The role of T cells expressing multiple co-inhibitory molecules during murine experimental malaria

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Basic

Research Article

T cells expressing multiple co-inhibitory molecules in acute malaria are not exhausted but exert a suppressive function in mice

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Overwhelming activation of T cells in acute malaria is associated with severe outcomes. Thus, counter-regulation by anti-inflammatory mechanisms is indispensable for an optimal resolution of disease. Using *Plasmodium berghei* ANKA (PbA) infection of C57BL/6 mice, we performed a comprehensive analysis of co-inhibitory molecules expressed on CD4$^+$ and CD8$^+$ T cells using an unbiased cluster analysis approach. We identified similar T cell clusters co-expressing several co-inhibitory molecules like programmed cell death protein 1 (PD-1) and lymphocyte activation gene 3 (LAG-3) in the CD4$^+$ and the CD8$^+$ T cell compartment. Interestingly, despite expressing co-inhibitory molecules, which are associated with T cell exhaustion in chronic settings, these T cells were more functional compared to activated T cells that were negative for co-inhibitory molecules. However, T cells expressing high levels of PD-1 and LAG-3 also conferred suppressive capacity and thus resembled type I regulatory T cells. To our knowledge, this is the first description of malaria-induced CD8$^+$ T cells with suppressive capacity. Importantly, we found an induction of T cells with a similar co-inhibitory rich phenotype in *Plasmodium falciparum*-infected patients. In conclusion, we demonstrate that malaria-induced T cells expressing co-inhibitory molecules are not exhausted, but acquire additional suppressive capacity, which might represent an immune regulatory pathway to prevent further activation of T cells during acute malaria.

Keywords: immune regulation · immunopathology · inhibitory receptors · malaria · T cells

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

With 229 million cases and 409,000 deaths worldwide in 2019, malaria remains a dangerous threat to humankind. Ninety-five percent of all malaria cases are due to an infection with *Plasmodium falciparum* [1]. A liver stage and a subsequent blood stage characterize the infection.

There are two major characteristics of T cell responses to *Plasmodium* infections. On the one hand, there is severe immunopathology caused by T cells. Severe malaria cases are predominantly described during the first years of contact with the parasite and are reduced after repeated malaria episodes [2]. On the other hand, there is a lack of a protective memory response. Multiple malaria episodes do not lead to long-lasting protective immunity. Hepatocytes present pathogen-derived antigens during the liver stage of the infection, which opens a window of opportunity for adaptive immunity to interfere. However, an immune response against the liver stage during the first infections fails to deliver long-lasting protection. T cells, responsible for the local anti-malarial response, can be detected in the liver after vaccination in murine models, but do not persist for more than one month [3].

In mouse models of severe malaria, there is strong evidence that T cells are responsible for immunopathology [4]. In humans, we and others could recently show that the severity of cerebral malaria correlates with the infiltration of $CD8^+$ T cells to the brain [5], and severe malaria is associated with an increase of granzyme B and activated cytotoxic T cells in the blood [6].

Interestingly, there is substantial evidence that T cell activation, induced by *Plasmodium* infection, concurs with the induction of co-inhibitory receptors [7, 8]. Co-inhibitory or immune-modulatory receptors like PD-1, LAG-3, CD39, T cell Ig and mucin-domain containing-3 (TIM-3), and T cell immunoreceptor with Ig and ITIM domains (TIGIT) are primarily associated with a gradual dysfunction of T cells, called exhaustion [9]. Exhaustion describes a mechanism to avoid tissue damage in organs under high antigen load [10].

Despite the high expression of co-inhibitory receptors in malaria [8], T cells mediate immunopathology and contribute to the development of malaria complications [6, 5], which is seemingly contradictory. Although a strong T cell response is initiated, a protective and long-lasting T cell memory is virtually absent in malaria.

We investigated these contradictions by avoiding the classic approach of analyzing malaria-specific T cell responses in a biased way. Using unbiased novel cluster analysis approaches, we identified the malaria-specific induction of distinct T cell populations with ambiguous properties. Apart from the expression of co-inhibitory receptors, such as PD-1, LAG-3, or TIM-3, the induced $CD4^+$ and $CD8^+$ T cells expressed high amounts of pro-inflammatory cytokines. For the first time, we also present that the same subsets of malaria-induced effector $CD4^+$ and cytotoxic $CD8^+$ T cells show suppressive capacity and can downregulate other $CD4^+$ and $CD8^+$ T cells. By identifying the dual function of malaria-specific T cells, we contribute to understanding the immunopathology in malaria.

Results

Malaria-induced T cells express receptors associated with an exhaustion phenotype

Patients with acute malaria show different clinical pictures of malaria disease. Recently, we demonstrated that distinct expression patterns of T cell markers associated with immune regulation, such as CD39 and TIM-3, indicate less severe pathology during infection [11]. Besides the activation of immunopathological T cells [12], we assume that the induction of immune-modulatory T cells occurs during infection with the parasite. To analyze the phenotype of malaria-induced T cells in the mouse model of *Plasmodium berghei* ANKA (PbA) infection, we isolated T cells from different organs 6 days post-infection (dpi). We analyzed the cells by flow cytometry and employed an unbiased approach of self-organizing maps (SOM) followed by a minimum spanning tree (MST) and automated clustering to visualize and cluster possible T cell subsets [13] (Figs. 1 and 2).

FlowSOM uses two-level clustering with an MST as visualization [13]. The first level is based on an artificial neural network, the SOM. Based on intracellular and surface protein expression (as indicated in Fig. 1B and 2B), the SOM is trained to assemble similar cells into a cluster grid. The topological information of the trained grid is visualized in a minimum spanning tree (Fig. 1A and 2A). The second level of clustering is indicated by the background color of the branches of the MST (Fig. 1A and 2A) as well as the cluster number (Fig. 1C and Fig. 2C). It is based on consensus hierarchical clustering as described previously [13, 14].

The artificial neural network was trained using isolated T cells from spleens of PbA-infected animals and naïve controls. We assume that T cells found in spleens of infected and uninfected animals reflect most naïve and activated T cell subsets throughout the organism. Therefore, T cells from livers of animals isolated from the indicated groups were also analyzed with the trained algorithm. We analyzed $CD4^+$ T cells and $CD8^+$ T cells separately.

Using the computed scaffold for $CD4^+$ T cells from the spleens of naïve animals, we identified distinct clusters representing the T cells found in naïve, uninfected animals (Fig. 1A). We found a similar cluster and distribution of naïve T cells in the liver of uninfected mice (Fig. 1A), indicating the distribution of naïve T cells between lymphoid organs and organs in the periphery.

Using the scaffold on $CD4^+$ T cells isolated from the liver of PbA infected animals, the induction of unique metaclusters was seen. These induced T cell clusters were also found in the spleens of infected animals, but not in the liver or the spleen of naïve, uninfected animals. Apart from a branch of classically activated $CD44^+CD62L^{low}$ T cells (Fig. 1A, blue circle), we identified another branch of induced T cells (Fig. 1A, red circle) that mainly consists of the metaclusters 4, 5, and 7. $CD4^+$ T cells from the metacluster 6 were $CD25^+FoxP3^+$ (Fig. 1B) and can be

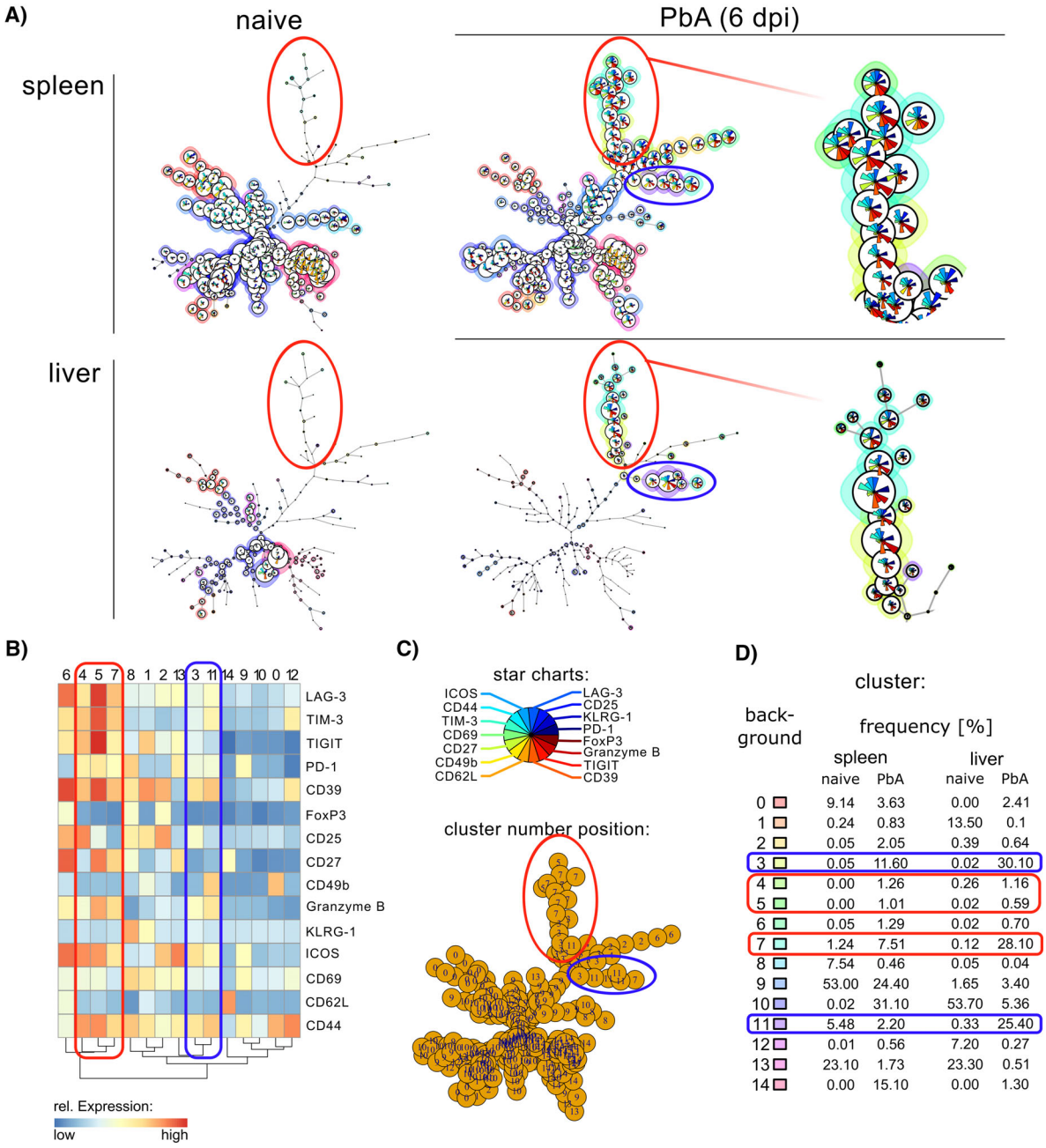

Figure 1. Malaria-induced CD4$^+$ T cell subsets are distinguished by the expression of co-inhibitory molecules. **(A)** FlowSOM trees of CD4$^+$ T cells from spleen and liver from naïve control and PbA-infected mice on day 6 post infection. Blue circle indicates conventional activated T cells, red circle marks co-inhibitory-rich T cells. **(B)** Expression heatmap of indicated markers of each metacluster. **(C)** Legends for FlowSOM tree; star charts show color code for the expression of each marker per cluster. **(D)** Cluster numbers with frequencies of distinct metaclusters. Cluster number position depicts metacluster number and position in the tree, cluster background shows the background color of each metacluster in the FlowSOM tree. Two to three individual mice per group out of one representative experiment.

