reproduction.
Introduction

Figure 2: Life cycle of malaria parasite
Description of the liver and blood phases the *Plasmodium* parasite goes through when residing in a vertebrate host, as well as the different sexual forms that fully develop inside the mosquito’s organism. (Winzeler 2008).

Sporozoites are introduced into the human by the bite of the *Anopheles* mosquito, the sporozoites go into the blood stream of the host, migrate to the liver to the parenchymal cells where they turn into schizonts. In the following five to ten days the schizonts undergo division and release around 30 000 merozoites per schizont. Since the parasite hides in vesicles called merosomes, it cannot be found by macrophages and thus this phase of the infection is asymptomatic. The parasite induces apoptosis of the parenchymal cells, migrates back into the bloodstream where the merozoites invade red blood cells and the blood stage begins. Once inside the erythrocytes, merozoites develop into trophozoites and afterwards into schizonts, which are capable of producing more merozoites. After approximately two to three days after infection, the red blood cells lyse and release merozoites into the bloodstream, which will invade new erythrocytes and continue the cycle. (Miller, Baruch et al. 2002).

In patients infected with *P. falciparum*, the erythrocytic cycles occur every 48 h. This time lapse corresponds to the manifestation of symptoms like fever, sweat and chills. (Miller, Baruch et al. 2002)

The sexual phase of the cycle can take place when trophozoites turn into male and female gametocytes which will be taken by the mosquito during a blood meal. The trophozoites develop into gametes in the mosquito’s gut, then fuse and form a zygote. In the following 24 h the zygotes turn into ookinetes, they encyst, become oocysts and produce around 1000 sporozoites each. After seven days, the oocysts lyse and release sporozoites which will migrate to the salivary glands of the mosquito and will wait to be transmitted to another host in the next blood meal. (Miller, Baruch et al. 2002).

**1.2.2 Immune response against malaria**
The outcome of the malaria infection depends mostly on factors that disturb the balance between anti and pro-inflammatory cytokines produced by the immune system of the host (Langhorne, *et al.* 2008).

When a person gets infected with malaria; antibodies are made against the surface antigens of the
parasite and against the proteins inserted into the infected erythrocytes membrane. One of these proteins is the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) which is encoded by *var* genes that lead to antigenic variants. When a host gets infected with a parasite variant that is not recognized by the existing antibodies, uncontrolled parasite replication might take place and thus a severe form of the malaria disease can be manifested. PfEMP1 binds mainly to intercellular adhesion molecule-1 (ICAM-1) or CD36, which is expressed on macrophages and DCs. Once bound to a receptor, PfEMP1 mediates phagocytosis of infected red blood cells (iRBCs) and inhibits the maturation of DCs, therefore reducing the DCs capacity to stimulate T cells. (Miller, Baruch et al. 2002). It is believed that repeated infections are needed to develop a diverse antibody repertoire and to become resistant to the parasite. There are malaria-specific antibodies which are able to inhibit cytoadherence and erythrocyte invasion by the parasite and can also mediate cytotoxicity. (Guevara Patiño, Holder et al. 1997).

The innate immune response elicited by a primary malaria infection is characterized by a production of IFNγ either by macrophages, NK T cells or γδ T cells or by pre-existing, cross-reactively primed effector memory T cells. It has been demonstrated that the malaria-reactive memory Th1 cells have the ability to respond to antigens like the tetanus toxoid, adenovirus, *Mycobacterium* and *Toxoplasma gondii*. (Currier, Sattabongkot et al. 1992). This cross-reactive Th1 cells can induce a stronger inflammatory response when the host is infected with malaria and elicit a more severe pathology (Artavanis-Tsakonas, Tongren et al. 2003).

The cell-mediated immunity during malaria infection is driven by the activation of macrophages, which leads to an increased phagocytosis and killing of iRBCs and also by the inhibition of the parasite growth mediated by IFNγ (Tsujii, Shiraki et al. 2003). The immunity against the malaria parasite is generated by the recognition of glycosylphosphatidylinositol (GPI) by TLR2 and TLR4. GPI is released when iRBCs rupture and it induces macrophages to secrete TNFα, nitric oxide and IL-1 (Schofield, Hewitt et al. 2002). When pro-inflammatory cytokines like IFNγ are secreted at low levels at the beginning of the infection, they retain parasite replication and thus avoid hyper-parasitaemia (Su and Stevenson 2000). Conversely, when there is rapid and uncontrolled production of IFNγ, it induces TNFα production and thus an excessive inflammatory reaction takes place which contributes to a more severe pathology. (Artavanis-Tsakonas, Tongren et al. 2003)

### 1.2.3 Pathogenesis of the malaria disease

As described before, the infection caused by the malaria parasite is divided in two phases; the liver-stage which is asymptomatic followed by the symptomatic blood-phase. This second stage elicits fever episodes, which occur when iRBCs rupture and release merozoites, allowing immune cells to make contact with parasite proteins. (Maitland and Marsh 2004).

Despite having a very similar symptomatic course with other tropical diseases, malaria can elicit grave manifestations like respiratory distress, cerebral malaria (CM), coma and shock (Maitland and Marsh
CM is a very severe form of the malaria disease that affects approximately 575,000 individuals annually, most of them children under the age of five years who live in endemic areas (Idro, et al. 2010). Patients with CM suffer from seizures and loss of consciousness caused by a reversible encephalopathy. This form of malaria disease can be acquired when a person is infected with *Plasmodium falciparum*.

The mechanism by which the *Plasmodium* parasite can cause CM is still poorly understood. There are two main hypothesis proposed for the development of CM. One mechanism is the sequestration of iRBCs. IRBCs can adhere to the cerebral vascular endothelium, to each other or to non-infected erythrocytes and form a rosette, which can obstruct a small blood vessel in the brain, lead to hypoxia and elicit cerebral symptoms (Kaul, et al. 1991). Even though hypoxia may lead to necrosis of neural tissues, it was demonstrated that children treated with anti-malarial drugs can reverse their coma and manifest no neurological sequels when cured of the infection. However, when an increased metabolic demand exists, due to seizures or fever episodes, the neural injury risk is higher and is not always reversible. (Idro, Marsh et al. 2010).

Another hypothesis proposed for the pathogenesis of cerebral malaria is the excessive secretion of pro-inflammatory cytokines. It is known that patients with CM have high levels of IFNγ, TNFα, IL-1, IL-6 and lymphotoxin-α (LT-α) (Engwerda, Mynott et al. 2002). Although IFNγ is essential for the control of parasitemia at the beginning of the malaria infection, it might also have detrimental effects by upregulating several pro-inflammatory cytokines like TNFα (Artavanis-Tsakonas, Tongren et al. 2003) TNFα contributes to the sequestration of iRBCs since it upregulates the ICAM-1 expression on cerebral endothelial cells, which captures PfEMP-1 with high affinity (Hunt and Grau 2003). When TNFα and IFNγ act synergistically, they can induce the production of nitric oxide (NO). Nitric oxide is able to cross the blood brain barrier and interfere with neurotransmission in the brain tissue; which could be the source of the reversible coma. (Clark, Rockett et al. 1992). It has also been demonstrated that IFNγ increases the quinolinic acid (QA) production, which is very neurotoxic and can lead to seizures. Vietnamese adults who suffered from cerebral malaria were tested and the results showed increased quinolinic acid levels above the threshold where neurotoxicity is known to occur. (Medana, Day et al. 2002).

A possible source of the increased IFNγ levels in patients could be the malaria-specific memory T cells produced during the first infection, which quickly react to the malaria antigens when these antigens are encountered for the second or subsequent time (Riley 1999).

The sequestration and obstruction of blood vessels does not only affect the brain but can also distress other organs like the spleen, intestine and fatty tissue. IRBCs as well as non-infected erythrocytes lose their deformability due to the highly oxidized membrane they possess. These defective erythrocytes are eliminated in the spleen by macrophages, which leads to severe anaemia. Before the abnormal erythrocytes are phagocysed, they become trapped in the spleen, and this organ becomes congested.
and soft. Over time the size of the spleen can increase (splenomegaly) or rupture and cause acute abdominal pain. (Del Portillo, et al. 2012).

### 1.2.4 Animal models for malaria infection

The *Plasmodium* parasite strain used to infect mice for this study was the *Plasmodium berghei* ANKA (PbA) mice strain. PbA was discovered by Ignace Vincke and Marcel Lips in 1948 in Central Africa (Cox 2010). This mouse strain elicits a neurovascular syndrome denominated experimental cerebral malaria (ECM) in C57BL/6J mice as early as 5 d.p.i.. If left untreated, mice can develop seizures, ataxia, paralysis and coma accompanied by a high parasitaemia. (Amani, Boubou et al. 1998). Not all mouse models have the same susceptibility to PbA. For example, BALB/c mice do not manifest any symptoms from cerebral malaria but die of severe anaemia around 3 weeks after infection (Hanum P., Hayano et al. 2003). Since the mice used for this study were from C57BL/6J genetic background, it was decided to work with the *Plasmodium berghei* ANKA strain.

### 1.2.5 Malaria disease and IL-22

The first study to demonstrate a correlation between IL-22 and the progression of malaria disease was the one made in 2005 by Koch *et al.* where an association between some single nucleotide polymorphisms (SNPs) and the severity of malaria was demonstrated. While studying the IFNγ genomic region, the IL-22 and IL-26 encoding genes were also investigated, since they are located nearby. On the IL22 +708T allele they found a correlation with protection against severe anaemia elicited by the malaria infection, while the IL22-1394G allele was associated with susceptibility to cerebral malaria. (Koch, Rockett et al. 2005).

Nowadays, it is known that IL-22 confers liver protection during several infection models like hepatitis B (Park, Wang et al. 2011) and concavalin A induced hepatitis (Radaeva, Sun et al. 2004). The study made by Mastelic *et al.* also found a correlation between IL-22 and liver protection during malaria disease (Mastelic, do Rosario et al. 2012). They demonstrated a significant increase in the alanine aminotransferase (ALT) levels on IL-22 infected mice compared to the wt mice infected with *P. chabaudi* at 7 d.p.i.. ALT is one of the markers known for liver damage, which is upregulated in viral hepatitis, liver cancer or fulminant liver failure, among other diseases (Berk and Korenblat 2012). An additional important finding made by Mastelic and colleagues was the fact that the absence of IL-22 lead to 50% mortality rate at 12d.p.i. compared to mice expressing IL-22 when infected with this malaria strain.

Another recent study investigated the immune response elicited by a co-infection with malaria and human immunodeficiency virus (HIV). For this purpose, the investigators infected macaques with malaria and simian-human immunodeficiency virus (SHIV). They discovered a hyper immune activation characterized by the expansion of CD4⁺ and CD8⁺ T cells producers of IFNγ and TNFα, which led to necrosis or depletion of lymphoid tissues. This exacerbated immune response elicited parasite replication and a severe virus-associated malaria disease in acutely SHIV infected macaques.
models. On the contrary, macaques that were chronically infected with SHIV without Acquired Immune Deficiency Syndrome (AIDS) were able to fight the malaria infection by expanding the TH17 and Th22 cells and thus suppressing the Th1 response. The upregulation of Th17 and Th22 cells correlated with protection against the fatal virus-associated malaria and did not speed up the progression of the viral disease. (Ryan-Payseur, Ali et al. 2011).

Since the malaria disease is still one of the leading causes of morbidity and mortality in underdeveloped countries (WHO 2013), it is imperative to get a better understanding at the physiopathology of the disease in order to develop better medication and to ameliorate the severe sequels malaria can cause.
1.3 Aim of the study

IL-22 is a member of the IL-10 family. In contrast to the IL-10 receptor, the IL-22 receptor is thought to be expressed only on parenchymal tissues. However, several data suggest that IL-22 can influence an ongoing immune response especially during infection. Thus, the aim of this study is to proof if IL-22 can modulate the host’s immune response driven by an infection while acting through parenchymal cells, which are known to express the receptor for IL-22.

