# Modulation of the $CD8^+$ T cell response by HVEM and CD160 during blood-stage malaria

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## Statutory declaration

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

Hamburg, den 21.12.17

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## Abbreviations

| | |
|---|---|
| 2B4 | Natural Killer Cell Receptor 2B4, CD244 |
| ADCI | Antibody-Dependent Cellular Inhibition |
| AP1 | Activator Protein 1 |
| BTLA | B and T Lymphocyte Attenuator, CD272 |
| CM | Cerebral Malaria |
| ConA | Concanavalin A |
| CRD | Cysteine-Rich Domaine |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| CTLA-4 | Cytotoxic T Lymphocyte Associated protein 4, CD152 |
| d p.i. | days post infection |
| DC | Dendritic Cell |
| $ddH_2O$ | double-destilled water |
| Dereg | Depletion of regulatory T cell |
| DNA | Deoxyribonucleic Acid |
| dNTP | Deoxyribonucleic Triphosphate |
| ECM | Experimental Cerebral Malaria |
| EDTA | Ethylenediaminetetraacetic Acid |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| ERK | Extracellular-signal Regulated Kinase |
| Fc | Fragment crystallizable region |
| FLT3L | FMS-Like Tyrosine kinase 3 Ligand |
| FMO | Fluorescence Minus One |
| FSC | Forward Scatter |
| GPI | Glycosylphosphatidylinositol |
| GrzB | Granzyme B |
| HIV | Human Immunodeficiency Virus |
| HRP | Horseradish Peroxidase |
| HSV | Herpes Simplex Virus |
| HVEM | Herpesvirus Entry Mediator |
| i.p. | intraperitoneal |
| ICAM1 | Intercellular Adhesion Molecule 1 |
| IFNγ | Interferon gamma |
| iIEL | intestinal Intraepithelial Lymphocytes |
| iRBC | infected Red Blood Cell |
| KLRG1 | Killer-cell Lectin like Receptor G1 |
| LIGHT | homologous to Lymphotoxin, exhibits Inducible expression and competes for HSV Glycoprotein D for binding to Herpesvirus entry mediator, a receptor expressed on T lymphocytes |
| MEK | Mitogen activated protein kinase kinase |
| MHCI | Major Histocompatibility Complex I |
| min | minutes |
| ml | millilitre |
| mRNA | messenger Ribonucleic Acid |
| NF-κB | Nuclear Factor kappa B |
| NHEJ | Non-Homologous End Joining |
| Pb | *Plasmodium berghei* |
| *Pb*A | *Plasmodium berghei* ANKA |

| | |
|---|---|
| PBMC | Peripheral Blood Mononuclear Cells |
| PBS | Phosphate Buffered Saline |
| PBSA | Bovine Serum Albumin in PBS |
| PCR | Polymerase Chain Reaction |
| PD-L1 | Programmed cell Death 1 Ligand 1, CD274 |
| PD1 | Programmed cell Death 1 |
| *Pf* | *Plasmodium falciparum* |
| PI3K | Phosphoinositide 3 Kinase |
| PKB | Protein Kinase B |
| PLD | Phospholipase D |
| PMA | Phytohaemaglutinin |
| *Py*NL | *Plasmodium yoelii* non-lethal |
| RBC | Red Blood Cell |
| RLFP | Restriction Length Fragment Polymorphism |
| RNA | Ribonucleic Acid |
| rpm | rounds per minute |
| RT | Room Temperature |
| sCD160 | soluble CD160 |
| sgRNA | single guide Ribonucleic Acid |
| SMAC | Supramolecular Adhesion Complex |
| SSC | Side Scatter |
| TGFβ | Transforming Growth Factor beta |
| Tim-3 | T-cell immunoglobulin and mucin-domain containing 3 |
| TLR9 | Toll-Like Receptor 9 |
| TNFa | Tumor Necrosis Factor alpha |
| Treg | regulatory T cell |
| uRBC | uninfected Red Blood Cell |

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# 1 Introduction

## 1.1 Malaria

The WHO Malaria Report 2015 has reported many promising trends concerning incidence and mortality rates as well as the distribution of malaria cases during the past 15 years. For example in 57 counties mainly from the Americas and Western Pacific Region a reduction of 75% of malaria cases was observed. In addition, 2015 has been the first year since 2000 without any autochthonous malaria case in the European Region. Nevertheless, incidence remains high in African countries. The geographical distribution of malaria in 2016 is shown in Figure 1.1.

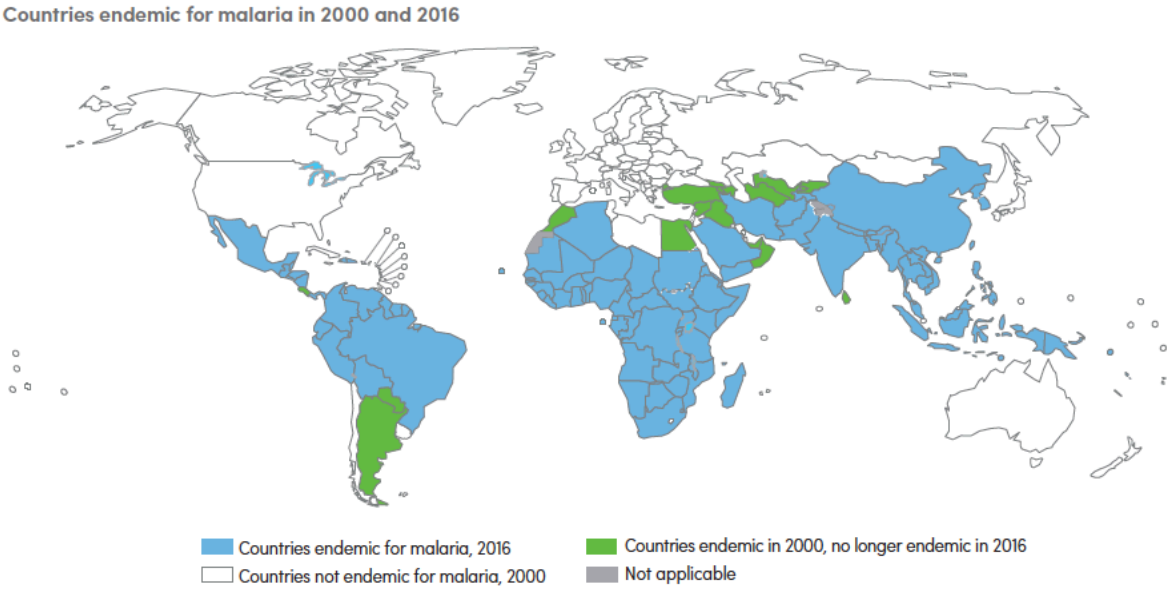



**Figure 1.1 Countries endemic for malaria in 2016 [1].** Countries endemic for malaria in 2016 are depicted in blue whereas countries, that have been endemic for malaria in 2000 but not in 2016, are highlighted in green.

Worldwide there are 3.2 billion people at risk of getting malaria and approximately 214 million new cases and 438,000 deaths occurred only in 2015 [2]. Mostly at risk for complicated malaria are children under five and pregnant women.

The causing agent of malaria is the intracellular parasite of the genus *Plasmodium* that is transmitted by the female anopheles mosquito. Six strains are known to be pathogenic in humans, namely *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium knowlesii*, *Plasmodium cynomolgi*, *Plasmodium falciparum* and *Plasmodium vivax*. Having acute fever in common, the disease differs depending on the causing strain in its course of infection concerning incubation time, parasite persistence, severity and chronicity of symptoms.

## 1.2 Plasmodium life cycle

Malaria parasites are injected into the human host as sporozoites by female anopheles mosquitos during their blood meal. Parasites travel to the liver via the blood stream where they pass Kupffer cells in order to reach hepatocytes [3-5]. After crossing several hepatocytes, sporozoites stay intracellular for approximately 7 days until they develop into schizonts. During maturation and replication of the parasite in the liver stage, the infection remains mainly asymptomatic. However, after the schizonts rupture and thereby release thousands of merozoites into the blood stream [6], immunopathology is induced. The blood-stage is characterised by strong inflammatory processes while the asexual replication of the parasite takes place. Merozoites enter red blood cells (RBCs) where they develop into the ring stage followed by the trophozoite stage and finally form new schizonts, which again release thousands of merozoites after the rupture of the RBC [7]. Some of the parasites differentiate also into the sexual form, the gametocytes, instead of completing the asexual cycle [8, 9]. Those can eventually be taken up by female anopheles mosquitos during their blood meal to undergo sexual replication. After the gametocytes have reached the stomach of the mosquito, the microgametocytes (male) penetrate macrogametocytes (female) forming zygotes that develop into ookinetes. Ookinetes are motile and can penetrate the midgut wall of the mosquito. After the penetration, ookinetes develop into oocysts, which, after maturation, finally release sporozoites. Those sporozoites need to find their way into the salivary glands so that they can be transmitted into the human host with the next blood meal to repeat the life cycle.

## 1.3 Cerebral malaria

Severe malaria, which is mainly caused by *Plasmodium falciparum,* is a multisystem disorder with different manifestations in children and adults. While complicated malaria in children mainly includes cerebral symptoms and anaemia, adults develop cerebral malaria (CM) acute respiratory distress syndromes, jaundice and acute liver failure [10].

Causes of the development of cerebral malaria have been analysed and discussed extensively and seem to be multifaceted. Initially, two hypothesises have been proposed. The sequestration hypothesis argues that parasitized red blood cells adhere in post capillary venules and subsequently mechanically disturb the blood flow. In addition, clogging of vessels was suggested to enrich metabolic waste products [11]. Due to the objection, that

not in all lethal cerebral malaria cases sequestered RBCs can be found in the brain, a second hypothesis was proposed, namely the cytokine theory [12]. The authors argue, that cytokines like TNFα and IL–1 produced by the host induce pathology due to their high levels in malaria patients. More recently, it has been suggested that a combination of sequestration of infected RBCs (iRBC), inflammation involving cytokines and also haemostasis leads to pathology [13, 14]. In Table 1, an overview of observations arguing for and against each of the proposed mechanisms behind the development of cerebral symptoms is assembled.

**Table 1 Overview of supporting and conflicting observations in CM patients concerning the proposed underlying processes.** According to [13].

| Process | Supporting | Not concurring |
|---|---|---|
| Sequestration | Only ring stages on blood films | High parasitemia does not necessarily result in death |
| | Histology, electron microscopy shows adherent iRBC | Sequestration occurs in all *P. falciparum*- infected individuals but CM only develops in about 1% |
| | Elevated blood lactate suggests hypoxia | Rapid improvement after treatment does not support hypoxia or stroke-like mechanisms |
| | Occluded post capillary venules detected following autopsy | Lactic acidosis is complex and does not necessarily reflect anaerobic glycolysis in tissue |
| | Infected and uninfected RBC deformability is an indicator of poor prognosis, suggesting plugging of capillaries | |
| Inflammation | High levels of proinflammatory cytokines and chemokines detected during CM | Anti-TNF monoclonal antibodies failed to protect, increased neurological sequelae |
| | Adhesion of leukocytes and platelets detected following autopsy of CM brains | Pentoxyfylline failed in small clinical studies |
| | Endothelial cell adhesion molecules elevated in tissues including brain | Dexamethasone failed to protect; increased disease |
| Haemostasis | Hemorrhage observed following autopsy of brain and other tissues | Brain vascular leak occurs in non-CM patients |
| | Retinal hemorrhage is an indicator of poor prognosis for CM | No increase in capillary leak detected by fluorescein angiography |
| | Platelet adhesion in brain correlates with CM | Thrombocytopenia occurs in non-lethal *P. vivax* malaria |
| | Consumption of coagulation factors results in a procoagulant state and a bleeding tendency that correlates with the development of CM | Oedema is observed in some but not all CM studies |

| | | |
|---|---|---|
| | Vascular leak occurs in brain during CM | Hypovolemia secondary to vascular leak is controversial |
| | Profound thrombocytopenia correlates with the development of CM | |

In summary, human cerebral malaria remains incompletely understood and further research is crucial for a better understanding of the underlying mechanism in order to provide adequate treatment.

### 1.3.1 Mouse model

As described above, malaria is characterised by diverse clinical manifestations, depending on parasite strains, age and immune status of the infected individual and many other factors. For most clinical manifestations, mouse models are available. Non-lethal models, e.g. *Plasmodium yoelii* NL (*Py*NL) or *Plasmodium chabaudi* infection of C57BL/6 mice, allow the analysis of the whole course of infection including clearance of the parasite. As this work focuses on *Plasmodium falciparum* malaria and cerebral outcomes, the corresponding model should be explained in more detail.

C57BL/6 mice infected with *Plasmodium berghei* ANKA develop a similar clinical outcome as observed in cerebral malaria in humans [15]. Beginning after 5-7 days of infection, mice show symptoms ranging from ataxia and convulsions to coma, finally leading to a lethal outcome [15, 16].

CM in humans is heterogeneous in pathogenesis between patients as well as between children and adults. It is caused by various mechanisms with sequestration of iRBC in brain vessels playing a central role [13]. The *Pb*A infection model cannot reflect all underlying mechanisms. However, it allows the analysis of the disruption of the blood brain barrier by cytotoxic T cells after cross-presentation of parasitic antigen on brain endothelial cells [17-20].

Research in humans is challenging due to a low CM incidence rate of about 1% of all malaria cases and mainly infants mainly being affected. Obviously, brain-tissue samples are in general only available post-mortem but even in fatal cases only rarely due to cultural reasons. Hence, the mouse model is important for a better understanding of the basic mechanisms of immune regulation during the course of the disease.

## 1.4 Remaining challenges in the fight against malaria

Despite huge efforts in research, treatment and prevention of infection remains a difficult task. Finding antigens that can be used as drug or vaccine targets is challenging because parasitic antigens vary greatly during the life cycle. In addition, parasites hide during their first developmental step in the liver, taking advantage of the immunotolerogenic environment.

All in all, a better understanding of how, when and where the immune system is able to fight efficiently against malaria parasites but at the same time to prevent an overwhelming, immunopathology inducing response and where it fails to do so, is highly important.

## 1.5 Immunology to *Plasmodia*

In terms of the protection against *Plasmodium* species, the immune system needs to deal with a protozoan parasite, which is a challenge in several ways. In contrast to viral or bacterial antigen, *Plasmodia* exhibit not only different repertoires of proteins depending on their developmental stage but also polymorphism of several proteins [21]. In addition, the intracellular parasite infects the liver and blood. Consequently, the parasite is able to hide either in an immunosuppressive environment (liver) or in RBCs lacking MHCI expression preventing cytotoxic T cells from detecting them. Furthermore, an enormous replication rate within infected cells enables a successful establishment of infection even in case of a low number of originally parasitized cells [22].

In general, the immune system can be subdivided into the innate and the adaptive branch, which will be discussed regarding their involvement in anti-*Plasmodial* immunity in the following chapters.

### 1.5.1 Immunity to sporozoites

To infect a mammalian host in the first place, foreign pathogens need to overcome physical and chemical barriers like epithelial surfaces e.g. of the skin, lung and gastrointestinal tract. *Plasmodium* sporozoites are injected into the host with the bite of a female anopheles mosquito thereby passing the epithelial surface [23]. After the parasite has passed the first line of defence, some parasites reach the liver, which is their target organ. However, others invade skin-draining lymph nodes, where they deliver antigen for resident dendritic cells (DC) [24]. Those dendritic cells in turn are crucial for the priming of sporozoite specific $CD8^+$ T cells that are required for elimination of parasitized hepatocytes [25, 26].

Despite the lack of clinical symptoms during the liver stage, innate as well as adaptive immune responses are initiated. Considering the innate arm, liver resident cells detect *Plasmodia* RNA, representing a pathogen-associated molecular pattern (PAMP), and a type I interferon response is induced [27]. Both, NK and NKT cells infiltrate the liver upon *Plasmodia* infection and can at least partially restrict parasite development in infected hepatocytes [28, 29]. More importantly, protective immunity against pre-erythrocytic parasites can be mediated by $CD8^+$ T cells and in part by antibodies [30, 31]. For the control of parasite growth and development in hepatocytes, $CD8^+$ T cells require the cytokines IFNγ and TNFα [30-32], whereas the relevance of cytotoxicity involving perforin or Fas/Fas-ligand is depending on the *Plasmodium* strain [32]. Strikingly, the number of liver stage-specific $CD8^+$ T cells needed for protective immunity is considerably higher compared to other intracellular pathogens [33]. During acute infection, depletion of $CD4^+$ T cells does not affect immunity, suggesting that they do not contribute to an inflammatory environment [30]. However, $CD4^+$ T cells are needed for memory establishment [34].

### 1.5.2 Immunity to blood stage parasites

In contrast to the liver stage, the blood stage is characterised by a significant inflammation and immunopathology. Early during the blood stage, macrophages accumulate in the spleen and start to produce IL-12, which is an important driver of Th1 immune response [35, 36]. In response to IL-12, NK cells produce IFNγ, which in turn is required for control of the parasitemia and induction of TNFα production e.g. by macrophages [37-41]. γδ T cells can contribute to the production of IFNγ [42] and *in vitro* assays with human peripheral blood mononuclear cells (PBMCs) demonstrated an inhibition of parasite growth in late or extracellular stages depending on γδ T cells rather than αβ T cells [43].

However, protective immunity largely depends on antibodies [44-47], which act in several ways. Opsonisation of merozoites mediates complement activation [48] or antibody-dependent cellular inhibition (ADCI) in collaboration with monocytes and macrophages [47, 49]. In addition, antibodies can prevent invasion of parasites into RBCs by targeting merozoite surface protein [50]. If directed against *plasmodial* proteins at the surface of infected RBCs (iRBC), antibodies can inhibit the sequestration of iRBCs, improving clearance of iRBCs in the spleen [51] or prevent aggregation of infected and uninfected RBCs (rosetting), which is related to severe malaria [52]. During rupture of iRBCs, *Plasmodia* toxins e.g. *Plasmodia* DNA or glycophosphatidylinositol (GPI) are released and enhance

inflammation via toll-like receptors [53, 54]. Neutralising antibodies directed against toxins can thereby restrict inflammatory responses [55].

Besides antibodies, $CD4^+$ T cells are able to provide partial protection, even in the absence of B cells [56]. Effector mechanisms of $CD4^+$ T cells during blood stage malaria are providing help for B cells as well as the production of Th1 cytokines [57, 58]. However, $CD4^+$ T cells without B cells are not sufficient to provide long-term protection as parasites are only completely cleared in the presence of B cells and protection in secondary infection is highly dependent on B cells [58, 59]. Considering the lack of MHCI expression by human RBCs, effectiveness of $CD8^+$ T cells in elimination of infected cells is questionable. However, some reports suggest a protective function of $CD8^+$ T cells in collaboration with macrophages [60, 61]. Importantly, this protection is described in mouse models, where young RBCs that still express MHCI are present and infected by parasites.

Despite the crucial function of IFNγ and TNFα in parasite clearance during the blood stage, the fulminate inflammatory response also induces immunopathology [62, 63]. In addition, activated and parasite-specific T cells are responsible for the development of experimental cerebral malaria by the disruption of the blood-brain-barrier due to cross-presentation of *plasmodial* antigen on brain-endothelial cells [19, 20].

Considering the fact that protective cytokines as IFNγ and TNFα can, if present at too high concentration, the wrong time or tissue, induce immunopathology, regulatory mechanisms are crucial to balance pro- and anti-inflammatory factors. Of special interest is the regulation of T cells, since $CD4^+$ T cells are the most important cytokine producers and $CD8^+$ T cells induce immunopathology in the brain.

Anti-inflammatory and suppressive mechanisms include cytokines, regulatory T cells and T cell intrinsic regulation. The most important anti-inflammatory cytokines in malaria are TGFβ and IL-10 [64-66]. Main sources of IL-10 during malaria are regulatory $CD4^+Foxp3^-$ T cells [67]. Since IL-10 suppresses pro-inflammatory cytokines (IFNγ, TNFα), it restricts the development of severe pathology including cerebral malaria [68], hypothermia and hypoglycaemia [69]. TGFβ, which can be produced by regulatory T cells, dendritic cells and $CD8^+$ T cells, enhances protective immunity and restricts pathologic effects during blood-stage, whereas it diminishes efficient $CD8^+$ T cell response in the liver stage [70-72].

If expanded *in vivo*, classical regulatory T cells ($CD4^+Foxp3^+$) can provide protection against cerebral malaria, while humoral and T cell immunity to *Plasmodia* is restricted [73, 74]. However, their deletion does not affect pathology in *Pb*A infected C57BL/6 mice [73, 75].
Despite the regulation by external and/or soluble factors during malaria, T cell functionality is fine-tuned by their expression of co-inhibitory receptors, which will be introduced in the following chapter.

## 1.6 The HVEM network

During the past years more and more attention was drawn to co-inhibitory receptors and their relevance in health and disease, especially as a potential therapeutic target. The first T cell regulatory receptors discovered are CTLA-4 (cytotoxic T-lymphocyte-associated protein-4; CD152) and PD-1 (programmed cell death protein-1; CD279), shortly followed by BTLA (B- and T- lymphocyte attenuator; CD272)[76-81]. In humans infected either with *Plasmodium vivax* or *Plasmodium falciparum*, CTLA-4 and PD-1 are highly expressed by T cells, especially $CD4^+$ T cells, in the peripheral blood [82, 83]. Furthermore, the impact of CTLA-4 and PD-1 on pathology was clearly demonstrated in experimental malaria. Both are crucial for the restriction of inflammation and blockade of either receptor aggravates experimental cerebral malaria [84, 85]. Consistent with this, engagement of BTLA by an agonistic antibody can reduce immunopathology in *Pb*A infected C57BL/6 mice [86]. Since the number of regulatory receptors discovered raises, the understanding of complex networks is of highest interest. Along this line, this thesis focuses on the dissection of the network consisting in addition to BTLA of the receptors HVEM (Herpesvirus Entry Mediator; CD270; TNFRSF14; ATAR; TR2), LIGHT (Homologous to **l**ymphotoxin, exhibits **i**nducible expression and competes for HSV **g**lycoprotein D for binding to **H**erpesvirus entry mediator, a receptor expressed by **T** lymphocytes; TNFSF14; CD258) and CD160 (BY55)[87-89].

### 1.6.1 HVEM, a molecular switch

Herpesvirus Entry Mediator, a member of the TNF receptor superfamily, acts as a co-stimulatory receptor, enhances T cell proliferation and provides pro-survival signals via NF-κB [90]. Due to its broad expression profile including T cells, B cells, endothelial and epithelial cells but also mast cells, HVEM is a central player in immune regulation [91-94]. Furthermore, HVEM can be considered as a molecular switch for T cell regulation because of its multiple interaction partners being either co-inhibitory (BTLA, CD160) or co-stimulatory

(LIGHT, CD160). Besides different function of the HVEM ligands, they also differ in their way of interaction. HVEM consists of four cysteine-rich domains (CRD), of which the CRD2/3 interacts with LIGHT while BTLA and CD160 compete for an overlapping binding region in CRD1 [87, 89, 95, 96]. Different binding regions allow the formation of cluster consisting of multiple HVEM-ligands e.g. LIGHT and BTLA or CD160 [87, 97].

LIGHT is able to bind HVEM in trans or as a soluble molecule (Figure 1.2 A, B)[88, 93]. In both cases, HVEM is clustered and T cells are co-stimulated [98]. Clustering of HVEM by soluble LIGHT enhances binding capacity of BTLA or CD160 presumably due to increased avidity (Figure 1.2 A)[87, 89, 98, 99]. However, interaction of membrane bound LIGHT with HVEM prevents BTLA from binding, most likely caused by steric hinderance (Figure 1.2 B)[99].

BTLA itself can interact both in *trans* and in *cis* [95, 100]. Depending on the interaction type, negative (BTLA) and positive (HVEM) signals are induced in the respective cells (Figure 1.2 D) or, in the case of *cis* interaction, no signal is induced at all (Figure 1.2 C)[95, 101]. By this, naïve T cells prevent signalling of BTLA and HVEM expressed by the same cell. Furthermore, soluble as well as membrane bound LIGHT is still able to bind to HVEM interacting in *cis* with BTLA but no signal transduction is induced upon LIGHT binding (Figure 1.2 C)[100].

The binding region of CD160 is distinct but overlaps with the BTLA binding region [89]. Due to this overlap, CD160 and BTLA compete for HVEM binding (Figure 1.2 D, E). The affinity of CD160 binding to HVEM is similar, but the dissociation rate is slower than for BTLA [102]. Furthermore, CD160 can be shed from the cell surface and might prevent interaction of HVEM and BTLA or membrane-bound CD160 (Figure 1.2 F)[103].

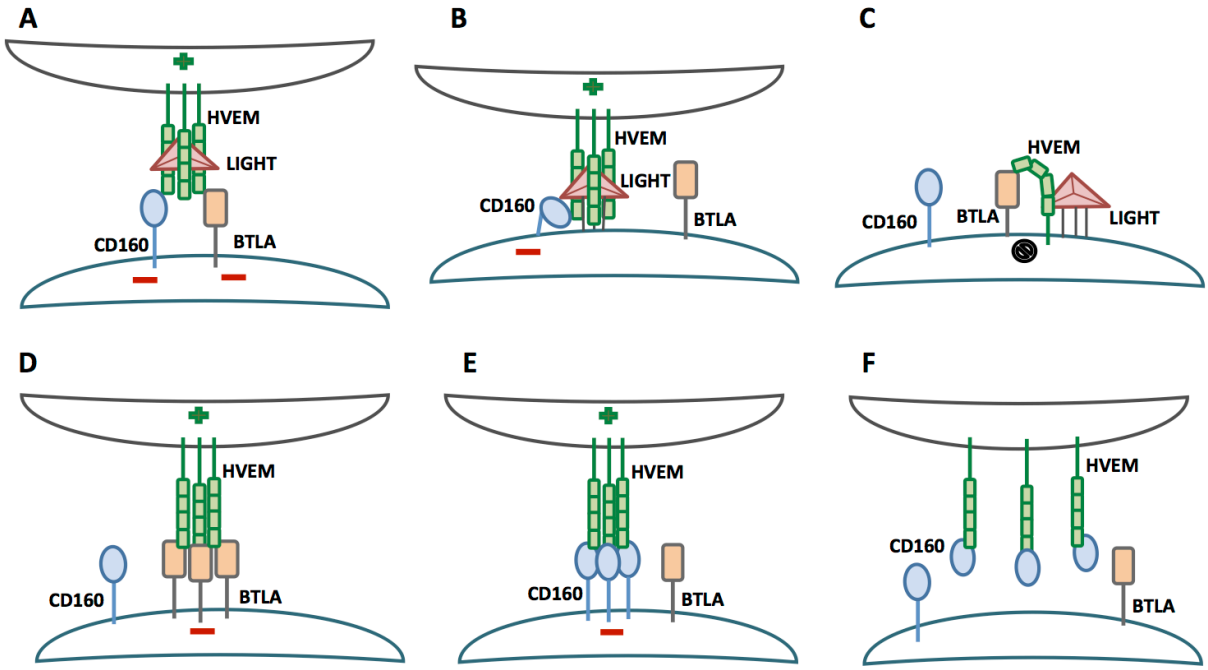



**Figure 1.2 Receptor-ligand interaction within the HVEM network. Possible Interactions of HVEM, LIGHT, CD160 and BTLA are depicted.** The interacting cells represent e.g. antigen-presenting cell-T cell, B cell-T cell, T cell-T cell. The outcome of signalling is indicated by plus (stimulatory) or minus (inhibitory). (A) Soluble LIGHT clusters HVEM, induces HVEM-signalling and enhances the binding capacity of CD160 or BTLA. (B) Membrane-bound LIGHT clusters HVEM, induces HVEM-signalling but prevents BTLA from binding due to steric hindrance. However, CD160 is able to bind, because of its higher flexibility. (C) *Cis* interaction of BTLA and HVEM prevents signalling of BTLA, HVEM, CD160 or LIGHT. (D, E) BTLA and CD160 compete for HVEM binding due to overlapping binding regions. (F) Soluble CD160 disrupts interaction of HVEM and membrane-bound CD160 or BTLA.

The complex network of modulatory molecules is influenced by the respective expression pattern on different cell types as well as the localisation and activation status of the cells. While LIGHT is involved in early activation of T cells [104, 105], BTLA is ubiquitously expressed on T cells [100] and CD160 can only be found on activated T cells [106].

In light of their function in experimental malaria, LIGHT and BTLA have been studied already. The absence of LIGHT-HVEM interaction does not influence the development of parasitemia or experimental cerebral malaria in *Pb*A infected C57BL/6 mice [107]. In contrast, BTLA can be utilised as an inhibitory switch due to reduced immunopathology by addition of an agonistic antibody [86]. However, the loss of BTLA in knockout mice enables the immune system to clear *Py*NL parasites significantly faster and more efficiently compared to wild type mice [108]. While research regarding BTLA has already shown promising results in the prevention of experimental cerebral malaria, CD160 might be an even more interesting target due to its restriction to activated T cells, promising higher specificity.