considered classical regulatory T cells [15]. In the liver, metaclusters 7 and 4 were most abundant (Fig. 1A). T cells represented in these clusters were FoxP3$^-$ (Fig. 1B). Apart from the high expression of CD44 or ICOS, which are associated with T cell activation [16], these clusters were characterized by their expression of immune regulatory molecules like TIM-3, CD39, TIGIT, PD-1, and LAG-3 (Fig. 1B, red frame). Only low amounts of the marker for finally differentiated T cells, Killer cell lectin-like receptor subfamily G member 1 (KLRG-1) [17], were detected. However, as granzyme B was expressed in these T cell subsets, we consider these T cells to be highly differentiated effector T cells.

Interestingly, by simultaneously analyzing CD8$^+$ T cells, we recognized a similar T cell activation and differentiation pattern. Specific clusters of CD8$^+$ T cells were identified in the spleen

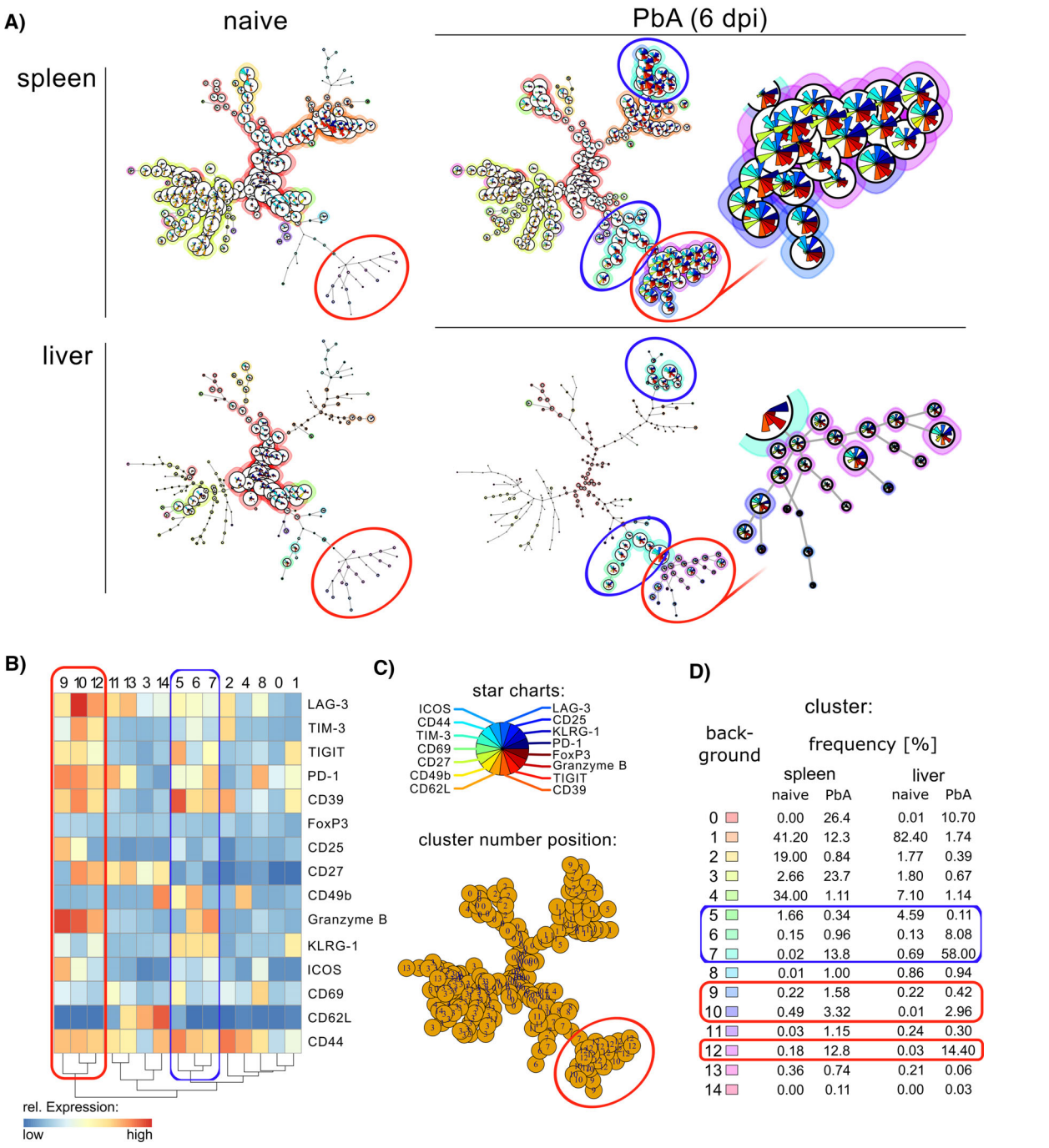

Figure 2. Malaria-induced CD8$^+$ T cell subsets are distinguished by the expression of co-inhibitory molecules. **(A)** FlowSOM trees of CD8$^+$CD3$^+$ T cells from spleen and liver from naïve control or PbA-infected mice on 6 dpi. Gray circle indicates T cells with naïve phenotype, blue circle indicates conventional activated T cells, red circle marks co-inhibitory-rich T cells. **(B)** Expression heatmap of indicated markers of each metacluster. **(C)** Legends for FlowSOM tree; star charts show color code for the expression of each marker per cluster. **(D)** Cluster numbers with frequencies of distinct metaclusters, cluster number position depicts metacluster number and position in the tree, cluster background shows the background color of each metacluster in the FlowSOM tree. 2–3 individual mice per group out of one representative experiment.

and liver of naïve mice. Besides these clusters, T cells from PbA-infected animals formed distinct clusters in the spleen and the liver (Fig. 2A, blue and red circle). Again, some of these PbA-induced clusters showed the expression of co-inhibitory molecules like TIGIT, TIM-3, PD-1, and LAG-3 (Fig. 2A, red circle, mainly clusters 9, 10, and 12). Furthermore, they were characterized by the expression of CD39. Again, we observed a high expression of granzyme B (Fig. 2B, red frame).

In conclusion, these data demonstrate that CD4$^+$ and CD8$^+$ T cells induced by PbA infection can be divided into two major

groups. One group is positive for CD44 but expresses lower levels of co-inhibitory molecules (Figs. 1A and 2A; blue circles). The second group (Figs. 1A and 2A; red circles) expresses CD44 and a high amount of anti-inflammatory receptors, including co-inhibitory molecules (PD-1, LAG-3) or functional receptors associated with T cell exhaustion in chronic infection models, like CD39 [18, 19]. Interestingly, we found a high expression of LAG-3 in these PbA-induced subsets of $CD4^+$ and $CD8^+$ T cells. Recently, LAG-3 was described as a reliable marker for induced potential regulatory B cells [20]. Moreover, when co-expressed with CD49b, it is described as a marker for type 1 regulatory T cells [21]. Strikingly, PbA-induced T cells with a high amount of co-inhibitory receptors also expressed the cytotoxic effector molecule granzyme B.

Blood stage-induced LAG-3^+ T cells are not exhausted

We observed the induction of T cells expressing high levels of LAG-3 and other co-inhibitory molecules by the PbA infection. To dissect immune responses to the liver and blood stage of infection, we experimented with three distinct treatment groups (Fig. 3A). C57BL/6 mice in group 1 were infected with viable sporozoites. These mice underwent a complete liver and blood stage. Group 2 was infected with viable sporozoites and treated with anti-parasitic pyrimethamine on day 5 post-infection. Pyrimethamine is toxic for extracellular parasites in the blood and shortens the blood stage [22]. Group 3 was infected with irradiated sporozoites. Irradiated sporozoites are able to infect hepatocytes, but they do not develop into schizonts and remain in the liver without provoking a blood stage [23]. Comparing the immune response to the liver and the blood stage, our data revealed that LAG-3 on $CD8^+$ and $CD4^+$ T cells was induced during the blood stage but not the liver stage of the disease (Fig. 3C and Fig. 4A). LAG-3^+ T cells could be found in blood, spleen, and in peripheral organs like the brain and liver (Fig. 3C), indicating a ubiquitous distribution during the infection with PbA. In addition to the increased frequency of LAG-3^+ T cells within the indicated subsets, the LAG-3 expression per cell was also increased during the blood stage of infection (Fig. 3D and 4B). Remarkably, when administering an anti-malarial treatment with pyrimethamine, which leads to delayed antigen withdrawal (experiment described in Fig. 3A and 3B), LAG-3^+ T cells disappeared rapidly (Fig. 3C and 4A), indicating a transient, antigen-specific induction of LAG-3^+ T cells.

We stained *ex vivo* for granzyme B in isolated T cells to validate that malaria-induced T cells are functional. We also detected the effector cytokine Interferon γ (IFNγ) and the anti-inflammatory cytokine IL-10 in restimulated splenocytes from infected animals. We observed that $CD4^+CD44^+$LAG-3^+ T cells expressed more granzyme B and higher amounts of IFNγ and IL-10 compared to activated $CD4^+CD44^+$LAG-3^- T cells (Fig. 3E and F). Indeed, our results show that the expression of granzyme B and IL-10 by $CD4^+$ T cells is associated with the expression of LAG-3 (Fig. 3). This indicates that the expression of co-inhibitory receptors during acute malaria is not associated with exhaustion.