For this objective; primary hepatocytes, hepatoma cells and dendritic cells are taken to stimulate antigen-specifically TCR-transgenic CD8⁺ or CD4⁺ in the presence or absence of recombinant IL-22 in order to study the effects of this cytokine on T cell activation in vitro.

Furthermore, the specific role of IL-22 during the malaria infection should be investigated more in detail since it was seen that IL-22⁻/⁻ mice have a lower parasitaemia in comparison to wild type mice infected with *P. berghei* ANKA (Sellau 2015). Thus, IL-22 must have the ability to influence the malaria-specific immune response.

To further study the effects of IL-22 in malaria, wild type and IL-22⁻/⁻ mice are infected with *P. berghei* ANKA. Splenocytes are isolated from the infected mice and are loaded with three different malaria-specific peptides. On another experiment setup, the CD8⁺ T cells are generated from the infected spleens and are given to Hepa 1-6 cells which were previously stimulated with the malaria-peptides. Both experiments are performed in the presence or absence of recombinant IL-22 and the immune response is determined by the cytokine production.
2 Materials and Methods

2.1 Instruments

Table 2: Instruments

<table>
<thead>
<tr>
<th>Material</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow cytometer Accuri C6</td>
<td>Accuri Cytometer Inc., Ann Arbor, USA</td>
</tr>
<tr>
<td>Flow cytometer LSRII</td>
<td>BD Biosciences, Heidelberg</td>
</tr>
<tr>
<td>Centrifuges 5415R / 5810R</td>
<td>Eppendorf, Hamburg</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt; incubator, Heracell 150</td>
<td>Heraeus Instruments, Hanau</td>
</tr>
<tr>
<td>Microscope</td>
<td>Nikon TMS, Japan</td>
</tr>
<tr>
<td>ELISA-photometer MRXII</td>
<td>Dynex Technologies Berlin</td>
</tr>
<tr>
<td>Laminar flow hood (Lamin Air HB 2448)</td>
<td>Heraeus Instruments, Hanau</td>
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<tr>
<td>Peristaltic Pump P-1</td>
<td>Pharmacia, GE Healthcare</td>
</tr>
<tr>
<td>Vortex Genie 2</td>
<td>Bender &amp; Hobe, Zurich</td>
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<tr>
<td>Fridge -20°C</td>
<td>Liebherr, Biberach</td>
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<tr>
<td>Water Bath</td>
<td>B. Braun, Melsungen</td>
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<td>pH meter MP225</td>
<td>Labortec, Wiesbaden</td>
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</table>


2.2 Glass and plastic

Table 3: Glass and plastic

<table>
<thead>
<tr>
<th>Material</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CellStrainer 70 µm &amp; 100 µm Nylon</td>
<td>Corning Incorporation, NC, USA</td>
</tr>
<tr>
<td>Disposable filters 50 µm</td>
<td>Sysmex Partec GmbH, Goerlitz</td>
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<tr>
<td>5 mL polystyrene round-bottom flow cytometry tubes</td>
<td>Sarstedt, Nümbrecht</td>
</tr>
<tr>
<td>6-well culture plates</td>
<td>Greiner, Frieckenhausen</td>
</tr>
<tr>
<td>96-well round-bottom / flat-bottom culture plates</td>
<td>Greiner, Frieckenhausen</td>
</tr>
<tr>
<td>96-well ELISA plates</td>
<td>Greiner, Frieckenhausen</td>
</tr>
<tr>
<td>Tissue culture dish 100 x 20 mm</td>
<td>Sarstedt, Nümbrecht</td>
</tr>
<tr>
<td>15 mL / 50 mL tubes</td>
<td>Falcon/BD, Heidelberg</td>
</tr>
<tr>
<td>1.5 mL / 2 mL Eppendorf tubes</td>
<td>Eppendorf, Hamburg</td>
</tr>
<tr>
<td>Glass pipettes 2 mL / 5 mL / 10 mL / 20 mL</td>
<td>Brand, Wertheim</td>
</tr>
<tr>
<td>Neubauer counting chamber 0.0025 mm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Brandt, Melsungen</td>
</tr>
<tr>
<td>Hypodermic needle 0.40 x 20 mm</td>
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<td>Insulin syringe</td>
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<td>Single-use syringe 5 mL / 10 mL / 20 mL</td>
<td>B. Braun, Melsungen</td>
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<tr>
<td>Petri dishes</td>
<td>Sarstedt, Nümbrecht</td>
</tr>
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</table>

2.3 Chemicals

2.3.1 Mice strains

All mice used were between 8 and 12 weeks old.

Table 4: Mice strains

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Manufacturer</th>
</tr>
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<tbody>
<tr>
<td><em>Mus musculus</em> C57BL/6J</td>
<td>Charles River, Köln</td>
</tr>
<tr>
<td><em>Mus musculus</em> IL-22&lt;sup&gt;−−&lt;/sup&gt;</td>
<td>BNI, Hamburg</td>
</tr>
<tr>
<td><em>Mus musculus</em> C57BL/6J OT1 / OT2</td>
<td>BNI, Hamburg</td>
</tr>
</tbody>
</table>
2.3.2 **Material for experiments with mice**

Table 5: Materials for experiments with mice

<table>
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<th>Material</th>
<th>Manufacturer</th>
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</thead>
<tbody>
<tr>
<td>Ketave® (100 mg/mL)</td>
<td>Pfizer Pharmacia, Berlin</td>
</tr>
<tr>
<td>Liberase TM Research Grade</td>
<td>Roche Diagnostics, Mannheim</td>
</tr>
<tr>
<td>Percoll</td>
<td>GE Healthcare, Freiburg</td>
</tr>
<tr>
<td>William’s E Medium with Glutamax™</td>
<td>Gibco, Carlsbad, USA</td>
</tr>
<tr>
<td>Xylazine (20 mg/mL)</td>
<td>Bayer, Leverkusen</td>
</tr>
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2.3.3 *Plasmodium berghei* epitopes

Table 6: *Plasmodium berghei* epitopes

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Sequence</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pb1</td>
<td>SQLLNKYL</td>
<td>Jerini Biotools GmbH, Berlin</td>
</tr>
<tr>
<td>Pb2</td>
<td>IITDFENL</td>
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<tr>
<td>F4</td>
<td>EIYIFTNI</td>
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2.3.4 Materials for cell-biologic experiments

Table 7: Materials for cell-biologic experiments

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<tr>
<td>Dulbecco’s Modified Eagle’s Medium (DMEM)</td>
<td>PAA, Pasching, Austria</td>
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<td>Fc-Block</td>
<td>BNI, Hamburg</td>
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<td>Fetal Calb Serum (FCS)</td>
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<tr>
<td>Fixation-Permeabilisation Diluent/Concentrate</td>
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<td>Gentamycin</td>
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<td>Ionomycin</td>
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<tr>
<td>L-Glutamine</td>
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<tr>
<td>Monensin</td>
<td>Biolegend, San Diego, USA</td>
</tr>
<tr>
<td>Mouse IL-22 Recombinant Protein</td>
<td>eBioscience Inc., San Diego, USA</td>
</tr>
<tr>
<td>Para-methoxy-amphetamine (PMA)</td>
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<td>Penicillin/Streptomycin</td>
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<td>Permeabilization buffer</td>
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<tr>
<td>Trypsin-EDTA</td>
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Table 8: Ovalbumin peptides

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<tbody>
<tr>
<td>Ova257-264</td>
<td>SIINFEKL</td>
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<tr>
<td>Ova223-339</td>
<td>ISQAVHAAHAEINEAGR</td>
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## 2.3.5 Antibodies

Table 9: Antibodies

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<th>Clone</th>
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<tbody>
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<td>α-mouse CD4-FITC</td>
<td>L3T4</td>
<td>BD, Biosciences, Heidelberg</td>
</tr>
<tr>
<td>α-mouse CD4-APC</td>
<td>RM4-5</td>
<td>Biolegend, San Diego, USA</td>
</tr>
<tr>
<td>α-mouse CD4-PE</td>
<td>GK1,5</td>
<td>BD, Biosciences, Heidelberg</td>
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<tr>
<td>α-mouse CD8-eFluor®450</td>
<td>53-6,7</td>
<td>Biolegend, San Diego, USA</td>
</tr>
<tr>
<td>α-mouse CD8-APC</td>
<td>53-6,7</td>
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</tr>
<tr>
<td>α-mouse CD8-AlexaFluor®488</td>
<td>53-6,7</td>
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</tr>
<tr>
<td>α-mouse CD11c-PE</td>
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<td>α-mouse CD19-APC</td>
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<td>6D5</td>
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<td>α-mouse CD44-PeCy7</td>
<td>IM7</td>
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<td>α-mouse CD62L-PE</td>
<td>MEL-14</td>
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<td>α-mouse CD80-PE</td>
<td>16-10A1</td>
<td>eBioscience Inc., San Diego, USA</td>
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<td>16-10A1</td>
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<td>α-mouse IFNγ-AlexaFluor®488</td>
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<td>α-mouse Ki67-FITC</td>
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<td>α-mouse TNFα-PE</td>
<td>MP6-XT22</td>
<td>eBioscience Inc., San Diego, USA</td>
</tr>
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</table>
2.3.6 Buffers and culture media

2.3.6.1 DMEM-culture medium

500 mL DMEM
50 mL FCS
5 mL L-Glutamine
2.5 mL Gentamycin

FCS was stored at -20°C, prior to use it was inactivated at 56°C for 45 min.

2.3.6.2 Culture medium for primary hepatocytes

500 mL William’s E Medium with Glutamax™
5 mL Penicillin/Streptomycin
50 mL FCS
5 mL L-Glutamine

2.3.6.3 Culture medium for BMDCs

500 mL DMEM
5 mL L-Glutamine
2.5 mL Gentamycin
50 mL FCS
50 mL GM-CSF

2.3.6.4 Freezing solution for stabilate (malaria infected red blood cells)

0.9 g NaCl
4.2 g Sorbitol

Diluted in 100 mL sterile H₂O with 35% glycerol
2.3.6.5 Liver perfusion medium

Table 10: Liver perfusion medium (PM)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>400 mg</td>
</tr>
<tr>
<td>MgSO₄ x 7 H₂O</td>
<td>190 mg</td>
</tr>
<tr>
<td>MgCl₂ x 6 H₂O</td>
<td>190 mg</td>
</tr>
<tr>
<td>Na₂HPO₄ x2 H₂O</td>
<td>60 mg</td>
</tr>
<tr>
<td>Hepes</td>
<td>2.38 g</td>
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<tr>
<td>NaCl</td>
<td>8 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>60 mg</td>
</tr>
<tr>
<td>Glucose</td>
<td>2 g</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>220 mg</td>
</tr>
<tr>
<td>BSA</td>
<td>2 g</td>
</tr>
</tbody>
</table>

Set the pH at 7.4 and sterilize the solution by filtrating.

2.3.6.6 Liver digestion medium

Table 11: Liver digestion medium (PPML)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>5.36 mM</td>
<td>400 mg</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.44 mM</td>
<td>58 mg</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>4.17 mM</td>
<td>350 mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>138 mM</td>
<td>8.06 g</td>
</tr>
<tr>
<td>Na₂HPO₄ x2 H₂O</td>
<td>0.38 mM</td>
<td>68 mg</td>
</tr>
<tr>
<td>Glucose</td>
<td>5 mM</td>
<td>1 g</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.5 mM</td>
<td>190 mg</td>
</tr>
<tr>
<td>Hepes</td>
<td>50 mM</td>
<td>11.91 g</td>
</tr>
</tbody>
</table>

Set the pH from the perfusion medium at 7.35 with 4M NaOH and sterilize the solution by filtrating.