### 1.6.2 CD160

CD160 was first described as an activating receptor on cytolytic NK cells, which can also be found on γδ cells and on a minor subset of αβ T lymphocytes in human [109]. Later on,

CD160 was found on the majority of intestinal intraepithelial lymphocytes (iIELs) [110] and, more recently, on mast cells [111]. In line with the distribution of CD160 on leukocytes in human, CD160 was found on murine NK cells, NKT cells, mast cells, iIELs and on a minor fraction of $CD8^+$ T cells [111-113]. Interestingly, CD160 not only binds to classical and non-classical MHCI but also to HVEM [89, 112, 114]. While MHCI binding is consistently reported to co-stimulate the CD160 expressing cell [115-117], engagement of CD160 by HVEM on human $CD4^+$ T cells was reported to act inhibitory [89] but on human NK cells to enhance cytotoxicity [91].

In human, a transmembrane (CD160-TM) and a GPI-anchored (CD160-GPI) isoform of CD160, resulting from alternative splicing, are described [118-120]. For CD160-TM an involvement of the ERK signalling pathway is suggested [118]. A remaining question is, how CD160 as a GPI anchored molecule can provide any downstream signalling. Regarding T cells, reduction of the tyrosine phosphorylation of CD3ζ in human $CD4^+$ T cells and association with the lymphocyte-specific tyrosine protein kinase p56lck in $CD8^+$ T cells was shown [89, 115]. In contrast, for NK cells and B cell chronic lymphocytic lymphoma (CLL), signalling via the PI3K-ERK-MEK pathway has been proposed [121, 122]. Furthermore, the GPI-anchored CD160 can be shed from the cell surface and prevent signalling of the membrane-bound CD160 [103, 111, 123].

The complex network involving two ligands potentially providing different immunomodulatory outcomes in addition to other co-inhibitory receptors like BTLA, which is competing with CD160 for the HVEM binding site [102], hinders the interpretation of data.

However, this also highlights the importance of *in vivo* studies, since *in vitro* experiments may not reflect the interaction of important receptors expressed by various cell types in the environment of a certain disease [112].

## 1.7 Aim of the study

Malaria is a life-threatening disease with many unanswered questions regarding underlying immunological mechanisms explaining pathology and poor memory development. The tight control of T cell effector mechanisms is crucial to allow protective anti-parasitic immune response without the drawback of immunopathology due to extensive inflammation. Co-inhibitory receptors are important regulators of T cell proliferation, survival and effector

function depending on activation and differentiation of the T cell as well as ligand expression in the environment.

Since BTLA was shown to be a promising target for prevention of immunopathology [86], this study aims to dissect the involvement of immunomodulatory receptors in the same network, namely HVEM and CD160. HVEM represents a master molecular switch, a ligand of both BTLA and CD160. In contrast, CD160 was described to exhibit an inhibitory function similar to BTLA, but with an expression pattern highly restricted to activated T cells. Considering this, CD160 might represent an even more appropriate drug target than BTLA.

This study aimed to further understand the involvement of HVEM and CD160 in T cell regulation during blood-stage malaria. To this end, experimental infection of C57BL/6J mice with *Pb*A or *Py*NL allowed the detailed immunological analysis concerning parasite clearance and development of experimental cerebral malaria. Most importantly, murine data were compared to data from human patients.

# 2 Material

## 2.1 Laboratory equipment

**Table 2 Laboratory Equipment**

| Name | Company |
|---|---|
| Agarosegel electrophoresis chamber | BioRAD, Munich, Germany |
| Analytical scale | Satorius AG, Göttingen, Germany |
| Benchtop centrifuge | Eppendorf, Hamburg, Germany |
| Cell sorter FACSariaIII | Becton Dickinson, Heidelberg, Germany |
| ChemiDoc Touch Imaging System | BioRAD, Munich, Germany |
| Centrifuge | Eppendorf, Hamburg, Germany |
| Freezer (-20/-70°C) | Liebherr, Biberach, Germany |
| Fridge | Liebherr, Biberach, Germany |
| Incubator | Heraeus instruments, Hanau, Germany |
| Laminar flow FlowSafe B-(MaxPro)$^3$-130 | Berner, Elmshorn, Germany |
| LSRII | Becton Dickinson, Heidelberg, Germany |
| Microwave | Panasonic, Wiesbaden, Germany |
| Milli-Q purification system | Merck Millipore, Burlington, USA |
| Mr. Frosty freezing device | Zefa Laborservice, Harthausen, Germany |
| Multichanel pipetts | Eppendorf, Hamburg, Germany |
| Multistepper pipetts | Eppendorf, Hamburg, Germany |
| Nanodrop 2000C | ThermoScientific. Waltham, USA |
| PCR maschine (peqstar 96x Universal Gradient) | Peqlab, Erlangen, Germany |
| pH meter | Labotec, Dillenburg-Manderbach, Germany |
| Pipettboy accu-jet pro | Brand, Wertheim, Germany |
| Pipetts (2, 10, 20, 100, 200, 1000 µl) | Gilson, Middleton, USA |
| Power supply | BioRAD, Munich, Germany |
| Tea stainer | WMF, Geislingen, Germany |
| Thermomix | Eppendorf, Hamburg, Germany |
| Water bath | Haake, Kahlsruhe, Germany |

## 2.2 Glass and plastic consumables

**Table 3 Glass and plastic consumables**

| Name | Company |
|---|---|
| Cellstrainer | BD Biosciences, Heidelberg, Germany |
| CellTrics (50 µm) | Partec, Görlitz, Germany |
| Cryo tubes | Nunc, Wiesbaden, Germany |
| Cell culture plate (6/96 well U/R bottom) | Greiner bio-one, Frickhausen, Gemany |
| Cannula, Sterican | Braun, Melsungen, Germany |
| ELISA plates (microplate high binding) | Greiner Bio-One, Kremsmünster, Austria |
| FACS tubes | Sarstedt, Nümbrecht, Germany |
| Falcons (15, 50 ml) | Sarstedt, Nümbrecht, Germany |
| Glasbottle (50, 100, 200, 500, 1000 µl) | Schott AG, Mainz, Germany |
| Glasspipetts (2, 5, 10, 20 ml) | Brand, Wertheim, Germany |
| Glass slides | Engelbrecht, Edermünde, Germany |
| Neubauer counting chamber | Hecht-Assistent, Sonderheim, Germany |
| Petridish | Greiner bio-one, Frickhausen, Gemany |
| Pipett tips (10, 20, 200, 1000 µl) | Sarstedt, Nümbrecht, Germany |
| Syringe (1, 5 ml) | Braun, Melsungen, Germany |
| Tubes (0.5, 1, 2, 5 ml) | Eppendorf, Hamburg, Germany |

## 2.3 Dissecting instruments

**Table 4 Dissecting instruments**

| Name | Company |
|---|---|
| Forcept | Neolab Migge, Heidelberg, Germany |
| Scissors | Neolab Migge, Heidelberg, Germany |
| Lancet (Accu check Softclix) | Roche, Basel, Switzerland |

## 2.4 Chemicals

All chemicals were purchased at Roth, Sigma-Aldrich or Merck.

## 2.5 DNA oligonucleotides

All oligonucleotides were synthesised by Eurofins.

**Table 5 DNA oligonucleotides**

| Name | Sequence | Application |
|---|---|---|
| CRISS_fw | GCAGTGTCCTTACTGTCATAGA | Genotyping |
| CRISS_rev | GCTGTTCTAAGTTGGTCTCAGG | Genotyping |
| sgRNA_fw | GAAATTAATACGACTCACTATAGGGAGAGCACAAGAAAGACGA | Generation |
| | AGCTGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC | sgRNA |
| sgRNA_rev | AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGA | Generation |
| | CTAGCCTTATTTTAACTTGCTATTTCTAGCTCT | sgRNA |

## 2.6 Peptides

Peptides were ordered at Jerini Biotools.

**Table 6 Peptides**

| Name | Sequence |
|---|---|
| Pb1 | SQLLNAKYL |
| Pb2 | IITDFENL |
| F4 | EIYIFTNI |

## 2.7 Enzymes

Enzymes were ordered at Thermo Fisher Scientific and were used with corresponding buffers.

**Table 7 Enzymes**

| Name | Restriction site |
|---|---|
| BpU10I | 5` CC ↓ TNAGC 3`; 3` GGANT ↑ CG 5` |
| DreamTaq | |

## 2.8 Antibodies

**Table 8 Antibodies**

| Epitope | Species | Fluorochrome | Dilution | Clone | Company |
|---|---|---|---|---|---|
| CD3 | mouse | AF488 | 1:200 | 145-2C11 | BioLegend |
| CD3 | human | APC-Cy7 | 0.025 µl/100 µl | HIT3a | BioLegend |
| CD4 | mouse | V500 | 1:400 | RM4-5 | BD |
| CD8 | mouse | AF700 | 1:200 | 53-6.7 | BioLegend |
| CD8 | mouse | V450 | 1:400 | 53-6.7 | eBioscience |
| CD8 | human | AF700 | 0.25 µl/100 µl | RPA-T8 | BioLegend |
| CD11a | mouse | PE | 1:500 | M17/4 | eBioscience |
| CD11a | mouse | V450 | 1:500 | M17/4 | eBioscience |
| CD19 | mouse | PE | 1:200 | 1D3 | BioLegend |
| CD28 | mouse | APC | 1:200 | 37.51 | BioLegend |
| CD28 | human | BV510 | 1 µl/100 µl | CD28.2 | BioLegend |
| CD44 | mouse | PE-Cy7 | 1:400 | IM7 | BioLegend |
| CD45 | mouse | Fitc | 1:200 | - | Caltag |
| CD69 | human | Fitc | 1:100 | FN50 | BioLegend |
| CD107a | mouse | BV421 | 1:200 | 1D4B | BioLegend |
| CD160 | human | PE-Cy7 | 5 µl/100 µl | By55 | BioLegend |
| CD160 | mouse | PerCP-Cy5.5 | 1:200 | 7H1 | BioLegend |
| BTLA | mouse | PE | 1:200 | 6F7 | eBioscience |
| CTLA-4 | human | PE | 0.625 µl/100 µl | L3D10 | BioLegend |
| γδ | mouse | Fitc | 1:200 | eBioGL3 | eBioscience |
| GrzB | mouse/human | AF647 | 1:300 | GB11 | BioLegend |
| HVEM | mouse | APC | 1:200 | LH1 | eBioscience |
| IFNg | mouse | AF488 | 1:100 | XMG1.2 | Biolegend |
| Ki67 | mouse/human | AF488 | 1:300 | Ki-67 | Biolegend |
| KLRG1 | mouse | eF780 | 1:200 | 2F1 | eBioscience |
| Live/dead | - | - | 1:1000 | - | ThermoFisher Scientific |
| NK1.1 | mouse | PE-Cy7 | 1:400 | PK136 | BD |

| | | | | | |
|---|---|---|---|---|---|
| PD-1 | mouse | PE-Cy7 | 1:200 | RMPI-30 | Biolegend |
| PD-1 | human | PerCP-Cy5.5 | 0.625 µl/100 µl | EH12.2H7 | Biolegend |
| Perforin | human | BV421 | 5 µl/100 µl | δG9 | BD |
| TCRβ | mouse | APC | 1:200 | H57-597 | BD |

## 2.9 Eukaryotic cell line

**Table 9 Eukaryotic cell line**

| Name | Origin | |
|---|---|---|
| Hepa1-6H | Hepatoma, transduced with HVEM | Julie Sellau, BNITM, Hamburg, Germany |

## 2.10 Parasites

**Table 10 Parasites**

| Strain | Origin |
|---|---|
| *Plasmodium berghei* ANKA (PbA) | BNITM, Hamburg, Germany |
| *Plasmodium yoelii* non-lethal (PyNL) | Jean Langhorne, London, England |

## 2.11 Mouse strains

**Table 11 Mouse strains**

| Name | Origin |
|---|---|
| C57BL/6J | BNITM, Hamburg, Germany |
| $HVEM^{-/-}$ | Prof. Dr. Klaus Pfeffer, breeding at BNITM, Hamburg, Germany |
| $CD160^{-/-}$ | BNITM, Hamburg, Germany |

## 2.12 Patients

Malaria patients were recruited at the BNITM Ambulance or the University Medical Center Hamburg-Eppendorf. Characteristics of patients are displayed in Table 12 (N/A= not available). Staff of the BNITM was recruited as healthy controls.

**Table 12 Patients**

| # | Age [years] | Gender female [F], male [M] | Parasitemia [%] |
|---|---|---|---|
| 1 | 39 | M | < 1 |
| 2 | 33 | F | < 1 |
| 3 | 37 | F | < 1 |
| 4 | 52 | F | < 1 |
| 5 | 33 | M | 2 |
| 6 | 45 | F | 3 |
| 7 | N/A | M | N/A |
| 8 | 36 | M | 1.5 |
| 9 | 69 | M | < 1 |
| 10 | N/A | M | N/A |
| 11 | 41 | M | N/A |
| 12 | 21 | F | < 1 |
| 13 | 62 | M | < 1 |
| 14 | 35 | M | N/A |
| 15 | N/A | N/A | 1 |
| 16 | 26 | M | 3 |
| 17 | 40 | M | 8 |
| 18 | 26 | M | < 1 |
| 19 | N/A | M | N/A |
| 20 | N/A | N/A | N/A |

## 2.13 Reagents

### 2.13.1 General

**Table 13 General reagents**

| Name | Company |
|---|---|
| Incidin Liquid | Ecolab, Düsseldorf. Germany |

### 2.13.2 Molecular biology

**Table 14 Reagents for molecular biology**

| Name | Company |
|---|---|
| Agarose | Biomol, Hamburg, Germany |
| Ampuwa, water | Fresenius, Graz, Austria |
| DNA loading dye (6x) | ThermoFisherScientific, Waltham, USA |
| dNTPs | ThermoFisherScientific, Waltham, USA |
| Ethidiumbromide | Sigma, Deisenhofen, Germany |
| GeneRuler 500 bp DNA ladder | ThermoFisherScientific, Waltham, USA |

### 2.13.3 Cell culture

**Table 15 Reagents for cell culture**

| Name | Company |
|---|---|
| Cohn II | Sigma-Aldrich, St Louis, USA |
| DMEM high glucose (4,5 g/L) with 25 mM | PAA, Pasching, Austria |
| DPBS | Pan Biotech, Aidenbach, Germany |
| EDTA | Merck Millipore, Burlington, USA |
| Fetal calf serum (FCS) | PAA, Pasching, Austria |
| Fc-Block | BNITM, Hamburg, Germany |
| Gentamycine | PAA, Pasching, Austria |
| Heparin-sodium | Ratiopharm, Ulm, Germany |
| Hepes Buffer Solution 1M | PAA, Pasching, Austria |
| L-Glutamine | PAA, Pasching, Austria |
| Percoll | GE Healthcare, Little Chalfont, England |

| | |
|---|---|
| RPMI1640 | PAA, Pasching, Austria |
| Trypan blue solution 0.4% | Sigma-Aldrich, St Louis, USA |
| Trypsin-EDTA | PAA, Pasching, Austria |

### 2.13.4 Buffer

**Table 16 Buffer**

| Name | Content | Application |
|---|---|---|
| TAE buffer | 40 mM Tris base<br>45 mM boric acid<br>0.5 mM EDTA<br>pH 8 in $H_2O$ | Gel electrophoresis |
| TBE buffer | 89 mM Tris base<br>89 mM boric acid<br>2 mM EDTA<br>pH 8 in $H_2O$ | |
| Ethidium bromide solution | 10 mg/ml in $H_2O$ | |
| Loading buffer | 10 mM sodium dihydrogen phosphate<br>0.25% bromophenol<br>50% glycerol | |
| Substrate buffer | 0.1 M $NaH_2PO4$<br>pH 5.5 with $Na_2HPO_4$ | ELISA |
| PBSA (1%) | 1 g/L BSA in PBS | |
| PBSA (0.1%) | 0.1 g/L BSA in PBS | |
| TMB | 6 mg/ml in DMSO | |
| RBC lysis buffer (murine) | 10 nM tris pH 7.2<br>0.15 M ammonium chloride | FACS |
| RBC lysis buffer (human) | Dilute 1-step Fix/Lyse solution (10x Stock; eBioscience) 1:10 in $H_2O$ | |
| Wright stain | 1 mg/ml in methanol | Parasitemia |
| Digestion buffer | 30 mM EDTA<br>10% FCS<br>in PBS | IEL isolation |

| | | |
|---|---|---|
| RPMI complete | RPMI1640 supplemented with<br>5% FCS<br>1% L-Glutamine<br>0.5% Gentamycine | Cell culture |

## 2.14 Kits

**Table 17 Kits**

| Name | Company |
|---|---|
| DuoSet ELISA Mouse IFNγ | RnD Systems, Minneapolis, USA |
| LIVE/DEAD cell mediated cytotoxicity Kit | ThermoFisherScientific, Waltham, USA |
| Intracellular Fixation/Permeabilization Buffer Set | ThermoFisherScientific, Waltham, USA |
| Qiaex II Gel Extraction Kit | Quiagen, Hilden, Germany |

## 2.15 Software

**Table 18 Software**

| Program | Purpose |
|---|---|
| Adobe Reader XI | Reading PDF files |
| FlowJo 10 | Analysis of flow cytometry data |
| GraphPad Prism 5.0 | Statistical analysis |
| Microsoft Office for Mac 2011 | Word and graphic processing |

# 3 Methods

## 3.1 Generation of CD160$^{-/-}$ mice by CRISPR/Cas9

The CRISPR/Cas9 technology is derived from the immune system of bacteria, which enables bacteria to remove foreign DNA. It consists of a RNA that specifically targets a DNA sequence of choice (sgRNA) leading to DNA cleavage by the sgRNA-binding enzyme Cas9 and subsequently to DNA repair mechanisms. Since DNA repair by non-homologous end joining (NHEJ) is error prone, indel mutations are introduced in the target region.

### 3.1.1 sgRNA templates

Oligos consisting of the T7 promotor and target region were ordered at Eurofins. The oligo design was performed according to a protocol designed by Kristoffer Rieken (UKE) and target regions were selected using the online tool CHOPCHOP.

Oligos were amplified by PCR (see cycles below), purified by agarose gel electrophoresis (1.5% agarose in TAE buffer) and extracted with the Qiaex II Gel Extraction Kit according to the supplier's protocol. Purification was again controlled by agarose gel electrophoresis (1.5% agarose in TAE buffer). The purified PCR products were used as templates for transcription into RNA (done by Irm Hermans-Borgmeyer, ZMNH).

**PCR cycles:**

| | | | |
|---|---|---|---|
| | 95°C | 2 min | Denaturation |
| 30x | 95°C | 30 s | Denaturation |
| | 58°C | 30 s | Annealing |
| | 72°C | 30 s | Elongation |
| | 72°C | 30 s | Final elongation |

### 3.1.2 Injection of sgRNA/Cas9 and implantation

The injection of sgRNA and Cas9 protein into cytoplasm and pronucleus of one-cell staged C57BL/6 mouse embryos and the implantation into foster mothers was performed by Irm Hermans-Borgmeyer (ZMNH).

### 3.1.3 Genotyping

Genotyping of mice was performed by restriction-length fragment polymorphism (RLFP). Mice were marked by ear punch and the ear tissue samples were collected for genotyping. To lyse the tissue, 100 µl lysis buffer were added and incubated at 56 °C and 150 rpm over night. Afterwards, genomic DNA sequences consisting the target region were amplified by PCR using the lysate as template (see PCR mixture and cycles below). Successful and specific amplification was checked by agarose gel electrophoresis (1% agarose in TBE buffer) Subsequently, PCR products were digested by an enzyme specific for the wild type sequence without indel mutations introduced by CRISPR/Cas9. Hence, wild type DNA is digested into two smaller fragments, while modified DNA remains at the original size. With this, the discrimination of wild type, heterozygous and homozygous mice by agarose gel electrophoresis (2% agarose in TBE buffer) is possible.

**PCR mixture**

| | |
|---|---|
| 10x green reaction buffer | 5 µl |
| dNTPs (2 mM) | 5 µl |
| Fw CRISS | 0.5 µl |
| Rev CRISS | 0.5 µl |
| Dream Taq | 0.25 µl |
| $H_2O$ | 37.75 µl |
| Lysate | 1 µl |

**PCR cycles**

| | | | |
|---|---|---|---|
| | 95°C | 5 min | Denaturation |
| 35x | 95°C | 30 s | Denaturation |
| | 58°C | 30 s | Annealing |
| | 72°C | 30 s | Elongation |
| | 72°C | 5 min | Final elongation |

**Digestion** with Bpu10I

| | |
|---|---|
| Reaction buffer (10x) | 1 µl |
| Bpu10I | 0.5 µl |
| PCR product | 5 µl |

## 3.2 Cell culture

### 3.2.1 Maintenance

Cells in culture were handled under sterile conditions. Adherend cells were diluted when reaching confluence. Detaching from the cell culture surface was mediated by trypsin and was stopped by adding complete medium. Cells were washed and seeded in fresh medium.

### 3.2.2 Counting

To assess the cell concentration in suspension, cells were counted using a *Neubauer* counting chamber. The concentration was calculated by the following formula:

Cell count/big square x dilution factor x $10^4$ = cells/ml

## 3.3 Cytotoxicity assay

The cytolytic effector function of $CD8^+$ T cells was analysed by a flow cytometry based assay (LIVE/DEAD cell mediated cytotoxicity Kit, ThermoFisherScientific) according to the supplier's protocol. First, target cells, namely splenocytes from naïve mice, were pulsed with 1 µg/ml of the *Pb*-peptides Pb1, Pb2 and F4 for 3 h at 37°C. Afterwards, cells were washed, diluted to 1x $10^6$ cells/ml and stained with 0.25 µl/ml DIOC for 20 min in PBS. To remove DIOC from the supernatant, cells were washed twice in PBS and suspended in complete medium to a concentration of 1x $10^6$ cells/ml. Second, effector cells from *Pb*A infected mice at day 6 p.i. were sorted for $CD44^{hi}CD160^{+/-}$ cells and diluted to a concentration of 7.7x $10^5$ cells/ml. To detect dead cells, a 1 µg/ml propidium iodide solution in complete medium was used.

**Assay setup:**

| | | |
|---|---|---|
| Effector cells | 130 µl | 1x $10^5$ cells/well |
| Target cells | 10 µl | 1x $10^4$ cells/well |
| PI solution | 130 µl | |

## 3.4 *Pb*A-specific *ex vivo* stimulation

Stimulation of *Pb*A specific $CD8^+$ T cells was performed with a mixture of *Pb*-specific peptides, namely Pb1, Pb2 and F4, which are MHCI restricted. In order to load the peptide on MHCI molecules of e.g. Hepa1-6H cells, cells were cultured in medium supplemented with 1 µg/ml of each peptide. After three to four hours the medium was removed and cells were washed with PBS. Subsequently, $CD8^+$ T cells were added in medium and co-cultured with the peptide-presenting cells.

## 3.5 ELISA (enzyme linked immunosorbent assay)

For detection of cytokines from cell culture supernatants, sandwich ELISAs were performed according to the supplier's protocol (RnD). Briefly, ELISA plates were coated with an antibody specific for the corresponding cytokine over night at room temperature in PBS. To avoid unspecific binding, plates were blocked with 1% PBSA for 2 h at room temperature. Blocked plates were either stored in blocking buffer at -20°C or directly incubated with samples and a serial dilution of standard for at least 4 h or over night at room temperature. Afterwards a second antibody against the specific protein was added for 2 h. For detection a horseradish peroxidase (HRP) was used, which catalyses the change of colour of a supplied substrate. The enzymatic reaction was stopped by acidification with 2 M sulphuric acid. After each incubation step, the plate was washed three times. Incubation was always performed at room temperature and in the dark.

## 3.6 Flow cytometry

Flow cytometry allows the analysis of the morphology by forward and side scatter (FSC/SSC) and the expression of extra- and intracellular molecules by detection via fluorescence labelled antibodies.

Cells derived from mice were stained according to the following protocol. Per sample 2-4 x $10^6$ cells were used. To discriminate living and dead cells, samples were first incubated with a live/dead staining reagent for 20 min in PBS at 4°C. After washing the cells once, antibodies directed against surface epitopes were incubated for 20-30 min in Fc blocking buffer at 4°C. By using Fc blocking buffer unspecific binding of antibodies to Fc receptors was avoided.

Antibodies were removed by washing with PBS and, in case intracellular epitopes were of interest, cells were fixed subsequently. For fixation, 500 µl or 100 µl Fix buffer was added to the tube or well, respectively. If tubes were used, buffer was added dropwise while the tube was mixed gently. In case of staining in plates, cells were suspended in fix buffer immediately. To permeabilise cells, they were washed twice with permeabilisation buffer. Antibodies directed against intracellular epitopes were diluted in permeabilisation buffer to ensure membrane permeability. Staining was performed for 30 min at 4°C. Afterwards cells were washed at least twice with permeabilisation buffer.

Samples were measured if not mentioned otherwise using the LSRII.

## 3.7 Cell sorting

For sorting, cells were isolated from spleens and stained for the surface molecules of interest as described above. The sort was performed using a BD FACSAria cell sorter. Cells were suspended in PBS and diluted to a final concentration of 2-4 x $10^7$ cells/ml. Sorted cells were collected in complete media at 4°C.

## 3.8 Mice

All experiments were approved of the office for consumer protection of the city of Hamburg (56/13; 32/15).

### 3.8.1 Isolation of organs

Mice were culled by slow application of $CO_2$. Reflexes were checked to ensure sufficient anaesthesia. Except for the experiments where heart puncture needed to be performed, a cervical dislocation was done. Tissues were collected in 3 ml cold PBS.

Dependent on further applications, blood was collected in PBS or undiluted in tubes with or without EDTA.

#### *3.8.1.1 Spleen*

Spleens were collected in 3 ml cold PBS and kept on ice. Splenocytes were isolated by mashing of the tissue structure with the stamp of a 5 ml syringe and filtration through a 70 µm cell strainer. Cell strainers were washed with 5 ml cold PBS. Afterwards, the filtered cells were centrifuged (300 g, 5 min, 4°C), supernatant removed and the pellet suspended in 3 ml RBC lysis buffer. RBCs were lysed at room temperature for 5 min. To stop the lysis, 3 ml medium was added, cells were centrifuged again, and pellets were suspended in PBS or medium depending on further applications.

#### *3.8.1.2 Blood*

In order to remove heparin or EDTA from blood samples for flow cytometry, the blood was diluted in PBS, centrifuged (500 g, 5 min, RT), and the supernatant carefully removed. The pellet was suspended in 1 ml RBC lysis buffer and incubated for 5 min at room temperature. The lysis was stopped by the addition of 1 ml PBS. Cells were centrifuged and the supernatant removed. If cells should not be fixed after staining, an additional lysis step was performed similar to the first one to increase efficiency of the RBC removal. Since the fixation procedure (see flow cytometry) leads to lysis of RBCs, a second incubation with RBC lysis buffer was not necessary. In order to remove residual lysis buffer, cells were washed twice with PBS before proceeding with staining protocol.

In order to receive plasma samples blood was collected in EDTA tubes, centrifuged (600 g, 15 min, RT) and the upper layer transferred into a fresh tube. Plasma was stored at -20°C.

#### *3.8.1.3 Brain*

Brains were collected in 3 ml cold PBS. The tissue and buffer were transferred into petri dishes, were they were cut into very small pieces using scissors. Afterwards, 5 ml PBS were added and cells from brain tissue pieces were suspended by pipetting with a 10 ml glass pipette until the buffer appears milky. In order to remove tissue residues, cells were filtered through a 70 µm cell sieve. Before cells were supplied to staining, they were washed twice.

#### *3.8.1.4 Intestinal intraepithelial Lymphocytes (iIEL)*

The intestine was dissected from the stomach to the ceacum and placed at a PBS drained tissue. Here, Peyer's patches and fatty tissue was removed, the intestine opened longitudinally, gently washed in PBS and cut into 1 cm pieces. The pieces were digested in 20 ml digestion medium for 30 min at 37°C. To improve the suspension of intraepithelial lymphocytes, the tubes were shaken regularly. After 30 min, the tubes were vigorously shaken 10x and the suspension filtered through a tea sieve. The washing step was repeated twice by adding 10 ml digestion medium to intestine pieces followed by 10x shaking and filtration. Since with the digestion not only the cells of interest but also the mucosa was brought into solution, a percoll gradient was performed. To this end, cells were centrifuged (5 min, 370 g, RT), the supernatant discarded, the pellet suspended in 5 ml 37% percoll in PBS and centrifuged at 520 g for 20 min at RT without beak. The supernatant containing percoll and mucosa was removed and the cell pellet washed twice in PBS.

### 3.8.2 Infection with *Plasmodia*

For the infection of mice with *Pb*A or *Py*NL for experiments, fresh blood of mice previously infected with stabilate was used. By using fresh blood rather than stabilate for infection, vitality and fitness of parasites are increased, which results in higher infection rates and better reproducibility.