Furthermore, the frequency of $CD8^+CD44^+CD62L^{low}$LAG-3^+ T cells was increased during the blood stage of infection. $CD8^+CD44^+CD62L^{low}$LAG-3^+ T cells could be found in peripheral organs (liver, brain), secondary lymphatic tissue (spleen), and the blood (Fig. 4A). Ex vivo staining of granzyme B revealed that $CD8^+CD44^+$LAG-3^+ T cells produce more cytotoxic granzyme B than T cells not expressing LAG-3 (Fig. 4C). Additionally, the expression of the effector cytokine IFN-γ (Fig. 4D) and the anti-inflammatory cytokine IL-10 was increased in the subset of $CD8^+CD44^+$LAG-3^+ T cells compared to their LAG-3^- counterparts (Fig. 4E). Interestingly, $CD4^+CD44^+CD62L^{low}$LAG-3^+ T cells as well as $CD8^+CD44^+CD62L^{low}$LAG-3^+ T cells expressing the regulatory cytokine IL-10 produced even more granzyme B (Fig. S1A and B) and IFN-γ in comparison to their IL-10 negative counterparts (Fig. S1C and D). However, the frequency of IL-10 producing $CD8^+$ T cells was lower compared to IL-10 producing $CD4^+$ T cells. Withdrawal of antigen by treatment with pyrimethamine led to a rapid reduction of LAG-3^+ T cells. On day 10 after infection or day 5 after pyrimethamine treatment, LAG-3 expression was strongly reduced and almost not detectable in any organ (Fig. 4A).

$CD8^+$LAG-3^+ and $CD4^+$LAG-3^+ T cells exert a suppressive capacity

Despite the expression of LAG-3 and other co-inhibitory molecules by malaria-induced T cells, our data imply that these cells are not exhausted, but develop a dual phenotype with pro- and anti-inflammatory characteristics. Indeed, inducible Type 1 regulatory T cells (Tr1) are also described to be induced during malaria [24]. Based on these results, showing the suppressive capacity of $CD4^+$LAG-3^+ T cells, we hypothesize that malaria-induced $CD8^+$LAG-3^+ T cells are suppressive as well. To this end, we conducted *in vitro* suppression assays through co-culture of naïve $CD4^+$ and $CD8^+$ T cells with different subsets of malaria-induced T cells. Immuno-suppressive Tr1 cells were described as being $CD4^+CD49b^+$LAG-3^+ T cells [21]. Thus, we sorted malaria-induced $CD4^+CD49b^+$LAG-3^+ T cells (described as Tr1 cells) and $CD4^+CD49b^-$LAG-3^+ T cells to compare their suppressive capacity (Fig. 5). Due to low amounts of classical $FoxP3^+$ regulatory T cells in these sorted T cell subsets (maximum of around 15% within LAG-3^-CD49b^+ T cells) (Fig. S2A and B), we considered their influence on the observed suppressive effect in the assays as negligible. Additionally, we performed suppression assays with the same LAG-3^+ and/or $CD49b^+$ subsets of $CD8^+$ T cells.

First, we investigated whether malaria-induced $CD4^+$ and $CD8^+$ T cells can suppress the proliferation of naïve $CD4^+$ T cells. $CD4^+$LAG-3^+ T cells suppressed the proliferation of $CD4^+$ T cells in a dose-dependent manner (Fig. 5A and B). We could not detect a significant difference in the regulatory capacity between LAG-3^+CD49b^+ T cells and LAG-3^+CD49b^- T cells; both subsets suppressed proliferating $CD4^+$ T cells equally. Like $CD4^+$LAG-3^+

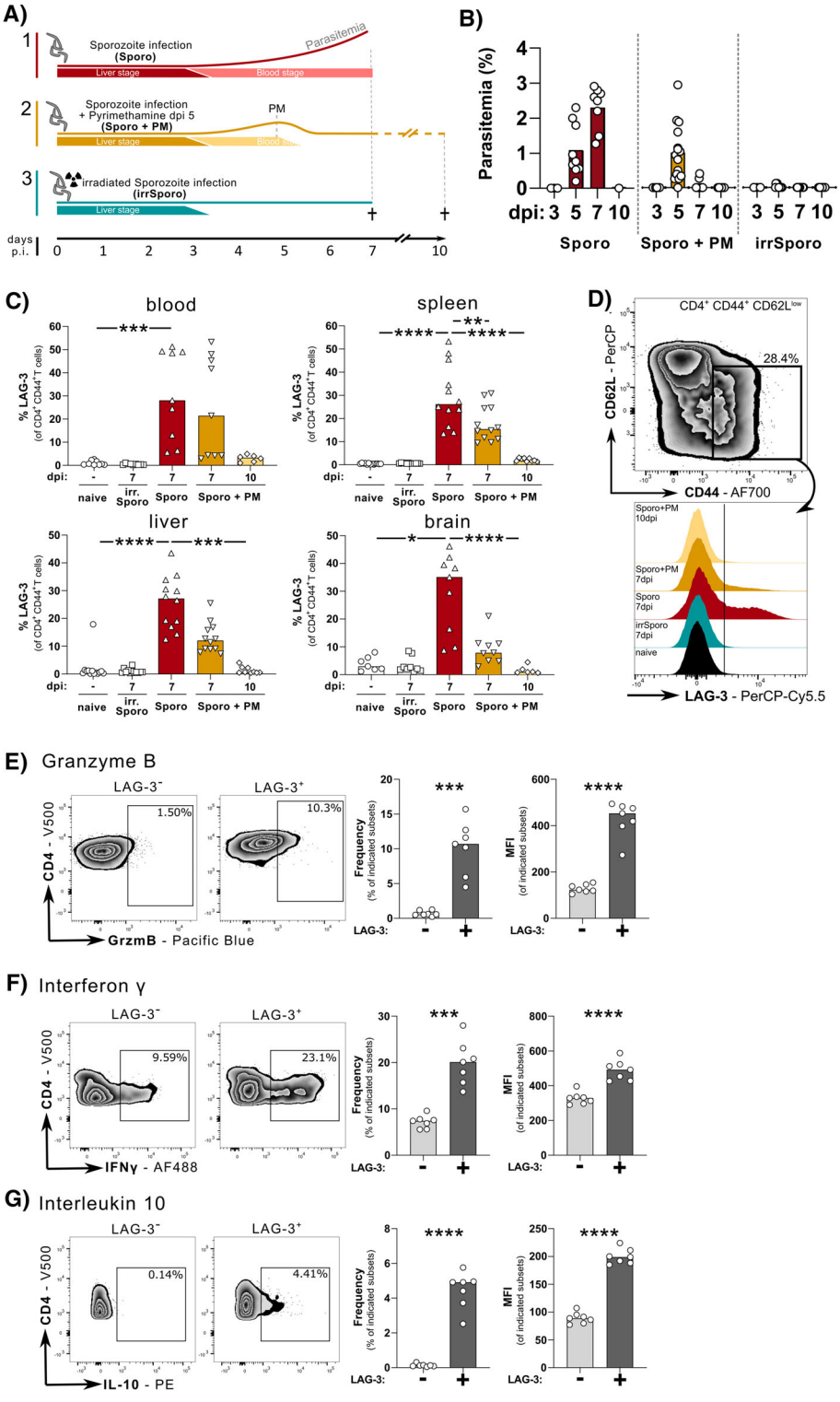

Figure 3. Blood stage-induced CD4$^+$LAG-3$^+$ T cells are not exhausted. **(A)** Experimental scheme to dissect impact on induced T cells of liver stage or blood stage of PbA infection. Group infected with viable sporozoites (Sporo) reflects liver and full blood stage (red). Group infected with viable sporozoites and treated with pyrimethamine (Sporo + PM) reflects a reduced blood stage (yellow). Group infected with irradiated sporozoites (irrSporo) are limited to a liver stage and do not show a blood stage (blue). **(B)** Blood parasitemia on indicated days post infection. **(C)** Frequency of LAG-3 expression by CD4$^+$CD44$^+$CD62L^{low} T cells in indicated organs and groups. Kruskal-Wallis test with Dunn's multiple comparisons test was used. For better readability, not all significances are depicted. **(D)** Representative dot plot of CD44$^+$CD62L^{low} CD4$^+$ T cells isolated from spleen and histograms of the LAG-3 expression in the different infection groups. Full gating strategy as described in Fig. S4 **(E)** Frequency and (MFI) of granzyme B expression in LAG-3$^+$ or LAG-3$^-$ CD4$^+$CD44$^+$ T cells measured *ex vivo* 7 dpi. **(F)** Frequency and MFI of IFNγ and **(G)** IL-10 stained after 5 h Phorbol 12-Myristat 13-Acetat (PMA)/Ionomycin (Iono) restimulation of T cells isolated from spleen 7 dpi. **(E-G)** Cells isolated from spleens and analyzed by paired *t*-test was applied. **(A-G)** Data were pooled from two to five experiments with 2–4 biological replicates each. Each dot represents one biological replicate. *P* values ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***) or ≤ 0.0001 (****) were considered statistically significant.

T cells, malaria-induced CD8$^+$LAG-3$^+$ T cells could also suppress CD4$^+$ T cells in a dose-dependent manner. Again, the co-expression of CD49b by CD8$^+$LAG-3$^+$ T cells was not associated with a significantly higher suppressive capacity (Fig. 5A and B).

As the induction of cytotoxic CD8$^+$ T cells drives the immunopathology in malaria, we assessed whether PbA-induced regulatory LAG-3$^+$ T cells could also suppress CD8$^+$ T effector cells. In vitro suppression assays showed that malaria-induced CD4$^+$LAG-3$^+$ and CD8$^+$LAG-3$^+$ T cells could suppress CD8$^+$ T cell proliferation. Again, the additional expression of CD49b did not lead to a more substantial suppressive capacity of LAG-3$^+$ T cells (Fig. 5C and D).