The formulas for the preparation of PM and PPML were taken from Elena Tasika’s Protocol (UKE).
2.3.6.7 Erythrocyte-lysis-buffer

10% 0.1 M Tris-HCl (pH 7.2)
90% 0.15 M NH₄Cl

2.3.6.8 FACS Buffer

50 mL 20 x PBS
10 mL FCS
10 mL NaN₃

2.3.6.9 MACS Buffer

2 mM EDTA
0.5% BSA
Diluted in 1 x PBS

2.3.6.10 Substrate Buffer for ELISA

15.6 g NaH₂PO₄2H₂O
Diluted in 500 mL H₂O (pH 5.5)

2.3.6.11 Blocking Buffer for ELISA

1% BSA diluted in 1 x PBS

2.3.6.12 Coating Buffer for TNFα ELISA

5 mM Na₂CO₃
35 mM NaHCO₃ (pH 9.6)
2.4 Methods

2.4.1 Methods in cell biology

2.4.1.1 General culture conditions
Cells were cultured in DMEM medium in a 9% CO₂ incubator at 37°C unless otherwise described.

2.4.1.2 Count of viable cells
Number of viable cells was determined by diluting cell concentration with tryptan blue solution and then counting the living cells under the microscope at 100 X magnification. The tryptan blue solution stains the dead cell and leaves the live ones unstained.

2.4.1.3 Culture of Hepatoma 1-6 cells
To prevent contamination of the cell culture, every step was performed under the laminar flow cabinet using sterile single-use plastic plates and dishes, as well as sterilized medium and solutions. The Hepa 1-6 cells were cultivated with DMEM medium +10% FCS, 4 mM L-Glutamine and 50 µg/mL Gentamycin. This cell line was cultured in 6-well sterile plates with 5 mL medium; the cells were split every third to fourth day. In order to detach the cells from the plate’s bottom, 700 µL Trypsin-EDTA were given to the cells for 10 min at 37°C. After this step, the cells were washed with 1 mL PBS, centrifuged 1200 rpm, 5 min at 4°C and finally counted and diluted into a new plate.

2.4.1.4 Preparation of spleen cells
The spleen was removed from the abdominal cavity, passed through a 70 µm cell-strainer with the help from a 5 mL syringe; afterwards it was washed twice with 5 mL PBS and centrifuged at 1200 rpm for 5 minutes at 4°C. The supernatant was thrown away and the cells were resuspended with 5 mL of erythrocyte lysis buffer for 5 min at room temperature. The reaction was stopped with 5 mL PBS and the cells were centrifuged again. After this step, the supernatant was discarded; the cells were resuspended in 10 mL DMEM and passed through a sterile disposable filter to remove rest of soft tissues. The cells were then diluted 1:10 in tryptan blue solution and counted under a 100 X magnification.

2.4.1.5 Preparation of bone marrow-derived dendritic cells
First, both legs of the mice were removed with scissors, the muscles were separated and discarded and the legs cut in half at the knees, then the femur and tibia were placed on a petri dish with 5 mL DMEM medium. The edges of the bones were cut off with scissors and the bone marrow was obtained by inserting a hypodermic needle on the end of each bone and passing 2.5 mL DMEM medium through each end. The cell suspension was collected on a reagent tube, centrifuged at 1200 rpm for 5 min at 4°C. Afterwards the supernatant was discarded and the cells resuspended with 5 mL of erythrocyte lysis buffer for 5 min at room temperature. After this step, the reaction was stopped with 5 mL DMEM medium and the cells were centrifuged again. Supernatants were discarded and cells were collected in 10 mL DMEM medium, then they were passed through a sterile disposable filter to remove clumps or rest of soft tissues. The viable cells were counted and resuspended in the right volume to achieve a cell
concentration of $3 \times 10^6$/mL. The cells were given in 1 mL per dish + 8 mL of DMEM medium and cultured for 7 days at 37°C and 9% CO$_2$. The medium used for the culture of BMDCs was DMEM +10% GM-CSF, 10% FCS, 5 mL Glutamine and 2.5 mL Gentamycin. On days 3 and 6 of culture, 8 mL medium was added per dish. On day 7 the bone marrow-derived dendritic cells (BMDCs) were removed from the dishes by washing them twice with 5 mL of cold PBS and then they were centrifuged, counted and used.

### 2.4.1.6 Isolation of CD4$^+$ or CD8$^+$ T cells by magnetic cell sorting

This procedure was performed by using the CD8a$^+$ T cell Isolation Kit mouse and the CD4$^+$ T cell Isolation Kit mouse from Miltenyi Biotec. Both kits follow the same protocol.

Spleen cells were prepared as described before. $1 \times 10^8$ cells were put in a reagent tube, centrifuged at 1200 rpm 5 min at 4°C, the supernatant was discarded and afterwards the splenocytes were resuspended in 400 µL MACS Buffer, followed by 100 µL Biotin antibody cocktail, the suspension was mixed by pipetting and cultivated for 5 min at 4°C. After this step the cells were washed with 300 µL MACS Buffer and 200 µL Biotin Microbeads were added and cultured for 10 min at 4°C.

In the meantime, a LS Column was placed on a magnetic field with a MACS separator and was equilibrated with 3 mL MACS Buffer. This 3 mL were discarded and then the cell suspension was put through the column and collected on a reagent tube. The cells were then centrifuged at 1200 rpm for 5 min at 4°C, supernatant was discarded and cells were resuspended with 3 mL DMEM medium. After this step, the T cells were counted and resuspended in the right volume to achieve a cell concentration of $1.5 \times 10^6$/mL.

### 2.4.1.7 Isolation of primary hepatocytes

This procedure was modified from the protocol made by Elena Tasika from AG Tiegs (Universitätsklinikum Hamburg-Eppendorf). The isolation procedure was done under her supervision.

The mice used for the primary hepatocyte isolation were male C57BL/6J around 8 weeks old.

First of all, the liver digestion medium (PPML) and the liver perfusion medium (PM) were warmed at 42°C in the water bath for 15 min.

The 4 mm wide hose was set in place in the pump with the left end on a reagent tube filled with 20 mL ddH$_2$O and the right end on an empty plastic container. The pump was started at 004%RS and the hose was cleaned with ddH$_2$O. The pump was turned up to 009%RS until a total of 10 mL ddH$_2$O was passed through it. Afterwards, the perfusion medium was taken out of the water bath and 10 mL of it were passed through the hose at 006%RS. The pump was turned down to 002%RS and the cannula was put at the right end of the hose, the pump was then stopped.

The Liberase was taken out of the refrigerator and left at room temperature for approximately 10 min and then it was diluted in 25 mL PPML. The solution was mixed gently and left at room temperature.
The mouse was anesthetized with 200 µL Sedaxylan i.p.. Sedaxylan was prepared by adding 1:200 PBS diluted Xylazin and 1:6.67 diluted Ketamin. To make sure that the mouse was well anesthetized its feet reflex were tested, then the mouse was cleaned with Incidin®, put in supine position and fixed by all four limbs with pins. A median incision was made in the lower abdomen with scissors and straight forceps, it was cut all the way from the hypogastrium to the thorax cavity, both fur and muscle layers were cut open as well as the peritoneum. The Vena Cava Superior was clamped with a 4-0 suture. The rest of the abdominal organs were moved away from the liver. A small incision was made on the fur and peritoneum at the right inguinal region to let the blood drain out.

The Portal Vein was identified and fixed with the cannula, the pump was set at 002%RS to let the medium with Liberase perfuse the liver, which should start to blanch immediately if the cannulation is performed properly. Once the perfusion of the liver takes place, the Vena Cava Inferior was cut open with the scissors to let the perfusate drain to waste.

By the time 5 mL from the Liberase solution had passed through the liver, the flow rate was increased at 003%RS. Another 5 mL were perfused through the liver and then the pump was turned up at 004%RS until all the solution was empty, when this happened the cannula was taken out of the Portal Vein.

The gall bladder was cut off the liver with the scissors. The liver was cut into half and moved to a petri dish containing 50 mL perfusion medium, then it was taken with the fine-tip forceps and shaken gently until it was dissolved into the solution. This suspension was filtered through a 100 µm sterile disposable filter into a 50 mL tube.

The hepatocytes were left in the tube for 20 min at room temperature to sediment. This was performed without closing the tube’s lid entirely in order to let oxygen enter the tube. After 20 min, the supernatant was aspirated until the falcon had only 25 mL cell suspension and it was mixed with 25 mL Percoll solution. Afterwards, the cell suspension was centrifuged at 550 rpm 10 min at 4°C. The supernatant was discarded and the cells were resuspended with 10 mL William’s E Medium with Glutamax™ with 10% FCS, 1% L-Glutamine and 1% Penicillin/Streptomycin. The viable cells were count with trypan blue solution. Since the isolation of primary hepatocytes is a stress factor for these cells, the culture medium needs to be changed 4 h after the isolation since hepatocytes secrete many hormones and proteins during this period. The cells were ready to use on the following day.

2.4.1.8 Stimulation of APCs with Ovalbumin-derived peptides and rIL-22
Depending on the experiment performed, primary hepatocytes, Hepa 1-6 cells or BMDCs were used as APCs to perform antigen presentation to CD8+ T cells and CD4+ T cells, respectively. The first step was to isolate the APCs, once they were cultivated in the plate; they were stimulated with 0.4 µg/mL SII or Ova323-339 with or without 50 ng/mL rIL-22 for 3 h at 37°C. Afterwards, the cells were washed with PBS to get rid of the non-binding peptide and the T lymphocytes were added in the presence or
absence of 15 ng/mL rIL-22. Depending on the experiment, the APCs and lymphocytes were co-cultivated for either 16 or 40 h. Next, the supernatants were taken and frozen at -20°C to perform LEGENDplex™ and ELISA.

In several experiments the remaining cells were then re-stimulated and stained intracellularly to measure cytokine production through flow cytometry.

2.4.1.9 **Stimulation of APCs with malaria-specific peptides**
Two different experiments were performed using the malaria-specific peptides Pb1, Pb2 and F4 from (Poh, Howland et al. 2014). For the first experiment, Hepa 1-6 cells were used as APCs and were stimulated with these malaria peptides, while in the second experiment whole splenocytes used as APCs.

2.4.1.9.1.1 **Stimulation of malaria-specific CD8^+ T cells by Hepa 1-6 cells**
Hepa 1-6 were placed in a plate 2x10^5/mL in 100 µL per well, they were incubated at 37°C 5% CO_2 for 24 h. Next, they were pulsed with 1 µg/mL from each malaria-specific peptide for 3 h in the presence or absence of 50 ng/mL rIL-22. Afterwards, the hepatoma cells were washed with 100 µL per well sterile PBS and were given either wt or IL-22^-/- CD8^+ T cells isolated from PbA-infected mice. 1x10^5 lymphocytes per well were given with or without rIL-22. After 24 h of co-cultivation, the supernatants were taken and IFNγ ELISA was performed.

2.4.1.9.1.2 **Stimulation of malaria-infected splenocytes**
Spleen cells from wt and IL-22^-/- mice at day 6 p.i. with PbA were taken and given on a round bottom 96-well plate 2x10^5 cells in 100 µL. The splenocytes were stimulated exactly like the hepatoma cells, with the exception that the spleen cells were not washed after 3 h of stimulation but were left incubated with the malaria peptide until the supernatants were collected after 24 h. For these experiments the supernatants were also collected to measure IFNγ through ELISA.

2.4.1.10 **Flow cytometry**
Fluorescence-activated cell sorting (FACS) allows the identification of different cellular populations by detecting fluorescent molecules positioned on the cells surface. This process was used to determine the T cells populations as well as their cytokine profile after *in vitro* culture. The cells were marked with monoclonal antibodies, which are labelled with fluorescent dyes. These antibodies get attached to the molecules on the cells and get detected by different lasers.