To obtain fresh blood with parasitized RBCs, mice were infected with 5x $10^6$ iRBC from stabilate in 200 µl PBS i.p. After 6-8 days, when parasitemia reached 0.5- 3%, mice were culled and a heart puncture was performed. The blood was drawn using a heparinised syringe. Afterwards, the blood was diluted in PBS (200 µl blood in 1800 µl PBS). Pending on parasitemia and RBC concentration, the blood was further diluted with PBS to achieve

5x $10^5$ iRBC/ml. Mice were infected with 1x $10^5$ iRBC in 200 µl PBS i.p. One day post infection, mice were monitored for swelling in the area of injection.

### 3.8.3 Survival

Incidence of cerebral malaria was analysed by survival experiments. Mice were infected as described in 3.8.2 and weight and the health status was monitored during the course of disease. Cerebral symptoms were assessed corresponding to the following scoring system.

| Score | Symptoms |
|---|---|
| 0 | no symptoms |
| 1 | ruffled fur, decreased activity |
| 2 | uncertain movement |
| 3 | apathetic, not able to grab grid, not reacting to touching, no movement |
| 4 | coma |
| 5 | death |

Mice showing a CM score equal or higher than 3 or a weight loss of more than 20% were culled.

### 3.8.4 Determination of the parasitemia

Since RBCs lack nuclei, parasitized RBCs can be detected by DNA staining. To this end, thin blood smears were incubated with approximately 8 drops of Wright stain solution. After 2 min of incubation 8 drops of $ddH_2O$ were added and incubated for 2 additional minutes. Staining solution was removed by washing with $ddH_2O$.

The parasitemia was determined by counting approximately 1000 total RBCs and the respective number of infected RBC.

## 3.9 Human

All experiments performed with human blood were approved of the ethics committee of the Medical Association Hamburg (PV4539).

### 3.9.1 Staining of whole blood samples

To analyse human blood lymphocytes for the expression of different markers by flow cytometry, 120 µl whole blood was used.

Surface epitopes were stained by addition of antibodies directly to the blood sample and incubation for 30 min at 4 °C. In addition, a live/dead staining reagent was added during surface staining. Afterwards, lymphocytes were fixed and RBCs were lysed by incubation

with 2 ml RBC lysis buffer for 12 min at room temperature. Lysis buffer was removed by washing with PBS. In case intracellular epitopes were also of interest, a second fixation with fix buffer for 30 min at room temperature was performed. Subsequently, cells were permeabilised by washing twice with permeabilisation buffer. To block unspecific binding of antibodies, 10 µl CohnII were added and incubated for 10 min at 4°C. After 10 min antibodies were added and the mix was incubated for 20 min. To remove unbound antibodies, cells were washed twice. Samples were analysed using the LSRII.

# 4 Results

The HVEM-BTLA-CD160-LIGHT pathway, consisting of co-inhibitory and co-stimulatory receptors and their ligands, provides the immune system with a fine-tuned molecular switch. As the pertinence of other co-inhibitory molecules like PD-1 and CTLA-4 has emerged during the past years, this work sheds light on the role of HVEM and its interaction partner CD160 during malaria.

Using the well-established model for cerebral malaria, infection of C57BL/6J mice with *Plasmodium berghei* ANKA (*Pb*A), mice deficient of HVEM were analysed for clinical outcome and the phenotype of their $CD8^+$ T cells. In addition, the expression pattern of CD160 during the course of the disease and the functionality of $CD160^+$ T cells was investigated.

Since comparability of experimental models and human disease is of major interest, the CD160 expression pattern was also analysed in human *Plasmodium falciparum* (*Pf*) malaria, confirming findings of the mouse model.

In the last part of this work the development of a $CD160^{-/-}$ mouse using the CRISPR/Cas9 technology is depicted, providing a highly valuable tool to further investigate the functional role of CD160.

## 4.1 The role of HVEM in T cell regulation during experimental malaria

Data from inflammatory diseases like colitis, autoimmune encephalitis or Crohns disease [124-127] emphasize the important role of HVEM in immune regulation, leading to the question how HVEM influences the progression of malaria disease.

To address this question, $HVEM^{-/-}$ mice were analysed during blood-stage *Pb*A infection. First of all, clinical data regarding parasitemia, the occurrence and the severity of cerebral symptoms was monitored. Mice were scored during the course of the infection and culled when they reached a score of 3 or higher. In this setting, $HVEM^{-/-}$ mice develop severe cerebral symptoms significantly later and to a lesser extend than wild type mice (Figure 4.1).

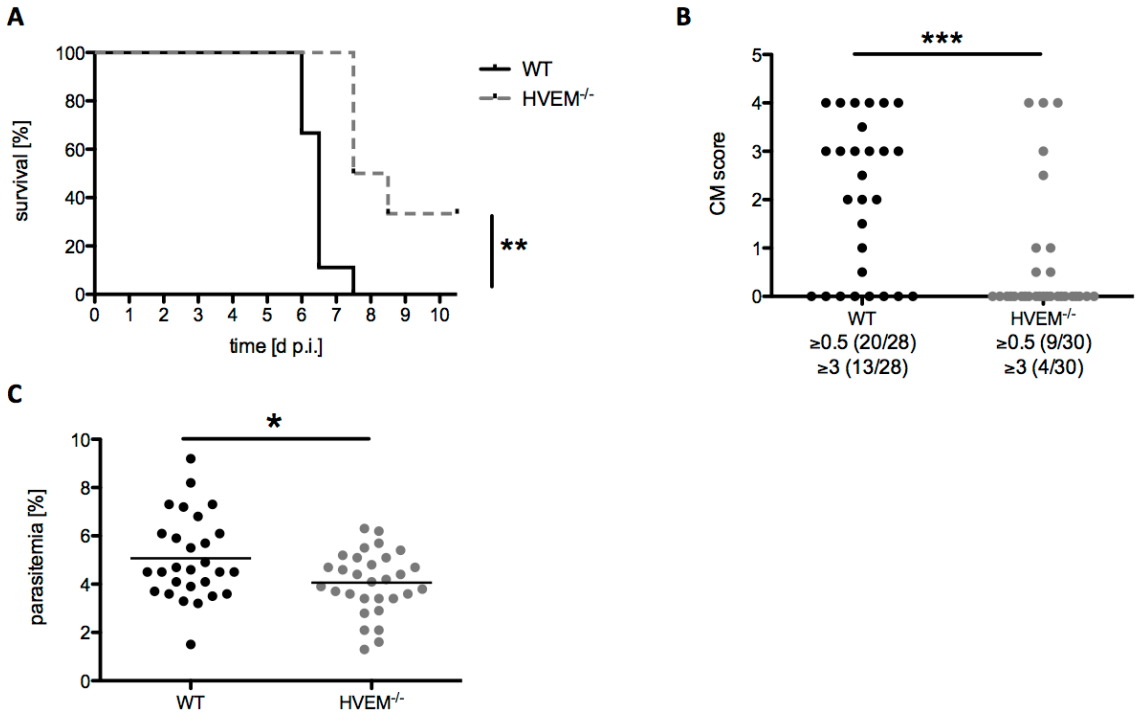



**Figure 4.1 HVEM$^{-/-}$ mice are less susceptible for cerebral malaria and develop a lower parasitemia.** (A) Survival of WT and HVEM$^{-/-}$ mice infected i.p. with $10^5$ *Pb*A iRBC. Mice showing a score ≥3 were euthanised. 8 (WT) or 6 (HVEM$^{-/-}$) mice were included in the experiment. Significance was calculated by Mantel-Cox Test, p=0.0013. (B) Incidence and severity of cerebral symptoms at day 6 post *Pb*A blood-stage infection was monitored. The number of mice showing at least mild symptoms (CM Score ≥0.5) and the number of severe cases (CM Score ≥3) of all analysed WT or HVEM$^{-/-}$ mice are annotated. Pooled data of 8 independent experiments are shown. Each circle represents one individual. Significance was calculated by unpaired t test, p=0.0009. (C) Parasitemia at day 6 post *Pb*A blood-stage infection was analysed in WT and HVEM$^{-/-}$ mice. Pooled data of 8 independent experiments are shown. Each circle represents one individual and means are depicted. Significance was calculated by unpaired t test, p=0.014.

With the objective to find the underlying immunological mechanisms by which HVEM favours the development of severe disease, T cells were analysed at the peak of their activation and concurrently the onset of cerebral malaria at day 6 p.i. Because the mouse model addresses the blood stage of the infection, tissues of interest were blood and spleen. In addition, the brain was analysed because of the cerebral pathology.

Interestingly, HVEM$^{-/-}$ mice showed a decreased number of CD8$^+$ T cells in the blood and at least in trend also in the brain, whereas CD4$^+$ T cell counts were higher in the spleen and in the blood (Figure 4.2).

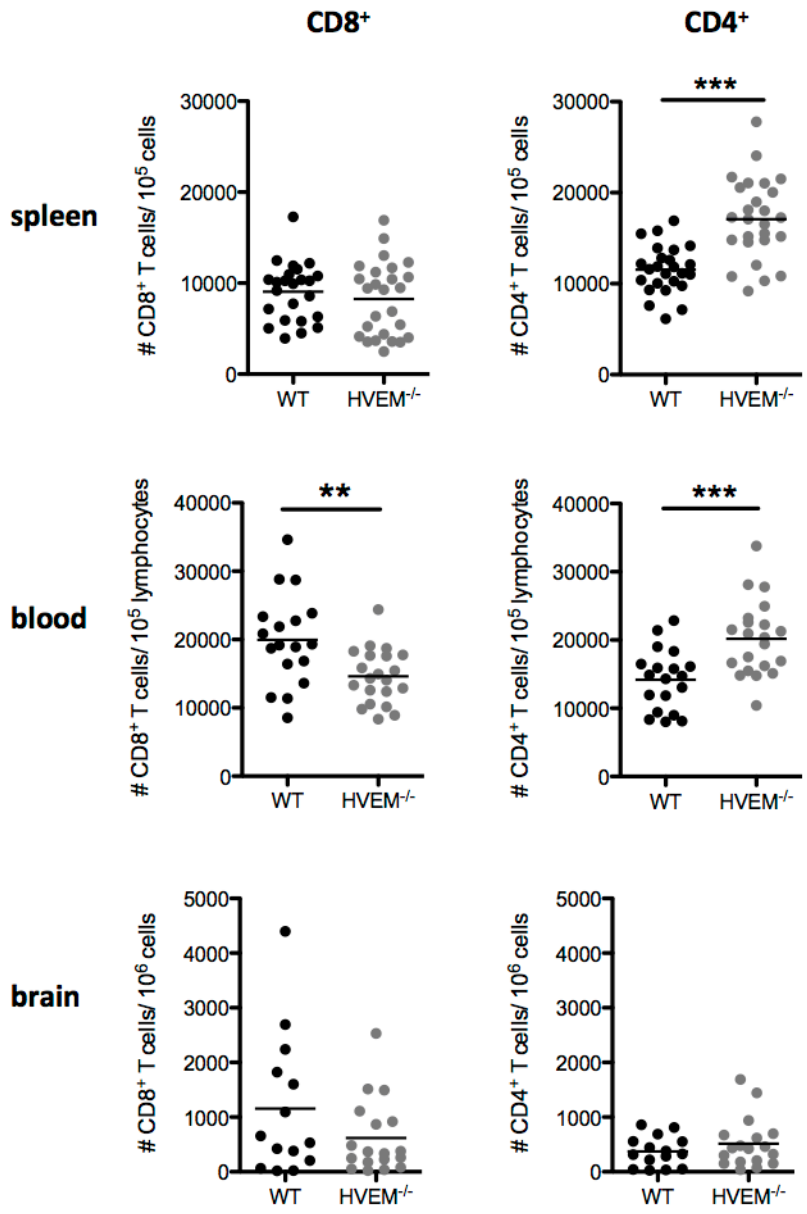



**Figure 4.2 HVEM$^{-/-}$ mice exhibit a smaller pool of CD8$^+$ T cells and more CD4$^+$ T cells than wild type mice.** WT and HVEM$^{-/-}$ mice were infected i.p. with $10^5$ *Pb*A iRBC. Cells isolated from spleen (A), blood (B) or brain (C) at d 6 p.i. were analysed by flow cytometry regarding the number of CD4 or CD8 expressing cells. Pooled data of 4-6 independent experiments are shown. Each circle represents one individual and means are depicted. Significance was calculated by unpaired t test: **p≤ 0.01; *** p≤ 0.001.

Cerebral malaria might be less pronounced in HVEM$^{-/-}$ mice due to lower number of cytotoxic T cells, which have been described as main mediators of pathology [128] or by a tight control of inflammation by regulatory CD4$^+$ T cells. To dissect the underlying mechanisms, the phenotype of CD4$^+$ and CD8$^+$ T cells was analysed.

While classical regulatory T cells (CD4$^+$Foxp3$^+$CD25$^+$) were shown to play only a minor role during *Pb*A blood-stage infection [75], acute malaria induces PD-1$^+$CTLA-4$^+$Foxp3$^-$ CD4$^+$ T

cells with regulatory properties [82, 85]. Both regulatory cell types were monitored during blood-stage *Pb*A infection of both wild type and HVEM$^{-/-}$ mice.

A similar frequency of CD4$^{+}$ T cells expresses the co-inhibitory receptors PD-1 and CTLA-4 in the spleen and the blood of wild type and HVEM$^{-/-}$ mice (Figure 4.3 A). Even though classical regulatory T cells (CD4$^{+}$Foxp3$^{+}$CD25$^{+}$) are only in trend higher in frequency in the blood with no alteration in the spleen, the count of regulatory T cells is significantly higher in HVEM$^{-/-}$ mice (Figure 4.3 B). This is in line with the enhanced CD4$^{+}$ T cell count (Figure 4.2). However, the number of PD-1 and CTLA-4 expressing cells is not altered in HVEM$^{-/-}$ compared to the wild type mice (data not shown).

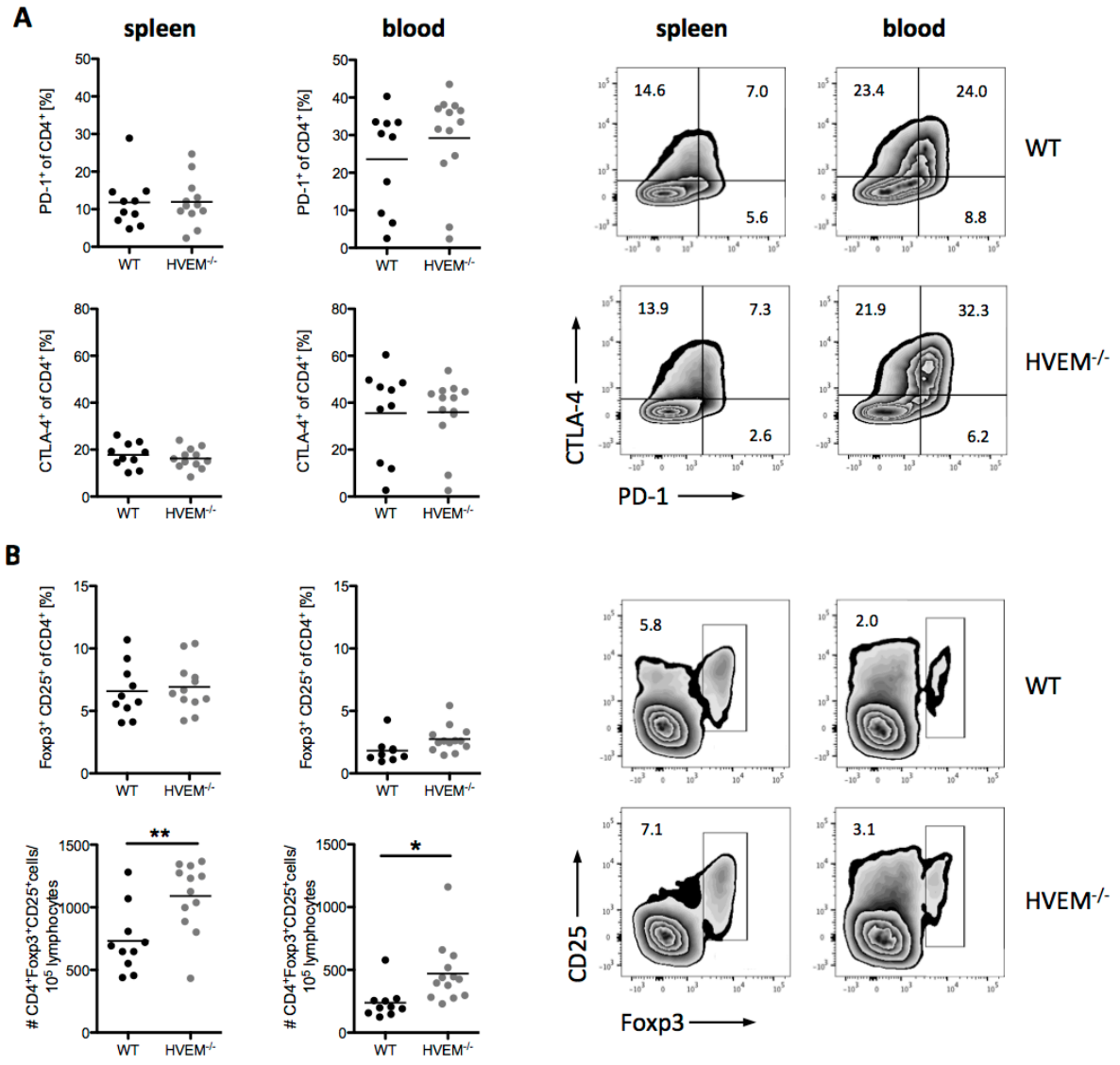
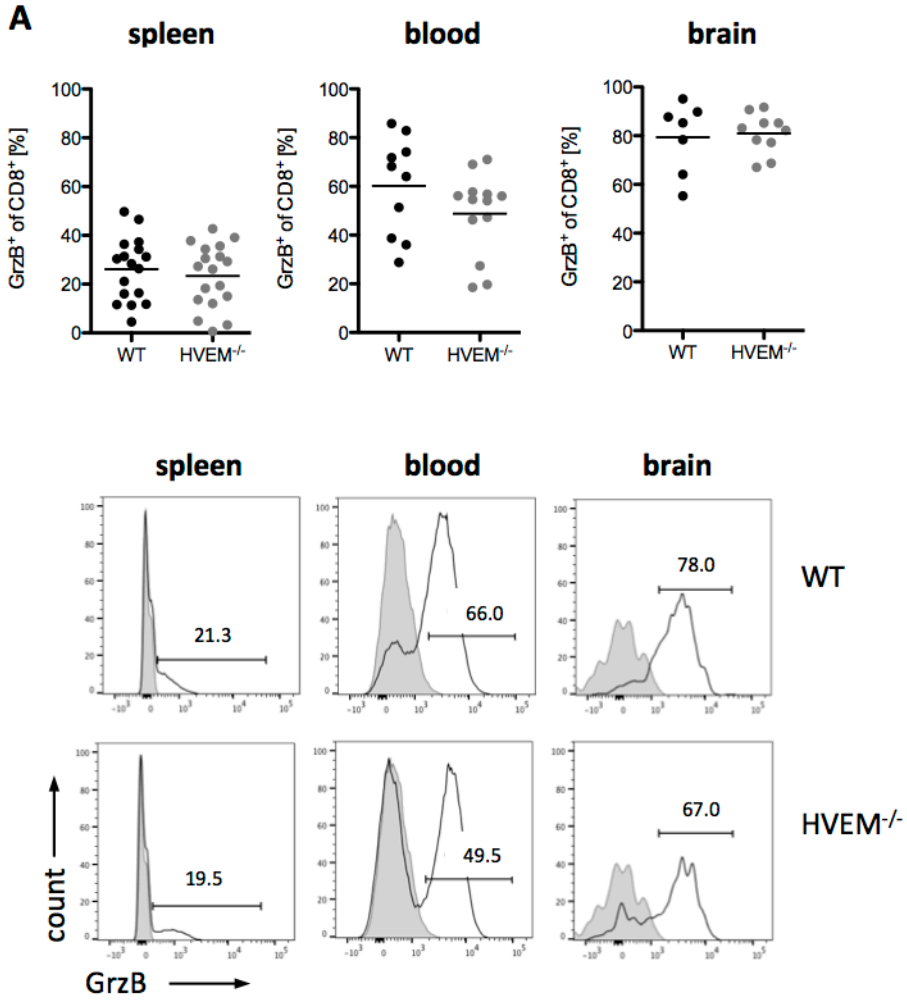



**Figure 4.3 HVEM$^{-/-}$ mice exhibit higher numbers of Foxp3$^{+}$CD25$^{+}$ Tregs compared to wild type mice.** WT and HVEM$^{-/-}$ mice were infected i.p. with $10^5$ *Pb*A iRBC and tissues were collected at d 6 p.i. Isolated cells were analysed for (A) PD-1 and CTLA-4 or (B) CD25 and Foxp3 expression of CD4$^{+}$ T cells. The quantitative overview shows pooled data of 3 independent experiments. Each circle represents one individual and means are depicted. Representative plots are shown. Significance was calculated by unpaired t test: * p≤ 0.05; **p≤ 0.01.

Taking into account that regulatory T cells are higher in number, the question arose, if subsequently $CD8^+$ T cells are less activated and functional. This was especially interesting, since $CD8^+$ T cells are also lower in number in $HVEM^{-/-}$ mice (Figure 4.2).

Effector functions of $CD8^+$ T cells include the production of pro-inflammatory cytokines naming IFNγ as one of the most important and cytotoxic molecules like granzyme B (GrzB). The release of cytotoxic molecules leads to the exposure of CD107a, a membrane bound molecule found in intracellular granules. Hence, staining of CD107a on the cell surface allows the detection of recently degranulated cells. In Figure 4.4 the frequency of $GrzB^+$ and $CD107a^+$ $CD8^+$ T cells is depicted. Both molecules are equally expressed in both mouse strains.



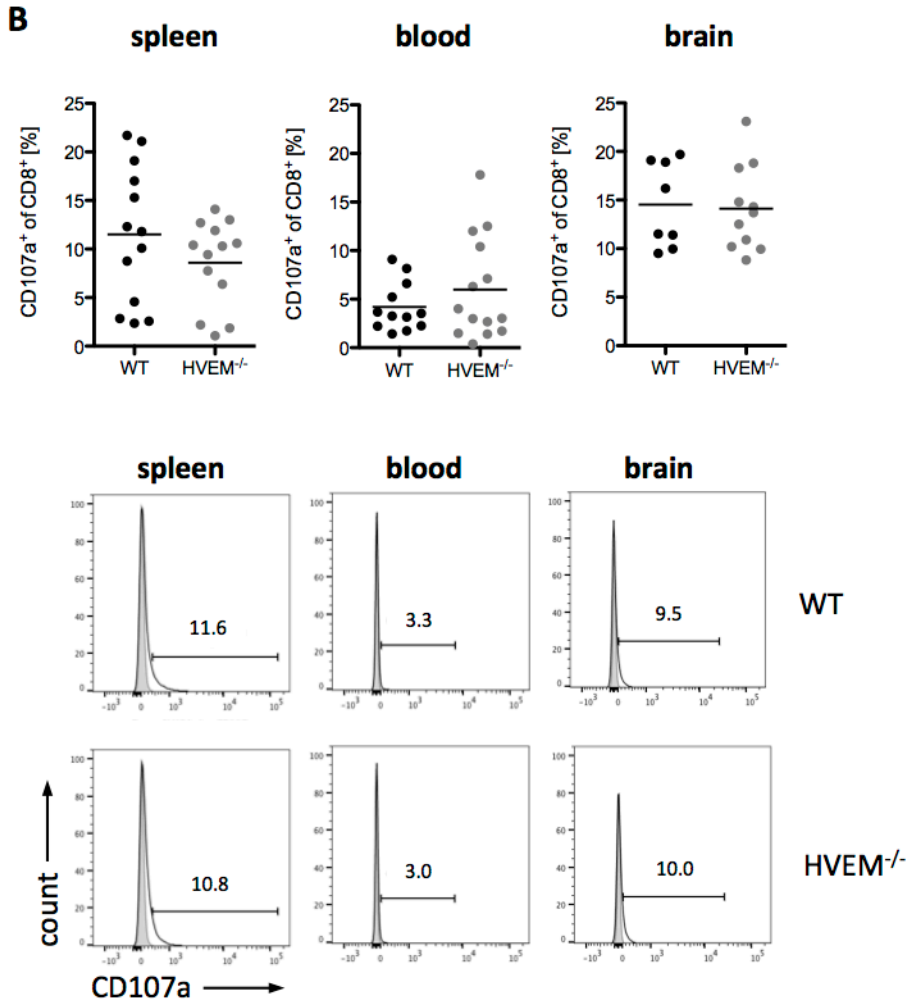



**Figure 4.4 Cytotoxicity of CD8$^+$ T cells is not impaired in HVEM$^{-/-}$ mice.** WT and HVEM$^{-/-}$ mice were infected i.p. with $10^5$ *Pb*A iRBC and tissues were collected at d 6 p.i. Isolated cells were analysed for intracellular GrzB expression (A) and for the degranulation marker CD107a (B) at the cell surface of CD8$^+$ T cells. The quantitative overview shows pooled data of 3 independent experiments. Each circle represents one individual and means are depicted. Representative histograms are shown.

Next, CD8$^+$ T cells from infected mice were analysed for intracellular IFNγ after stimulation with PMA/ionomycin *in vitro*. Only CD8$^+$ T cells showing an activated phenotype (CD44$^{hi}$) produced IFNγ, as shown in the representative plots in Figure 4.5, and, again, both mouse strains behaved akin.

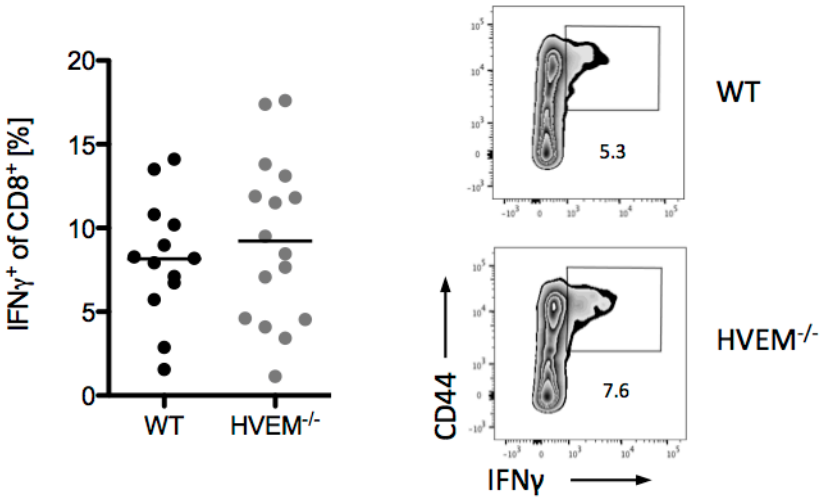



**Figure 4.5 The capacity of CD8$^+$ T cells to produce IFNγ is not impaired in HVEM$^{-/-}$ mice.** WT and HVEM$^{-/-}$ mice were infected i.p. with $10^5$ *Pb*A iRBC and spleens were collected at d 6 p.i. Splenic cells were *in vitro* stimulated by PMA/ionomycin in the presence of monensin. Intracellular IFNγ of CD8$^+$ T cells was analysed by flow cytometry. The quantitative overview shows pooled data of 3 independent experiments. Each circle represents one individual and means are depicted. Representative plots are shown.

In summary, CD8$^+$ T cells of HVEM$^{-/-}$ mice are not altered in either their expression of cytotoxicity marker or their capacity to produce IFNγ, indicating that HVEM$^{-/-}$ CD8$^+$ T cells posses no defect in their effector function.

However, not only the effector function but also the ability of the effector cells to bind to brain endothelial cells might contribute to the CD8$^+$ T cell mediated pathology [129]. For this reason, the expression of CD44 and CD11a was analysed on CD8$^+$ T cells of *Pb*A blood-stage infected WT and HVEM$^{-/-}$ mice at day 6 p.i. While no difference between WT and HVEM$^{-/-}$ could be observed in the spleen, a significant lower frequency of CD8$^+$ T cells in the blood of HVEM$^{-/-}$ mice express CD11a and CD44 (Figure 4.6). In addition, it becomes clear, that the frequency of CD44$^{hi}$/CD11a$^+$ cells is highly dependent on the analysed tissue. Both markers are highly abundant on CD8$^+$ T cells in the brain with intermediate level in the blood and lowest frequency in the spleen (Figure 4.6).