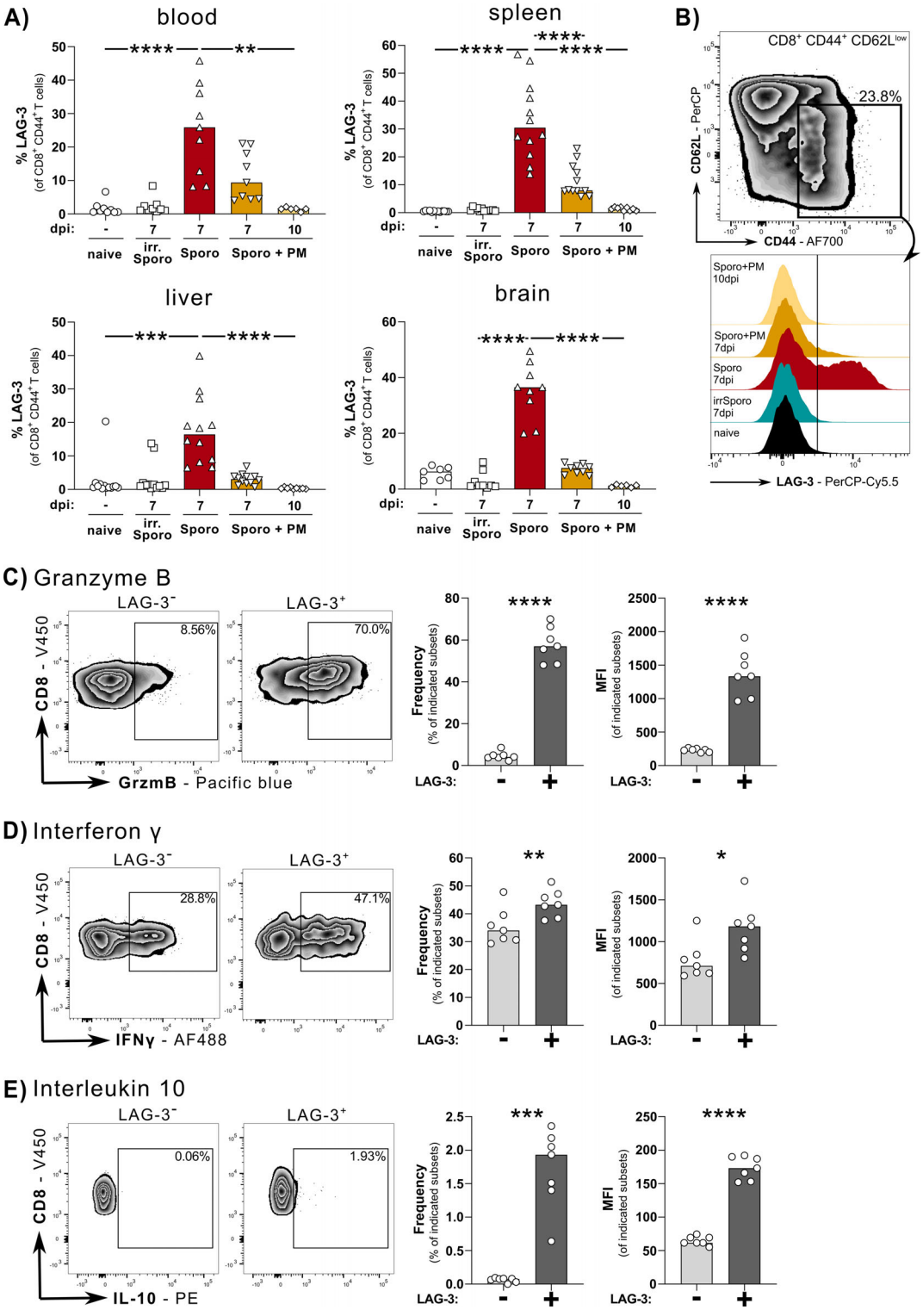

Figure 4. Blood stage-induced CD8$^+$LAG-3$^+$ T cells are not exhausted. **(A)** Frequency of LAG-3 expression by CD8$^+$CD44$^+$ T cells in indicated organs and groups. Kruskal-Wallis test with Dunn's multiple comparisons test was used. For better readability, not all significances are depicted. **(B)** Representative dot plot of CD44$^+$CD62L^{low} CD8$^+$ T cells isolated from spleen and histograms of their LAG-3 expression in the different infection groups. Full gating strategy described in Fig. S4 **(C)** Granzyme B expression in LAG-3$^+$ or LAG-3$^-$ CD4$^+$CD44$^+$ T cells measured *ex vivo* 7 dpi. **(D)** IL-10 and IFN-γ stained after 5 h PMA/Iono restimulation of T cells isolated from spleen 7 dpi. Data in **(C)** and **(D)** were analyzed with a paired *t*-test. For the comparison of the MFIs of IFN-γ expression **(F)**, the Wilcoxon Test was applied. **(A-D)** Data were pooled from two to five experiments with 2–4 biological replicates each. Each dot represents one biological replicate. P values ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***) or ≤ 0.0001 (****) were considered statistically significant.

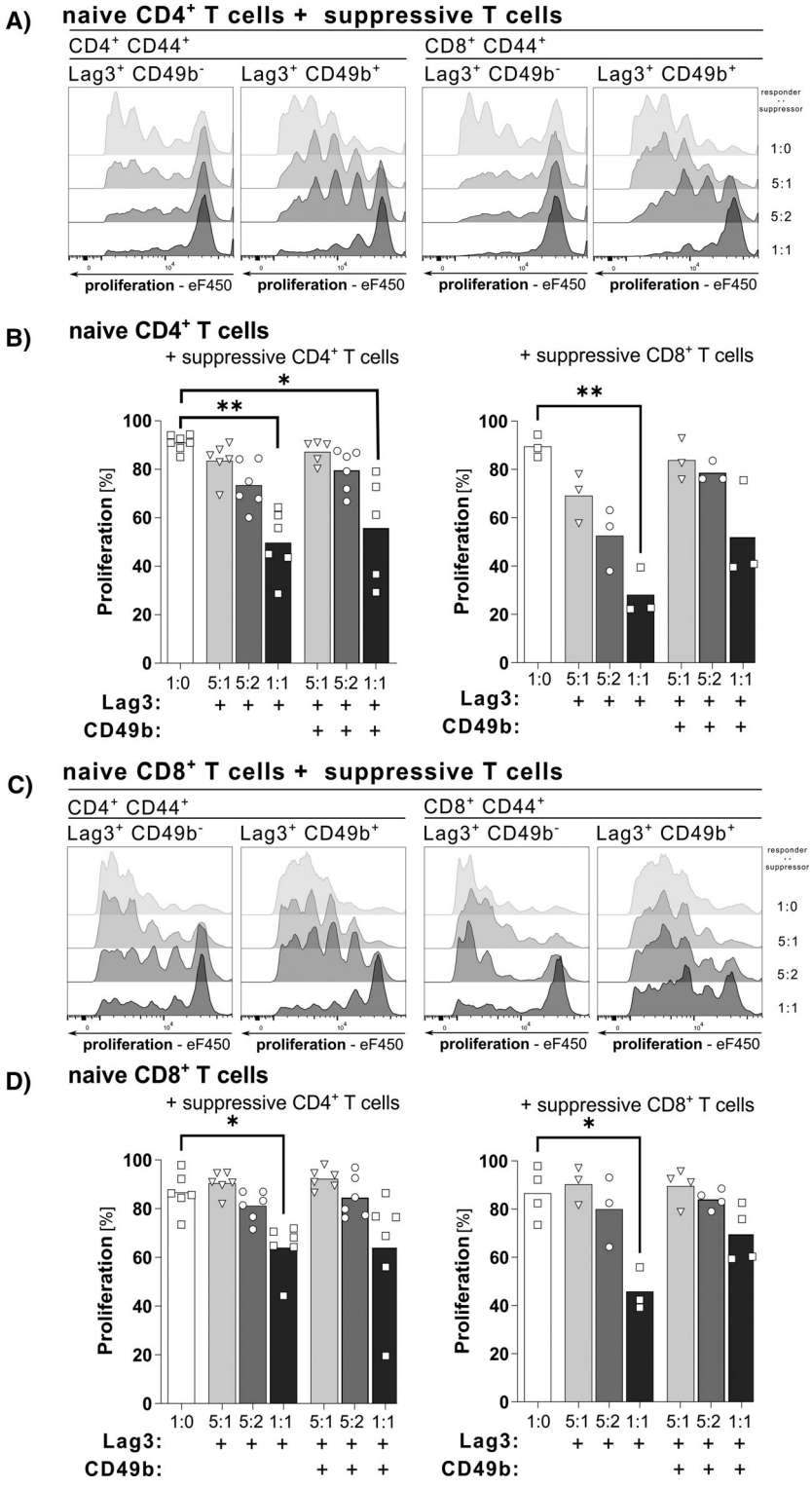

Figure 5. Malaria-induced T cells show regulatory capacity. PbA-induced potentially regulatory T cells were isolated 6 dpi and sorted for indicated subsets. Co-culture of effector T cells and potential regulatory T cells was performed for 3 days. Effector T cells were stained with eFluor450 to track proliferation. **(A)** Histogram of proliferated $CD4^+$ T cells in indicated co-culture. Each panel shows a respective histogram out of 4–6 technical replications from three to six individual experiments. **(B)** Quantification of suppression of $CD4^+$ effector T cells. Each dot represents one individual experiment with 2–3 technical duplicates. **(C)** Histogram of proliferated $CD8^+$ T cells in indicated co-culture. Each panel shows a respective histogram from 2–3 technical repetitions out of 3–7 individual experiments. **(D)** Quantification of suppression of $CD8^+$ effector T cells. Each dot represents one individual experiment with 2–3 technical repetitions. **(B and D)** A mixed-effects analysis with a Geisser-Greenhouse correction or a Friedman test was performed. P-values ≤ 0.05 (*) and ≤ 0.01 (**) were considered statistically significant.

In conclusion, malaria-induced LAG-3^+CD4^+ T cells show a suppressive effect on $CD4^+$ and $CD8^+$ T cells. Remarkably, we demonstrated that malaria-induced LAG-3^+CD8^+ T cells also displayed a suppressive capacity towards naïve $CD4^+$ and $CD8^+$ T cells.

Malaria-induced $CD8^+$LAG-3^+ T cells are still cytotoxic

The infection by PbA induces T cells co-expressing a variety of co-inhibitory molecules. Interestingly, we saw that the induced

$CD4^+$ and $CD8^+$ T cells are not exhausted, but develop suppressive capacity. Further, we questioned whether induced $CD8^+$ T cells are still cytotoxic, regardless of the expression of multiple inhibitory surface receptors and their suppressive capacity (Fig. 6). For the implementation of an in vitro cytotoxicity assay, we infected OT-I mice with a transgenic PbA (PbOVA) strain, expressing the MHCI epitope SIINFEKL [25]. All $CD8^+$ T cells in the OT-I mouse strain are specific for SIINFEKL [26]. We isolated the previously examined CD49b/LAG-3 $CD8^+$ T cell subsets by cell sorting and co-cultivated PbOVA-induced sorted splenocytes with SIINFEKL-pulsed splenocytes (Fig. 6A-D). Surprisingly, the co-inhibitor-rich cells (here sorted as LAG-3 positive) demonstrated a higher cytotoxic capacity than their LAG-3^- counterparts by killing more antigen-loaded target cells in a dose-dependent manner (Fig. 6E). Taken together, by investigating the expression of granzyme B, IFN-γ, and IL-10, we demonstrated that malaria-induced $CD4^+$LAG-3^+ and $CD8^+$LAG-3^+ T cells are not exhausted, but rather highly activated. Moreover, malaria-induced T cells seem to develop a dual phenotype by simultaneously producing pro- and anti-inflammatory molecules.