For this analysis 1x10^5 cells were incubated with the fluorescent antibodies at 4°C in the dark for 30 min. To avoid unspecific binding of the antibodies with other molecules from the cells, this incubation was set with Fc-Block. Afterwards, the cells were washed with FACS-Buffer, vortexed and measured.

When intracellular molecules were analysed, the cells were re-stimulated before staining them. The re-stimulation was prepared with 50 ng/mL PMA and 500 ng/mL ionomycin for 4 h. After 1 h incubation with PMA and ionomycin, monensin 0.2 µL was added per well plate. Subsequently, cell
Materials and Methods

Surface staining was performed as described above. Afterwards, the cells were washed and fixed with FoxP3 Fix/Perm Buffer Set diluted 1:4 for 30 min at room temperature. After this step, the cells were washed 3 times with Permwash, diluted 1:10 in ddH$_2$O. At this point, the cells are permeabilized and fixed and therefore molecules that reside on the inside of the cell can be stained with fluorescent antibodies. After incubating the cells with the intracellular antibodies for 30 min, the cells were washed twice and measured through flow cytometry.

The flow cytometry analyses were performed with either the Accuri C6 cytometer (Accuri Cytometer Inc., Ann Arbor) or the LSRII (BD, Biosciences, Heidelberg).

2.4.1.11 ELISA

This assay was used to measure the IFN$\gamma$ and TNF$\alpha$ concentration in cell culture supernatants collected from all of the experiments.

The different reagents (coating and detection antibodies and standard proteins) were obtained from R&D Systems (Minneapolis, MN) for IFN$\gamma$ ELISA and from Biolegend (San Diego, CA) for the TNF$\alpha$ ELISA.

2.4.1.11.1 IFN$\gamma$ ELISA

On the first day, a flat bottom 96-well ELISA plate was coated with 50 µL/well from capture antibody diluted in PBS, it was placed on the platform shaker for 30 min at room temperature and then kept overnight at 4°C.

The next day, the plate was washed 3 times with 0.05% Tween in 1X PBS solution, the plate was then inverted to remove any rest of washing solution and blotted against clean paper towels, afterwards it was blocked with 1% BSA 200 µL/well and left in the platform shaker 2 h at room temperature. After this step, the plate was washed again 3 times as explained before and standard was added 50 µL/well in duplets (maximum concentration of 2000 pg/mL) and diluted in BSA 0.1% 1:2 in 7 steps. The supernatants from the cell culture were given in the plate diluted in BSA 0.1% and the plate was left in the platform shaker for another 30 min and then incubated overnight at 4°C.

On the following day, the plate was washed 3 times, detection antibody diluted in BSA 0.1% was given 50 µL/well and left at room temperature in the platform shaker for 1 h. After this step, the plate was washed as mentioned above and 50 µL/well from streptavidin diluted in BSA 0.1% was given to the plate and left on the platform shaker for 30 min. The plate was washed one last time and given 100 µL/well from substrate solution. Depending on the strength of the optical density seen, the reaction was stopped at 15 min or a couple of minutes later with Stop Solution. The optical density was measured by the spectrophotometer at 450 nm.

2.4.1.11.2 TNF$\alpha$ ELISA

This assay was developed exactly like the IFN$\gamma$ ELISA with the following exceptions:
Materials and Methods

- The capture antibody was diluted in TNFα Coating Buffer.
- Standard protein, detection antibody as well as Streptavidin were diluted in 1% BSA.
- The standard was diluted to achieve a top concentration of 500 pg/mL.

2.4.1.12 LEGENDplex™
This assay was used to measure various cytokines like IFNγ, TNFα, IL-2 and IL-6 among others in cell culture supernatants collected from several experiments.

The LEGENDplex™ Mouse Th Cytokine Panel (13-plex) from Biolegend was used to perform this test. The procedure was followed as written in the protocol from Biolegend, afterwards the samples were measured with the ACCURI C6 cytometer.

2.4.2 Ex vivo experiments

2.4.2.1 Induction of malaria infection in mice with P. berghei ANKA strain
The asexual form of blood stage Plasmodium berghei ANKA was kept in liquid nitrogen as stabilate. The stabilate was resuspended in 800 µL sterile PBS to infect mice. Each mouse was injected 200 µL from the stabilate solution intraperitoneally. At day 6 p.i. blood samples were taken from the mice by puncturing the tail and heart. The blood was collected in a heparinized syringe to avoid coagulation. The mice were euthanized with CO2 afterwards. The parasitaemia was determined by preparing a thin blood smear and staining it with the Wright’s Stain. Subsequently, wild type and IL-22-/- mice were infected with 1x10^5 iRBC in 200 µL i.p.. At day 6 p.i. the mice were euthanized with CO2, blood samples were taken to count the parasitaemia and the spleens were taken to perform the respective experiments.

2.4.3 Statistical analysis
The statistical analysis was performed by using the Prism 5.00 software (GraphPad Software). The tests used to evaluate significant differences between two groups were two-way ANOVA or the unpaired two tailed t test. In all of the experiments p < 0.05 was considered significant.

The flow cytometry analysis was made with the FlowJo Software, version 10 (BD, Biosciences).
3 Results

IL-22 has the capacity to regulate the host defence at barrier surfaces as well as promote tissue regeneration in different organs like the skin, the liver and the lung, among others (Dudakov, Hanash et al. 2012). For this study, it was of interest to clarify if IL-22 is able to regulate the host’s immune response during infection and if different cells which express the receptor for IL-22 can influence the immune response elicited by CD4+ and CD8+ T cells in a malaria disease model.

Primary hepatocytes, Hepa 1-6 cells, and dendritic cells were taken to stimulate TCR-transgenic CD8+ or CD4+ T cells. These experiments were done in the presence or absence of recombinant IL-22 (rIL-22) in order to study the effects in vitro of this cytokine on T cell activation. The APCs were pulsed for 3 h with either SII or Ova peptide or with medium as a control in the presence or absence of rIL-22. Afterwards, CD8+ or CD4+ T cells were added, respectively and cytokine production was measured by different assays.

The effect of rIL-22 on T cells was evaluated in a model in which the APCs were incubated with T lymphocytes in the presence of their specific antigen, which simulated the in vivo antigen presentation mechanism. When studying the cytotoxic immune response, splenocytes from TCR transgenic OT I mice were used to purify CD8+ T cells by a magnetic isolation kit. These CD8+ T lymphocytes are MHC I restricted, they recognize the epitope from the Ova257-264 peptide (SIINFEKL) and thus get activated with the recognition of the peptide. The CD8+ T cells were used and co-cultivated with primary hepatocytes, Hepa 1-6 cells or dendritic cells that were loaded with SII in the presence or absence of rIL-22. CD4+ T cells were also magnetically enriched but from the transgenic TCR OT II mice. The CD4+ T lymphocytes from these mice have the ability to respond to the epitope Ova323-339 peptide and are MHC II restricted. As a result, CD4+ T cells get activated by cells previously pulsed with this Ova peptide.

The main cytokines measured for this study were IFNγ and TNFα, since they belong to the Th1 immune response, which is thought to be the dominant immune response elicited in the malaria infection in humans as well as in mice. (Sedegah, Finkelman et al. 1994).

To evaluate the total amount of IFNγ and TNFα produced by the T cells in the supernatants of the experiments, an ELISA was performed. To calculate the concentration of other cytokines like IL-2, IL-6, IL-10 and IL-17 in the supernatants, the LEGENDplex™ Kit was used. Only the concentrations of IFNγ, TNFα, IL-6 and IL-2 are shown in the figures. It should be noted that a statistical analysis of the LEGENDplex™ assay was not performed since the results given by this test are single values. In order to assess the cytokine phenotype from the different splenocytes; the flow-cytometry staining was performed with fluorescent antibodies. For all of the experiments CD44 was used as a marker for T cell activation.
3.1 Biological effect of IL-22 on CD8+ T cells in vitro

3.1.1 Cytokine production of CD8+ T cells stimulated by Hepa 1-6 cells or primary hepatocytes in the presence or absence of rIL-22

Since the liver plays an important role during the malaria disease and since hepatocytes express the receptor for IL-22, it was of interest to find out if liver cells could modulate the immune response elicited in an infection model with the help of rIL-22. For this task, both primary hepatocytes and Hepa 1-6 cells were chosen to act as APCs to display SII to CD8+ T cells and hence activate these lymphocytes. It has been proved that Hepa 1-6 cells express the IL-22 receptor, and also induce the STAT 3 phosphorylation when given rIL-22, which is the IL-22 receptor’s main transcription factor. (Weber, Schlautköter et al. 2007). In comparison, primary hepatocytes also express the IL-22 receptor complex and thus IL-22 can elicit a down-stream signalling on these cells as well (Dambacher, et al. 2008).

In the experiments using primary hepatocytes as APCs, these cells were pulsed with SII for 3 h and then co-cultivated with CD8+ T cells for 16 h. On the other hand, when using Hepa 1-6 cells to stimulate the CD8+ T lymphocytes, both cell populations were co-cultivated for 40 h. The IFNγ and TNFα expression by the T cells was determined by FACS analysis as shown in Fig. 3A and B. There is a tendency of a decrease in the percentage of IFNγ+ and TNFα+ T cells when given SII and rIL-22 together, in comparison to the cells only stimulated with SII (Fig. 3C and D).
Results

Figure 3: CD8⁺ T cells from OT I mice stimulated by pulsed primary hepatocytes or Hepa 1-6 cells

1x10⁵ primary hepatocytes or Hepa 1-6 cells were pulsed with SII or medium for 3 h in the presence or absence of rIL-22. Subsequently, 2.5x10⁵ purified CD8⁺ T cells were added with or without rIL-22 and were co-cultivated for 16 h and 40 h, respectively. Afterwards, the CD8⁺ T cells were re-stimulated for 4 h with PMA/Ionomycin, the last 3 h together with monensin, and then the cells were stained intracellularly. (A) and (B) are representative flow cytometry plots of CD8⁺ CD44⁺ IFNγ⁺ and CD8⁺ CD44⁺ TNFα⁺ T cells. (C) Percentages of CD8⁺ T cells CD44⁺ and IFNγ⁺ from four different setups. (D) Results are presented as percentages of CD8⁺ T cells CD44⁺ and TNFα⁺ from four different setups. Data is shown from three independent experiments. Results were analysed by two-way ANOVA, n.s.: no statistical significance.
Results

Figure 4: IFNγ concentration in the supernatant of CD8⁺ T cells from OT I mice stimulated by pulsed primary hepatocytes or Hepa 1-6 cells

1x10⁵ primary hepatocytes or Hepa 1-6 cells were pulsed with SII or medium for 3 h in the presence or absence of rIL-22. Subsequently, 2.5x10⁵ purified CD8⁺ T cells were added with or without rIL-22 and were co-cultivated for 16 h and 40 h, respectively. Afterwards, the supernatants were collected and tested by ELISA. IFNγ concentration in the supernatants of cells stimulated by primary hepatocytes (A) or Hepa 1-6 cells (B) from four different setups is exemplified. Results are representative from one out of three independent experiments. Data was analysed by unpaired t test, n.s.: no statistical significance, n.d.: not detectable.