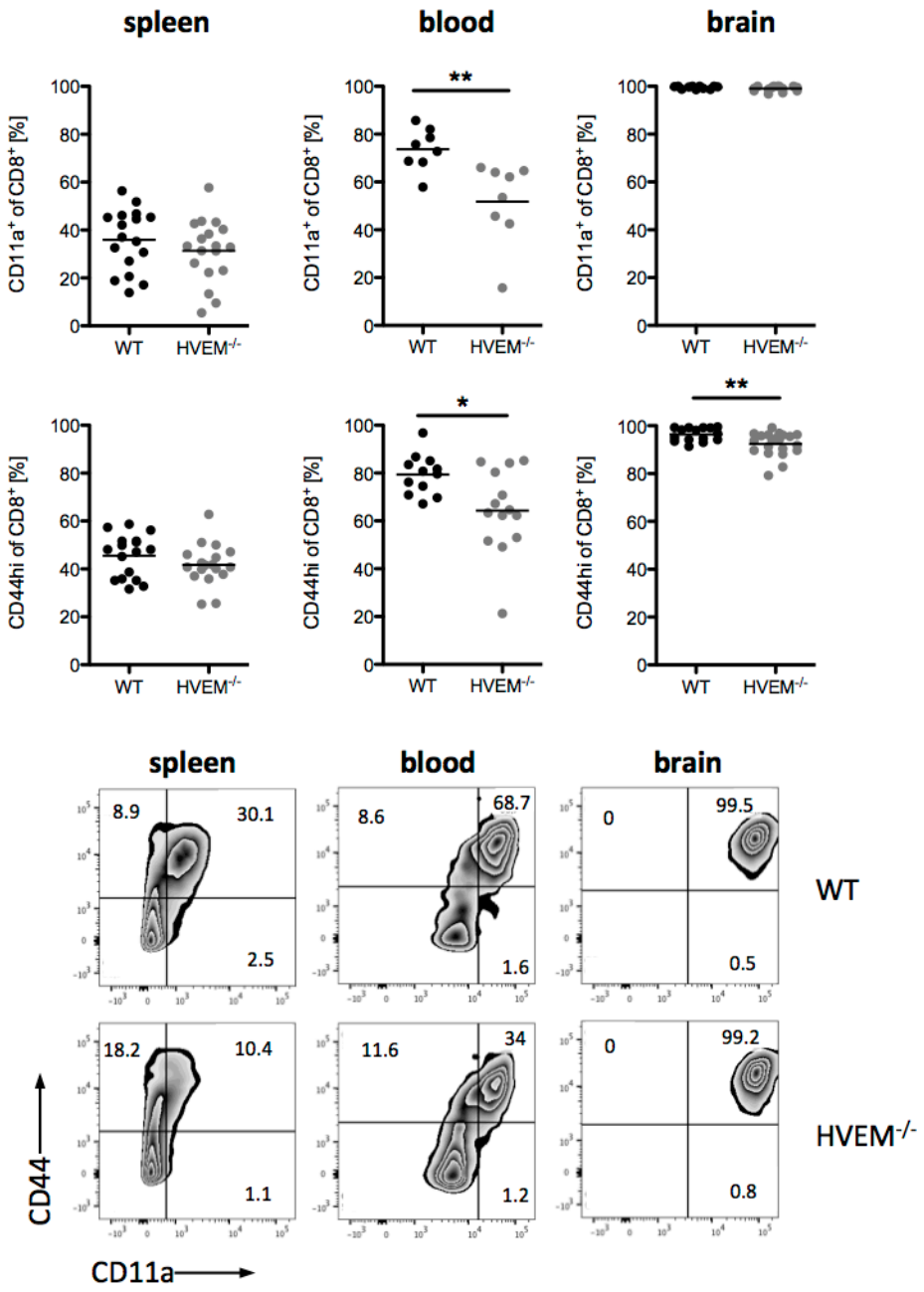



**Figure 4.6 In HVEM$^{-/-}$ mice a lower frequency of CD8$^+$ T cells expresses CD44 and CD11a in the blood.** WT and HVEM$^{-/-}$ mice were infected i.p. with $10^5$ *Pb*A iRBC and tissues were collected at d 6 p.i. Isolated cells were analysed for CD44 and CD11a expression by CD8$^+$ T cells. The quantitative overview shows pooled data of 2-5 independent experiments. Each circle represents one individual and means are depicted. Representative plots are shown. Significance was calculated by unpaired t test: * p≤ 0.05; ** p≤ 0.01.

As mentioned above (Figure 4.2), the total number of CD8$^+$ T cells is lower in HVEM$^{-/-}$ mice compared to wild type mice, which might be due to a lower proliferation rate. To investigate this, proliferation of CD8$^+$ T cells of infected mice was analysed using the marker Ki67. Surprisingly, the expression of the proliferation marker Ki67 was neither in the spleen nor in blood or brain altered in HVEM$^{-/-}$ mice (Figure 4.7).

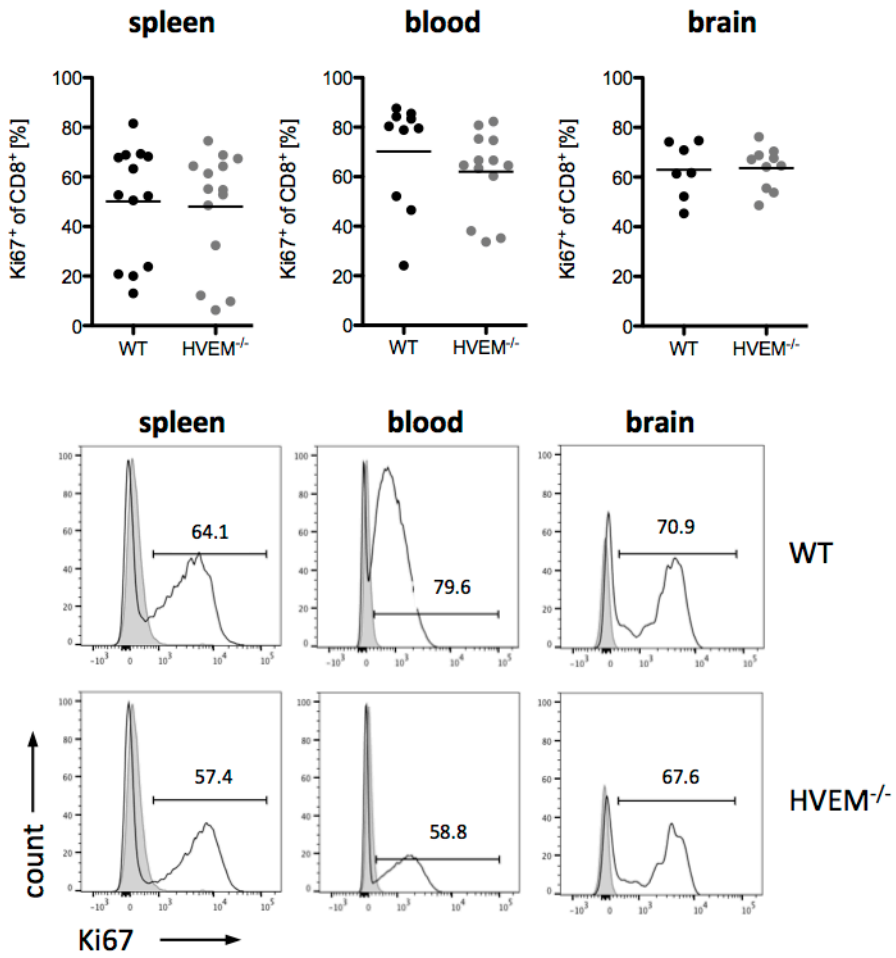



**Figure 4.7 HVEM$^{-/-}$ CD8$^{+}$ T cells show no defect in proliferation.** WT and HVEM$^{-/-}$ mice were infected i.p. with $10^5$ *Pb*A iRBC and tissues were collected at d 6 p.i. Isolated cells were analysed for Ki67 expression by CD8$^{+}$ T cells. The quantitative overview shows pooled data of 3 independent experiments. Each circle represents one individual and means are depicted. Representative plots are shown.

While Ki67 is commonly used as a marker for all cells being in the active phase of the cell cycle, only excluding the G0 phase, it may not allow to detect a defect in initial clonal expansion of recently activated cells. To assess, whether CD8$^{+}$ T cells are able to respond to IL-2, which is needed for clonal expansion, the high-affinity IL-2 receptor chain, namely CD25, was of interest. Actually, the percentage of CD25$^{+}$ CD8$^{+}$ T cells was significantly higher in the blood and higher in trend in the spleen of HVEM$^{-/-}$ compared to wild type mice (Figure 4.8).

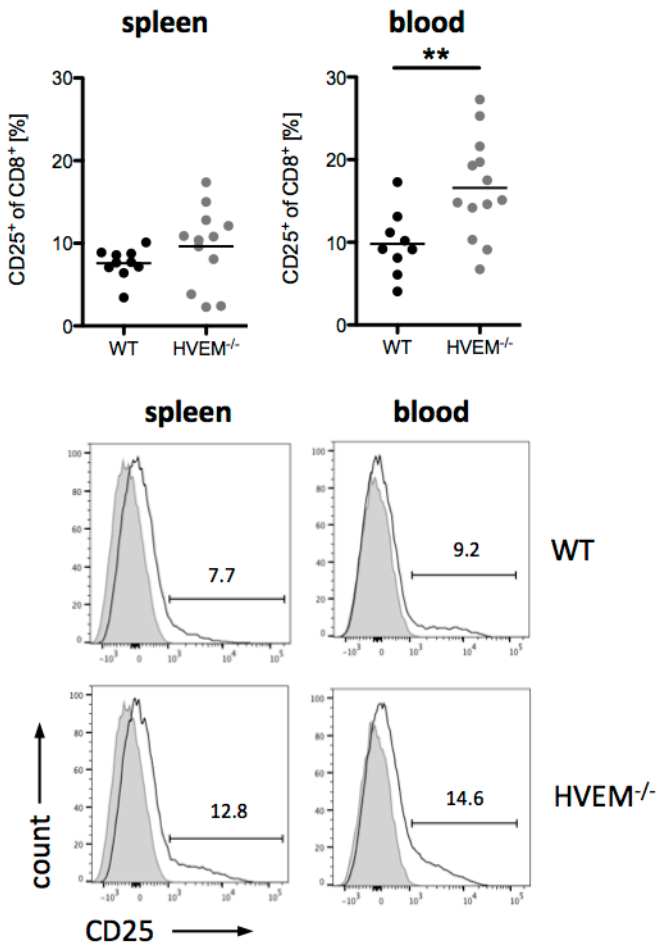



**Figure 4.8 HVEM$^{-/-}$ mice express high level of CD25 on CD8$^{+}$ T cells.** WT and HVEM$^{-/-}$ mice were infected i.p. with $10^5$ *Pb*A iRBC and tissues were collected at d 6 p.i. Isolated cells were analysed for CD25 expression by CD8$^{+}$ T cells. The quantitative overview shows pooled data of 3 independent experiments. Each circle represents one individual and means are depicted. Representative plots are shown. Significance was calculated by unpaired t test: **p≤ 0.01.

If not defective for proliferation, another explanation for a lower number of cells might be their failure in survival. Important evidence for this hypothesis is the differentiation status of cells. To this end, the activation and differentiation marker CD160 and KLRG1 were analysed on CD8$^{+}$ T cells of blood-stage *Pb*A infected wild type and HVEM$^{-/-}$ mice. Both are expressed to a significantly lower extent on CD8$^{+}$ T cells in blood and brain of HVEM$^{-/-}$ mice, while KLRG1 is also lower in frequency in the spleen of HVEM$^{-/-}$ mice (Figure 4.9).

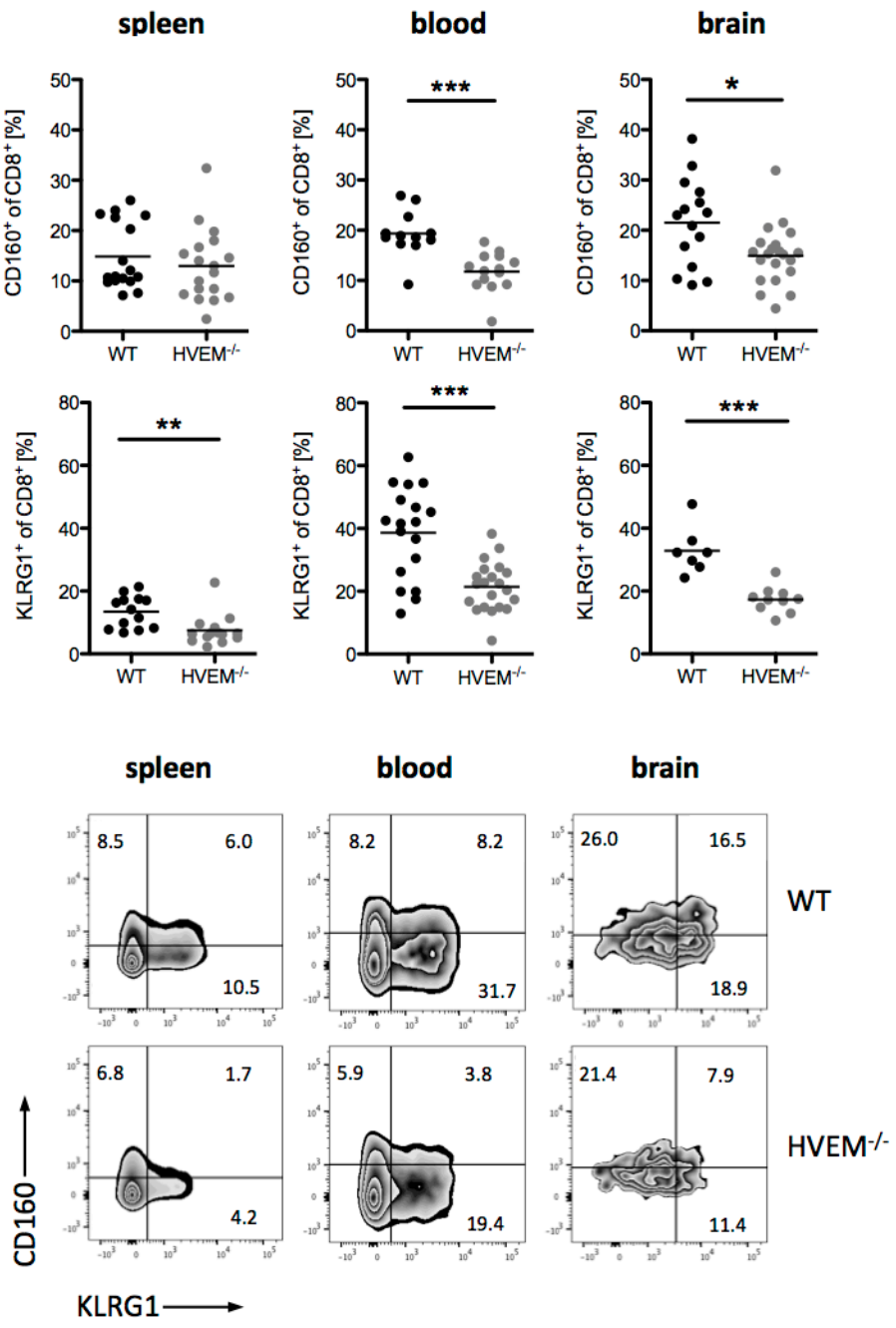



**Figure 4.9 HVEM$^{-/-}$ mice exhibit a lower frequency of terminally differentiated CD8$^{+}$ T cells.** WT and HVEM$^{-/-}$ mice were infected i.p. with $10^5$ *Pb*A iRBC and tissues collected at d 6 p.i. Isolated cells were analysed for CD160 and KLRG1 expression by CD8$^{+}$ T cells. The quantitative overview shows pooled data of 3-5 independent experiments. Each circle represents one individual and means are depicted. Representative plots are shown. Significance was calculated by unpaired t test: *p≤ 0.05; ** p≤ 0.01; *** p≤ 0.001.

Analysis of HVEM$^{-/-}$ mice revealed the importance of the HVEM network on disease progression and severity. Nevertheless, it is not known, whether this effect is mediated by one distinct ligand or by the interaction with several. Previously, the co-inhibitory function of BTLA during experimental malaria was studied in detail [86, 108], but little is know about

CD160. The second part of this thesis focuses on CD160 and its role in experimental malaria and in *Plasmodium falciparum* infection of humans.

## 4.2 Characterisation of CD160 during experimental malaria

The role of CD160 in experimental malaria has not been studied yet. Hence, the first question that arose was, which T cell subtype expresses CD160 during blood-stage infection with *Pb*A. Of special interest were T cells for two different reasons. First, T cells are known to express CD160 upon activation [106] and second, $CD4^+$ as well as $CD8^+$ T cells were found to be crucial for the development of cerebral malaria [128].

To this end, C57BL/6J mice were infected with *Pb*A iRBC and CD160 expression was analysed on $CD4^+$ and $CD8^+$ T cells at day 6 p.i. In addition, naïve mice were used to show whether CD160 is constitutive expressed or up-regulated upon infection. Furthermore, the expression pattern of CD160 was compared to well-known co-inhibitory receptors (PD-1, CTLA-4) typically expressed by $CD4^+$ T cells, to its ligand (HVEM) and to its competitor (BTLA).

In comparison to all analysed receptors, CD160 exhibits a distinct expression pattern. It is, in contrast to PD-1 and CTLA-4, which are mainly expressed on $CD4^+$ T cells of infected mice, specifically found on $CD8^+$ T cells (Figure 4.10). In addition, CD160 can only be detected on a small fraction of cells after infection, while HVEM and BTLA are highly expressed on T cells of both naive and infected mice (Figure 4.10).

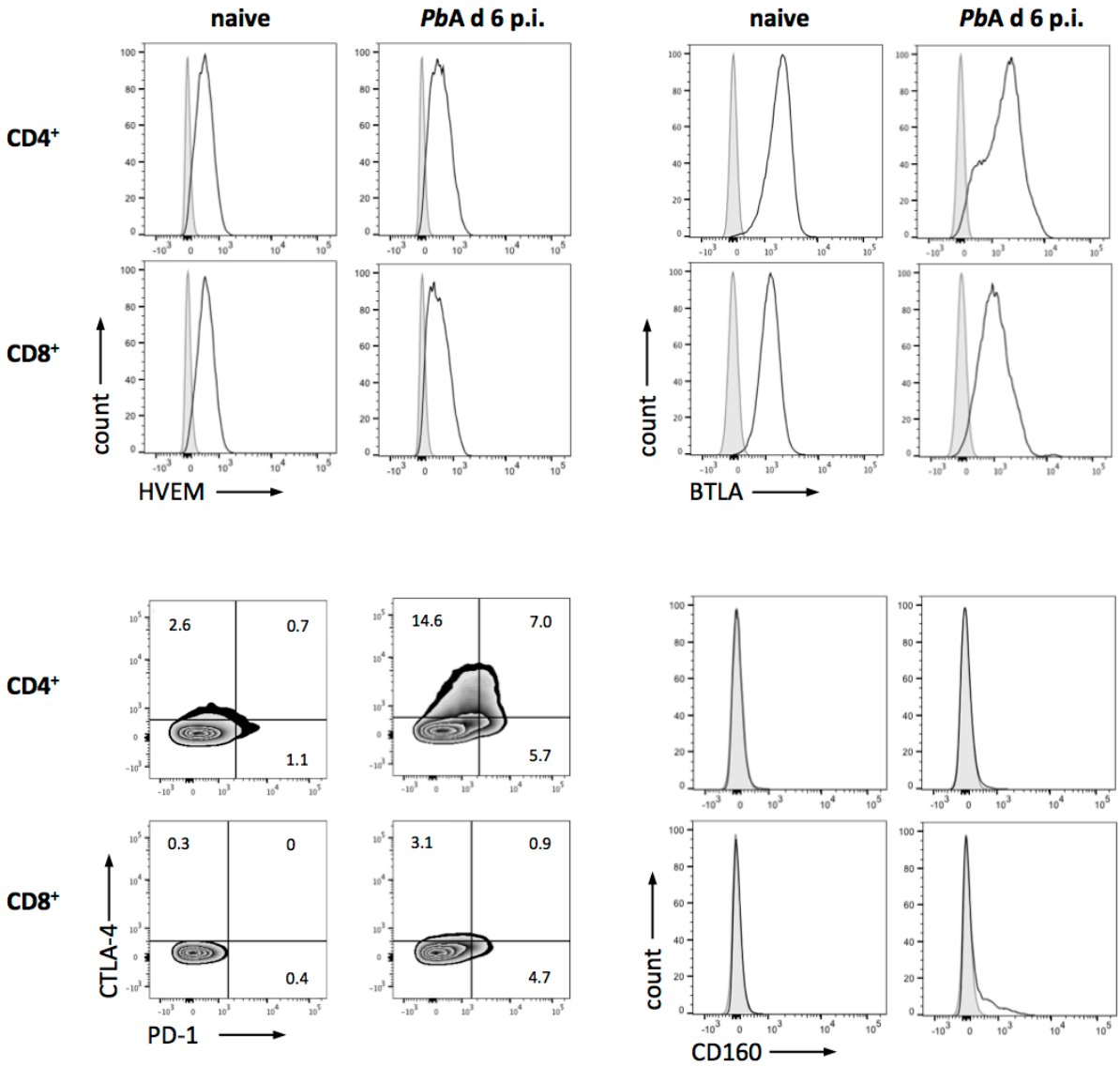



**Figure 4.10 Expression of CD160 on T cells is restricted to CD8$^+$ T cells during *Pb*A infection.** C57BL/6J mice were infected i.p. with $10^5$ *Pb*A iRBC or mock infected with PBS and spleens were collected at d 6 p.i. Isolated cells were analysed for the indicated markers expressed by CD4$^+$ or CD8$^+$ T cells. Histograms show FMO controls in grey and stained samples in black. PD-1 and CTLA-4 plots are gated according to FMO controls. Representative histograms or plots are shown.

After CD160 expression was shown at the late phase of blood-stage *Pb*A infection, namely day 6, the kinetic of CD160 upregulation during the course of the disease was analysed. Hence, blood samples of with *Pb*A iRBC infected C57BL/6J mice were taken at day 3, 5, 6 and 7 p.i. and stained for CD160 and CD8. Indeed, CD160 remains absent until day 5 post infection, is clearly induced at day 6 p.i. and stays high at day 7 p.i. (Figure 4.11 A). The kinetic of CD160 expression on CD8$^+$ T cells in blood and spleen is akin (data not shown). Of note, symptoms of cerebral malaria and expression of CD160 on CD8$^+$ T cells start to occur simultaneously, namely at day 6 p.i. (data not shown). Since CD160 is a GPI anchored protein, which can be shed from the cell surface, serum level of soluble CD160 from *Pb*A

iRBC infected mice were also analysed at day 3, 5, 6 and 7 p.i. Interestingly, the serum level of the soluble protein is already higher compared to naïve control mice at day 5 p.i. that is to say one day earlier than CD160 can be detected on the surface of $CD8^+$ T cells (Figure 4.11 B).

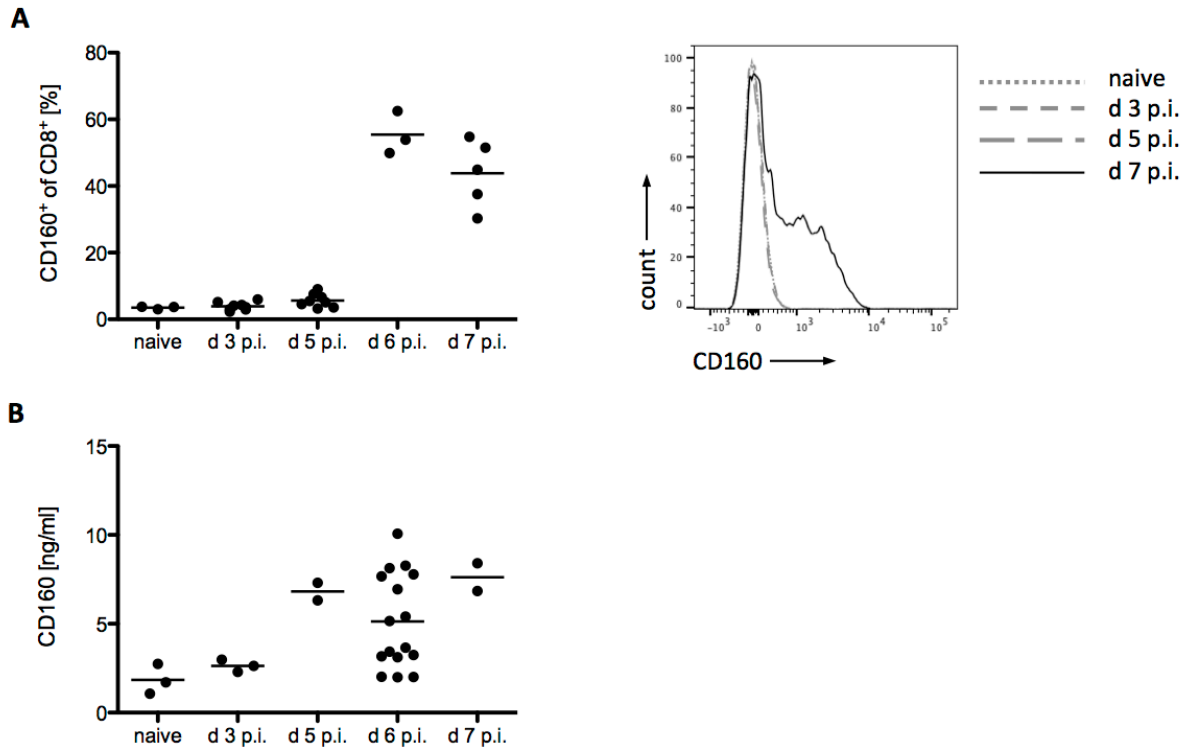



**Figure 4.11 Expression of CD160 on $CD8^+$ T cells is induced in the late stage of the *Pb*A infection.** (A) C57BL/6J mice were infected i.p. with $10^5$ *Pb*A iRBC or mock infected with PBS and blood was collected at the indicated days post infection. The experiment was terminated when mice developed severe cerebral symptoms or at day 7 p.i. Isolated cells were analysed for CD160 expression by $CD8^+$ T cells. The histogram shows CD160 expression of one representative infected mouse during the course of infection and a naïve control. The quantitative overview shows pooled data of 2 independent experiments. Each circle represents one individual and means are depicted. (B) Serum samples of naïve or infected mice at the indicated days post infection were collected. Soluble CD160 was detected by ELISA. Each circle represents one individual and means are depicted.

As mentioned above, affected tissues in mice suffering from experimental cerebral malaria are spleen, blood and brain. This is why the frequency of $CD160^+$ $CD8^+$ T cells was compared within the respective organs. Both, blood and brain exhibit significantly higher frequencies of $CD160^+$ $CD8^+$ T cells compared to the spleen (Figure 4.12).

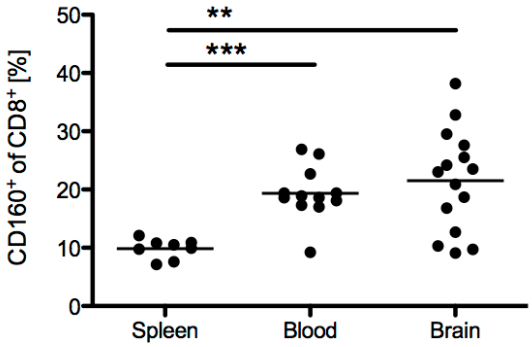



**Figure 4.12 The frequency of CD160$^+$ CD8$^+$ T cells is highest in blood and brain.** C57BL/6J mice were infected i.p. with $10^5$ *Pb*A iRBC and tissues were collected at d 6 p.i. Isolated cells were analysed for CD160 expression by CD8$^+$ T cells. The quantitative overview shows pooled data of 6 independent experiments. Each circle represents one individual and means are depicted. Significance was calculated by unpaired t test: **p≤ 0.01; *** p≤ 0.001.

Taking the kinetic of the CD160 expression and occurrence of CD160$^+$ CD8$^+$ T cells in the brain into account, the question arouse if there is a correlation between the expression of CD160 and severity of the disease. Severity of disease was rated by parasitemia and severity of cerebral symptoms specified by a scoring system (Chapter 3.8.3). Both parameters were compared to the frequency of CD160$^+$ CD8$^+$ T cells on the one hand and to the concentration of soluble CD160 in the serum on the other hand.

Indeed, the percentage of CD8$^+$ T cells expressing CD160 at day 6 p.i. correlates with the severity of cerebral symptoms, whereas soluble CD160 level correlate with parasitemia but not vice versa (Figure 4.13).