$CD4^+$LAG-3^+ and $CD8^+$LAG-3^+ T cells are induced in patients with acute *P. falciparum* malaria

We showed that infection of C57BL/6 mice with PbA led to solid activation of adaptive immunity. A specific population of LAG-3^+ T cells was induced, and these T cells were characterized by their expression of other co-inhibitory molecules like PD-1, CD39, TIGIT, or TIM-3. Interestingly, we observed that these cells also expressed granzyme B and IFN-γ. Conversely, we demonstrated the expression of anti-inflammatory IL-10 by those malaria-induced T cells. Finally, we showed malaria-induced LAG-3^+ T cells from the CD4 and CD8 subsets exert regulatory capacity toward $CD4^+$ and $CD8^+$ T cells. These results demonstrated the induction of potential regulatory $CD4^+$ and $CD8^+$ T cells in a mouse model of cerebral malaria.

To validate the expansion of these regulatory T cells in human malaria, we investigated blood samples of *P. falciparum*-positive travelers in Hamburg, Germany, treated at the University Medical Centre Hamburg Eppendorf. In concordance with the previous data in the murine PbA model, malaria patients showed a significantly higher frequency of $CD4^+$LAG-3^+ T cells than healthy donors (Fig. 7A). Comparable to the induced regulatory $CD4^+$ T cells in the murine model, induced $CD4^+$ T cells in malaria patients expressed activation markers, like C-C chemokine receptor type 5 (CCR5), as well as surface markers associated with exhaustion, like PD-1, TIGIT, or TIM-3 (Fig. 7C). Interestingly, $CD4^+$LAG-3^+ T cells expressed high amounts of the immune regulatory ectonucleotidase CD39 (Fig. 7C).

Furthermore, when investigating $CD8^+$ T cell subsets in malaria patients, we also observed the induction of $CD8^+$LAG-3^+ T cells (Fig. 7B). Following our findings in the mouse model of PbA infection, induced $CD8^+$LAG-3^+ T cells were characterized by the expression of other co-inhibitory receptors like PD-1 and TIGIT (Fig. 7D). In contrast to the mouse model, only the $CD8^+$LAG-3^+CD49b^+ T cells also expressed TIM-3. Moreover, the $CD8^+$LAG-3^+CD49b^+ T cells expressed higher levels of the activation marker CCR5 and the immune regulatory ectonucleotidase CD39 (Fig. 7D) compared to their CD49b^- counterparts, indicating a higher suppressive capacity.

Collectively, by investigating the T cell response in malaria patients, these results show the induction of potentially regulatory $CD4^+$ and $CD8^+$ T cells in humans. Human LAG-3^+CD49b^- and LAG-3^+CD49b^+ T cells share specific characteristics with the formerly described, PbA-induced, regulatory T cells in mice.

Discussion

The blood stage of *P. falciparum* malaria is associated with robust activation of $CD4^+$ as well as $CD8^+$ T cells producing pro-inflammatory cytokines and cytotoxic molecules, respectively [27]. Recent data suggest that an overwhelming T cell response contributes to a severe clinical outcome [6, 5], indicating that tight control of T cells is of significant importance for optimal resolution of a malaria episode. Thus, activation of T cells appears to be a tightrope walk between protection and pathology.

Acute malaria is associated with strong induction of co-inhibitory molecules on T cells. This was demonstrated in humans [11, 28] as well as in animal models [29, 30]. In chronic antigen exposure settings, the expression of these molecules is associated with T cell exhaustion, especially of $CD8^+$ T cells [10, 31]. The function of these molecules in acute inflammatory settings is not well characterized, and it is not even clear whether these co-inhibitory molecules on $CD4^+$ T cells are linked to their exhaustion as is described for $CD8^+$ T cells. Using mouse models of acute malaria, it was already shown that blocking the exhaustion marker CTLA-4 could exacerbate the disease [30].

In contrast, a combined blockade of PD-1 and LAG-3 is associated with better control of parasitemia in a more chronic model of malaria [32]. It is tempting to speculate that induction of co-inhibitory molecules in acute malaria regulates T cell activation. They either limit overwhelming T cell activation and immunopathology or limit parasite control in more chronic situations. Moreover, induction of co-inhibitory molecules might be, on the one hand, an immune escape mechanism of the parasite [33] or, on the other hand, an emergency brake of the host preventing overactivation of the immune system [10]. To further pinpoint the function of these molecules, a comprehensive picture of the induction and co-expression of these molecules on T cells and an association with their function is of crucial importance to further unravel immune regulatory networks in malaria.

To this end, we analyzed T cell activation after infection of C57BL/6 mice with *P. berghei* ANKA, a widely used model of experimental cerebral malaria, using the unbiased clustering approach FlowSOM [13]. These allowed us to compare

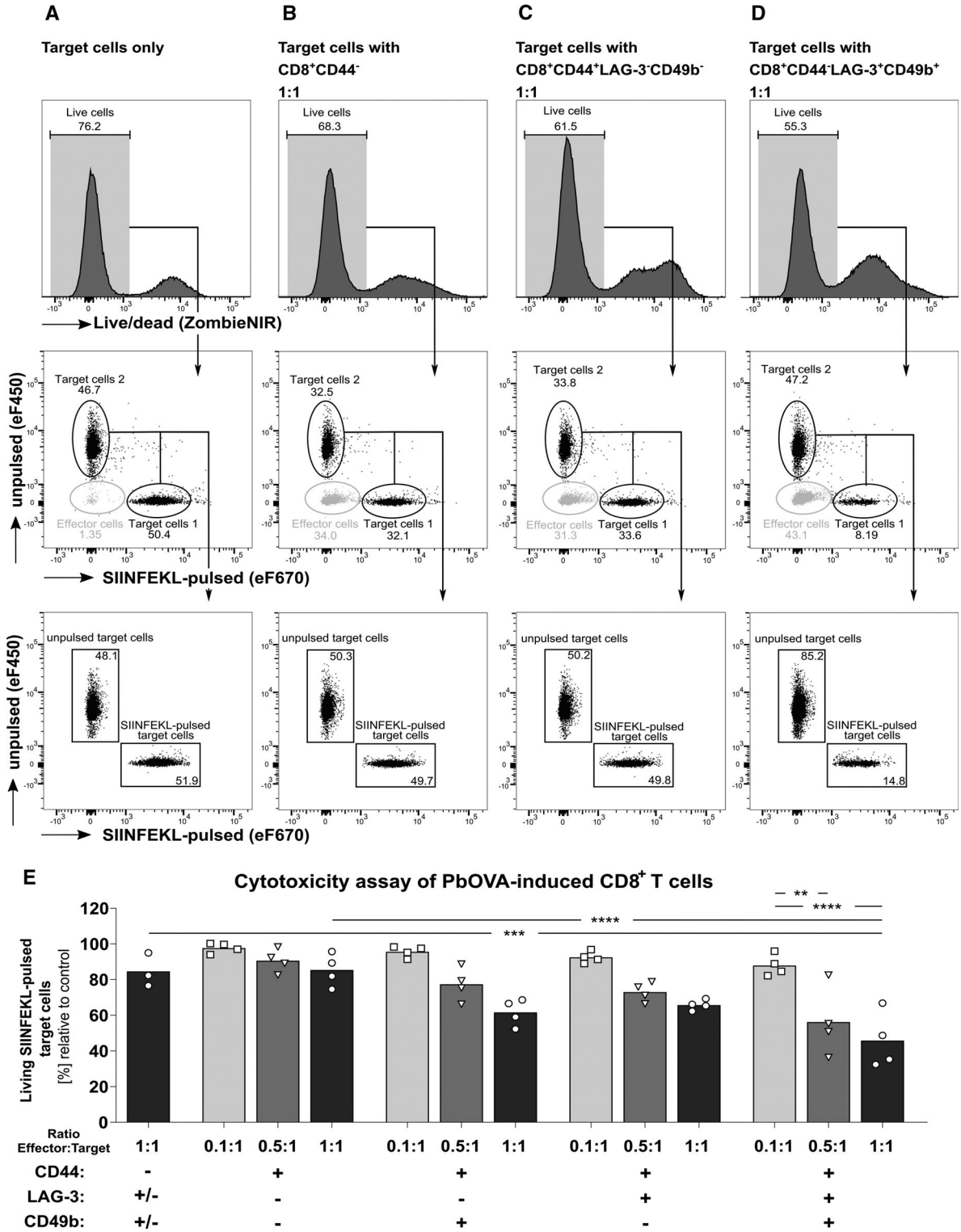

Figure 6. Malaria-induced regulatory CD8$^+$ T cells are still cytotoxic. CD8$^+$ T cells were isolated and sorted from PbOVA-infected OT-I mice 6 dpi and co-cultured with SIINFEKL-pulsed target cells to determine cytotoxic capacity of indicated subsets. **(A-D)** Part of the gating strategy to calculate the proportion of living SIINFEKL-loaded target cells after the co-culture with effector cells. Representative histograms and dot plots of **(A)** a target cell control, **(B)** target cells co-incubated with CD8$^+$CD44$^-$, **(C)** CD8$^+$CD44$^+$LAG-3$^-$CD49b$^-$ T cells and **(D)** CD8$^+$CD44$^+$LAG-3$^+$CD49b$^+$ T cells. **(E)** Antigen-specific killing of different CD8$^+$ T cell subsets of OT-I mice infected with PbOVA. Ratios of effector to target cells in co-culture are indicated. Each dot represents an individual experiment (4 experiments in total) with a minimum of two technical replicates. Ordinary one-way ANOVA with subsequent Tukey´s multiple comparison; *P*-values ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***), or ≤ 0.0001 (****) were considered statistically significant.