The difference seen in cytokine production on cells pulsed with and without rIL-22 is not of statistical significance (Fig. 4A and B). Through the ELISA it was possible to measure the total amount of cytokine produced by all T lymphocytes and not their individual expression as shown during flow cytometry.
Results

Figure 5: TNFα concentration in the supernatant of CD8+ T cells from OT I mice stimulated by pulsed primary hepatocytes or Hepa 1-6 cells

1x10⁵ primary hepatocytes or Hepa 1-6 cells were pulsed with SII or medium for 3 h in the presence or absence of rIL-22. Subsequently, 2.5x10⁵ purified CD8+ T cells were added with or without rIL-22 and were co-cultivated for 16 h and 40 h, respectively. Afterwards, the supernatants were collected and tested by ELISA. TNFα concentration in the supernatants of cells stimulated by primary hepatocytes (A) or Hepa 1-6 cells (B) from four different setups is exemplified. Results are representative from one out of three independent experiments. Data was analysed by unpaired t test, n.s.: no statistical significance, n.d.: not detectable.

As demonstrated in Fig. 5A there seems to be a decreasing tendency in TNFα production on SII-pulsed cells in the presence of rIL-22. The difference seen is very subtle and is not statistically significant. On the other hand, when measuring TNFα production on cells stimulated by Hepa 1-6 cells, there is a slightly increase in cytokine concentration when given rIL-22 (Fig. 5B). There is also a lot less TNFα and IFNγ produced on cells stimulated by Hepa 1-6 cells than those stimulated with primary hepatocytes.
Results

Figure 6: Cytokine concentration in the supernatant of CD8⁺ T cells from OT I mice stimulated by primary hepatocytes

1x10⁴ primary hepatocytes were pulsed with SII or medium for 3 h in the presence or absence of rIL-22. Subsequently, 2.5x10⁷ purified CD8⁺ T cells were added with or without rIL-22. After 16 h, the supernatants were collected and pooled for each setup. The cytokines concentration was determined by using the LEGENDplex™ Kit. Results represent one out of three independent experiments.

The diagram shown in Fig. 6 demonstrates a tendency to less cytokine production on cells stimulated with SII and rIL-22 compared to cells that were only given the SII peptide. This is the case for IFNγ and IL-2. TNFα concentration did not change on pulsed cells in the presence or absence of rIL-22 and the IL-6 concentration did not variate within the four different setups.

3.1.2 Cytokine production of CD8⁺ T cells stimulated by wt or IL-22⁻/⁻ BMDCs in the presence or absence of rIL-22

Even though it is believed that the IL-22 receptor is not expressed on immune cells, there is a study proving its expression on peripheral blood mononuclear cells (PBMCs) on patients with primary Sjögren Syndrome (Ciccia, et al. 2015). In congruence with this finding, Julie Sellau found an induction of the IL-22Rα1 chain on DCs in PbA-infected mice at day 6 p.i. in comparison to the naïve DCs (Sellau 2015). In order to verify if this finding could be reproduced in vitro by simulating an infection, BMDCs were used to stimulate CD8⁺ T cells. The BMDCs were cultured in the presence of GM-CSF for 6 days and used for antigen presentation at day 7. These immune cells were pulsed with either SII or medium for 3 h in the presence or absence of IL-22. Consequently, the non-binding SII peptide was washed away and CD8⁺ T cells were added to the BMDCs. Both cell populations were co-cultivated for 40 h with or without rIL-22.
Results

Figure 7: Expression of CD86 and CD80 on wt and IL-22<sup>-</sup> BMDCs

BMDCs were isolated from wt and IL-22<sup>-</sup> mice and were cultivated for 6 days with GM-CSF, at day 7 the BMDCs were taken from the culture plates, their phenotype was determined by FACS analysis and then they were used to display antigens to either CD4<sup>+</sup> or CD8<sup>+</sup> T cells. (A) Exemplifies the wt BMDCs and (B) represents IL-22<sup>-</sup> BMDCs.

The BMDCs used for the stimulation of T lymphocytes were stained with fluorescent-labelled antibodies to determine their phenotype. In Fig. 7 it is demonstrated that the IL-22<sup>-</sup> BMDCs express more CD80 and CD86 than wt BMDCs.
Figure 8: CD8+ T cells from OT I mice stimulated by wt or IL-22+/− BMDCs

1x10⁵ wild type or IL-22+/− BMDCs were stimulated with SII or medium for 3 h in the presence or absence of rIL-22. Afterwards, 2.5x10⁵ purified CD8+ T cells were added with or without rIL-22 and were co-cultivated for 40 h. Subsequently, the CD8+ T cells were re-stimulated for 4 h with PMA/Ionomycin, the last 3 h together with monensin and then the cells were stained intracellularly. (A) and (B) representative flow cytometry plots of CD8+ CD44+ IFNγ+ and CD8+ CD44+ TNFα+ T cells. (C) Percentages of CD8+ T cells CD44+ and IFNγ+ stimulated by either wt or IL-22+/− DCs from four different setups. (D) represents the percentages of CD8+ T cells CD44+ and TNFα+ from the same experiments. Data is shown from one out of three independent experiments. Results were analysed by two-way ANOVA, n.s.: no statistical significance.

These graphics represent the gating schema for CD8+ T cells stimulated by wt BMDCs (Fig. 8A) and IL-22+/− BMDCs (Fig. 8B). The percentage of CD8+ CD44+ IFNγ+ T cells is represented in Fig. 8C and the percentage of CD8+ CD44+ TNFα+ T cells in Fig. 8D from an OT I mouse after being stained intracellularly and measured through flow cytometry. While there is a tendency towards decrease of TNFα+ cells on lymphocytes stimulated with rIL-22 (Fig. 8D), in Fig. 8C there is no clear tendency, since T cells stimulated by IL-22+/− BMDCs produce different amounts of cytokines.
Figure 9: CD8\(^+\) T cells from OT I mice stimulated by wt or IL-22\(^+\) BMDCs
1x10\(^5\) wild type or IL-22\(^+\) BMDCs were pulsed with SII or medium for 3 h in the presence or absence of rIL-22. Subsequently, 2.5x10\(^5\) purified CD8\(^+\) T cells were added with or without rIL-22 and were co-cultivated for 40 h. Afterwards, the supernatants were collected and tested by ELISA. Results illustrate the TNF\(\alpha\) concentration in the supernatants of cells stimulated by wild type or IL-22\(^+\) BMDCs from four different setups and are representative from one out of three independent experiments. Data was analysed by unpaired t test, n.s.: no statistical significance, n.d.: not detectable.

The TNF\(\alpha\) production of CD8\(^+\) T cells after stimulation with BMDCs did not decrease in the presence of rIL-22. There is no clear tendency in the cytokine production within the different setups in both lymphocytes populations stimulated with wild type and IL-22\(^+\) BMDCs (Fig. 9).

Figure 10: Cytokine concentration in the supernatant of CD8\(^+\) T cells from OT I mice stimulated by wt BMDCs
1x10\(^5\) wt BMDCs were pulsed with SII or medium for 3 h in the presence or absence of rIL-22. Subsequently, 2.5x10\(^5\) purified CD8\(^+\) T cells were added with or without rIL-22. After 40 h, the supernatants were collected and pooled for each setup. The cytokines concentration was determined by using the LEGENDplex\textsuperscript{TM} Kit. Results represent one out of three independent experiments.
Results

Figure 11: Cytokine concentration in the supernatant of CD8\(^+\) T cells from OT I mice stimulated by IL-22\(^+\) BMDCs

1x10\(^7\) IL-22\(^+\) BMDCs were pulsed with SII or medium for 3 h in the presence or absence of rIL-22. Subsequently, 2.5x10\(^5\) purified CD8\(^+\) T cells were added with or without rIL-22. After 40 h, the supernatants were collected and pooled for each setup. The cytokines concentration was determined by using the LEGENDplex\textsuperscript{TM} Kit. Results represent one out of three independent experiments.

Unlike the results obtained by ELISA and by FACS analysis, where no difference in cytokine production by CD8\(^+\) T cells in the presence and absence of rIL-22 was found. When using the LEGENDplex™ Kit, a tendency to decrease cytokine production was found when given rIL-22 on SII-pulsed cells (Fig. 10 and Fig. 11).

3.1.3 Cytokine production of CD4\(^+\) T cells stimulated by wt or IL-22\(^+\) BMDCs in the presence or absence of rIL-22.

Since CD4\(^+\) T cells are also involved in the development of severe malaria but at an earlier time point than the CD8\(^+\) T cells (Yanez, Manning et al. 1996), it was of interest to find out if IL-22 had the capacity to regulate the immune response evoked by the CD4\(^+\) T cells. For this purpose, the same wt and IL-22\(^+\) BMDCs were isolated to display the MHC II peptide Ova\(_{323-339}\) to the CD4\(^+\) T cells using the same protocol performed for the experiments with CD8\(^+\) T cells and BMDCs.
Results

Figure 12: CD4⁺ T cells from OT II mice stimulated by wt or IL-22⁻/⁻ BMDCs

1x10⁵ wild type or IL-22⁻/⁻ BMDCs were pulsed with Ova,23,339 or medium for 3 h in the presence or absence of rIL-22. Afterwards, 2.5x10⁵ purified CD4⁺ T cells were added with or without rIL-22 and were co-cultivated for 40 h. Subsequently, the CD4⁺ T cells were re-stimulated for 4 h with PMA/Ionomycin, the last 3 h together with monensin and then the cells were stained intracellularly. (A) and (B) Representative flow cytometry plots of CD4⁺ CD44⁺ IFNγ⁺ and CD4⁺ CD44⁺ TNFα⁺ T cells. (C) Percentages of CD4⁺ T cells CD44⁺ and IFNγ⁺ stimulated by either wt or IL-22⁻/⁻ BMDCs from four different setups, (D) represents the percentages of CD4⁺ T cells CD44⁺ and TNFα⁺ from the same experiments. Data is shown from one out of three independent experiments. Results were analysed by two-way ANOVA, n.s.: no statistical significance, *p<0.05.

Fig. 12A and B represent the gating schema used for CD4⁺ T cells stimulated by wt and IL-22⁻/⁻ BMDCs, respectively. Fig. 12C illustrates the percentage of CD4⁺ CD44⁺ IFNγ⁺ T cells and Fig. 12D the percentage of CD4⁺ CD44⁺ TNFα⁺ T cells taken from an OT II mouse after being stained intracellularly and measured through flow cytometry. In Fig. 12C and D, a marked decrease of cytokine expression can be seen on stimulated cells given rIL-22. This difference is of statistical significance in Fig. 12C on CD4⁺ T cells stimulated by wt BMDCs.
Even though there was a marked decrease in the cells positive for IFNγ and TNFα in the presence of rIL-22 when measured through flow cytometry, this decline cannot be reproduced in the total amount of cytokines produced when measured by ELISA (Fig 13).

Consistently with the ELISA results, there was no marked tendency observed in cytokine production measured by the LEGENDplex™ Kit for wt or IL-22−/− BMDCs as shown in Fig. 14 and 15. For the CD4+ T cells stimulated by wt BMDCs, there seems to be an increase in cytokine concentration when given rIL-22. On CD4+ T cells stimulated by wt or IL-22−/− BMDCs, there is a higher amount of IL-2 measured than IFNγ and TNFα.

**Figure 13: CD4+ T cells from OT II mice stimulated by wt or IL-22−/− BMDCs**

1x10⁶ wild type or IL-22−/− BMDCs were stimulated with Ova132-139 or medium for 3 h in the presence or absence of rIL-22. Subsequently, 2.5x10⁶ purified CD4+ T cells were added with or without rIL-22 and were co-cultivated for 40 h. Afterwards, the supernatants were collected and tested by ELISA. IFNγ (A) and TNFα (B) concentration in the supernatants of cells stimulated by wild type or IL-22−/− BMDCs from four different setups. Results are representative from one out of three independent experiments. Data was analysed by unpaired t test, n.s.: no statistical significance, n.d.: not detectable.
Results

Figure 14: Cytokine concentration in the supernatant of CD4$^+$ T cells from OT II mice stimulated by wt BMDCs
1x10^5 wt BMDCs were pulsed with Ova$_{323-339}$ or medium for 3 h in the presence or absence of rIL-22. Subsequently, 2.5x10^5 purified CD4$^+$ T cells were added with or without rIL-22. After 40 h, the supernatants were collected and pooled for each setup. The cytokines concentration was determined by using the LEGENDplex™ Kit. Results represent one out of three independent experiments.