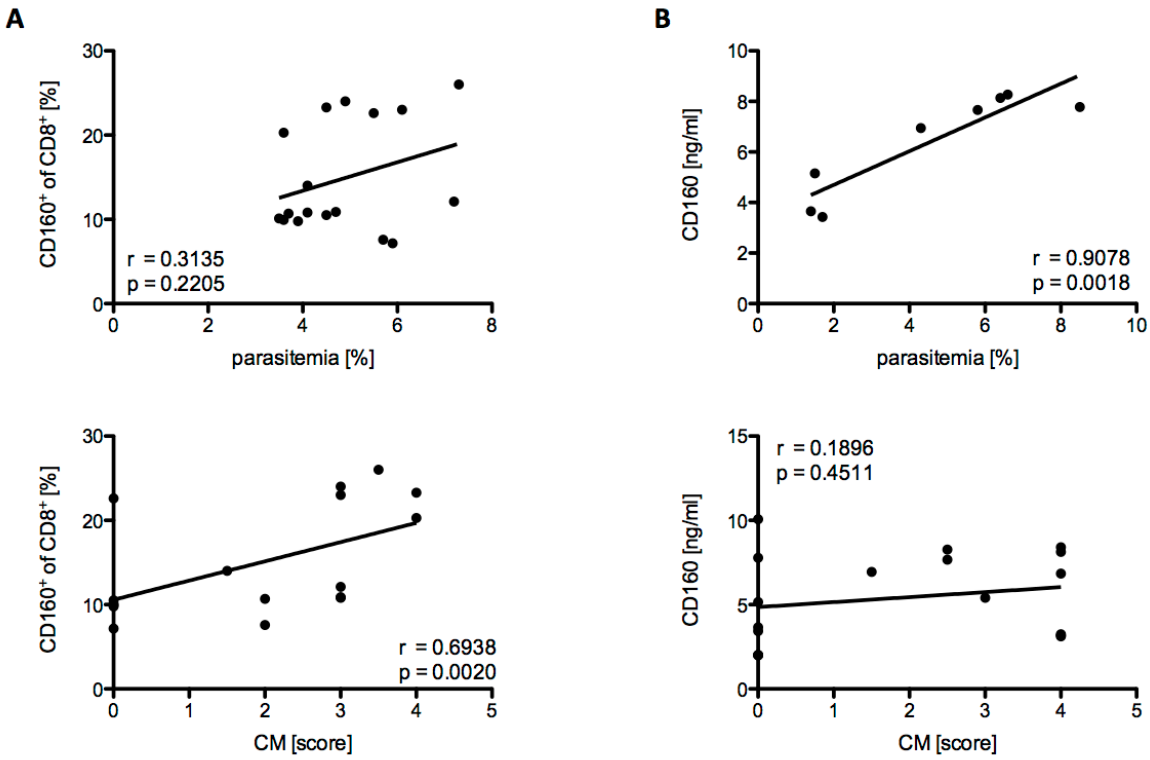



**Figure 4.13 CD160 expression correlates with parasitemia and severity of cerebral symptoms.** The frequency of CD160$^+$ CD8$^+$ T cells (A) or the concentration of soluble CD160 in the sera (B) of *Pb*A infected C57BL/6J mice were analysed for correlation with the parasitemia or the CM score. Correlation was calculated by Pearson (Parasitemia) or Spearman (CM score). Data of 2 (B) or 4 (A) pooled independent experiments are shown. Each circle represents one individual.

Aiming to further understand the impact of CD160 on pathology, *Plasmodium yoelii* non-lethal (*Py*NL) parasites were utilised. Even though *Py*NL causes high parasitemia and splenomegaly (data not shown), C57BL/6J mice do not develop cerebral malaria and are able to clear the infection. Therefore, infection with *Py*NL allows the analysis of CD160 expression during strong inflammation but without immunopathology. Strikingly, CD8$^+$ T cell activation determined by CD44 and CD11a expression is even more pronounced in *Py*NL than in *Pb*A infected mice (Figure 4.14). However, the frequency of CD160 expressing cells within the CD44$^{hi}$CD11a$^+$ population is significantly lower in the non-lethal model (Figure 4.14).

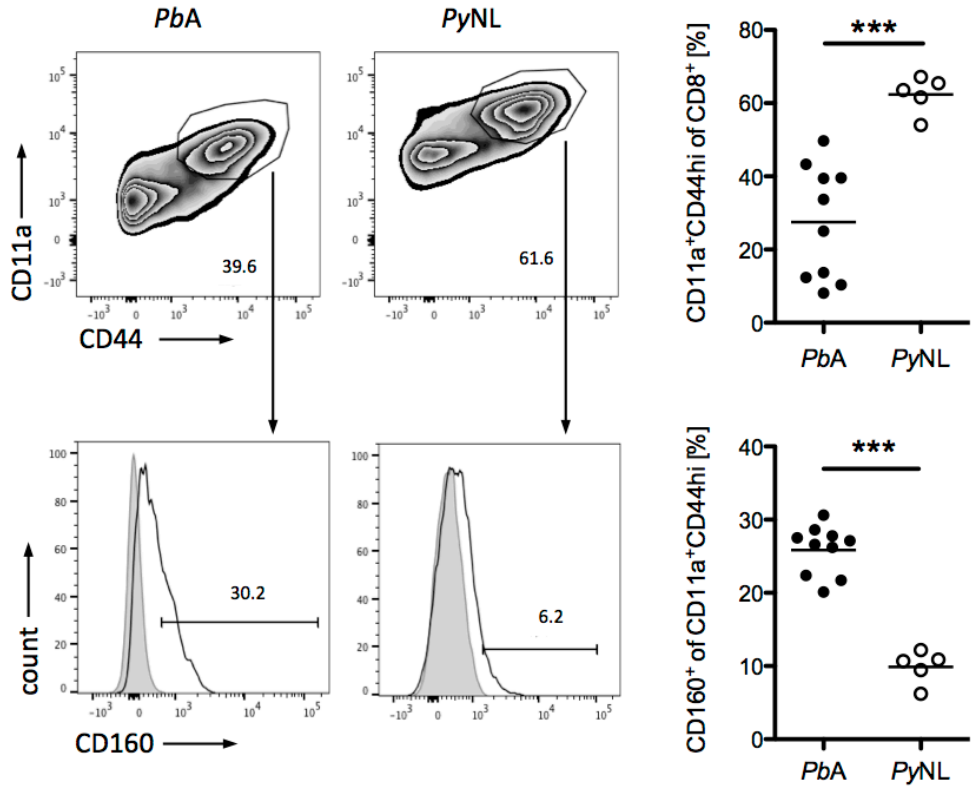



**Figure 4.14 CD160 is less abundant on activated, antigen-specific CD8$^+$ T cells in the non-lethal *Py*NL infection compared to the lethal *Pb*A infection.** C57BL/6J mice were infected i.p. with $10^5$ *Pb*A or *Py*NL iRBC and spleens were collected at d 6 p.i. The frequency of activated, antigen-specific CD8$^+$ T cells was determined by expression of CD44 and CD11a. CD160 expression was analysed within the CD44$^{hi}$CD11a$^+$ population. The quantitative overview shows pooled data from 2 independent experiments. Each circle represents one individual and means are depicted. Representative plots are shown. Significance was calculated by unpaired t test: ***p≤ 0.001.

As C57BL/6J mice can clear *Py*NL infections, T cell phenotypes cannot only be analysed during the acute phase of the infection but also after parasite clearance. Hence, *Py*NL infected mice were used to monitor CD160 expression at a later time point (d 12/20 p.i.) and determine the persistence of CD160$^+$ CD8$^+$ T cells. In addition, the expression of proliferation (Ki67), cytotoxicity (GrzB, CD107a), activation (CD28, CD44, CD11a) and differentiation (KLRG1) markers during the course of infection was of interest. All analysed markers are highly expressed at day 6 p.i. but subsequently their kinetic can be divided into two groups. One, including GrzB, Ki67 and KLRG1, is characterised by a lower frequency already at day 12 p.i. (Ki67, GrzB) and at day 20 p.i. by level comparable to naïve mice. The second, including CD44, CD11a and CD28, showed high expression level at day 12 and day 20 p.i. as well. CD160 was expressed at similar level at day 6, 12 and 20 p.i., thereby accounting to the second group (Figure 4.15). The observed expression pattern indicates that CD8$^+$ T cells, which were highly activated and cytotoxic at day 6 p.i., lose their ability to proliferate (Ki67)

and to kill target cells (GrzB) until day 20 p.i. Nevertheless, once activated, $CD8^+$ T cell populations expressing CD160, $CD44^{hi}$, CD11a or CD28 persist at least until d 20 p.i.

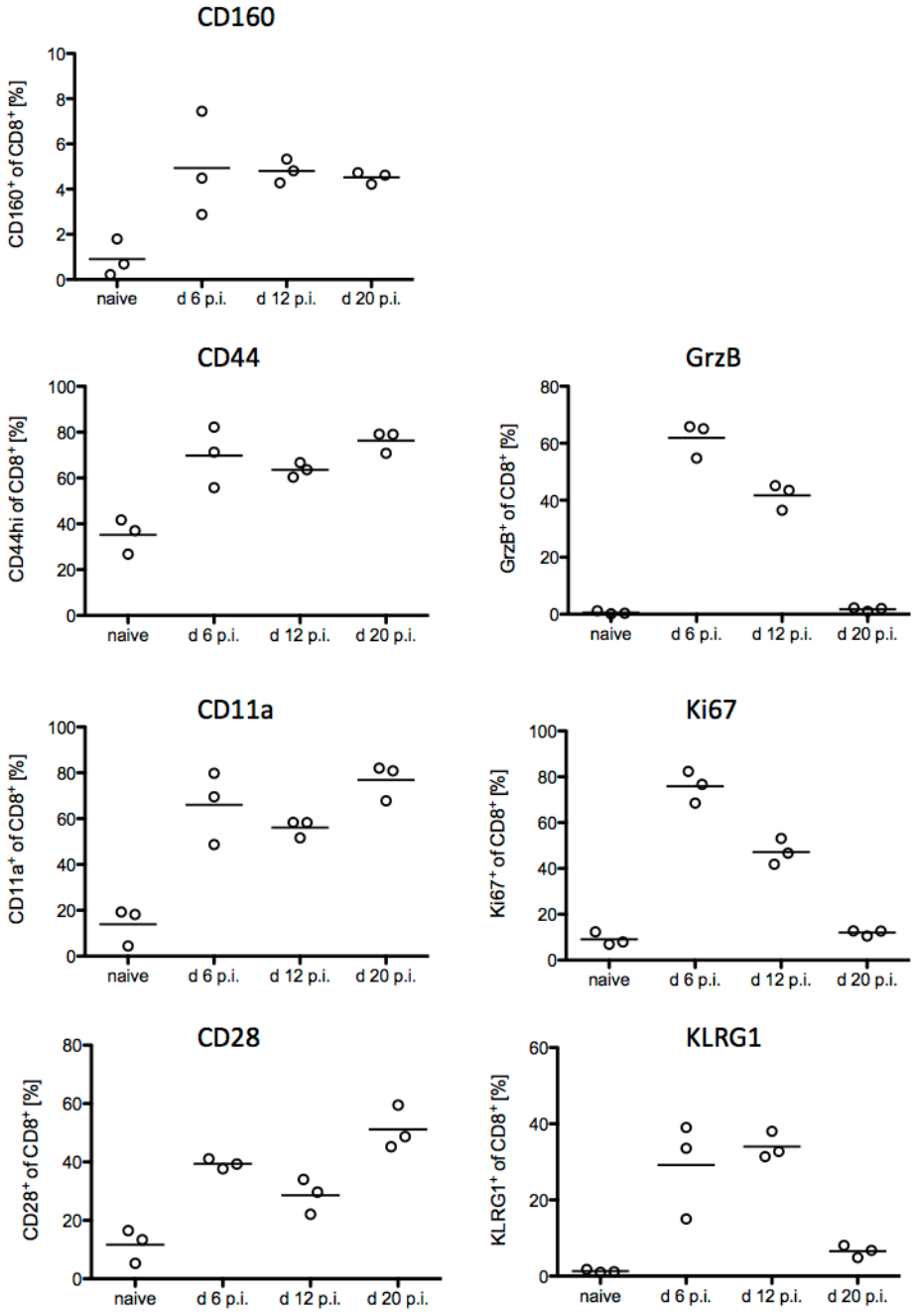
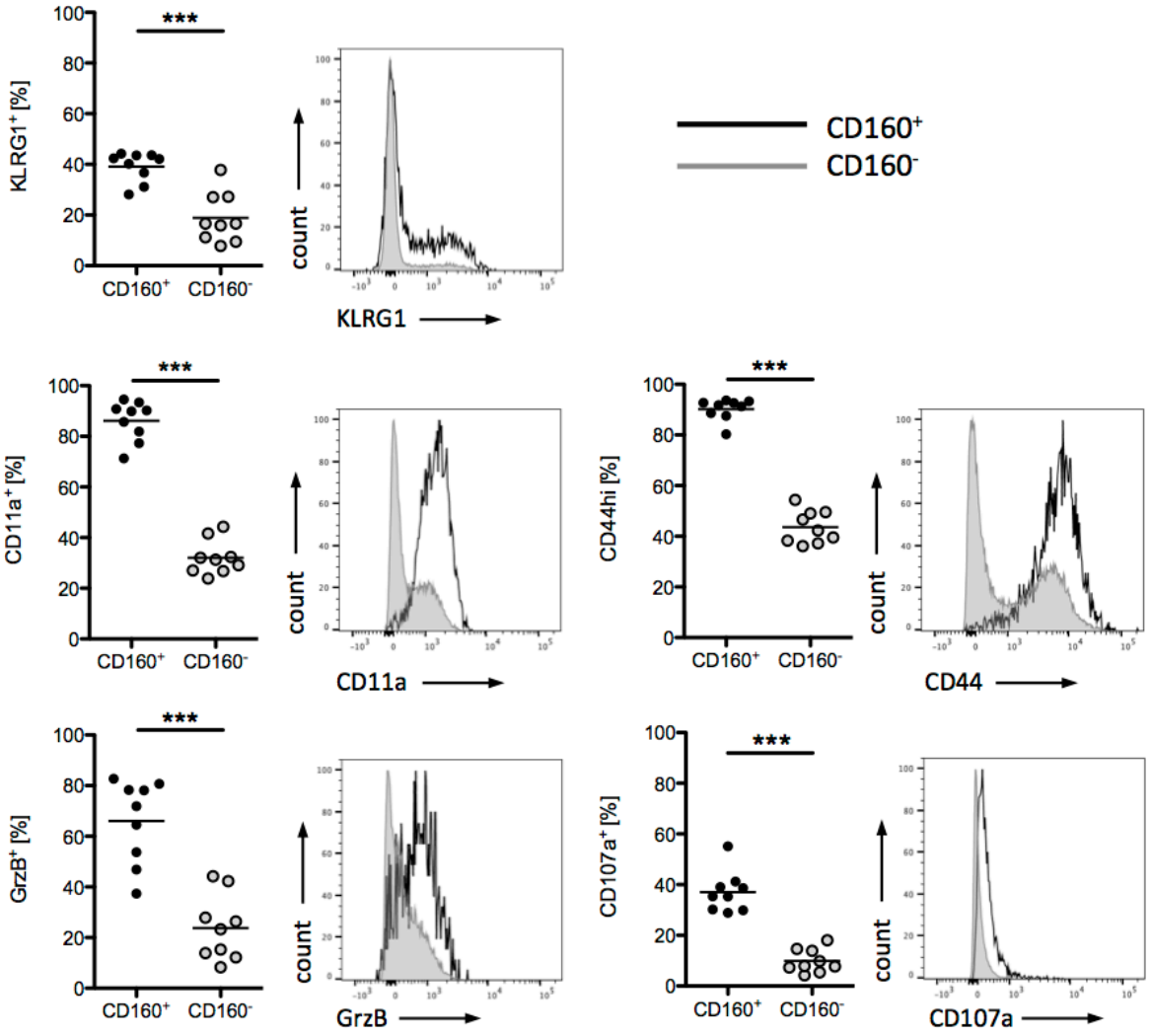



**Figure 4.15 $CD160^+$ $CD8^+$ T cells can be found in *Py*NL infected mice even after parasite clearance from peripheral blood.** C57BL/6J mice were infected i.p. with $10^5$ *Py*NL iRBC and blood was collected at the indicated days post infection. $CD8^+$ T cells were analysed by flow cytometry for the expression of the indicated markers. One representative experiment of two experiments is shown. Each circle represents one individual and means are depicted.

As shown above, CD160 expression is related to pathogenicity of experimental cerebral malaria. For this reason, the phenotype of CD160 expressing $CD8^+$ T cells at the peak of T cell activation, namely d 6 p.i., was analysed in detail. The obtained multi-colour flow cytometry

data was analysed as the following: $CD8^+$ T cells were divided into $CD160^+$ and $CD160^-$ populations and both populations were characterised concerning their activation, proliferation, cytotoxicity and differentiation status. Strikingly, almost all $CD160^+$ cells do also express markers for activation (CD44), for antigen-experienced cells (CD11a) and proliferation (Ki67), the co-stimulatory molecule CD28 and the co-inhibitory molecule BTLA (Figure 4.16). Furthermore, a higher frequency of cells of the $CD160^+$ population express the marker for cytotoxicity (GrzB), degranulation (CD107a), terminal differentiation (KLRG1) and the co-inhibitory molecule PD-1 compared to the $CD160^-$ population (Figure 4.16).



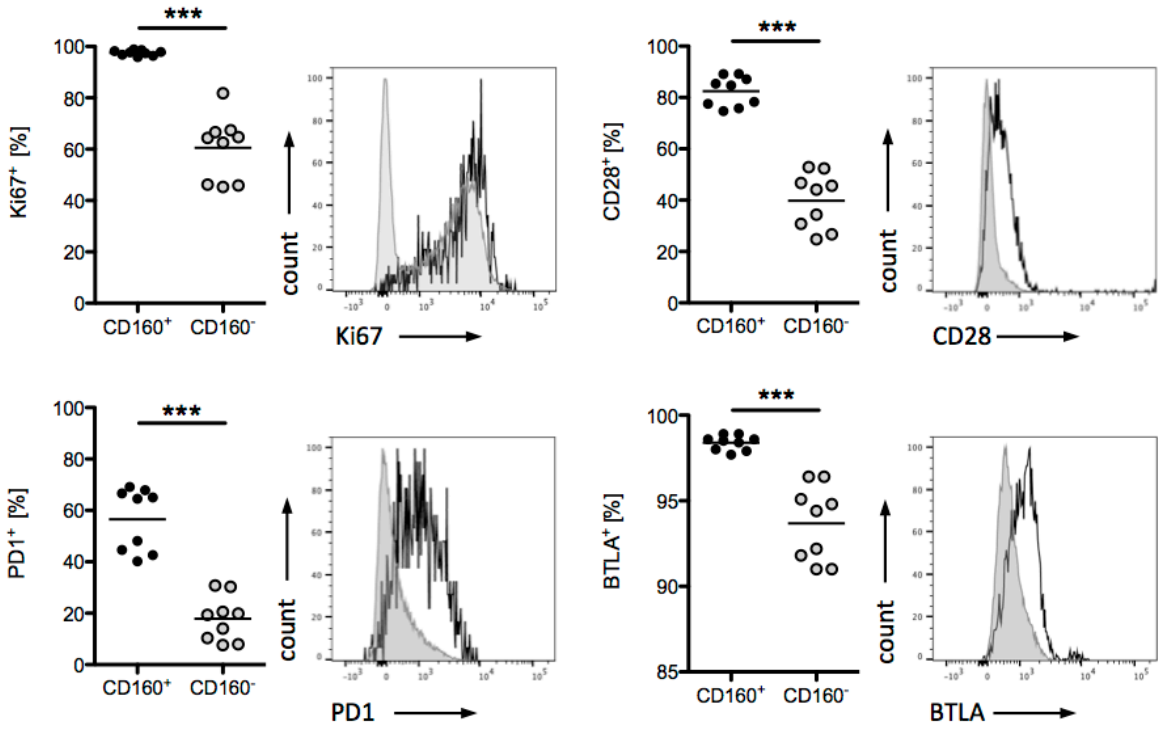
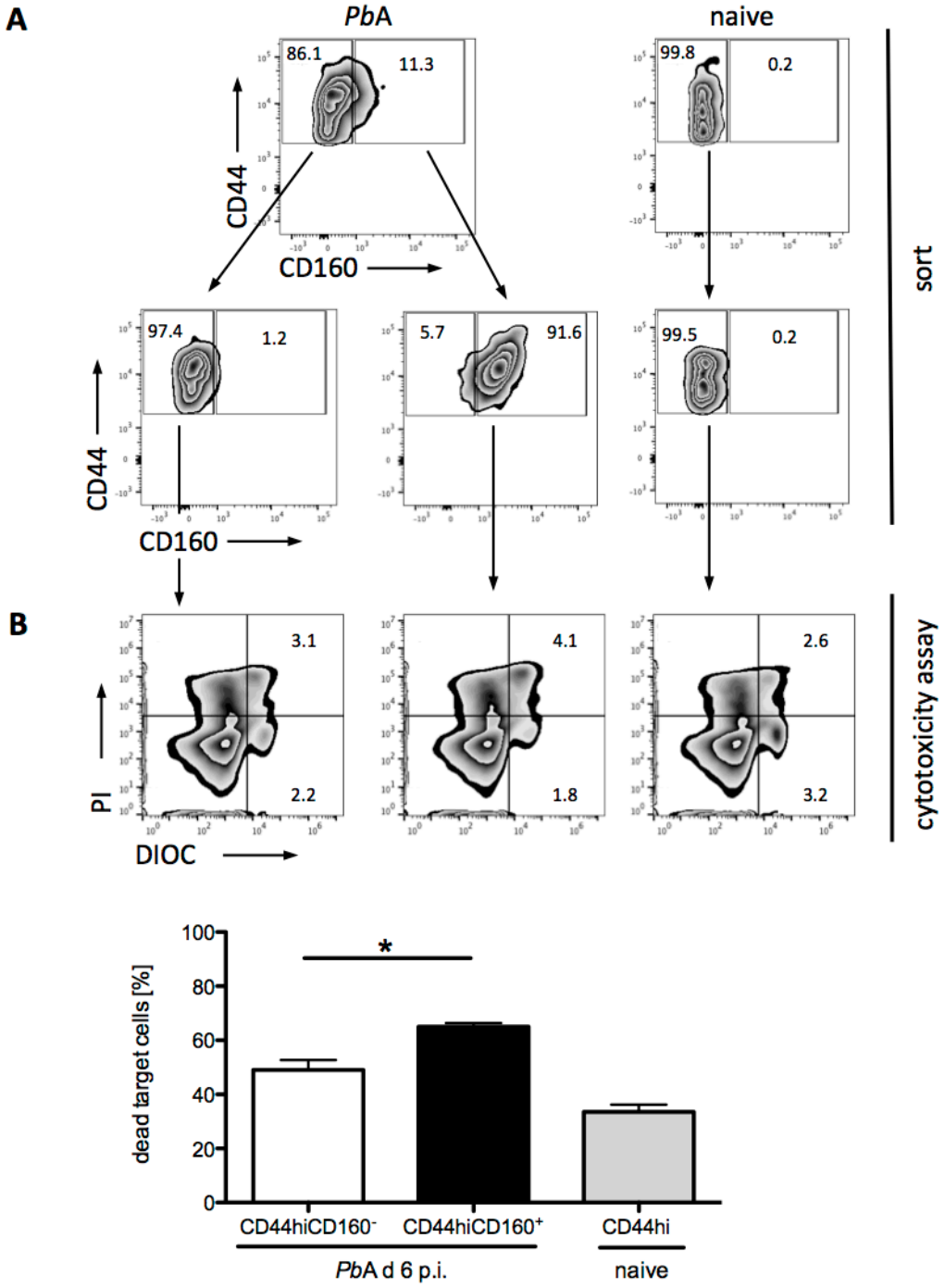



**Figure 4.16 CD160$^+$ CD8$^+$ T cells co-express markers for activation, proliferation, cytotoxicity, terminal differentiation and co-inhibitory molecules.** C57BL/6J mice were infected i.p. with $10^5$ *Pb*A iRBC and spleens were collected at d 6 p.i. The expression of the indicated markers was analysed within the CD8$^+$CD160$^+$ or CD8$^+$CD160$^-$ population. The quantitative overview shows pooled data of 2 independent experiments. Each circle represents one individual and means are depicted. The histograms show the marker expression by the CD8$^+$CD160$^-$ (grey) and CD8$^+$CD160$^+$ (black) population of one representative mouse. Significance was calculated by unpaired t test: ***p≤ 0.001.

By flow cytometry, an activated, cytotoxic phenotype of CD160$^+$ CD8$^+$ T cells was clearly described. Subsequently, to provide evidence of the actual functionality of these CD160$^+$ CD8$^+$ T cells, *in vitro* assays were used. To this end, CD8$^+$ T cells from *Pb*A infected mice (d 6 p.i.) were sorted for activated (CD44$^{hi}$) cells either expressing CD160 or being CD160$^-$. Naïve control CD8$^+$ T cells were sorted for CD44$^{hi}$ expression (Figure 4.17 A). Functionality of the sorted cell populations was determined by their ability to kill target cells and to produce the pro-inflammatory cytokine IFNγ. To test for cytotoxicity, whole splenocytes from naïve mice were pulsed with *Pb*A-specific peptides and labelled with a fluorescent dye (DIOC). Subsequently, sorted effector cells (CD160$^+$ or CD160$^-$) were co-cultured with the target cells for two hours in presence of propidium iodide (PI). Flow cytometry allowed the detection of dead target cells (DIOC$^+$PI$^+$). As already suggested by the phenotype, CD160$^+$ cells were more sufficient in killing of target cells, even though they were compared to similar activated (CD44$^{hi}$) cells (Figure 4.17 B).

To test for cytokine production, hepatoma cells (Hepa1-6H) were pulsed with *Pb*A-specific peptide and co-cultured with sorted effector cells (CD160$^+$ or CD160$^-$). After 24 hours of

stimulation, the cell culture supernatant was analysed for IFNγ by ELISA. Again, CD160$^{+}$ cells produced higher amounts of IFNγ compared to the CD160$^{-}$ counterpart (Figure 4.17 C).



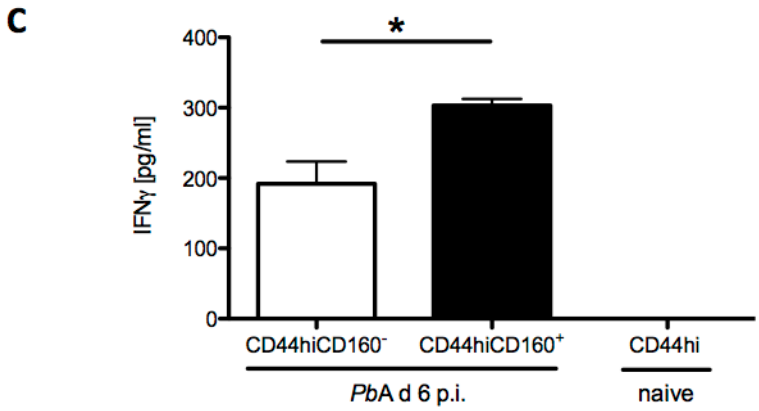



**Figure 4.17 CD160$^{+}$ CD8$^{+}$ T cells act cytotoxic and produce the pro-inflammatory cytokine IFNγ.** C57BL/6J mice were infected i.p. with $10^{5}$ *Pb*A iRBC and spleens were collected at d 6 p.i. Splenic cells were sorted for CD8$^{+}$CD44$^{hi}$ cells being either CD160$^{+}$ or CD160$^{-}$ (A). (B) To determine cytotoxic capacity, sorted cells were co-cultured with *Pb*A-peptide pulsed and DIOC labelled target cells. Dead cells were detected by propidium iodide (PI) incorporation. Both, PI and DIOC staining were detected by flow cytometry. Representative plots are shown. The quantitative analysis shows representative data of 1 out of 6 independent experiments (mean ± SEM). Significance was calculated by unpaired students t test: *p≤ 0.05. (C) Sorted cell populations were co-cultured with *Pb*A-peptide pulsed Hepa1-6H cells. IFNγ from cell culture supernatant was measured by ELISA. The quantitative analysis shows representative data of 1 out of 4 independent experiments (mean ± SEM). Significance was calculated by unpaired students t test: *p≤ 0.05.

Although all data shown support the hypothesis of a co-stimulatory involvement of CD160 in CD8$^{+}$ T cell activity during experimental malaria, no direct functional involvement was proven. In order to address this, a CD160$^{-/-}$ mouse was developed using the CRISPR/Cas9 system (Chapter 3.1).

## 4.3 Characterisation of CD8$^+$ T cells in human malaria

To verify the findings of the experimental model and to determine the importance of CD160 during *Pf* infection of humans, malaria patient samples were analysed and compared to healthy controls.

While there is comprehensive data about CD4$^+$ T cells in human malaria [82, 83, 130-132], CD8$^+$ T cells are less characterised during acute blood-stage infection. Hence, effector molecule expression and activation, proliferation and differentiation markers were stained in whole blood samples. Since CD69 rather than CD44 is commonly used as a marker for activated T cells in human, it was also used in this study.

In contrast to the mouse model, CD160 is not significantly higher expressed on CD8$^+$ T cells in infected individuals compared to healthy controls. The cytotoxic effector molecules perforin and GrzB are significantly or in trend, respectively, higher in patients than in healthy individuals. Furthermore, in line with the experimental model, there are more proliferating (Ki67$^+$) and activated (CD69$^+$) CD8$^+$ T cells in malaria patients than in healthy controls. While the frequency of cells expressing the co-inhibitory molecule CTLA-4 is not altered, PD-1 is expressed by a higher frequency of CD8$^+$ T cells of malaria patients compared to healthy controls (Figure 4.18).