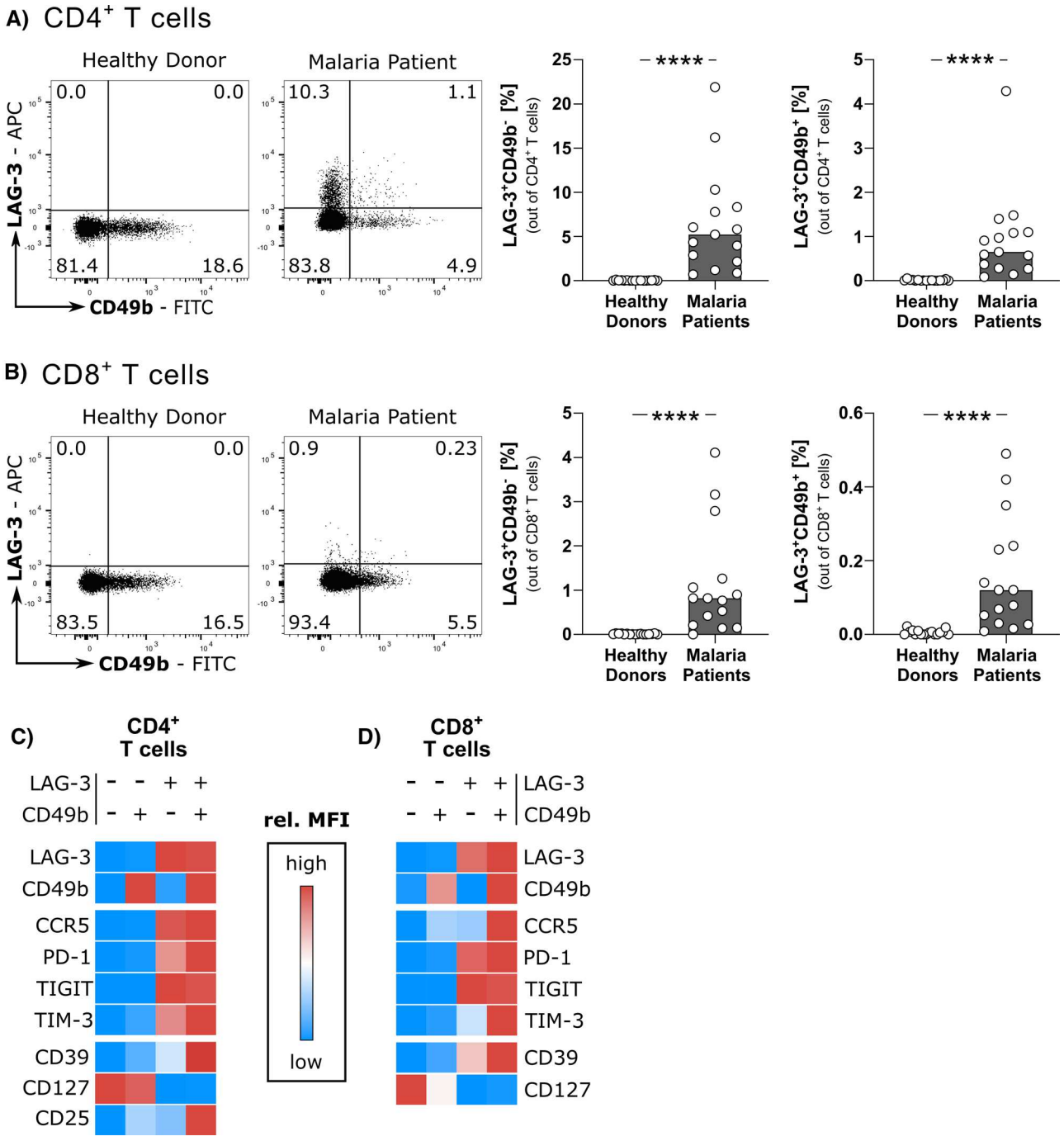

Figure 7. Malaria-induced LAG-3$^+$ T cells in malaria patients. Whole blood samples of healthy donors and malaria patients were stained *ex vivo* and analyzed by flow cytometry. Frequencies of LAG-3$^+$CD49b$^-$ and LAG-3$^+$CD49b$^+$ cells within **(A)** CD4$^+$ and **(B)** CD8$^+$ T cells of healthy donors (n = 17) and malaria patients (n = 15). Exemplary dot plots of LAG-3 and CD49b stainings of **(A)** CD4$^+$ T cells and **(B)** CD8$^+$ T cells of each one healthy donor and one malaria patient are shown. Bars represent the median. **(A-B)** Each dot represents one individual patient/ donor as indicated (healthy donors n = 17, malaria patients n = 15). Mann-Whitney Test; *P*-values ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***) or ≤ 0.0001 (****) were considered statistically significant. Relative MFI of surface markers of indicated LAG-3 and CD49b expressing **(C)** CD4$^+$ and **(D)** CD8$^+$ T cells of malaria patients (n = 7-9).

T cells from different groups in an unbiased way, but on the same scaffold. Interestingly, a significant proportion of activated CD4$^+$CD44$^+$ as well as CD8$^+$CD44$^+$ T cells in the spleen and liver were PD-1$^+$ and LAG-3$^+$. Despite expressing these co-inhibitory molecules, they appeared to be fully functional T effector cells producing pro-inflammatory cytokines and cytotoxic molecules. Thus, CD8$^+$CD44$^+$LAG-3$^+$ T cells were capable of lysing peptide-pulsed target cells.

Furthermore, LAG-3$^+$ T cells were more cytolytic than their LAG-3$^-$ counterparts. Thus, in acute malaria, the expression

of co-inhibitory molecules delineates strongly activated T cells instead of being indicators of T cell dysfunction. However, we cannot exclude that the expression of co-inhibitory molecules restricts the function of these T cells or that in this acute model, with its very fast and robust induction of T cells, a prolonged expression would be needed to attenuate T cell exhaustion.

Interestingly, $CD4^+LAG\text{-}3^+$ and $CD8^+LAG\text{-}3^+$ T cells produce both pro-inflammatory cytokines like IFNγ and anti-inflammatory cytokines like IL-10 and thus resemble Tr1 cells [34]. To further decipher their function, we analyzed their suppressive capacity and demonstrated that $CD4^+LAG\text{-}3^+$ T cells could suppress $CD4^+$ and $CD8^+$ T cells, regardless of CD49b expression. We have to emphasize here that suppressive $CD4^+LAG\text{-}3^+$ T cells contain less conventional Tregs (3-6%) when compared to much less suppressive $CD4^+LAG\text{-}3^-$ T cells (5 to 14%). Furthermore, $LAG\text{-}3^-CD49b^+$ cells, a subset that shows only a minor suppressive capacity, is even more enriched in Tregs (14%). This indicates that the suppressive effect of malaria-induced co-inhibitor-rich T cells is not exerted by conventional Tregs. We also found that $CD8^+LAG\text{-}3^+$ T cells suppress $CD4^+$ and $CD8^+$ T cells, independently of CD49b expression. This is, to our knowledge, the first description of malaria-induced $CD8^+LAG\text{-}3^+$ T cells with suppressive capacity. Tr1 cells in chronic inflammatory conditions are characterized by co-expression of LAG-3 and CD49b [21]. In our work, describing an acute disease, the suppressive capacities of $LAG\text{-}3^+CD49b^+$ and $LAG\text{-}3^+CD49b^-$ T cells are alike. However, as an integrin, CD49b might be associated with tissue localization and might not be functionally involved in their suppressive activity, and differences might be due to different disease models.

To confirm whether similar T cell subsets were induced in acute human malaria, we analyzed LAG-3-expressing T cells in acute *P. falciparum* malaria. Indeed, we found strong induction of LAG-3 on $CD4^+$ and $CD8^+$ T cells, and similar to the mouse model, a minor fraction of them co-express CD49b. However, $LAG\text{-}3^+CD49b^+$ and $LAG\text{-}3^+CD49b^-$ $CD4^+$ and $CD8^+$ T cells are very similar regarding co-expression of other co-inhibitory molecules and express PD-1, TIGIT, and TIM-3. Thus, experiments to characterize the suppressive capacity of the different subsets of malaria-induced T cells expressing co-inhibitory receptors should be done. It is tempting to speculate that these T cells can modify the course of the disease by having a dual function in expressing pro-inflammatory cytokines and at the same time being suppressive to other T cells. It could be interesting to study these T cell populations in patients with either severe or uncomplicated course of the disease. Recently, we described the induction of $CD4^+$ T cells in acute *P. falciparum* malaria co-expressing PD-1 and CTLA-4 [8]. Interestingly, these cells can suppress other $CD4^+$ T cells as efficiently as $FoxP3^+$ Treg.

In summary, we show the induction of regulatory $CD4^+$ T cells and $CD8^+$ T cells during infection of C57BL/6 mice with PbA. These malaria-induced T cells co-expressing LAG-3 and several other co-inhibitory molecules can suppress T cell proliferation and share characteristics with Tr1 cells. Functional assays revealed that the $LAG\text{-}3^+CD8^+$ T cell subset could lyse antigen-pulsed target cells and suppress the function of other T cells. In addition, we provide evidence that T cells with similar characteristics are also induced in acute *P. falciparum* malaria in humans. This novel immune regulatory pathway may have evolved to prevent and/or control prolonged inflammation in the setting of strong immune activation as they are found in acute malaria.

Methods

Animals

C57BL/6J and BALB/c mice were bred at the animal facility of the BNITM, Hamburg, Germany in individual ventilated cages (IVC) with food and water ad libidum. All animal experiments were performed in accordance with German law and registered with the "Behörde für Gesundheit und Verbraucherschutz (BGV)" under license 83/17; O012/2018; N005/2019.

Plasmodium berghei ANKA (PbA) Infection

Infected red blood cells (iRBC) were stored with 15 % glycerol (Merck, Darmstadt, Germany), 2.1 % sorbitol (Sigma Aldrich, St. Louis, Missouri) in PBS in liquid nitrogen. To achieve consistent blood-stage infections and avoid bias by frozen parasites, pre-experimental mice were infected with stabilate. Infected blood was obtained from pre-experimental mice after 5–7 days, and C57BL/6J mice were injected with 1×10^5 iRBC in 200 μl PBS intraperitoneally (i.p.). For the generation of sporozoites, BALB/c mice were treated with 1.25 mg phenylhydrazine to stimulate erythrocyte generation 2 days before infection with 70–120 μl infected blood from pre-experimental mice. On day 3–4 post-infection, mice were fed as blood meals for 30 min to *Anopheles stephensi* mosquitos. After the blood meal, mosquitos were held at 21°C, 80% humidity, and a 14 h light/10 h dark day-night cycle. After 18–21 days, sporozoites were isolated from the salivary glands of infected mosquitos. Sporozoites were irradiated by exposure to a caesium-137 source. Age-matched (7-15 weeks) C57BL/6J were injected with 1000–5000 PbA sporozoites. Indicated groups were treated with 70 μg/ml pyrimethamine in drinking water 5 days after initial infection [35].