Figure 15: Cytokine concentration in the supernatant of CD4$^+$ T cells from OT II mice stimulated by IL-22$^+$ BMDCs
1x10^5 IL-22$^+$ BMDCs were pulsed with Ova$_{323-339}$ or medium for 3 h in the presence or absence of rIL-22. Subsequently, 2.5x10^5 purified CD4$^+$ T cells were added with or without rIL-22. After 40 h, the supernatants were collected and pooled for each setup. The cytokines concentration was determined by using the LEGENDplex™ Kit. Results represent one out of three independent experiments.
3.1.4 Cytokine production of OT I derived splenocytes stimulated with SIINFEKL

To further investigate the effect of rIL-22 *in vitro*, it was chosen to work with SII stimulated splenocytes. It was suggested that DCs derived from the spleen could perform a better antigen presentation than BMDCs, and it was also supposed that other cells like parenchymal cells or regulatory T cells from the spleen could function as receptors for IL-22 and hence regulate the T cell activation in presence of IL-22.

**Figure 16: Splenocytes from OT I mice stimulated by SIINFEKL**

2x10^5 splenocytes were pulsed with SII or medium in the presence or absence of rIL-22. The supernatants were collected after 40 h. Subsequently, the splenocytes were re-stimulated for 4 h with PMA/Ionomycin, the last 3 h together with monensin and then the cells were stained intracellularly. (A) and (B) are representative flow cytometry plots of CD8^+ CD44^+ IFNγ^+ and CD8^+ CD44^+ TNFα^+ T cells. (C) Percentages of CD8^+ T cells CD44^+ and IFNγ^+ from four different setups. (D) represents the percentages of CD8^+ T cells CD44^+ and TNFα^+ from the same experiments. Data is shown from one out of three independent experiments. Results were analysed by two-way ANOVA, n.s.: no statistical significance.
Results

Figure 17: IFNγ concentration in the supernatant of spleen cells from OT I mice

2x10⁵ splenocytes were pulsed with SII or medium in the presence or absence of rIL-22. The supernatants were collected after 40 h and IFNγ concentration was measured by ELISA. Results represent one out of three independent experiments. Data was analysed by unpaired t test, n.d.: not detectable, * p<0.05.

For the experiments performed by stimulating whole splenocytes, there is no difference in cytokine production in the presence or absence of rIL-22 when measured by flow cytometry (Fig. 16C and D). On the contrary, there is a significant decrease seen in IFNγ production on splenocytes stimulated in the presence of rIL-22 when determined by ELISA (Fig. 17).

3.2 Ex vivo effect of IL-22 during malaria

It has been documented that IL-22 confers protection to malaria-infected hosts. The study performed by Mastelic et al. presented a 50% increased mortality rate in IL-22⁻/⁻ infected mice with *P. chabaudi* at day 12 p.i., compared to infected wt mice (Mastelic, do Rosario et al. 2012). Furthermore, Ryan-Payeuser et al. found an expansion of Th22 cells with a decrease in Th1 cells in macaques infected with both malaria and simian-human immunodeficiency virus (Ryan-Payeuser, Ali et al. 2011). These Th1 cells are responsible for producing cytokines like IFNγ and TNFα, which are mediators for the progression of severe malaria (Amani, Boubou et al. 1998). To confirm that IL-22 could evoke such a protective effect on malaria-infected mice, wild type and IL-22⁻/⁻ mice were infected with PbA and at day 6 p.i., the spleens were taken to perform the experiments. For one assay, whole splenocytes were pulsed with 3 different PbA-specific peptides (Poh, Howland et al. 2014) with or without rIL-22. Meanwhile, to study the liver regulation of the immune response during malaria, CD8⁺ T cells were isolated from the spleens from the same mice and were given to peptide-stimulated Hepa 1-6 cells in the presence or absence of rIL-22. For both experiment setups, the supernatants were collected after 24 h to measure IFNγ by ELISA.
Figure 18: CD8⁺ T cells from wt of IL-22⁻/⁻ naïve or PbA-infected mice

1x10⁵ CD8⁺ T cells from naïve or PbA-infected mice were isolated from both wt and IL-22⁻/⁻ mice and were stained with fluorescent antibodies. Representative flow cytometry plots of CD8⁺ CD44⁺ T cells from naïve (A) and PbA-infected mice (B).
As seen in Fig. 18A, there is a higher percentage of activated CD8\(^+\) T cells as marked by the fluorescent antibody CD44 on IL-22\(^{-/-}\) mice in comparison to wt mice. A similar phenomenon is observed in Fig. 18B from PbA-infected mice at day 6 p.i..

### 3.2.1 IFN\(\gamma\) production of CD8\(^+\) T cells from PbA-infected wt or IL-22\(^{-/-}\) mice stimulated by Hepa 1-6 cells

![Graph showing IFN\(\gamma\) concentration](image)

**Figure 19:** IFN\(\gamma\) concentration in the supernatant of CD8\(^+\) T cells from wt or IL-22\(^{-/-}\) PbA-infected mice stimulated by Hepa 1-6 cells

2x10\(^4\) Hepa 1-6 cells were stimulated with 3 PbA-specific peptides or medium for 3h in the presence or absence of rIL-22. Subsequently, 1x10\(^5\) purified CD8\(^+\) T cells from naïve or PbA-infected wt and IL-22\(^{-/-}\) mice were added with or without rIL-22 and were co-cultivated for 24 h. Subsequently, the supernatants were collected and the IFN\(\gamma\) concentration was measured by ELISA. Results represent one out of three independent experiments. Data was analysed by unpaired t test, ***p<0.0001.

As shown in Fig. 19 there is a significant increase of IFN\(\gamma\) production by CD8\(^+\) T cells stimulated by Hepa 1-6 cells on IL-22\(^{-/-}\) infected mice compared to wt infected mice, both in the setups with and without rIL-22. The presence of rIL-22 does not influence the production of IFN\(\gamma\).

### 3.2.2 IFN\(\gamma\) production of splenocytes from wt or IL-22\(^{-/-}\) PbA-infected mice

On the experiments performed with whole spleen cells from infected mice; the splenocytes were stimulated with 3 PbA peptides with or without rIL-22 for 24 h. The IFN\(\gamma\) concentration was then measured by ELISA. Since there was no washing step performed to get rid of unspecific binding to the peptide, splenocytes from naïve mice were taken as control groups.
**Results**

**Figure 20: IFNγ concentration in the supernatant of spleen cells from wt or IL-22−/− PbA-infected mice**

2x10⁵ splenocytes were stimulated with 3 PbA-specific peptides or medium in the presence or absence of rIL-22. The supernatants were collected after 24 h and IFNγ concentration was measured by ELISA. Results represent one out of three independent experiments. Data was analysed by unpaired t test, *** p<0.0001.

Similar to the results obtained on cells stimulated by Hepa 1-6 cells, in Fig. 20 a significant increase (3-fold) can be seen in IFNγ production on splenocytes from IL-22−/− infected mice compared to wt infected mice.
4 Discussion
The present study is dedicated to investigate the effect of IL-22 during the malaria disease. Koch et al. were the first group of scientists that found an involvement of IL-22 in malaria. They demonstrated several SNPs in the IL-22 encoding gene; the IL22 +708T allele was linked to protection against severe anaemia while the IL22-1394G allele was associated with susceptibility to cerebral malaria (Koch, Rockett et al. 2005). A few years later, another study attributed a protection role to IL-22 in the malaria disease. In this study an up-regulation of Th17/Th22 cells was associated to the down-regulation of the pathological Th1 response (Ryan-Payseur, Ali et al. 2011). These findings correlate with data obtained by Julie Sellau from our laboratory group. She demonstrated that IL-22−/− mice infected with Plasmodium berghei ANKA had a decreased parasitaemia but developed more severe symptoms of malaria than infected wild type mice (Sellau 2015). This data led to the conclusion that lack of IL-22 might be involved in the severeness of the malaria disease.

IL-22 is expressed in a wide variety of tissues like the skin, pancreas, thymus and kidney, among others (Sonnenberg, Fouser et al. 2011). To analyse the function of IL-22 in the different tissues, it is imperative to consider the cytokines IL-22 is expressed with as well as the tissue microenvironment, since IL-22 can act both pro- and anti-inflammatory. For example, when co-expressed with TNFα and IL-17, IL-22 can cause a pathogenic effect on tissues like the skin (Guilloteau, Paris et al. 2010). On the other hand, IL-22 can act protective and inhibit pro-inflammatory cytokines in diseases like allergic airway inflammation (Nakagome, Imamura et al. 2011). Overall, IL-22 is in charge of reinforcing epithelial barrier functions as well as repairing tissues and healing wounds. Nevertheless, it is vital to control excessive or prolonged production of IL-22 since it can lead to diseases like psoriasis, type 1 diabetes and Crohn’s disease, among others. (Wolk, Witte et al. 2007).

It is believed that the main receptor chain of IL-22 is present on a few parenchymal cells but absent on immune cells (Wolk, Kunz et al. 2004). This raises the question if and how can IL-22 modulate the immune response although IL-22Rα1 seems to be expressed only on parenchymal cells. In contradiction to Wolk’s study, Ciccia et al. revealed the expression of IL-22Rα1 on PBMCs in patients with primary Sjögren’s Syndrome when compared to PBMCs from patients with non-specific chronic sialo-adenitis (Ciccia, Guggino et al. 2015). This discovery questions if it is possible to induce IL-22Rα1 on immune cells under particular conditions and if this is the case, how does this happen.

An organ where IL-22 plays a defensive role is the liver. Hepatocytes have the ability to react to IL-22, since they express the IL-22Rα1 chain on their surface (Sabat, Ouyang et al. 2014). It has been proved that IL-22 stimulates the secretion of anti-apoptotic proteins in the liver like Bcl-2 and Bcl-xL (Radaeva, Sun et al. 2004) as it also induces liver regeneration by promoting the proliferation of liver stem cells (Ren, Hu et al. 2010). In the course of the malaria disease, the liver undergoes several changes, both during the liver- and blood-phase. The study by Viriyavejakul et al. demonstrated that patients infected with Plasmodium falciparum suffer from liver damage, since they found an increase
Discussion

of transaminases levels in the plasma of these patients (Viriyavejakul, Khachonsaksumet et al. 2014), which represent either liver inflammation or injury (Berk and Korenblat 2012). The investigation made by Viriyavejakul and colleagues also documented hyperplastic and hypertrophic Kupffer cells (KC) with haemozoin deposition and portal inflammation as the most common liver changes in patients with severe malaria. Since the KC eliminate iRBCs and phagocyte the parasite’s antigens, they become enlarged or hyperplastic. Besides acting as macrophages, KC can also display antigens to the hepatic circulating lymphocytes and thus elicit a specific immune response against the malaria parasite. (Viriyavejakul, Khachonsaksumet et al. 2014).

The aim of this study was to analyse the function of IL-22 in an infection model by using different APCs to stimulate antigen-specifically TCR-transgenic CD8+ or CD4+ T cells. The APCs used were considered to express IL-22Rα1 and thus be able to respond to rIL-22. The immune response induced by the T lymphocytes was measured by cytokine production through different assays.