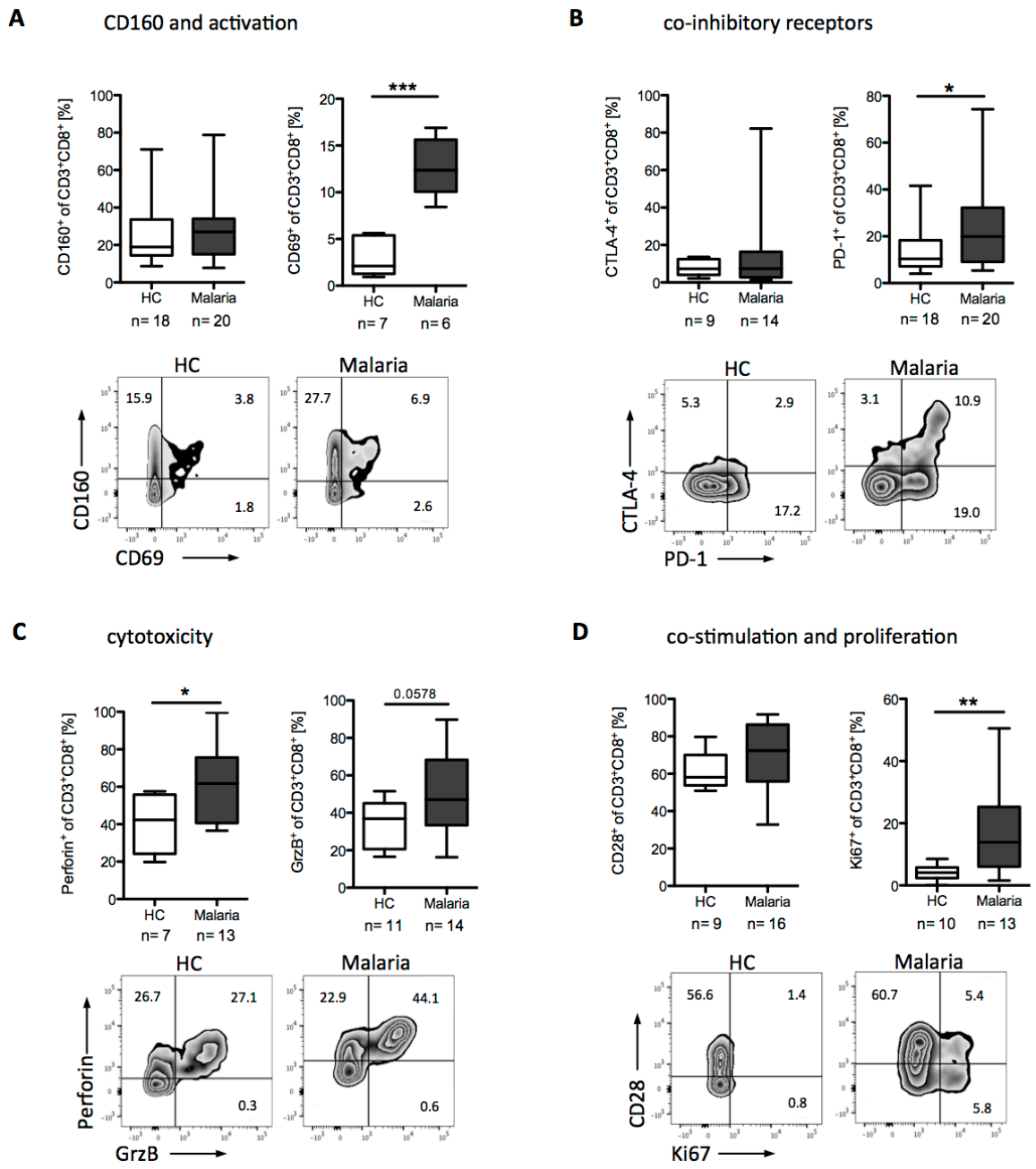
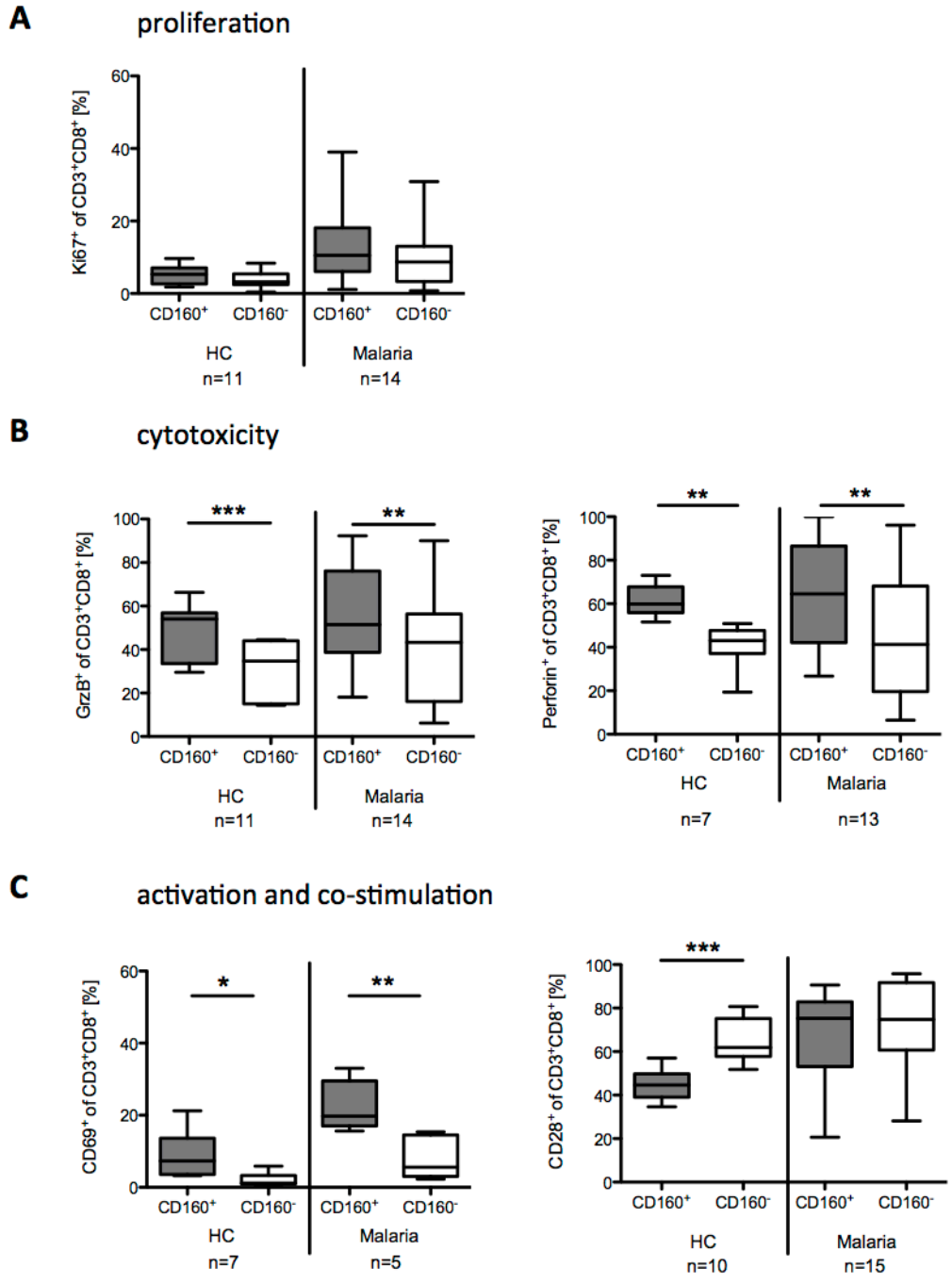



**Figure 4.18 CD8$^+$ T cells from *Pf* infected patients are characterised by enhanced expression of activation, cytotoxicity and proliferation markers and higher frequency of PD-1$^+$ cells compared to healthy controls.** Whole blood samples from malaria patients (Malaria) or healthy controls (HC) were stained for the indicated activation, cytotoxicity and proliferation marker and co-inhibitory molecules and analysed by flow cytometry. The number of included individuals is annotated. Representative plots are shown. Significance was calculated by unpaired t test: *p≤ 0.05; **p≤ 0.01; ***p≤ 0.001.

Even though the frequency of CD160 expressing CD8$^+$ T cells is not higher in malaria patients, cells were analysed for co-expression with functional markers, as it was shown for the experimental infection (Figure 4.16). Similar to data obtained from *Pb*A infected mice, CD160$^+$ CD8$^+$ T cells from humans express higher level of activation (CD69), proliferation (Ki67) and cytotoxicity (GrzB, Perforin) markers. This is true for malaria patients but also for

healthy controls. In contrast to the experimental model, the frequency of $CD28^+$ $CD8^+$ T cells is lower in the $CD160^+$ population compared to the $CD160^-$ counterpart (Figure 4.19).



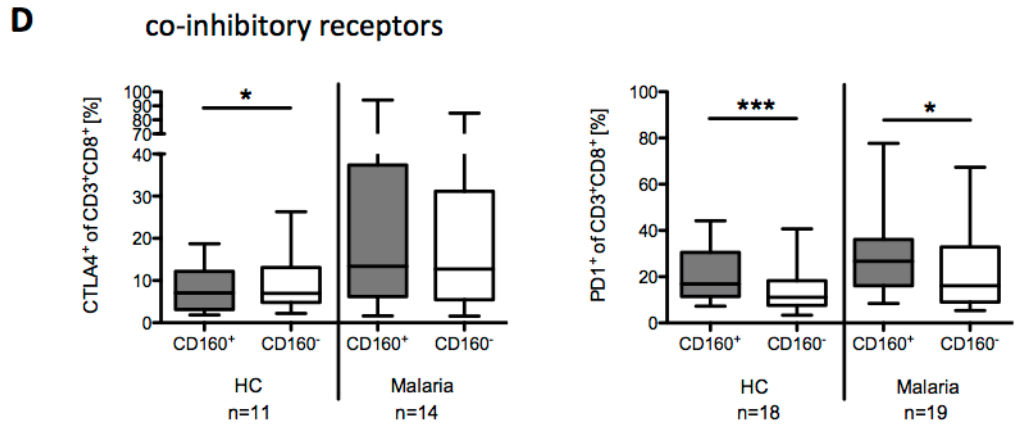



**Figure 4.19 CD160$^+$ CD8$^+$ T cells from human blood show an activated and cytotoxic phenotype.** Whole blood samples from malaria patients (Malaria) or healthy controls (HC) were stained for activation, cytotoxicity and proliferation markers and co-inhibitory molecules and analysed by flow cytometry. CD8$^+$ T cells were gated for CD160$^+$ and CD160$^-$ populations and the expression of the indicated markers was analysed within both populations. Significance was calculated by paired t test *p≤ 0.05; **p≤ 0.01; ***p≤ 0.001.

While experimental infections were performed in young (7-10 weeks), female mice, patients enrolled in the human study include males as well as females having a wide range in age (21-69 years). Hence, CD160 expression was analysed comparing gender, age and parasitemia. In malaria patients, CD160 expression positively correlates with age (Figure 4.20 A). Regarding the gender of enrolled subjects, no differences in CD160 expression were found (Figure 4.20 B). Taking into consideration, that the number of patients having at least a parasitemia of 1 % is low, only a trend for lower CD160 expression in patients with higher parasitemia could be shown (Figure 4.20 C).

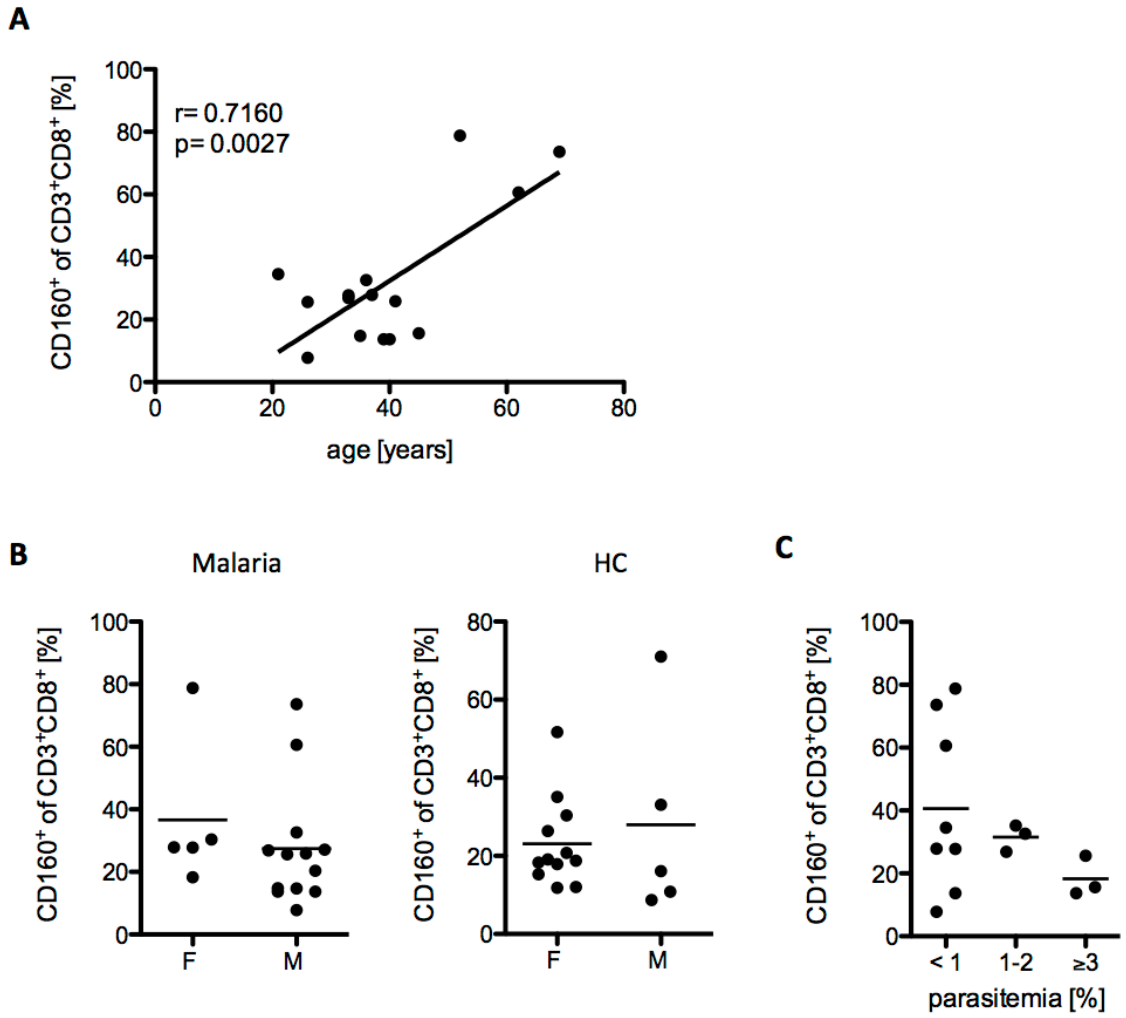



**Figure 4.20 Expression of CD160 by CD8$^+$ T cells is related to the age of malaria patients.** The frequency of CD160$^+$ CD8$^+$ T cells from malaria patients (Malaria) or healthy control (HC) was compared to the age (A), sex (B) and the parasitemia (C). Correlation was calculated by Pearson. Each circle represents one individual and (B,C) means are depicted.

## 4.4 Generation of a CD160$^{-/-}$ mouse

Knockout (KO) mice have been designed in the past mainly by utilizing Zinc Finger Nucleases (ZFN) or Transcription Activator-Like Effectors (TALEN). In 2013 the CRISPR/Cas9 technology was published as an additional tool for gene editing and a convenient alternative [133]. Since gene editing by CRISPR/Cas9 is easy, fast and efficient, this technology was used for the generation of a CD160 KO mouse.

The CRISPR/Cas9 technology includes two components: First the sgRNA, which binds to the target DNA sequence and thereby guides the second component, the DNA-cleaving enzyme Cas9, to the site of choice. In general, the only requirement for a target site is a 20 nt sequence followed by a so called protospacer adjacent motif (PAM), namely NGG, without any off-target effects. However, the target site should also be located as close as possible to the beginning of the first exon, enhancing the probability of a detrimental frame shift and a premature stop codon. As the genotyping of mice for the CD160 KO should to be performed by Restriction Fragment Length Polymorphisms (RFLP), a restriction site surrounding the Cas9 cleavage site (upstream of 18 nt of the target sequence) was needed. A suitable target site for *cd160* was designed with the help of the design tool CHOPCHOP [134].

Injection of sgRNA and the Cas9 into embryonic stem cells with C57BL/6 background was performed in cooperation with Irm Hermans-Borgmeyer at the ZMNH.

Founder animals (F0) were genotyped by RFLP. For mice showing a heterozygous phenotype, PCR fragments were cloned into a TA plasmid and clones were sequenced. Cloning was necessary since founder animals were mosaics. Because the mutation was introduced by non-homologous end joining during DNA repair, the resulting sequence differs between the founder animals. Two founder mice (line B and line D) were chosen for backcrossing with wild type C57BL/6J mice. The two lines differ in the number of deleted nucleotides (B=6, D=5) and thereby exhibit only a deletion of the amino acids AE (line B) or a frameshift and premature stop-codon (line D). An overview of the amino acid sequence and posttranslational modifications of wild type and both edited lines is depicted in Figure 4.21.

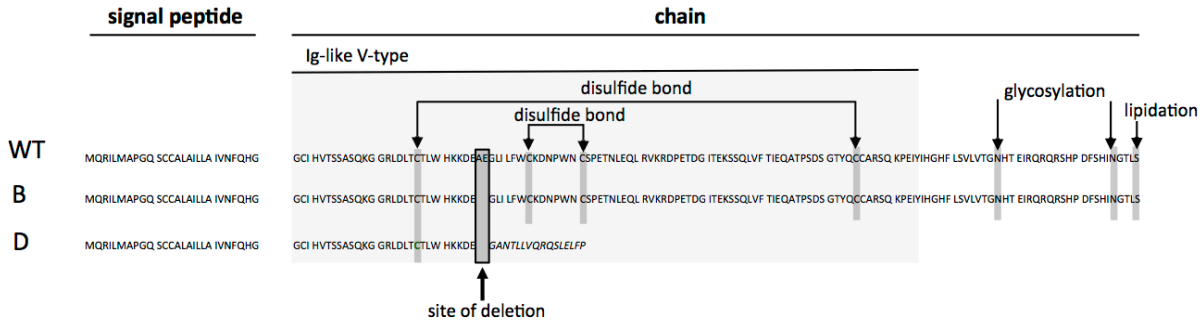



**Figure 4.21 Protein sequence and post-translational modifications of CD160 in wild type and CD160$^{-/-}$ line B and D mice.** WT protein sequences, disulfide bounds, glycosylation sites, the lipidation site and known structural domains are depicted according to uniprot data. For line D, the protein sequence subsequent of the site of deletion was calculated by the ExPASy translation tool.

F1 animals were again genotyped, sequenced and heterozygous animals were bred. Within the offspring (F2), homozygous mice were identified, sequenced and used for further breeding. In Figure 4.22 an example for the genotyping by RFLP is shown. Briefly, a 297 bp DNA fragment comprising the target sequence was amplified from ear tissue lysate by PCR. Subsequently, the PCR product was digested with the enzyme Bpu10I. Digestion resulted for wild type sequences in two fragments, namely 228 bp and 69 bp. In gene modified sequences DNA size remained 297 bp due to a destroyed restriction site.

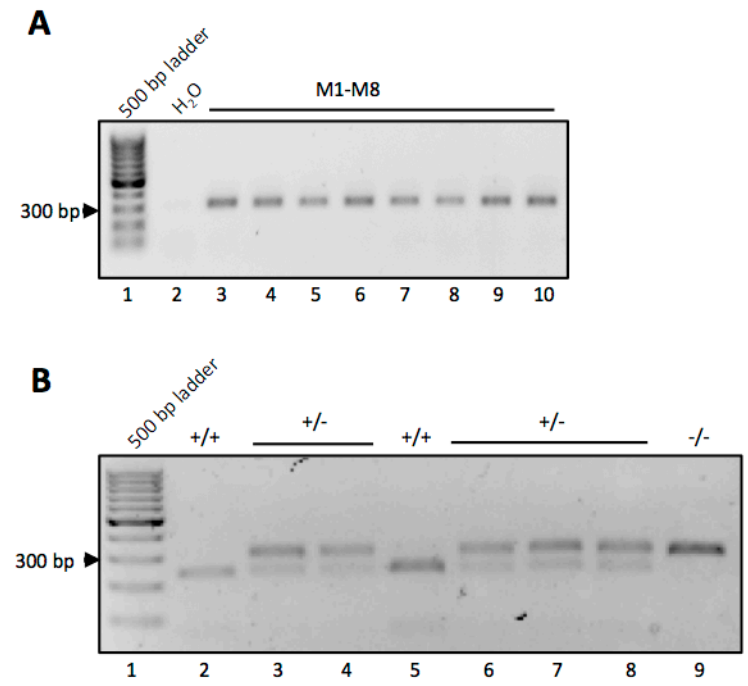



**Figure 4.22 Genotyping of CD160$^{-/-}$ mice.** (A) Ear tissue lysates were used for amplification of the DNA sequence including the CRISPR/Cas9 target site by PCR. The size of the PCR products was analysed by agarose gel electrophoresis. An exemplary gel with samples from eight mice (M1-M8, lane 3-10) and a negative control without template ($H_2O$, lane 2) is shown. The PCR product size is annotated according to the 500 bp ladder (lane 1). (B) PCR products were digested by Bpu10I and the size again analysed by agarose gel electrophoresis. The genotype referring to the analysed mice is annotated: +/+ wild type, +/- heterozygous, -/- homozygous knockout.

Beside the verification of the successful modification of the *cd160* gene, the loss of the protein was analysed by flow cytometry. Two different cell types were of interest. First, since this study focuses on T cell regulation and CD160 is expressed by activated $CD8^+$ T cells of *Pb*A infected mice at day 6 p.i. (Figure 4.11), T cells of the spleen and blood of infected mice were analysed (Figure 4.23 A,B). Second, a majority of intraepithelial lymphocytes (IEL) expresses CD160 [110]. Considering the fact, that IEL do not require previous activation to express CD160 and, most importantly, provide a source of $CD160^+$ cells independently of the study focus, they are a valuable control (Figure 4.23 C). In comparison to wild type mice, only a minor fraction of $CD8^+$ T cells and IELs derived from $CD160^{-/-B}$ mice expresses CD160. However, line B does not represent a complete KO, rather a knockdown. In contrast, $CD160^{-/-D}$ mice represent a complete knockout shown by the absence of CD160 on activated $CD8^+$ T cells from spleen and blood and on IEL derived from the small intestine of naïve mice.

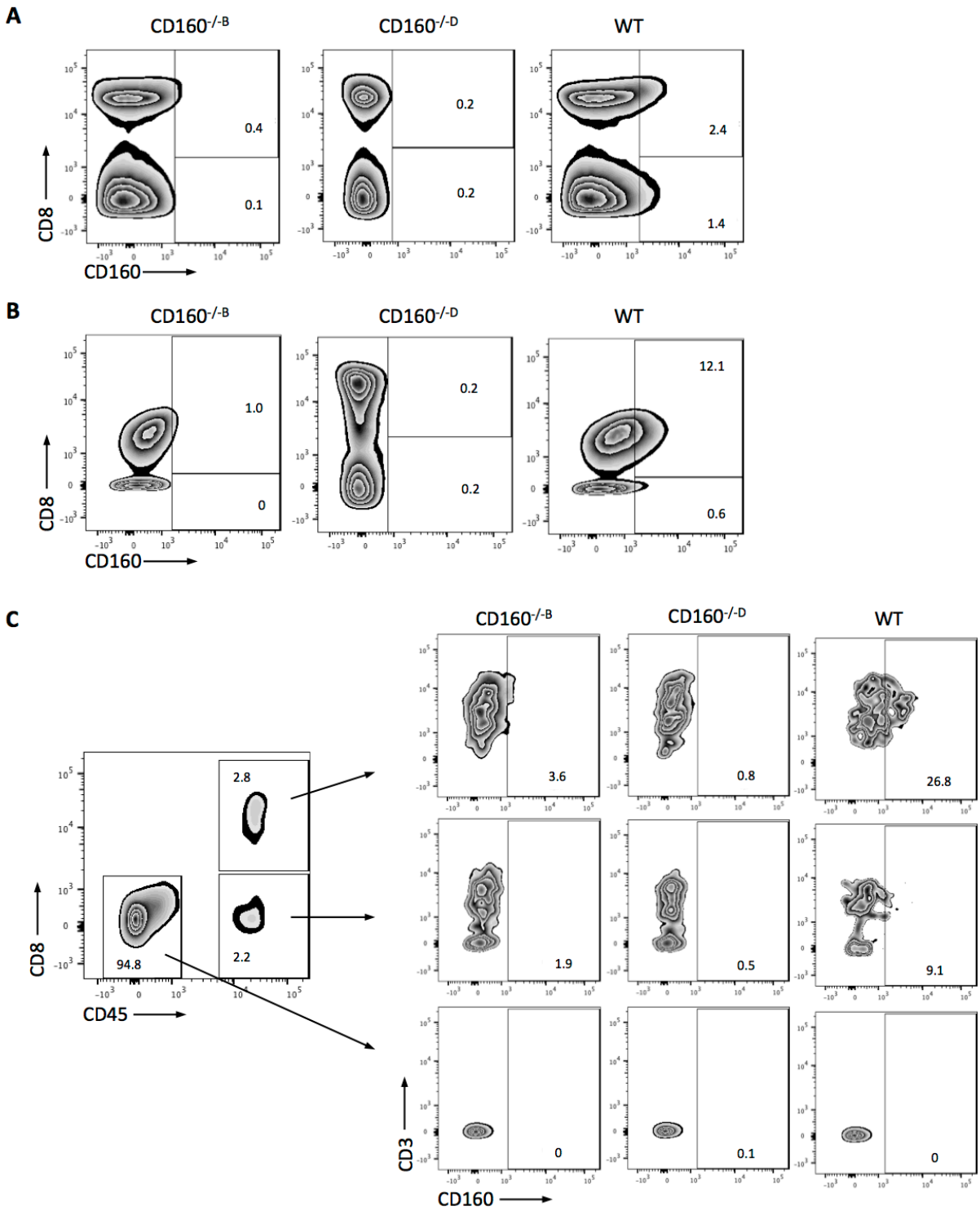



**Figure 4.23 Verification of the loss of CD160 protein in CD160$^{-/-}$ mice.** WT, CD160$^{-/-B}$ and CD160$^{-/-D}$ mice were infected i.p. with $10^5$ *Pb*A iRBC and organs were collected at d 6 p.i. CD3$^+$ cells from the spleen (A) or blood (B) were analysed by flow cytometry for CD160 expression. Representative plots of two independent experiments are shown. (C) Intestinal intraepithelial cells from naïve WT, CD160$^{-/-B}$ and CD160$^{-/-D}$ mice were analysed by flow cytometry for CD160 expression on non-hematopoietic cells (CD8$^-$CD45$^-$) and hematopoietic cells (CD45$^+$), being positive or negative for CD8. Representative plots of two independent experiments are shown.

In order to exclude a phenotype of CD160$^{-/-}$ mice mediated by a reduced or enhanced frequency of an immunological compartment e.g. NK cells or altered T cell maturation in the

thymus, splenocytes and thymocytes were analysed by flow cytometry (Figure 4.24). All analysed compartments are similar in WT, CD160$^{-/-B}$ and CD160$^{-/-D}$ mice. No differences in the frequency of different T cell subsets (TCRβ, γδ, CD4$^{+}$, CD8$^{+}$), NK cells (NK1.1$^{+}$) or B cells (CD19$^{+}$) from spleens of WT, CD160$^{-/-B}$ and CD160$^{-/-D}$ mice could be observed. In addition, thymuses of WT, CD160$^{-/-B}$ and CD160$^{-/-D}$ mice contained a similar frequency of single or double positive CD4/CD8 T cells.