Parasitemia

PbA infection rate was determined by counting infected erythrocytes. A thin blood smear from infected mice was stained with Wright Giemsa Stain. A minimum of 1000 erythrocytes in minimum three fields of views were counted.

T cell isolation

Mice were euthanized, and 300–500 μl blood was drawn from the *vena cava* and mixed with 20 μl Heparin-Natrium-25000 (Ratiopharm, Ulm, Germany). Following the blood draw, mice were directly perfused with PBS/2% FCS/1 mM EDTA and liver digest medium (ThermoFisher Scientific/ Gibco, Waltham, Massachusetts) through the vena cava. Leukocytes were isolated from blood, brain, liver, and spleen. Centrifugations were done for 5 min, 400 × *g* at 4°C unless otherwise stated.

Blood: Whole blood was taken from the *vena cava* and mixed with heparin. Erythrocytes were lysed two times with 20 ml lysis buffer (150 mM NH_4CL, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2-7.4). Erythrocytes-free single cells were directly used for staining.

Spleen: Prepared spleens were disrupted through a 100 μm sieve with PBS/2% FCS/1 mM EDTA. The resulting suspension was centrifuged. Erythrocytes were lysed using lysis buffer. Cells were washed with PBS/2% FCS/1 mM EDTA and filtered through another 100 μm cell strainer.

Liver: Lymphocytes from liver tissue were obtained as described previously [31]. Briefly, livers were cut into small pieces and digested. Single cells were separated from debris by 40% Percoll (SIGMA, St. Louis, Missouri) in PBS/2% FCS/1mM EDTA, and centrifugation was done for 20 min at 600 × *g* at room temperature (RT). Erythrocytes were lysed.

Brain: The brain was strained through a 100 μm sieve, washed with PBS/2% FCS/1 mM EDTA. Pellet was resuspended in 40% Percoll in PBS/2% FCS/1 mM EDTA and centrifuged at 600 × *g*, RT. Erythrocytes were lysed before staining.

Restimulation of lymphocytes

Cells were restimulated to induce cytokine production. Briefly, 2×10^6 cells were incubated for 5 h with Brefeldin A (BioLegend, San Diego, California) and Monensin (BioLegend, San Diego, California) at 37°C, 5 % CO_2. For restimulation, cells were incubated either with 1 mg/ml PMA and 0.25 mg/ml Ionomycin (Iono) or 1 μg/ml PbA-peptide mix (SQLLNAKYL; IITDFENL; EIYIFTNI from Jerini Biotools [36]) or left unstimulated.

Suppression assay

To investigate the suppressive capacity of the different T cell subpopulations, suppression assays were performed. Splenocytes from naïve C57BL/6 mice were obtained as described previously. $CD4^+$ or $CD8^+$ T cells (target cells) were obtained using the respective negative selection kit (Miltenyi, Bergisch Gladbach, Germany) and were labeled with 1 μM of proliferation dye eFluor450 (ThermoFisher, Waltham, Massachusetts). Potential suppressive T cell populations were obtained from spleens of infected C57BL/6 mice at day 6 post-infection and sorted using a FACS Aria II (BD Bioscience, Franklin Lakes, New Jersey) (Fig S3). 5×10^4 naïve target $CD4^+$ or $CD8^+$ T cells were seeded in complete RPMI 1640 (RPMI 1640 + 5 % FCS + 2 mM L-glutamine + 10 mM HEPES + 50 μg/mL Gentamicin) in a round-bottom 96-well plate and co-cultivated with different ratios of potential suppressor T cells (1:0, 5:1, 5:2 and 1:1 responder: suppressor T cells). The cells were cultured with αCD3/αCD28 beads (Invitrogen, Carlsbad, California) at a ratio of 1:1 (beads: total T cells) and incubated at 37°C, 5 % CO_2 for 3 days.

$CD8^+$ cytotoxicity assay

$CD8^+$ T cells were isolated and sorted as described above from spleens of OT-I mice infected with PbOVA. Splenocytes from naïve C57BL/6 mice were split into two groups, one of which was pulsed with 1 μg/ml SIINFEKL peptide in complete RPMI 1640 for 1 h at 37°C, 5 % CO_2. The groups (pulsed and unpulsed) were labeled with eFluor450 or eFluor670 (ThermoFisher, Waltham, Massachusetts), respectively, and then mixed. Mixed target cells were co-incubated with the sorted $CD8^+$ T cells in indicated ratios for 6 h. Afterwards, living cells were identified by ZombieNIR Live/Dead staining (BioLegend, San Diego, California). By calculating the proportion of SIINFEKL-loaded target cells from the remaining living target cells compared to a control without any sorted $CD8^+$ T cells, the cytotoxic capacity of each sorted subset was determined.

Flow cytometry analysis of murine cells

The following antibodies were used for surface staining at RT for 20 min: αCD3 BUV395 (145-2C11), αCD4 V500 (RM4-5), αCD8 V450 (53-6.7), and αCD223 (LAG-3) BUV737 (C9B7W) purchased from BD Bioscience, Franklin Lakes, New Jersey. αCD25 AlexaFluor 700 (PC61), αCD27 FITC (LG4.A10), αCD39 PE-Cy7 (Duha59), αCD44 AlexaFluor 700 or BV650 (IM7), αCD49b PE or AlexaFluor 488 (DX5), αCD62L PE-Cy5 or PerCP (MEL-14), αCD69 BV785 (H1.2F3), αCD223 (LAG-3) PerCP-Cy5.5 (C9B7W), αCD278 (C398.4A), αCD279 (PD-1) APC or PE-Cy7 (RMP1-39), αCD366 (Tim-3) BV711(RMT3-23), and αTIGIT PE-Dazzle594 (2G9) were purchased from BioLegend, San Diego, California, and αKLRG-1 APC-eFluor780 (2F2) was purchased from ThermoFisher, Waltham, Massachusetts. According to the manufacturer's instruction, intracellular staining was performed using the FoxP3 transcription factor staining kit (eBiosience, San Diego, California). Fixation/ permeabilization was followed by intracellular staining for 20 minutes at RT. αgranzyme B Pacific blue (GB11, BioLegend, San Diego, California), αCD3 BUV395, and αFoxP3 PerCP-Cy5.5 (FJK-16s, ThermoFisher, Waltham, Massachusetts) were used. Samples were acquired on a BD LSRII or BD LSRFortessa (BD Bioscience, Franklin Lakes, New Jersey). FACS data were analyzed using FlowJo v10 (BD Bioscience, Franklin Lakes, New Jersey). Gates were set with the help of uninfected controls, as well as cell populations with a known lack of expression of specific markers.

Table 1. Clinical data of enrolled malaria patients

#	Sex	Age	Country of infection	Parasitemia at the day of diagnosis	Parasitemia at the day of sampling	Day (sampling) after the start of treatment
1	F	47	Togo	5%	Negative	2
2	M	45	Togo	<1%	< 1 %	3
3	f	26	Togo	<1%	Negative	3
4	m	26	Guinea-Bissau	<1%	<1%	3
5	m	41	Nigeria	<1%	Negative	3
6	m	33	Benin	<1%	Negative	4
7	m	43	n.a.	2.50%	Negative	2
8	f	18	Cameroon	1.50%	<1%	2
9	f	61	Tanzania	21%	Negative	16
10	m	49	Nigeria	<1%	<1%	1
11	f	39	Cameroon	6%	Negative	4
12	m	51	Ivory Coast	0.50%	Negative	4
13	m	50	Togo	0.50%	<1%	3
14	m	41	Togo	<1%	Negative	4
15	f	55	Ghana	5%	Negative	3
	f/m = 6/9	mean = 41.7				

Data processing and clustering

Lymphocytes were cleaned from doublets and gated for CD3 to identify T cells. $CD3^+$ T cells were subsequently gated for the expression of CD4 and CD8. $CD8^+$ T cells or $CD4^+$ T cells were downsampled, and respective groups were concatenated (2-3 individual mice per group). Clustering was performed by FlowSOM (package v2.6; random starting seeds) in FlowJo [13]. In total, 15 markers were included (LAG-3, TIM-3, TIGIT, PD-1, CD39, FoxP3, CD25, CD27, CD49b, granzyme B, KLRG-1, ICOS, CD69, CD62L, CD44). The grid size of the self-organizing map (SOM) was set to 15×15. Metacluster number was set to 15. Concatenated expression data from splenocytes from PbA-infected animals and naïve controls were used to generate a master cluster grid and a minimum-spanning tree (MST). To identify differences in the expression pattern, the individual groups were computed based on the master grid. $CD4^+$ and $CD8^+$ T cells were analyzed individually.

Human study population

In total, 15 malaria patients (Table 1) and 17 healthy adults (Table 2) between the age of 18–61 were enrolled in this study. All malaria patients were returning travelers infected with *P. falciparum* in a malaria-endemic area, but developed their malaria symptoms after returning to Germany. The malaria patients were enrolled during their malaria treatment at the University Medical Centre Hamburg Eppendorf (Hamburg, Germany). The healthy adults were enrolled at the Bernhard Nocht Institute for Tropical Medicine in Hamburg. Ethical approval was obtained for the study from the Ethics Committee Hamburg, Germany (PV 5537). Written informed consent was given from all study participants before inclusion in the study.

Determination of *Plasmodium falciparum* infection

P. falciparum infection and parasitemia were determined by thick and thin blood film stained with Giemsa and examined with an oil immersion microscope (100x magnification).