4.1 In vitro effect of rIL-22 using liver cells as antigen presenting cells

The liver is a vital organ in charge of protecting itself from both endogenous and exogenous toxins as well as secreting different hormones and storing them (Fromenty and Pessayre 1995). When a hepatocyte gets infected with a pathogen, becomes injured by toxic metabolites or when the DNA of the hepatocyte is damaged, this cell can undergo necrosis or apoptosis through various mechanisms (Fau, Lekehal et al. 1997). On the one hand, CTLs can directly kill abnormal hepatocytes through the perforin/granzyme and Fas pathways (Sad et al., 1996). On the other hand, both CD4+ and CD8+ T cells can secrete high concentrations of IFNγ, which elicits several antimicrobial pathways (Krishnan, Guilbert et al. 1996).

IFNγ is a soluble pro-inflammatory cytokine in charge of activating macrophages and increasing antigen presentation by up-regulating the MHC molecules (Schroder, Hertzog et al. 2004). The antimicrobial function of IFNγ is regulated by the IFN regulatory factor-1, which mediates cell arrest and apoptosis of infected cells such as hepatocytes. If IFNγ is secreted at high concentrations and not regulated, an uncontrolled cytotoxic immune response against hepatocytes can occur, leading to apoptosis. (Kano, Haruyama et al. 1999). When a chronic state of inflammation is present in the liver without proper regulation, DNA damage occurs and malignant cells can develop. (Horras, Lamb et al. 2011).

Another pro-inflammatory cytokine involved in pathogen eradication on hepatocytes is TNFα. TNFα has the capacity to induce the nuclear factor-kappa beta (NF-κB). NF-κB can lead to proliferation of hepatic malignant cells as well as the progression of fatty liver diseases (Wullaert, van Loo et al. 2007).

Although both IFNγ and TNFα participate in the apoptosis of pathogenic hepatocytes, if there is no proper regulation of these cytokines, an excessive inflammatory response might take place in the liver, causing death of normal hepatocytes (Morita, Watanabe et al. 1995). In order to avoid this detrimental
effect in the liver, it is imperative to control excessive cytokine production elicited during an immune response.

One possible regulator of IFNγ and TNFα could be the IL-10 family member, IL-22. This cytokine has the ability to control IFNγ-mediated inflammation by suppressing the up-regulation of MHC. Additionally, IL-22 impairs the pro-inflammatory chemokines driven by IFNγ, such as chemokine C-C motif ligand 5 and interferon gamma-inducible protein 10. As a result, less immune cells are recruited to the site of infection and the inflammatory response finishes. (Pennino, Bhavsar et al. 2013).

The suppression of IFNγ by IL-22 correlates with the results obtained from this study, where it was shown that the IFNγ produced by T cells stimulated by primary hepatocytes was decreased in the presence of rIL-22 compared to cells stimulated in the absence of rIL-22. However, the difference seen is not significant according to the unpaired t test. Even though there are several studies supporting the hypothesis that IL-22 can regulate the pathologic inflammation induced by IFNγ (Justa, Zhou et al. 2014), the mechanism by which IL-22 is able to achieve this is still not understood.

Concerning TNFα levels, it was demonstrated by Lu et al. that IL-22 was able to decrease the concentration of different pro-inflammatory cytokines in the liver, such as IL-6, IL-1β and TNFα. The reduction of these cytokines leads to attenuation of hepatic inflammation, necrosis and fibrosis. (Lu, Guo et al. 2015). In the experiments made for this study, a decreasing tendency was seen in the TNFα levels in the presence of rIL-22. Nevertheless, the decrease was even smaller than the one obtained in the IFNγ ELISA.

As demonstrated in the flow cytometry plots, the difference of IFNγ and TNFα producing lymphocytes is minimal when comparing groups of cells stimulated with or without rIL-22. This subtle difference could be due to the generalized restimulation the T lymphocytes went through before being stained intracellularly. It is known that the application of PMA/Ionomycin is a strong stimulator and is also very toxic to cells (Baran, Kowalczyk et al. 2001). Perhaps the stimulation with PMA/Iono achieved by bypassing the TCR could result in an excessive and antigen-unspecific stimulation which induces the loss of the rIL-22 effect within the different cell populations.

Even though it was shown by Weber et al. that Hepa 1-6 cells express the IL-22Rα1 chain as demonstrated by real time polymerase chain reaction (Weber, Schlautkötter et al. 2007), the effect of rIL-22 is minimal when using Hepa 1-6 cells as APCs in this study. When comparing the amount of cytokines produced by primary hepatocytes and Hepa 1-6 cells, it is noticeable that T cells that were stimulated by primary hepatocytes produce higher amounts of both IFNγ and TNFα. A hypothesis for this difference could be that the primary hepatocytes are better at antigen presentation than Hepa 1-6 cells or it could be due to the longer co-cultivation of T cells and primary hepatocytes (40 h) compared to the 16 h that Hepa 1-6 cells are cultivated together with the T lymphocytes.
4.2 **In vitro effect of rIL-22 on T cells stimulated by bone marrow derived dendritic cells**

Dendritic cells are denominated professional antigen presenting cells since their main function is to display antigens to naïve T cells and create a link between the innate and adaptive immune responses. Depending on their maturation status, the DCs can either induce tolerance to certain antigens or elicit a specific immune response. (Abbas, Lichtman et al. 2012). The DCs used for this study are BMDCs, which originate from a myeloid lineage. BMDCs are not only able to activate both CD4⁺ and CD8⁺ T cells through antigen presentation but also help in the development of B cells into antibody-producing cells. (Steinman 2007).

Even though it is believed that immune cells do not express the IL-22Rα1 chain (Wolk, Kunz et al. 2004), Julie Sellau revealed on her thesis that IL-22Rα1 can be induced on splenic DCs from PbA-infected wt mice (Sellau 2015). The expression of IL-22Rα1 was also demonstrated on peripheral blood mononuclear cells under pathologic conditions, while being absent under physiological circumstances (Ciccia, Guggino et al. 2015).

For this study, BMDCs were generated from wt and IL-22⁻/⁻ mice to perform antigen presentation to T lymphocytes enriched from splenocytes of transgenic OT I or OT II mice. The IL-22⁻/⁻ and the wt BMDCs were chosen as Julie Sellau demonstrated on her experiments that IL-22⁻/⁻ BMDCs express higher amounts of the co-stimulator CD86 upon stimulation (Sellau 2015) and can therefore perform a better T cell activation than wt BMDCs (Kronin, Hochrein et al. 2000). In contrast to this data, on the results obtained by ELISA and intracellular staining for this study, there was no increase in cytokine production by T cells stimulated by IL-22⁻/⁻ BMDCs compared to wt BMDCs. When measuring cytokine concentration of different cytokines by CD8⁺ T cells with LEGENDplex™, there is a tendency towards less cytokine production in the presence of rIL-22. However, a statistical analysis could not be performed with this data, since the LEGENDplex™ assay provides the results in single values and this assay was performed only once for every experiment.

Moreover, there is no congruent trend in cytokine production by CD4⁺ T cells in the presence or absence of rIL-22 as tested by LEGENDplex™, which agrees with the IFNγ and TNFα ELISA results. Surprisingly, there is a tendency to decrease in cytokine production in the presence of rIL-22 when measured by intracellular staining for both IFNγ and TNFα. The difference seen is of statistical significance in the IFNγ expression of CD4⁺ T cells stimulated by wt BMDCs.

Taken together, throughout the experiments in vitro performed with BMDCs, there is no difference in cytokine production between cells stimulated by wt and IL-22⁻/⁻ BMDCs. Additionally, the effect of rIL-22 in regulating the immune response seems to be absent, since there is no consistent decrease in cytokine production in the presence of rIL-22. A reason for this phenomenon could be the expression of IL-22BP by the BMDCs. It was recently shown that IL-22BP is expressed by immature DCs (Martin, Bériou et al. 2014). This statement leads to the assumption that even if the IL-22Rα1 chain
was induced on BMDCs, IL-22BP would block the function of IL-22 since IL-22BP has a much higher affinity to IL-22 than IL-22Rα1 does (Huber, Gagliani et al. 2012). An alternative explanation for the loss of effect of rIL-22 could be the high number of BMDCs used to stimulate T cells. As established by our laboratory group, 1x10^3 BMDCs were sufficient to perform antigen presentation to 2x10^5 T lymphocytes (Krause 2015). In the experiments made for this study, 1x10^5 BMDCs were taken to stimulate 2.5x10^5 T lymphocytes. The high number of BMDCs used, might evoke a generalized activation of naïve T cells and thus discrete differences in cytokine production within the different experiment setups might get lost.

Another interesting outcome of the experiments using BMDCs is the fact that CD4^+ T lymphocytes produce less cytokine than CD8^+ T cells do. One reason for this finding could be the fact that the helper T lymphocytes need 6 h to become stimulated while cytotoxic T lymphocytes only take 2 h (Iezzi, Karjalainen et al. 1998). These results agree with the data obtained by the LEGENDplex™ assay, where it is shown that CD4^+ T cells produce higher amounts of IL-2 than IFNγ and TNFα. This could indicate that the CD4^+ T cells were still going through the clonal expansion phase and were not differentiated into effector cells by the time the cells were removed from the culture plate and the supernatants were collected (Abbas, Lichtman et al. 2012). An additional motive for the lesser IFNγ and TNFα production by CD4^+ T cells may be that the Ova peptide alone is not enough to stimulate and produce Th1 cells. As seen in another study, in order to increase the Th1 cell induction in vitro, naïve T cells were stimulated with Ova_{323-339} and in the presence of IL-2, IL-12 and IFNγ (Nishimura, Iwakabe et al. 1999).

4.3 *In vitro* effect of rIL-22 on splenocytes

The spleen is the largest lymphoid organ in the human body (Steiniger and Barth 2000). It is in charge of protecting the organism from invading pathogens and from senescent, damaged or aberrant cells. The spleen has two main components, the red pulp and the white pulp. The white pulp is mainly composed of immune cells. It is organized in periarteriolar lymphoid sheaths, where T lymphocytes become in contact with DCs or B cells displaying pathogenic antigens. (Mebius and Kraal 2005). The migration of different immune cells to the lymphoid sheaths is mediated by several chemokines, which are controlled by cytokines like TNFα and lymphotoxin-α (Ngo, Korner et al. 1999). The spleen is important for the immune homeostasis, since both the innate and adaptive immune responses can take place in this organ.

Even though it has been proved by several studies that the IL-22 receptor is absent on immune cells; Justa *et al.* demonstrated that IL-22Rα1 can be induced on CD4^+ T cells from the spleen under inflammatory conditions (Justa, Zhou et al. 2014). Concordantly, the study made by Weber *et al.* demonstrated the expression of IL-22Rα1 in the spleen, kidney and liver by quantitative PCR (Weber, Schlautkötter et al. 2007). These findings encouraged the inquiry if IL-22 can elicit an immunomodulatory effect on the spleen, as both IL-22 and its main receptor chain are expressed on
this organ (Dudakov, Hanash et al. 2012). For this purpose, splenocytes were enriched from wt mice and these cells were pulsed with SII to activate CD8⁺ T cells. Afterwards, the IFNγ produced by the T lymphocytes was measured by ELISA. With this assay, it was demonstrated that rIL-22 leads to a significant decrease in the IFNγ production on pulsed CD8⁺ T cells, which confirms that IL-22 has the ability to modulate the immune response in the spleen.