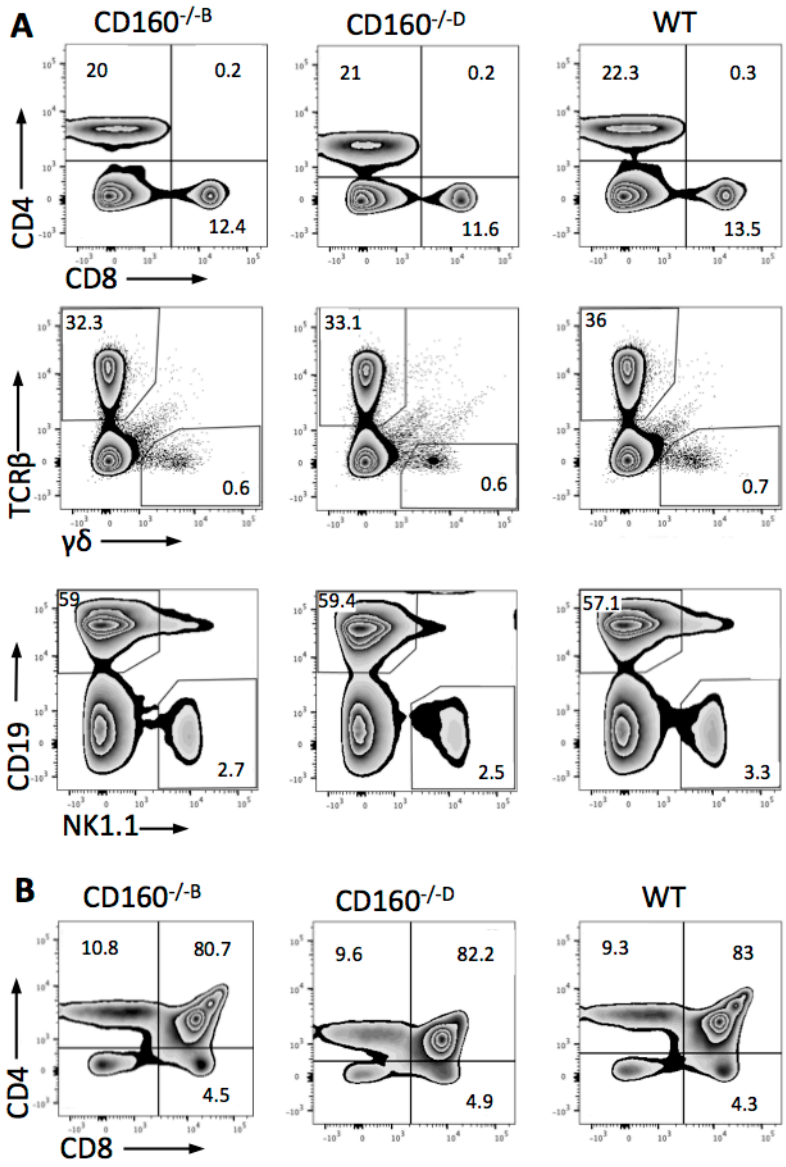



**Figure 4.24 Characterisation of the immunological compartments in CD160$^{-/-}$ mice.** (A) Splenocytes from naïve WT, CD160$^{-/-B}$ and CD160$^{-/-D}$ mice were analysed for the frequency of CD4$^{+}$, CD8$^{+}$, TCRβ$^{+}$, γδ$^{+}$, CD19$^{+}$ and NK1.1$^{+}$ cells by flow cytometry. Representative plots of two independent experiments are shown. (B) Thymocytes from naïve WT, CD160$^{-/-B}$ and CD160$^{-/-D}$ mice were analysed by flow cytometry for the frequency of single and double positive CD8$^{+}$ and CD4$^{+}$ cells. Representative plots of two independent experiments are shown.

## 4.5 Outlook: CD160 restricts immunopathology during *Pb*A infection

Due to time limitation, the data of the following chapter is only preliminary. As indicated, experiments were performed with a limited number of mice and repetitions. However, the chapter aims to give an outlook on future research.

The previous data obtained during the characterisation of CD160 expressing $CD8^+$ T cells suggests an immunomodulatory role of CD160 especially in highly activated and cytotoxic $CD8^+$ T cells. Since $CD8^+$ T cells are known to play a key role in the development of cerebral malaria in *Pb*A infected C57BL/6J mice, the question arose how the loss of CD160 influences the progression of cerebral symptoms. To this end, wild type and $CD160^{-/-B}$ mice were infected with *Pb*A and the parasitemia, incidence of cerebral symptoms at day 6 p.i. and the survival of the mice was monitored. While the parasitemia is not altered in $CD160^{-/-B}$ mice (Figure 4.25 A), the loss of CD160 leads to an increase in the severity of cerebral symptoms at day 6 p.i. (Figure 4.25 B). In line with this, $CD160^{-/-B}$ mice succumb earlier to immunopathology compared to wild type mice (Figure 4.25 C).

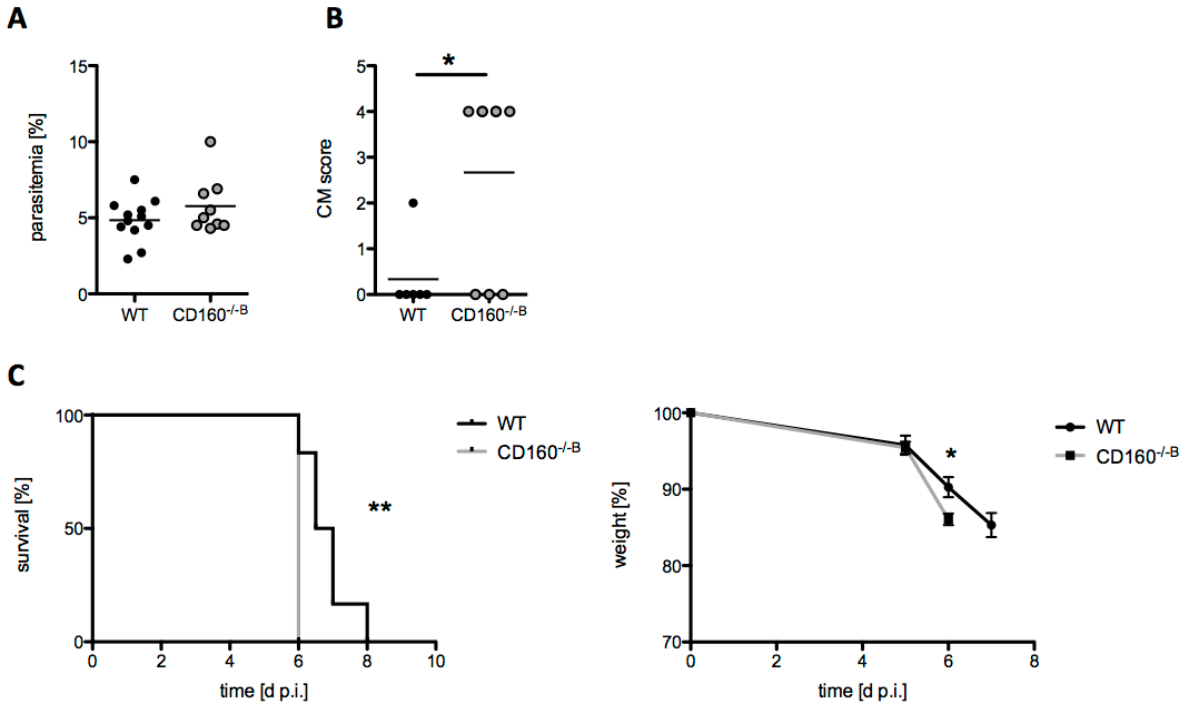



**Figure 4.25 CD160 restricts immunopathology in *Pb*A infected mice.** WT and $CD160^{-/-B}$ mice were infected i.p. with $10^5$ *Pb*A iRBC. The parasitemia (A) and severity of cerebral symptoms indicated by the CM Score (B) were determined at d 6 p.i. The quantitative overview shows pooled data of 2 independent experiments. Each circle represents one individual and means are depicted. Significance was calculated by unpaired t test: ns $p>0.05$, *$p\leq 0.05$. (C) Survival of WT and $CD160^{-/-B}$ mice infected i.p. with $10^5$ *Pb*A iRBC. Mice were monitored for cerebral symptoms and weight loss. Mice showing a score ≥3 were euthanised. For both groups, 6 mice were included in the experiment. One representative out of two independent experiments is shown. Significance was calculated by Mantel-Cox Test (survival), **p= 0.0051 or unpaired t test (weight loss): *$p\leq 0.05$.

In summary, the first data obtained with $CD160^{-/-}$ mice indicates that CD160 acts inhibitory on $CD8^{+}$ T cells to restrict immunopathology.

# 5 Discussion

This study aimed to shed light into the regulation of the immune response during malaria with the focus on the molecular switch of HVEM-BTLA-CD160-LIGHT interaction and special interest in the immunomodulatory properties of CD160. To this end, mechanistically data were obtained utilising an experimental infection in mice with the objective to subsequently verify the observed phenotype in human malaria patients. As regulatory mechanisms and pathology during the blood-stage of disease were of interest, experimental infections were performed by injection of infected RBCs. However, the immune activation during the liver-stage and the release of a more natural amount of merozoites rather than injection of an artificially high amount of iRBCs with parasites in different stages might influence the immunological outcome. Nonetheless, as discussed in detail, special interest of this study lays on the regulation of $CD8^+$ T cells and immunopathology induced by $CD8^+$ T cells, occurring at a late stage of the infection. In conclusion, minor differences regarding e.g. antigen-load during the beginning of the infection may play a less important role in this context. In addition, the infection with a standardised number of infected RBCs allows the analysis under highly controlled conditions in comparison to infection via the bite of infected mosquitos. Taken together, the blood stage infection was chosen due to technical advances and a negligible biological bias.

The dissection of important receptor-ligand interactions remains challenging due to multiple binding partners, bi-directional signalling, the possibility of cis- and trans- interactions, multiple cell types involved, different kinetics of ligand expression and the involvement of soluble factors.

## 5.1 HVEM provides important survival signals for $CD8^+$ T cells

To address the functional involvement of the molecular switch HVEM in immunomodulation during blood-stage malaria, *Pb*A infected HVEM knockout mice were characterised. In these mice, the loss of stimulatory (HVEM, LIGHT, CD160) and inhibitory (BTLA, CD160) pathways is combined, which allows to analyse the importance of the whole network. Interestingly, $HVEM^{-/-}$ mice showed a significantly lower parasitemia and attenuated cerebral symptoms (Figure 4.1). Previous data from our lab already suggested a reduced occurrence of experimental cerebral malaria (ECM) in $HVEM^{-/-}$ mice without reaching significance

presumably due to a different infection protocol [86]. The 10 fold higher dose of iRBC used in the previous study might overrule or at least reduce marginal effects observed in Figure 4.1.

At least in the experimental infection of C57BL/6 mice with *Pb*A, T cells were shown to play a central and detrimental role in the development of cerebral malaria [128]. In conclusion, this study focused on the analysis of T cells, even though it is important to mention, that T cells are not the only cells expressing HVEM. HVEM is also abundant on B cells, endothelial, epithelial and mast cells [91-94].

The findings of a milder cerebral symptomatic and a lower parasitemia in HVEM$^{-/-}$ mice seem to be in conflict, since parasite clearance requires inflammation whereas cerebral symptoms are induced by immunopathology. However, the magnitude of inflammation does not necessarily correlate with pathology, considering the fact that *Py*NL infected C57BL/6 mice develop no cerebral symptoms even though a massive and even stronger T cell activation (CD44$^{hi}$CD11a$^{+}$) compared to *Pb*A infections takes place (Figure 4.14). Most importantly, the main mediators in parasite clearance are antibodies, CD4$^{+}$ T cells, γδ T cells, macrophages and NK cells rather than CD8$^{+}$ T cells, which are the main drivers of pathology [49, 50, 58, 135-137]. Of note, HVEM-BTLA interaction inhibits B cell activation [138, 139], providing a possible explanation for a decreased parasitic load in HVEM$^{-/-}$ mice mediated by enhanced antibody production.

Analysis of the CD8$^{+}$ and CD4$^{+}$ T cell pool in infected HVEM$^{-/-}$ mice showed a decrease in the CD8$^{+}$ T cell number while CD4$^{+}$ T cells are increased in number compared to infected wild type mice (Figure 4.2). A lower number of CD8$^{+}$ T cells can be explained in three different ways. First, a lower antigen-load as shown by the reduced parasitemia (Figure 4.1) and a less inflammatory microenvironment might result in less expansion of T cells. Second, cells might show a defect in activation and clonal expansion, or third, they undergo apoptosis to a higher extend than under wild type conditions.

The influence of the antigen-load and inflammation on the CD8$^{+}$ T cell expansion was analysed by our group by the adoptive and competitive transfer of OT-1 and OT-1xHVEM$^{-/-}$ CD8$^{+}$ T cells into wild type hosts [140]. Even though both CD8$^{+}$ T cell populations encountered the same microenvironment, OT-1xHVEM$^{-/-}$ CD8$^{+}$ T cells were outcompeted by

wild type $CD8^+$ T cells, confirming the findings of a reduced number of $CD8^+$ T cells in the full body $HVEM^{-/-}$ mice (Figure 4.2).

Both, the defect in expansion or survival, are reasonable considering the signal transduction induced by HVEM. HVEM is known to provide co-stimulatory signals after trans-interaction with BTLA, CD160 and LIGHT [98, 141]. Molecules involved in HVEM signal transduction are TRAF proteins, which are associated to the cytoplasmic tail, and the transcription factors NF-κB and AP-1 [98, 141, 142]. Subsequently, activation leads to the induction of pro-survival signals, progression in the cell cycle and Th1 associated cytokine production [90, 98, 104, 105].

Staining of the proliferation marker Ki67 in *Pb*A infected $HVEM^{-/-}$ mice at day 6 p.i. revealed no difference compared to wild type mice regarding the frequency of $Ki67^+$ $CD8^+$ T cells (Figure 4.7). Supporting this finding, our group showed in adoptively and competitively transferred $CD8^+$ T cells of OT-1 and OT-1x$HVEM^{-/-}$ mice no difference in the dilution of proliferation dyes until day 5 p.i. [140]. Most importantly, the adoptive transfer provides exactly the same microenvironment including parasitic load to both $CD8^+$ T cell populations. In addition, *in vitro* stimulation of $CD8^+$ T cells of OT-1 or OT-1x$HVEM^{-/-}$ mice with SIINFEKL showed an even more pronounced proliferation in $HVEM^{-/-}$ $CD8^+$ T cells [140]. Supporting this, $HVEM^{-/-}$ T cells are hyper responsive for anti-CD3 induced proliferation *in vitro* compared to wild type controls [125]. Taken together, the data implicates that $CD8^+$ T cells can compensate for the loss of pro-proliferative signals corresponding to the loss of HVEM. One possible mechanism could be IL-2 signalling. Indeed, a significantly higher frequency of $HVEM^{-/-}$ $CD8^+$ T cells express the IL-2 high affinity receptor chain CD25 (Figure 4.8). Interestingly, it was shown, that stimulation of BTLA expressing cells with an agonistic antibody reduced the expression of CD25 and IL-2 [101, 126]. It is tempting to hypothesise, that HVEM as the natural ligand of BTLA does also induce the down-regulation of CD25 on BTLA expressing cells. Since BTLA is prominent on the majority of T cells (Figure 4.10), this mechanism could apply to all T cells. Moreover, microarray analysis of human $CD4^+$ T cells, stimulated with anti- CD3, CD28 and CD160 antibody, revealed a reduction of *IL2RA* (CD25) mRNA [89]. Even though the impact of crosslinking of CD160 was shown on $CD4^+$ T cells, in case of the *Pb*A infection the same mechanism might preferentially apply on $CD8^+$ T cells, since in this model CD160 is highly and specifically up-regulated on $CD8^+$ T cells (Figure 4.10). Taken together, the loss of HVEM-BTLA/CD160 interaction might lead to the loss of

CD25 and IL-2 inhibition resulting in enhanced pro-proliferative signalling. If CD25 regulation is indeed mediated by HVEM interaction with BTLA and/or CD160 expressing T cells, this needs to be a T cell- T cell interaction occurring in a cluster of proliferating T cells, since the effect can also be observed in $HVEM^{-/-}$ $CD8^+$ T cells transferred in wild type hosts.

While no evidence for a defect in proliferation and expansion of $CD8^+$ T cells or an impact of the microenvironment could be found, analysis of the terminally differentiation markers CD160 (discussed in 5.6) and KLRG1 clearly indicated a defect in survival of $HVEM^{-/-}$ $CD8^+$ T cells (Figure 4.9). Supporting this, an important role of HVEM for the persistence of memory $CD4^+$ T cells (Th2 and Th1) was reported [143]. In this study, survival of $CD4^+$ T cells could be restored by a constitutively active protein kinase B (PKB, Akt), which showed reduced activity in $HVEM^{-/-}$ $CD4^+$ T cells. This data supports our findings of a defect in survival due to a lack of HVEM but is in contrast to the observed enlarged $CD4^+$ T cell pool (Figure 4.2). A possible explanation could be, that in the study of Soroosh and colleagues the phenotype only occurred after recall response with a model antigen whereas the *Pb*A model deals with an acute infection. $HVEM^{-/-}$ mice might also be defective in establishment of a $CD4^+$ T cell memory compartment after *Pb*A infection, which could be studied in detail for the liver stage by infection with attenuated sporozoites or for the blood-stage by treatment of blood-stage parasites with a drug like pyrimethamine and subsequent reinfection.

The difference in the dependency of $CD4^+$ and $CD8^+$ T cells on HVEM signalling might also be explained by a different stability in homeostasis. $CD4^+$ T cells are less susceptible for aging than $CD8^+$ T cells [144].

## 5.2 HVEM has no impact on the expression of GrzB, degranulation and IFNγ production

As shown by others, experimental cerebral malaria induced by *Pb*A is favoured by high level of IFNγ [145] and the cytotoxic effector function of $CD8^+$ T cells in the brain is absolutely crucial. Both, GrzB and perforin knockout mice are protected against ECM [17, 18].

Along this line, a diminished effector function of $CD8^+$ T cells in $HVEM^{-/-}$ mice might contribute to a milder course of disease. However, no difference in the frequency of $GrzB^+$ $CD8^+$ T cells could be observed (Figure 4.4), which was confirmed by data from adoptively transferred $CD8^+$ T cells in wild type host performed by our lab group [140]. As in both experiments only one time point during the course of disease was analysed, the absence of a

difference could be a matter of the kinetic. Wild type $CD8^+$ T cells could have already released the GrzB molecules stored intracellular in granules to a certain amount, leading to an underestimation of cytotoxic T cells. However, the release of effector molecules by degranulation measured by the surface expression of CD107a was equal in wild type and $HVEM^{-/-}$ mice (Figure 4.4). In addition, adoptively transferred $CD8^+$ T cells were analysed at day 5 p.i. and $CD8^+$ T cells in chapter 4.1 at day 6 p.i., therefore, including the day before (d 5 p.i.) and the day of onset of cerebral malaria (d 6 p.i.), the critical time point for the development of immunopathology in the brain is covered.

Just as the cytotoxic function seems not to be altered in $HVEM^{-/-}$ $CD8^+$ T cells, the same frequency of $HVEM^{-/-}$ $CD8^+$ T cells is able to produce IFNγ upon *ex vivo* stimulation of splenocytes from *Pb*A infected mice with PMA/ionomycin compared to wild type $CD8^+$ T cells (Figure 4.5). Nevertheless, the strong activation of cells might overrule marginal differences in IFNγ production *in vivo*. Hence, a better approach is to stimulate with parasite specific epitopes [19, 20]. Interestingly, $HVEM^{-/-}$ splenocytes secrete significantly less IFNγ into the cell culture supernatant after stimulation with *Pb*A-peptides [146]. This seems to be in conflict with the data obtained from the PMA/ionomycin stimulation. First, it needs to be considered, that analysis of supernatant cannot distinguish between different types of IFNγ producing cells. Even though the MHCI restricted peptides used should specifically stimulate $CD8^+$ T cells, a non-peptide control revealed cytokine production even without stimulation, presumably due to previously *in vivo* activated cells. Second, the use of whole splenocytes is biased by the lower number of effector cells in the $HVEM^{-/-}$ mice as shown in chapter 4.1. To address both concerns, a better approach would be the use of sorted, activated effector cells (e.g. $CD11a^+CD44^{hi}$ $CD8^+$ T cells), stimulated with *Pb*A-peptide pulsed brain endothelial cells, mimicking the cross-presenting brain endothelial cells in ECM mice.

## 5.3 Expression of activation markers is reduced in $HVEM^{-/-}$ $CD8^+$ T cells

Besides the number of potential pathogenic CTLs, their activation status might be detrimental for the development of cerebral malaria. Activation of T cells is commonly specified by the expression of adhesion molecules, which are required for the sequestration into inflamed tissue or loss of lymphoid homing receptors. In this study, the molecules CD44 and CD11a were used. CD11a is important for the interaction of T cell and antigen-presenting cell and can be found in the supramolecular activation cluster (SMAC) in the

external subregion [147, 148]. Even though it is not the only receptor involved in cytoadherence, expression of the CD11a ligand ICAM-1 coincides with vascular leakage and the absence of ICAM-1 protects C57BL/6 mice from *Pb*A induced ECM [149, 150]. Along this line, the blockade of CD11a leads to a significant decrease in experimental cerebral malaria [151]. Together with the observed expression of CD11a on all $CD8^+$ T cell isolated from the brain of CM and non-CM mice (Figure 4.6), CD11a can be considered as an important marker for activation and cytoadherence. In contrast to CD11a, CD44 is not associated to experimental cerebral malaria but is considered as a general marker for effector cells [152]. Interestingly, $HVEM^{-/-}$ mice indeed show a lower frequency of $CD44^{hi}$ and $CD11a^+$ $CD8^+$ T cells in the blood, providing a smaller pool of activated cells, being able to attach to brain endothelial cells. The difference observed in the blood is not found in spleen and brain, which is perfectly in line with the hypothesis of a lack of survival signals in $HVEM^{-/-}$ $CD8^+$ T cells. Due to a high frequency of naïve or recently activated $CD8^+$ T cells in the spleen, T cells in this organ mainly depend on stimulation by professional antigen-presenting cells leading to their activation and clonal expansion. However, in the blood effector cells strongly depend on survival signals for their persistence. As all T cells in the brain express CD44 and CD11a (Figure 4.6), both molecules might be required for cytoadhesion. Hence, T cells from the blood are selected for the phenotype $CD44^{hi}$/$CD11a^+$.

## 5.4 $HVEM^{-/-}$ mice exhibit a higher number of Tregs than wild type mice

The current study showed a significantly higher number of $Foxp3^+$ Tregs in *Pb*A infected $HVEM^{-/-}$ mice compared to wild type mice (Figure 4.3). In contrast, Sharma and colleagues observed a diminished Treg response in Herpes-Simplex virus-1 (HSV) infected $HVEM^{-/-}$ mice compared to wild type mice [153]. Importantly, the infection with HSV provides an additional ligand for HVEM, namely the viral gD protein, which stimulates HVEM expressing regulatory T cells [153]. Hence, wild type Tregs receive stimulation during HSV infection, which is absent in $HVEM^{-/-}$ mice due to the lack of the ligand for gD. The absence of viral gD protein in the *Pb*A infection might explain the different outcome in Treg frequency in the *Pb*A model compared to the HSV infection. Nonetheless, the question arises whether the increased number of regulatory T cells in $HVEM^{-/-}$ mice has an impact on the reduction of immunopathology. The role of regulatory T cells in immunosuppression during experimental cerebral malaria is controversially discussed. While depletion of $CD25^+$ cells by monoclonal

antibodies was published to reduce the development of ECM [154], the depletion of $Foxp3^+$ T cells in Dereg mice did not influence pathogenesis [75]. Since the depletion of $CD25^+$ cells not only targets Tregs but also a population of effector T cells, that is induced during *Pb*A infection [75], data obtained with Dereg mice appeared to be more convincing. Even though *Pb*A infected $HVEM^{-/-}$ mice showed higher numbers of Tregs compared to wild type mice, the frequency of Tregs was not altered (Figure 4.3). Hence, the regulatory/effector T cell ratio is unchanged. In addition, one effector mechanism of Tregs is the interaction of HVEM with the co-inhibitory molecule BTLA expressed by T effector cells [155], suggesting a diminished regulatory capacity of $HVEM^{-/-}$ Tregs. Taking the limited role in the *Pb*A infection, unchanged frequency and presumable diminished effector function of $HVEM^{-/-}$ Tregs into concern, a major immunosuppressive role of Tregs in $HVEM^{-/-}$ mice is not conclusive.

## 5.5 CD160 is embedded in a complex network of regulatory receptors

The HVEM network consists of several immunomodulatory players with a distinct role of each receptor on different cell types depending on the stage of infection or health status. Regarding CD160, it is exclusively expressed by $CD8^+$ T cells of infected mice but not by $CD4^+$ T cells (Figure 4.10). In contrast, BTLA is, like HVEM, highly abundant on $CD4^+$ and $CD8^+$ T cells (Figure 4.10). In a naïve state, BTLA was shown to interact with HVEM in cis and thereby prevents stimulatory signalling of HVEM [100]. Both, CD160 and BTLA, compete for binding to HVEM due to overlapping binding regions and provide inhibitory signalling upon receptor binding [102]. However, the occurrence of CD160 only during infection on a specific $CD8^+$ T cell subset suggests a different role in immunomodulation.

Moreover, most other well-known co-inhibitory receptors like PD-1 and CTLA-4 are more prominent on $CD4^+$ T cells during infection than on $CD8^+$ T cell and their function in T cell regulation during malaria was predominantly described on $CD4^+$ T cells in human and mice [82, 84, 156]. Nonetheless, CTLA-4 and PD-1 are also expressed on $CD8^+$ T cells (Figure 4.10) and data obtained in studies using knockout mice or blocking antibodies cannot discriminate between effects on $CD4^+$ and $CD8^+$ T cells [85, 157].

## 5.6 Expression of CD160 characterises highly activated and cytotoxic $CD8^+$ T cells

As discussed in section 5.3, CD44 and CD11a allow the discrimination of activated, antigen-experienced cells. The clear co-expression of CD160 with CD44 and CD11a shows its

restriction to activated cells, going in line with the absence of CD160 in naïve mice and the late induction during the course of infection (Figure 4.11). Supporting this, CD160 is induced upon ConA *in vitro* stimulation especially on $CD44^{hi}CD62L^{low}$ effector/memory $CD8^+$ T cells [123]. Since CD44 and CD11a seem to be indispensable for the sequestration into the brain due to their ubiquitous expression on $CD8^+$ T cells in this tissue (Figure 4.6), CD160 is found on cells, that is the potential source for immunopathology.

Not only the localisation of $CD160^+$ $CD8^+$ T cells in the brain, but also their co-expression of the cytotoxic molecule GrzB (Figure 4.16) highlights the relevance of this cell subset, since immunopathology in experimental cerebral malaria strictly depends on the expression of granzymes [18]. As shown in 4.2 and by others, GrzB is specifically induced in the acute stage of *Py*NL infection and expression decreases after parasite clearance (Figure 4.15)[158]. Furthermore, also $CD8^+$ T cells that have recently degranulated according to their surface expression of CD107a co-express CD160. In conclusion, $CD160^+$ $CD8^+$ T cells not only store cytotoxic molecules but also release them.

Interestingly, Figure 4.16 also showed a co-expression of CD160 and CD28, while others described CD160 as a co-stimulatory molecule in the absence of CD28 [114, 115, 123]. However, differences should be made regarding the differentiation status of the cells of interest. This study showed an induction of CD28 after infection. In conclusion, activation of the $CD8^+$ T cells might first lead to the expression of the co-stimulatory molecule CD28 and subsequently to the induction of CD160. While CD28 and CD160 are co-expressed at the acute stage of the infection, CD28 is down regulated with proceeding T cell differentiation while cells remain $CD160^+$. Persistence of $CD160^+$ $CD8^+$ T cells is supported by data obtained from *Py*NL infection of C57BL/6 mice, were the frequency of $CD160^+$ $CD8^+$ T cells remains unchanged after 20 days of infection even after clearance of the parasite (Figure 4.15). In addition, the frequency of $CD160^+$ $CD8^+$ T cells correlates with age in malaria patients (Figure 4.20), indicating an accumulation of $CD160^+$ effector/memory T cells in humans as well. Furthermore, CD160 is at least partially co-expressed with KLRG1, a common marker for terminally differentiated cells lacking proliferative capacity [159]. Interestingly, the receptor KLRG1 shares several features with CD160. First, it is expressed by the same cell types namely NK cells, antigen-specific $CD8^+$ T cells as well as γδ T cells [160, 161]. Second, KLRG1 can act co-inhibitory if engaged by its ligand E-cadherin [162]. Third, the frequency of KLRG1 positive cells rises with age in human [163].

Besides KLRG1, the co-inhibitory receptors PD-1 and BTLA are enriched in the $CD160^+$ $CD8^+$ population. A diversity of regulatory molecules can be considered as a further hint for terminal differentiation [164, 165].

PD-1 is well studied on $CD4^+$ T cells in experimental and human malaria, where it characterises a cell type with regulatory properties [82, 85]. In addition, PD-1 is induced in *Py*NL as well as in *Pb*A infection during the acute phase on $CD8^+$ T cells were it restricts T cell function [85, 158]. Accordingly, *in vivo* blockade of PD-L1 enhances the occurrence of cerebral malaria and the production of IFNγ by $CD8^+$ T cells [85]. While beneficial in acute malaria, PD-1 is considered as an exhaustion marker in viral infection [166] and co-expression with CD160 was shown on functionally impaired $CD8^+$ T cells of HIV patients [167].

Along this line, treatment with an agonistic antibody targeting BTLA restricts the development of experimental cerebral malaria [86], while clearance of *Py*NL parasites is improved in $BTLA^{-/-}$ mice involving innate and adaptive immune responses [108]. Since BTLA und CD160 interact with overlapping regions of HVEM, they compete for their binding partner [102]. Assuming CD160 exhibits a co-inhibitory function, it might compensate for BTLA in case of a loss of BTLA signalling. At first view, the observed co-expression argues against such a compensatory effect. In contrast, it rather implies additive effects maybe also involving the second CD160-ligand namely MHCI. However, CD160 might indeed act compensatory in case of unavailability of BTLA for trans-interaction with HVEM due to established cis-binding.

The phenotype of CD160 expressing $CD8^+$ T cells points to a highly cytotoxic function of those cells. In order to prove that CD160 expressing cells are indeed able to efficiently kill target cells and to produce pro-inflammatory cytokines *in vitro* assays were utilized. Aiming to exclude any bias due to a high proportion of naïve T cells within the $CD160^-$ control, effector cells were sorted according to their CD44 expression (Figure 4.17).

Even though $CD160^+$ cells co-express known co-inhibitory molecules like PD-1, BTLA or KLRG1, their ability to kill target cells and to produce IFNγ is not impaired but even more pronounced compared to CD160 negative cells (Figure 4.17). Surprisingly, the effector T cells used in this assay are highly specialized for the production of IFNγ while they are not able to secrete e.g. TNFα or IL-2 (data not shown), although they were isolated from infected

mice only 6 days after infection. In conclusion, both assays argue against “exhaustion” or dysfunction of CD160 expressing $CD8^+$ T cells. Importantly, no conclusions about the functional involvement of CD160 in killing or cytokine production can be drawn. Others have shown a (co-) stimulatory effect of CD160 for NK and T cell mediated cytotoxicity and IFNγ production using crosslinking antibodies or CD160-ligand [112, 115-117]. However, also contrasting results meaning a (co-) inhibitory function of CD160 was observed using a similar *in vitro* stimulation of NK or T cells with cross-linking antibodies, natural ligand or chemicals [89, 112]. Overall, the respective cell type, culturing and stimulation conditions seem to have a great impact on the outcome. Unfortunately, until now the context, which drives inhibition or stimulation, is not understood. Hence, the interpretation of *in vitro* assays regarding the function of CD160 remains challenging, further emphasising the importance of *in vivo* experiments and knockout mouse models.

## 5.7 CD160 expression is linked to pathology

Strikingly, expression of CD160 by $CD8^+$ T cells is first detected at day 6 p.i., the day of onset of cerebral malaria (Figure 4.11). In addition, the highest abundance of $CD160^+$ $CD8^+$ T cells is in the brain of infected mice (Figure 4.12), where immunopathology is induced pointing again to a linkage of CD160 and pathology.

The occurrence and development of experimental cerebral malaria is tightly linked to cytotoxic T cells and IFNγ [168-171]. Interestingly, analysis of phenotype and function of CD160 expressing $CD8^+$ T cells revealed their predominant cytotoxicity and IFNγ production compared to their $CD160^-$ counterpart (Figure 4.17). Taking only the analysis of the phenotype of $CD160^+$ $CD8^+$ T cells and functional assays into concern, no conclusion about the direct involvement of CD160 in killing or cytokine production could be drawn. However, since CD160 defines a highly cytotoxic subset, which is most importantly highest in frequency in the brain compared to spleen and blood (Figure 4.12) a higher abundance of potential pathologic cells could explain the correlation with the severity of pathology. Clearance of *Plasmodia* infected RBCs is mediated by several mechanisms including antibodies, reactive oxygen species and cytokines [49, 50, 58, 135-137]. However, depletion of αβ T cells has no impact on the parasitemia in *Pb*A infected C57BL/6 mice [168], showing a dispensable role in parasite clearance. The fact that T cells are not the main effector cells

for the control of the parasitemia may be the reason for the lack of correlation of the frequency of $CD160^+$ $CD8^+$ T cells and parasitemia.

The non-lethal infection of C57BL/6 mice with *Py*NL leads to more pronounced splenomegaly and early IFNγ production compared to *Pb*A infected mice [172], suggesting strong immune activation without the drawback of immunopathology. Indeed, in *Py*NL infected mice, the frequency of activated ($CD44^{hi}CD11a^+$) $CD8^+$ T cells is significantly higher than in the *Pb*A infection (Figure 4.14). Nonetheless, within the activated $CD8^+$ T cell compartment, the frequency of $CD160^+$ cells is clearly lower in the non-lethal than in the lethal infection (Figure 4.14). Accordingly, one explanation for the absence of immunopathology in *Py*NL infected mice could be the diminished frequency of potentially pathologic cells. However, a more important factor for protection might be a lack of cross-presentation of *Py*NL antigen on endothelial cells, which is described for the non-CM causing strains *Py*17X, *Py*17XNL and *Pb*NK65 [19, 20].

## 5.8 Potential interaction partners for CD160 in the brain

Since all data discussed above pinpoint $CD160^+$ $CD8^+$ T cells to the severity of cerebral malaria, the question arises if CD160 signalling is a driving force for pathology. To answer this question, one important point is the availability of CD160-ligand in the brain. Until today, two interaction partners of CD160 are described, namely classical and non-classical MHCI and HVEM [89, 112, 114]. For the development of experimental cerebral malaria, the interaction of $CD8^+$ T cells with cross-presenting brain endothelial cells is crucial [20], emphasising the relevance of CD160-ligand expression by those endothelial cells. MHCI is expressed on all nucleated cells but interaction with CD160 is weak, requiring clustering of molecules [112, 114]. In contrast, HVEM is less broadly expressed but its affinity for CD160 is higher compared to MHCI [89]. Interestingly, HVEM was found in the brain expressed at low level in naïve mice and a strong induction occurs during infection with highest level of HVEM mRNA in mice suffering from cerebral malaria [86, 173]. It is important to mention, that the analysis was performed on whole brain tissue and HVEM expression could not be connected to a certain cell type. The source of HVEM mRNA might be sequestered T cells. Nevertheless, as HVEM is expressed on brain endothelial cells [93], one may hypothesise that HVEM is present or even enhanced expressed on brain endothelial cells during experimental malaria. In consequence, due to the induction during infection and the high binding affinity, the

interaction of HVEM expressed by the cross-presenting endothelial cells with CD160 on $CD8^+$ T cells might play a predominate role in T cell regulation compared to the interaction of CD160 and MHCI.

## 5.9 Source and relevance of soluble CD160

Until today only few data exists about soluble CD160 and its functional role and cellular source during malaria is still elusive. Hence, cell types, which might contribute to the presence of sCD160 in sera of *Pb*A infected mice will be discussed in this chapter.

Tissue resident mast cells of mice and human were found to store CD160 in intracellular granula [111]. Interestingly, inflammation in skin tissue reduced or abolished the intracellular CD160 of resident mast cells, whereas CD160 can be found in cell culture supernatants of mast cell cultures [111]. Moreover, mast cells were not only shown to be a potential source of sCD160, but they were also identified as an important stimulator of $CD8^+$ T cell activation in malaria. During infection with *Plasmodia*, mast cells are activated in a type I IFN and TRL9 dependent manner and produce the cytokine FLT3L by which CD8-$\alpha^+$ dendritic cells are induced. CD8-$\alpha^+$ dendritic cells are key players for the activation and expansion of $CD8^+$ T cells but not for $CD4^+$ T cells [174]. However, mast cells as a component of the innate immune system are activated at a very early stage of infection. Already at day 2 p.i., elevated level of FLT3L were observed [174]. Moreover, CD160 was shown to be stored in granula of naive mast cells [111], implying a fast release upon activation. In contrast, the level of sCD160 was not enhanced earlier than day 5 p.i., arguing against mast cells as the main producer of soluble CD160. Another explanation could be a certain level of "shedding-capacity" of the phospholipase D (PLD), which is responsible for cleavage of the GPI anchor [103]. CD160 might already be present at low level at the cell surface of $CD8^+$ T cells but is cleaved immediately afterwards. At a certain stage of activation, the amount of CD160 on the cell surface might overrule the capacity of PLD for cleavage of the protein or PLD might be down regulated. To address this, mRNA level of CD160 in $CD8^+$ T cells during the course of infection as well as expression and activity of the PLD need to be analysed. In NK cells, shedding of CD160 is induced by stimulation with IL-15. IL-15 induces the expression of PLD and inhibition of the enzymatic activity of PLD at least partially prevents CD160 shedding [103]. In contrast to e.g. IL-12, malaria patients do not exhibit detectable IL-15 serum level [175]. However, the lack of IL-15 leads to an impaired innate and adaptive

immune response in *Plasmodium chabaudi* AS infected mice, indicating that IL-15 does play a role during infection, even though it might not be present at high level in serum [176]. Hence, release of CD160 by NK cells seems reasonable.

Finally, γδ T cells could be a source of sCD160, since they are known to express CD160. At least in human malaria, γδ T cells expand transiently during infection, presumably enhancing their possible impact in the production of sCD160 [177].

In conclusion, soluble CD160 might be released by several cell types. Hence, the serum level of CD160 might represent a broader view on the immune status involving also innate (NK cells, mast cells, γδ T cells) immune cells and their individual response to the parasite.

It was proposed, that sCD160 might disrupt the interaction of membrane bound CD160 expressed by cytotoxic effector cells with its ligand in order to suppress effector function [111]. Since $CD160^+$ $CD8^+$ T cells appear rather late during *Pb*A infection and they exhibit a terminally differentiated phenotype, an important function might be the influence on $CD8^+$ T cell interaction with brain-endothelial cells.

## 5.10 CD160 characterises a similar $CD8^+$ T cell subset in human and mice

Even though mouse models are highly important for the analysis of complex immune regulatory mechanisms, the main goal remains the understanding of the human immune system. Hence, verification of data generated in mouse models with human samples is crucial. For this reason, the phenotype of $CD8^+$ T cells was not only analysed in mouse but also in human samples. Functional markers expressed by $CD8^+$ T cells and their induction upon *Plasmodium falciparum* infection in human was analysed using whole blood samples. While most human studies struggle with complicated accessibility of the affected tissue, in the blood of malaria patients with ongoing blood-stage infection, the cells of interest can be analysed. Most studies analysing $CD8^+$ T cells in malaria focus on memory T cells specific for the liver stage, since $CD8^+$ T cells are the main effector cells in parasite clearance during this stage of the infection [30, 178-180]. In order to assess the $CD8^+$ T cell phenotype during acute blood-stage infection, whole blood samples were stained *ex vivo*. As expected, in acute malaria activation (CD69), proliferation (Ki67) and cytotoxicity markers (GrzB, Perforin) and the co-inhibitory molecule PD-1 are expressed by an increased frequency of $CD8^+$ T cells compared to healthy controls (Figure 4.18). However, the frequency of $CD160^+$ $CD8^+$ T cells is not altered in the patient group compared to healthy control. This is in contrast to the

induction of CD160 on $CD8^+$ T cells during the experimental infection of mice with *Pb*A or *Py*NL parasites. Most importantly, healthy controls cannot be considered similar to naïve mice especially regarding the phenotype of $CD8^+$ T cells. Since people enrolled in this study are adults, they have already encountered several infections during the past years. It is known, that viral infections induce effector cells which express co-inhibitory molecules and persist long term [165, 181-185]. In HIV infected patients CD160 is co-expressed with PD-1 and 2B4 [186]. While co-expression with PD-1 is associated with dysfunctional $CD8^+$ T cells [167], a population of 2B4 and CD160 co-expressing $CD8^+$ T cells was specifically found in HIV elite controllers [187]. Independent of the respective function, literature points to an accumulation of antigen-specific $CD160^+$ T cells. Indeed, the frequency of $CD160^+$ $CD8^+$ T cells positively correlates with the age of malaria patients. Considering the fact that the basal level of co-inhibitory positive $CD8^+$ T cells is not known for the patients enrolled in the study and the detection of malaria specific $CD8^+$ T cells was not possible, an induction during acute infection might not be detected even though it was present.

Irrespective of the healthy status of the individual, the human $CD160^+$ subset showed the same phenotype compared to the expression pattern observed in murine $CD160^+$ T cells regarding their cytotoxic capacity. In conclusion, CD160 defines a $CD8^+$ T cell subset with a highly cytotoxic phenotype, which is non-specific for malaria but can be described as general T cell activation status.

The only markers in the human data set (Figure 4.19), which do not mirror the mouse data, are CD28 and Ki67. While CD28 decreases on $CD8^+$ T cells in human with age [144], it is not present in naïve mice, but is induced on effector T cells during infection. In conclusion, the co-expression of CD160 and CD28 can be explained by the recent activation of $CD8^+$ T cell in mice upon infection. In contrast, $CD160^+$ $CD8^+$ T cells accumulate in human, accompanied by a loss of CD28 expression over time. It was proposed that CD160 acts as a co-stimulatory molecule in the absence of CD28 signalling [114]. However, preliminary data (Figure 4.25) suggests a co-inhibitory function of CD160 on $CD8^+$ T cells, arguing against a compensatory role after the loss of CD28 expression.

Ki67 is commonly used as a marker for proliferating cells, because it is expressed by all cells which have entered the cell cycle [188]. In line with the hypothesis that CD160 is present on terminally differentiated T cells, $CD160^+$ $CD8^+$ T cells in human peripheral blood do not

express Ki67 to a higher extend than $CD160^-$ $CD8^+$ T cells. In contrast in mice, were all $CD8^+$ T cells are recently activated by malarial antigen, $CD160^+$ T cells still proliferate.

In summary, in humans, $CD8^+$ T cells lose the expression of CD28 and their capacity to proliferate after terminal differentiation, but still store cytolytic and cytotoxic effector molecules and express CD160.

## 5.11 Preliminary data suggests a co-inhibitory function of CD160 on $CD8^+$ T cells

This thesis is focused on the regulation of T cell function and immunopathology induced by T cells in experimentally *Pb*A infected mice. However, CD160 is not exclusive expressed by T cells but also by NK, NKT, γδ T cells and mast cells. In conclusion, data obtained with $CD160^{-/-}$ mice considering the development of parasitemia and cerebral malaria need to take the effector function of all CD160 expressing cell types into account. Subsequently, the role of CD160 expressed by different cell types in view of potential effects during malaria should be discussed.

As mentioned above, mast cells are a source of soluble CD160, which was proposed as a regulatory mechanism of $CD8^+$ T cell activation by the disruption of CD160 signalling mediated by the soluble CD160 [111]. Nonetheless, no function of soluble CD160 in malaria could be proven yet.

NK cells are part of the innate immune system and can be, dependent on parasite and mouse strain used, important players or dispensable. In C57BL/6 mice infected with *Plasmodium chabaudi* or *yoelii*, NK cells contribute to parasite clearance in a IFNγ dependent and cytotoxicity independent manner [38] whereas in infection with *Plasmodium berghei* ANKA the involvement of NK cells in parasite clearance is less well described. Regarding malaria in humans, NK cells are the major source of early IFNγ within PBMCs of *Pf* infected patients [137]. Indeed, CD160 expressed by NK cells was shown to be crucial for an IFNγ dependent tumour control and remission [189]. Interestingly, killing of tumour cells was established in MHCI and in HVEM negative tumour cell lines, raising the question, which ligand expressed by which cell type induces CD160 signalling in this model.

Similar to NK cells, γδ T cells provide IFNγ during the early stage of malaria and thereby take part in parasite clearance. However, little is known about the function of CD160 expressed by γδ T cells.

While the impact of γδ T cells and NK cells on the development of cerebral malaria is controversially discussed [128, 168, 190], $CD4^+$ and $CD8^+$ T cells are clearly crucial for pathology [128].

Even though a detailed phenotyping of CD160 expressing $CD8^+$ T cells could link this cell subset to severity of disease, which is in line with their highly cytotoxic capacity, the question remained which role CD160 plays in the regulation of those cells. There is strong evidence for the hypothesis of a co-stimulatory signalling in literature providing an explanation for the observed activation of $CD160^+$ $CD8^+$ T cells [106, 114, 115, 191]. However, recent publications reported an inhibition via CD160-HVEM interaction [89, 185]. Considering other co-inhibitory receptors like PD-1 or Tim-3, which are induced in response to T cell activation in order to restrict T cell function to prevent overwhelming responses, it seems also reasonable to suggest such a function for CD160. Nevertheless, both functions namely co-inhibition and co-stimulation might play a role in malaria depending on the cell type, environment and differentiation status of the respective cells. Dual functionality is a phenomenon, which is also described for 2B4. Similar to CD160, 2B4 is a receptor expressed by virus-specific $CD8^+$ T cells and by NK cells [192]. In the case of 2B4, stimulatory or inhibitory signalling depends on the expression level of the receptor and its downstream signalling molecules [192]. Indeed, even for CD160 expressed by murine NK cells a dual functionality could be observed dependent on the respective stimulus [112].

All data obtained until now in $CD160^{-/-}$ mice infected with *Pb*A support the idea of a dual function, since they point to a co-inhibitory function in $CD8^+$ T cells and at the same time to a co-stimulatory role in NK cell activation. Incidence of experimental cerebral malaria mediated by $CD4^+$ and $CD8^+$ T cells is enhanced after loss of CD160. Of note, the parasitemia is also slightly enhanced in $CD160^{-/-}$ mice (Figure 4.25). In conclusion, $CD8^+$ T cells induced immunopathology seems to be not longer restricted by CD160 while innate cells e.g. NK cells might be impaired in the production of early IFNγ due to the loss of CD160 mediated stimulation.

# 6 Future perspectives

## 6.1 HVEM

As shown in this thesis, the molecular switch HVEM provides important pro-survival signals to $CD8^+$ T cells during acute *Pb*A infection in C57BL/6J mice. Until now, an enlargement of the $CD4^+$ T cells pool in the absence of HVEM could be shown. However, other studies suggest a similar pro-survival effect of HVEM for $CD4^+$ memory T cells [143]. Hence, experiments addressing the $CD4^+$ memory T cell development and maintenance in $HVEM^{-/-}$ mice would be of interest. To this end, different approaches could be used. First, vaccination of mice with irradiated sporozoites and subsequent challenge with replication competent sporozoites can be utilised. With this, the memory response against the liver stage can be analysed, which is especially of interest for vaccination strategies. Second, treatment of the blood-stage with anti-parasitic drugs until parasites are completely eliminated can be followed by infection with blood-stage parasites. Because it is also using blood-stage parasites, this setting represents an approach closely related to the data obtained in this thesis, which would enhance the comparability of results. A third option would be the infection of C57BL/6J mice not with the lethal *Pb*A but with a parasite strain, which can be cleared e.g. *Plasmodium yoelii* or *Plasmodium chaubaudi*, and a second infection afterwards. The advantage would be a longer lasting first infection compared to *Pb*A infected and drug treated mice allowing proper memory formation. In addition, protective immunity depends more on T cells in *Plasmodium yoelii* or *chaubaudi* infections compared to *Pb*A. However, different parasite strains are difficult to compare to previous data.

This thesis could show but not explain a reduction of parasitemia in $HVEM^{-/-}$ mice. As discussed above, increased B cell function due to a lack of inhibitory signalling via BTLA could lead to higher anti-parasitic antibody titer, resulting in a better control of parasitemia. Hence, antibody production should be monitored in $HVEM^{-/-}$ mice.

## 6.2 CD160

The $CD160^{-/-}$ mouse generated in this thesis is highly valuable for the analysis of several unanswered questions.

Preliminary data suggests an inhibitory effect of CD160 on $CD8^+$ T cells in experimental malaria. The direct proof of functional improvement of $CD8^+$ T cells lacking CD160 is difficult

to show due to the lack of a surrogate marker for CD160. Without the possibility to identify the cells, that would normally have upregulated CD160, in the knockout mouse, direct comparison of e.g. cytokine production or killing capacity is at least difficult. In the spleen only approximately 10 % of $CD8^+$ T cells express CD160 in wild type mice. Hence, even if the lack of CD160 in those cells improves e.g. IFNγ production, a difference to wild type cells is most likely overruled by the fraction of $CD160^-$ cells. However, regarding the correlation of CD160 expression of $CD8^+$ T cells and severity of cerebral malaria, immunopathology in the brain seems the most appropriate read-out for the change of T cell functionality. Besides the development of cerebral symptoms, the number of brain-infiltrating $CD8^+$ T cells, disruption of the blood-brain-barrier measured by Evans blue staining and histological assessment of the severity of haemorrhages can be considered.

In contrast to the proposed co-inhibitory signalling induced in $CD8^+$ T cells by CD160, enhanced parasitemia in $CD160^{-/-}$ suggests a co-stimulatory effect in IFNγ producing NK cells early during infection. For further analysis an infection with *Py*NL parasites allowing the observation of the parasitemia without the development of cerebral malaria might be more appropriate than infection with *Pb*A. Depletion of NK cells should abrogate any differences in parasitemia observed between $CD160^{-/-}$ and wild type mice. In addition, the use of $CD160^{-/-}$ Rag mice can exclude any impact of cells derived from the adaptive immune system like $CD8^+$ T cells in parasite clearance.

In terms of human data, a further comparison of CD160 expression level on $CD8^+$ T cells related to age would be of interest. Along this line, a method that allows to distinguish persistent $CD8^+$ T cells and recently, malaria-specifically activated $CD8^+$ T cells would be highly valuable. The analysis of samples from experimentally infected humans would allow the comparison of pre-, during and after infection samples, providing an opportunity to subtract non-malarial activation. In order to dissect $CD8^+$ T cell populations specifically found in patients, comparison of $CD8^+$ T cell phenotypes with bioinformatical tools in large groups of healthy and infected humans could be used. Certainly, a malaria parasite-specific staining (e.g. tetramers) would be most interesting.

# 7 Abstract

The causative agent of most severe malaria cases, such as cerebral malaria in human is *Plasmodium falciparum*. Due to limitations in the analysis of underlying immunological mechanisms leading to pathology in human brains, mouse models are important. Experimental infection of C57BL/6 mice with *Pb*A induces a T cell-dependent immunopathology during the blood stage, leading to cerebral malaria. Recognition of cross-presented *plasmodial* antigen on brain-endothelial cells leads to the disruption of the blood-brain barrier by T cells [19, 20]. A tight control of T cell function by co-inhibitory receptors such as CTLA-4 and PD-1 is essential to prevent fatal symptoms [84, 85]. Due to the reported involvement of the co-inhibitory receptor BTLA in T cell control [86], its ligand (HVEM) and competitor (CD160) were analysed in this thesis.

The first part of this study focuses on HVEM. Even though HVEM represents a main regulatory element due to its interaction with the immunomodulatory receptors BTLA, CD160 and LIGHT, no alteration of T cell functionality in HVEM$^{-/-}$ CD8$^{+}$ T cells could be observed. However, HVEM$^{-/-}$ mice developed lower parasitemia, showed less severe cerebral symptoms and survived longer if infected with *Pb*A compared to wild type mice. This partial protection can be explained by a reduced CD8$^{+}$ T cell number in combination with a reduced frequency of activated and antigen-specific CD8$^{+}$ T cells. A lack of stimulation via HVEM during activation of CD8$^{+}$ T cells seems to be compensated by enhanced IL-2 signalling, leading to an equal initial expansion. However, persistence of terminally differentiated CD8$^{+}$ T effector cells depends on pro-survival signals provided by HVEM. Interestingly, CD4$^{+}$ T cells are increased in number in HVEM$^{-/-}$ mice, which is accompanied by an enlarged pool of regulatory T cells, providing an additional explanation for a balanced immune response.

The second part of this study characterises the phenotype of CD160 expressing CD8$^{+}$ T cells. Expression of CD160 is induced on activated, antigen-experienced CD8$^{+}$ T cells in the late stage of *Pb*A infection but is absent in naïve T cells and CD4$^{+}$ T cells in mice. CD160 is not only co-expressed with cytotoxicity markers but it could also been shown that CD160$^{+}$ CD8$^{+}$ T cells are highly efficient in killing of target cells and production of the pro-inflammatory cytokine IFNγ. Along this line, CD160$^{+}$ CD8$^{+}$ T cells could be linked to immunopathology because their frequency correlates with the severity of cerebral symptoms, is highest in the brain and reduced in non-lethal *Py*NL infection. Most importantly, CD160 characterises a

similar subset in human and mice, highlighting the relevance of the data obtained in the mouse model. In order to dissect the functional involvement of CD160 in $CD8^+$ T cell regulation, a $CD160^{-/-}$ mouse was generated using the CRISPR/Cas9 technology. Preliminary data obtained from *Pb*A infected $CD160^{-/-}$ mice suggest a co-inhibitory function of CD160 on $CD8^+$ T cells.

In summary, HVEM and CD160 play distinct but crucial roles in $CD8^+$ T cell regulation during blood-stage malaria. While HVEM represents a target broadly affecting all T cells undergoing several rounds of replication hence requiring pro-survival signals, CD160 is highly specific for the control of a subset of $CD8^+$ T effector cells, which is related to immunopathology.

# 8 Zusammenfassung

Die meisten Fälle von schwerer Malaria wie z.B. zerebrale Malaria werden im Menschen durch *Plasmodium falciparum* ausgelöst. Die Analyse der Immunpathologie im menschlichen Gehirn ist nur sehr eingeschränkt möglich, weshalb Maus-Modelle ein wertvolles Werkzeug darstellen. Bei der experimentellen Infektion von C57BL/6 Mäusen mit *Pb*A wird Immunpathologie durch Hirn-sequestrierende T-Zellen induziert. Erkennen T-Zellen *Plasmodien* Antigen, welches auf Hirnendothelzellen kreuzpräsentiert wird, kommt es zur Durchlässigkeit der Blut-Hirn-Schranke. Eine strenge Kontrolle der T-Zellfunktion durch koinhibitorische Rezeptoren wie beispielsweise PD-1 und CTLA-4 ist essentiell zur Vermeidung von schweren Symptomen. Da auch für den Koinhibitor BTLA eine Rolle in der T-Zell Regulation während der Malaria Blutphase gezeigt werden konnte, hat sich diese Arbeit mit dem BTLA-Liganden (HVEM), sowie dem BTLA-Kompetitor (CD160) beschäftigt.

Der erste Teil dieser Arbeit untersucht den molekularen Schalter HVEM. Obwohl HVEM auf T-Zellen in Abhängigkeit von seinem Liganden sowohl stimulatorisch als auch inhibitorisch wirken kann, hat der Verlust von HVEM keinen Einfluss auf die Funktionalität von $CD8^+$ T-Zellen in der experimentellen Malaria. Trotzdem zeigen *Pb*A infizierte $HVEM^{-/-}$ Mäuse deutlich mildere zerebrale Symptome, überleben die Infektion länger und entwickeln eine geringere Parasitämie als Wildtyp-Mäuse. Das Fehlen von stimulatorischen Signalen durch HVEM während der initialen Expansion von $CD8^+$ T-Zellen kann vermutlich durch verstärkte IL-2 Signale kompensiert werden. Nach mehreren Proliferationsschritten sind terminal differenzierten $CD8^+$ T-Effektorzellen allerdings von Überlebenssignalen durch HVEM abhängig. Interessanterweise ist die Anzahl an $CD4^+$ T-Zellen in *Pb*A infizierten $HVEM^{-/-}$ Mäusen signifikant höher als in Wildtypen und damit liegen auch mehr regulatorische T-Zellen vor, was eine weitere Erklärung für die verringerte Pathologie geben könnte.

Im zweiten Teil der Arbeit werden $CD8^+$ T-Zellen, die CD160 exprimieren, näher charakterisiert. Die Expression von CD160 wird induziert auf aktivierten, Antigen-erfahrenen $CD8^+$ T-Zellen, aber nicht in naiven oder $CD4^+$ T-Zellen in Mäusen. $CD8^+$ T-Zellen, die CD160 exprimieren, zeigen nicht nur eine Koexpression von Zytotoxizitätsmarkern, sondern sind auch in der Lage effizient Zielzellen zu zerstören und das pro-inflammatorische Zytokin IFNγ zu produzieren. Weiterführend konnten die $CD160^+$ $CD8^+$ T Zellen mit zerebraler Pathologie in Zusammenhang gebracht werden, da ihre Frequenz mit der Schwere der Symptome

korreliert, am höchsten im Gehirn ist und in der nicht-letalen Infektion mit *Py*NL im Vergleich zur letalen *Pb*A Infektion deutlich reduziert ist. Der Phänotyp von $CD160^+$ $CD8^+$ T Zellen ist ähnlich in murinen und humanen Geweben, sodass eine Vergleichbarkeit der im Mausmodell generierten Daten mit Malaria Patienten gewährleitet ist.

Zur Untersuchung des Einflusses von CD160 auf die T-Zellfunktion wurde eine $CD160^{-/-}$ Maus mit der CRISPR/Cas9 Technologie generiert. Vorläufige Ergebnisse aus der Infektion von $CD160^{-/-}$ Mäusen mit *Pb*A weisen auf eine koinhibitorische Funktion von CD160 hin.

Zusammenfassend lässt sich feststellen, dass sowohl HVEM als auch CD160 auf unterschiedliche Weise eine wichtige Rolle in der T-Zellregulation während der Malaria Blutphase spielen. Während HVEM von allen $CD8^+$ T-Zellen, die wiederholte Proliferationsschritte durchlaufen haben, als Überlebenssignal benötigt wird, ist CD160 hoch spezifisch für die Kontrolle von $CD8^+$ T-Zellen, die mit der Pathologie assoziiert sind.

# 9 Literature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