The mechanism by which IL-22 could act on splenic immune cells is unknown. One hypothesis could be attributed to the co-stimulators CD80 and CD86. As seen by Julie Sellau, on IL-22⁺BMDCs there is an up-regulation of both CD80 and CD86 after being stimulated for 24 h by LPS (Sellau 2015). CD86 and CD80 bind to CD28 from naïve T cells to activate the lymphocytes and induce cytokine production (Abbas, Lichtman et al. 2012). Therefore, it is possible that when rIL-22 is given to BMDCs, these cells decline the expression of the co-stimulators and as a consequence the T lymphocytes turn into a state of anergy and produce no cytokines (Villadangos, Cardoso et al. 2001). Another hypothesis for the lesser IFNγ production in the presence of rIL-22 could be that other cells in the spleen also produce IL-22 under pathologic conditions (Colonna 2009). DCs for example, produce cytokines like IL-7, IL-12 and IL-23, which help in the elicitation of the immune response and also induce IL-22 production. IL-23 is one of the primary inducers of IL-22 on cells like NKT cells and γδ T lymphocytes, among others (Dudakov, Hanash et al. 2012), while IL-12 activates NKT cells and therefore help in the IL-22 production by these cells (Zenewicz and Flavell 2008). IL-7 is crucial for the differentiation of IL-22-producing cells and it’s also essential for Il22 gene expression (Qiu, Heller et al. 2012). Altogether these other cytokines might induce higher levels of IL-22 and thus reinforce the function of IL-22 in the spleen.

When analysing the flow cytometry data, there are high percentages of stimulated CD8⁺CD44⁺ T cells positive for both IFNγ and TNFα after intracellular staining. These results demonstrate that the stimulation induced by the SII peptide activates CD8⁺ T cells and that these cells are in charge of producing the IFNγ measured by ELISA. Even though there are significant differences in the cytokine production in the presence and absence of rIL-22 within the different cell populations as tested by ELISA, these differences are not detected in the flow cytometry plots. This finding correlates with the theory that PMA/Ionomycin re-stimulates T cells in a universal manner and therefore discrete differences in T cell cytokine production get lost.

4.4 Ex vivo effect of IL-22 during the malaria infection
Malaria remains a health threat in underdeveloped countries, especially in Africa. Even though there exist enough antimalarial drugs affordable to most individuals, the competence of these medicines has been compromised by the high drug resistance rate. Since the efficacy of the treatment depends to some extent, on the patient’s capacity to create a proper immune response against the parasite, it is imperative to get a better understanding at the host’s immune system during the malaria disease. (Hyde 2005).
Although the human body is able to establish a proper specific immune response against the malaria parasite, there are many patients specially children that fail to combat the infection and develop cerebral symptoms or even die from this disease (Artavanis-Tsakonas, Tongren et al. 2003). It is known that the immune system tries to eradicate the parasite through the humoral and cellular immune responses. The humoral immune response is of limited help since the malaria antigens are polymorphic or develop antigen variations. When the organism gets infected with a new parasite variant, uncontrolled parasite replication takes place and the person infected can suffer from the malaria disease. (Bull, Berriman et al. 2005). On the other hand, the cellular immunity can attack the different antigens in a more direct way. During this immune response, macrophages are activated by NK cells, γδ T cells or Th1 cytokines like IFNγ. (Artavanis-Tsakonas, Tongren et al. 2003). The activation of macrophages leads to enhanced phagocytosis and also to production of nitric oxide. NO is able to kill the malaria parasite when secreted at high concentrations. (Balmer, Phillips et al. 2000).

It has been established that IFNγ is involved in the onset of ECM together with TNFα during the late phase of the malaria disease (Amani, Boubou et al. 1998), therefore both of these cytokines have become targets for the antimalarial drugs. One cytokine that might able to down-regulate the concentration of IFNγ and thus of TNFα, is IL-22. In the ex vivo experiments performed for this study, where wt and IL-22−/− mice were infected with PbA, the protective function of IL-22 during the malaria infection became evident. Splenocytes were isolated from PbA infected mice, were stimulated with PbA-specific peptides (Poh, Howland et al. 2014) and their IFNγ production was measured by ELISA. A 3-fold increase in IFNγ production was demonstrated on splenocytes derived from infected IL-22−/− mice compared to infected wt mice. Similar results were obtained by the experiments performed using Hepa 1-6 cells as APCs and co-cultivating them with PbA-infected CD8+ T cells and then stimulating both cell types with the malaria-specific peptides. Moreover, on the experiments using Hepa 1-6 cells as APCs, the presence or rIL-22 does not change the production of IFNγ. On the other hand, when stimulating infected splenocytes with the malaria peptides, a decrease in IFNγ production was seen on IL-22−/− pulsed cells in the presence of rIL-22 compared to the cells stimulated without rIL-22. This difference seen (p = 0.0586) would support the hypothesis that spleen cells act as a third party in priming T cells and therefore the effect of rIL-22 can become enhanced.

The ex vivo results obtained by this investigation also associate to the study from Ryan-Payseur et al., where the scientists co-infected macaques with Plasmodium fragile and with simian-human immunodeficiency virus (SHIV) (Ryan-Payseur, Ali et al. 2011). They demonstrated a link between the development of Th17/Th22 cells and the immunity against fatal virus-associated malaria in chronically infected SHIV patients. The expansion of Th22 cells was correlated with the decrease of pathological Th1 cells. During the acute co-infection, the immune response went through a hyper activation of Th1 cells with high production of cytokines like IFNγ and TNFα which led to progression of both, SHIV and malaria.
Both IFNγ and TNFα seem to play a dual role during the malaria disease; as they evoke ECM during the late phase of the infection and control the parasitaemia in the early phases (Amani, Boubou et al. 1998). CD8+ T cells are able to elicit a specific immune response against the malaria parasite during the blood phase of the infection even though erythrocytes lack the MHC molecules. It is believed that red blood cells can act as a source of antigen cross-priming and therefore CD8+ T cells can recognize the parasitic antigens (Lundie, de Koning-Ward et al. 2008). The immune response evoked by the CD8+ T lymphocytes might lead to one of the most severe manifestations of the malaria disease; CM. It was shown by Howland et al. that CD8+ T cells can damage the blood-brain barrier, sequestrate in the brain micro-vessels and cause ischemia, which evokes ECM on PbA infected mice (Howland, Poh et al. 2013). This conclusion agrees with a preliminary experiment performed for this study, where it was shown that IL-22−/− infected mice produce more IFNγ and have a higher percentage of activated CD8+ T cells than wt mice, making the IL-22−/− mice more susceptible to severe malaria (Amani, Boubou et al. 1998). The amount of activated CD8+ T lymphocytes was measured by staining the cells with anti-CD44. A reason for IL-22−/− mice having more IFNγ producing CD8+ T cells could be explained by the finding from Julie Sellau, where she demonstrated that IL-22−/− BMDCs express higher amounts of the co-stimulator CD86 than wt BMDCs upon stimulation (Sellau 2015), and can therefore perform a better T cell activation (Abbas, Lichtman et al. 2012). However, to confirm these preliminary results further experiments are needed.

In conclusion, on the experiments performed with the malaria-specific peptides, the protective role of IL-22 during the malaria disease could be proved. The absence of IL-22 in mice leads to a higher concentration of IFNγ, which is correlated to the severity of the malaria disease (Amani, Boubou et al. 1998). On the other hand, the decrease in IFNγ production in the presence of rIL-22 could only be reproduced on the in vitro experiment performed with whole splenocytes, while in the other in vitro experiments the effect of rIL-22 is minimal. A hypothesis for these results could be that cells other than DCs express the IL-22Rα1 in the spleen under inflammatory conditions and thus, the effect of IL-22 becomes more evident. Another reason could be that other cytokines are released in the spleen that reinforces the function of IL-22, like IL-23, IL-7, IL-12 (Dudakov, Hanash et al. 2012), which would indicate that the non-lymphoid cells from the spleen can act as a third party in supporting T cell priming and therefore the effect of IL-22 becomes enhanced.

4.5 Future directions
It was demonstrated by this study, that IL-22 has a protective role in the malaria disease. Nevertheless the mechanism by which IL-22 achieves this task, is still poorly understood. Some scientists claim that tissue cells that express the receptor for IL-22 can modulate the immune response (Sonnenberg, Fouser et al. 2011), while others state that there is an induction of the receptor for IL-22 on immune cells during an infection (Justa, Zhou et al. 2014), and therefore the immune regulation can take place when IL-22 is present.
To confirm if BMDCs can respond to IL-22, the experiments performed by using IL-22–/– and wt BMDCs should be repeated by using IL-22BP–/– and wt mice, since it has been demonstrated that IL-22BP is found on DCs (Martin, Bériou et al. 2014), which could block the effect that IL-22 induces on these cells. If there is still not a significant difference in the production of IFNγ on wt and IL-22–/– mice, maybe instead of using SII to restimulate the naïve CD8+ T cells, it would be better to enrich both BMDCs and CD8+ T cells from PbA-infected mice and restimulate these cells with the PbA-specific peptides.

To proof if IL-22 is the cytokine responsible for the protection against severe malaria by acting on immune cells, the Cre/lox system should be used to knockout the IL-22Rα1 chain on DCs, B and T lymphocytes. If the Cre/lox mice produce higher amounts of IFNγ than wt mice, this finding would demonstrate that IL-22 does not play an important role on immune cells in malaria. This would agree with the study by Wolk et al. where they stated that IL-22 can act only on non-immune cells and another hypothesis for the protection against ECM in IL-22–/– mice should be searched for (Wolk, Kunz et al. 2004). On the other hand, if the difference seen is lost, it would indicate that immune cells can respond to IL-22 and the mechanism by which IL-22 acts on immune cells should be confirmed with further investigations.
5 References


Krishnan, L., L. J. Guilbert, T. G. Wegmann, M. Belosevic and T. R. Mosmann (1996). T helper 1 response against Leishmania major in pregnant C57BL/6 mice increases implantation failure and fetal
resorptions. Correlation with increased IFN-gamma and TNF and reduced IL-10 production by placental cells. *Journal of Immunology* 156(2): 653-662.


6 Abstract

IL-22 is a member of the IL-10 family of cytokines and is an important regulator of tissue cells. IL-22 mediates its effects through a transmembrane heterodimeric receptor complex, consisting of the ubiquitously expressed subunit IL-10R2 and IL-22Rα1, which is thought to be absent on immune cells.

The aim of this study was to proof if IL-22 has the capacity to regulate the immune response elicited during an infection model, by acting through parenchymal cells, which express the IL-22 main receptor subunit, IL-22Rα1.

It was demonstrated in the in vitro experiments where several APCs were used as targets for IL-22, that IL-22 has no clear effect in modulating the immune response during an infection model, since there was no change statistically significant in the IFNγ or TNFα concentration in the presence or absence of IL-22. On the other hand, when using whole splenocytes as receptors for IL-22, the effect of IL-22 in decreasing the production of IFNγ becomes evident. Furthermore, the protective function that IL-22 plays during the malaria infection could be demonstrated, since there was a significant increase in the production of the inflammatory cytokine IFNγ in PbA-infected mice lacking IL-22.

Since the mechanism by which IL-22 is able to modulate the immune response by down-regulating IFNγ is still unknown, more studies are needed to clarify this matter and be able to develop more efficient therapies for the malaria disease.
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Full Form</th>
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<td>Celsius degree</td>
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<tr>
<td>aa</td>
<td>Amino acids</td>
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<td>Aryl hydrocarbon receptor</td>
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<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>BMDCs</td>
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<td>ca</td>
<td>Circa</td>
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<td>Cluster of Differentiation</td>
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<td>Dendritic cell</td>
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<tr>
<td>ddH₂O</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>ELISA</td>
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<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<tr>
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<td>IL-TIF</td>
<td>IL-10-related T cell-derived Inducible Factor</td>
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<td>iNOS</td>
<td>Inducible Nitric-Oxide Synthase</td>
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<tr>
<td>i.p.</td>
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<td>Phosphate Buffered Saline</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PDC</td>
<td>Plasmacytoid dendritic cell</td>
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<td>wt</td>
<td>Wild Type</td>
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Zuallererst bedanke ich mich herzlichst bei der AG Jacobs für die tolle Arbeitsatmosphäre, das mir entgegen gebrachte Vertrauen und die vielen leckeren Kuchen. Ich bin sehr glücklich darüber, dass ich euch alle kennen lernen durfte.


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9 Curriculum vitae

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