Ex vivo staining of human peripheral blood

Venous blood was collected into Lithium-Heparin tubes and processed within 5 h. One hundred microliters of whole blood were incubated at 4°C for 30 min with the following surface

Table 2. Clinical Data of healthy donors

#	Sex	Age
1	F	59
2	F	27
3	M	27
4	M	26
5	M	34
6	F	25
7	F	57
8	F	51
9	F	27
10	M	35
11	F	31
12	M	29
13	M	53
14	F	26
15	F	24
16	F	21
17	F	39
	F/M = 11/6	Mean = 34.8

antibodies: CD3 APC/Cy7 (HIT3a), CD4 BV510 (RPA-T4), CD8 AF700 (RPA-T8), CD127 AF488 (A019D5), CD25 PE/Cy7 (BC96), PD-1 PerCP/Cy5.5 (EH12.2H7), TIGIT BV605 (A15153G), TIM-3 BV650 (F38–2E2), CD39 PE/Dazzle594 (A1), CD49b FITC (P1E6-C5), CCR5 PE (J418F1) (all BioLegend, San Diego, California), and LAG-3 APC (3DS223H) (eBioscience, San Diego, California). Afterwards, the cells were lysed and fixed with 2 ml of 1× RBC Lysis/Fixation Solution (Biolegend) for 15 min at RT and washed with cold FACS-buffer (PBS with 1 % FCS). Cells were analyzed on an LSRFortessa (BD Bioscience, Franklin Lakes, New Jersey) or a CytoFLEX S (Beckman Coulter, Brea, California) flow cytometer and analyzed with FlowJo v10 (BD Bioscience, Franklin Lakes, New Jersey). Lymphocyte doublets were excluded by FSC-A/FSC-H gating. Afterwards, T cells were gated by their expression of CD3. $CD3^+$ T cells were subsequently gated on CD4 and CD8. $CD4^+$ and $CD8^+$ T cells were gated for LAG-3 and CD49b. LAG-$3^{+/-}$CD49$b^{+/-}$ populations were gated to determine the median fluorescence intensities (MFI) of all analyzed surface markers on these populations. This was done separately for $CD4^+$ and $CD8^+$ T cells, respectively. According to fluorescence minus one (FMO) controls, gates were set to identify false positives and spillover.

Statistics

Mouse data were checked for normality by the D'Agostino & Pearson test or the Shapiro-Wilk test for small group size. If a Gaussian distribution is assumed, ordinary one-way ANOVA with subsequent Tukey's multiple comparison test was performed. Otherwise, the Kruskal-Wallis test with Dunn's multiple comparisons test was performed. If the samples were paired and the Shapiro-Wilk test showed a normal distribution, a mixed-effects analysis with a Geisser-Greenhouse correction was performed. If the Shapiro-Wilk test did not show a normally distribution a Friedman test was performed Comparison of just two groups was made by Mann-Whitney test if normal distribution was assumed; otherwise, samples were analyzed with a *t*-test. The specific test used is indicated in each figure. Statistic calculations were done with Graphpad Prism v8 (Graphpad Software, San Diego, USA).

For human samples, frequencies of different T cell subsets of the two groups were compared using unpaired, nonparametric Mann-Whitney tests (GraphPad Prism v8).

P values ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***), or ≤ 0.0001 (****) were considered statistically significant.

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Guidelines for flow cytometry analysis

The authors adhered to the guidelines for analysis of flow cytometry studies [37]

Data availability statement: The data that support the findings of the study are available from the corresponding author (riehn@bnitm.de) upon reasonable request.

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Author contributions: J.B., C.L., and L.C.K. designed and performed experiments and analyzed data. J.S.W., M.A., and Mi.R. provided patient samples and scientific input. M.M conceptualized and supervised the human study part and provided scientific input. M.R. designed experiments, analyzed data, and wrote the first draft of the manuscript. T.J. and M.R. conceptualized the study, supervised the project, and wrote the manuscript. All authors contributed to the manuscript.

Conflict of interest: The authors have declared no conflict of interest.

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Abbreviations: **CD**: cluster of differentiation · **dpi**: days post-infection · **LAG-3**: lymphocyte activation gene 3 · **KLRG-1**: Killer cell lectin-like receptor subfamily G member 1 · **MFI**: mean fluorescence intensities · **MST**: minimum spanning tree · **PbA**: *Plasmodium berghei* · **PD-1**: programmed cell death protein 1 · **PM**: pyrimethamin · **SOM**: self-organizing map · **TIGIT**: T cell immunoreceptor with Ig and ITIM domain · **TIM-3**: T cell Ig and mucin-domain containing-3;

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II. Supplementary material for: T cells expressing multiple co-inhibitory molecules in acute malaria are not exhausted but exert a suppressive function in mice

Johannes Brandi, Cari Lehmann, Lea-Christina Kaminski, Julian Schulze zur Wiesch, Marylyn Addo, Michael Ramharter, Maria Mackroth, Thomas Jacobs, Mathias Riehn

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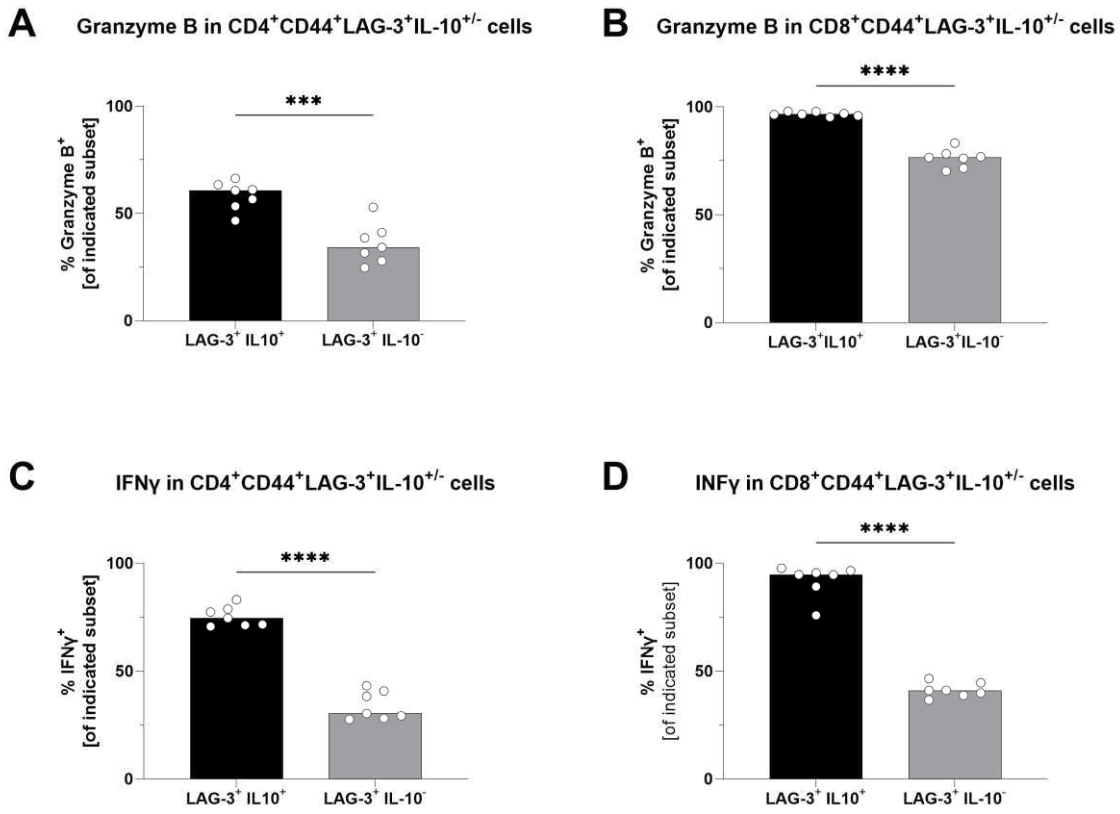
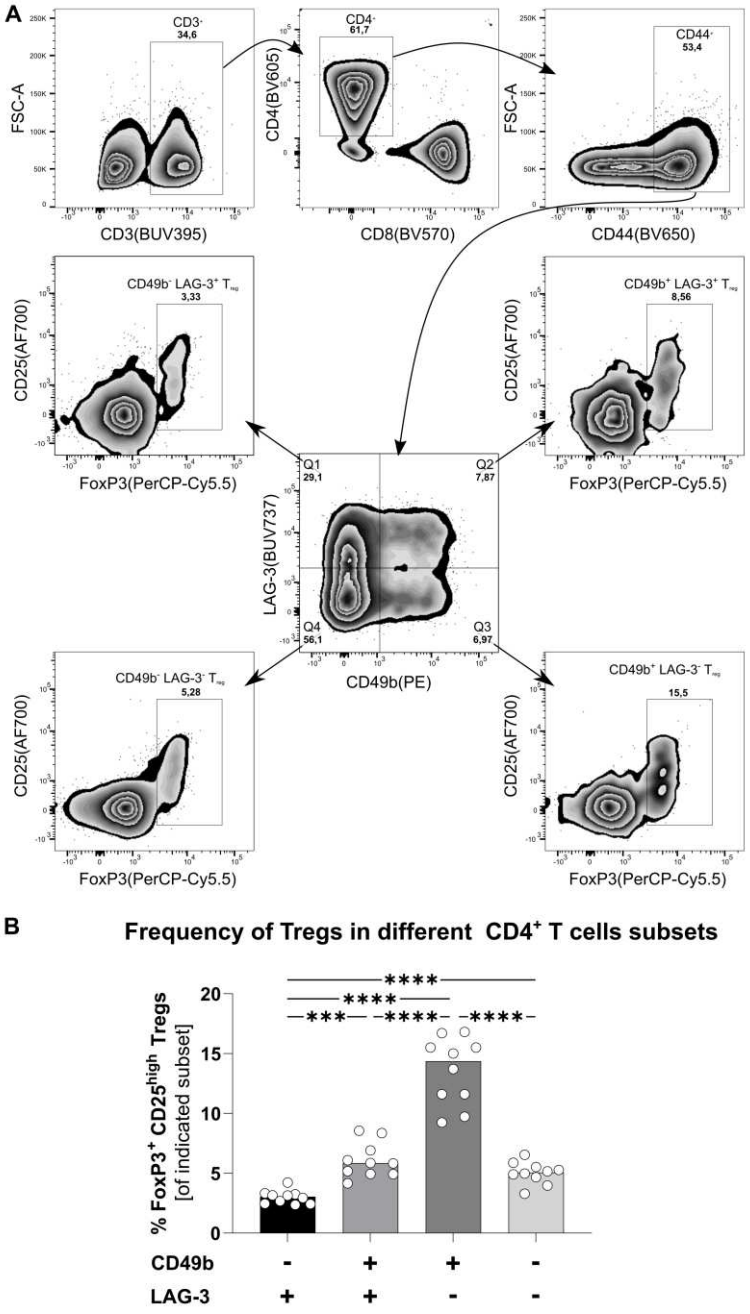
Supplementary figure 1:



Co-expression of IL-10 with Granzyme B or IFNγ in CD4$^+$CD44$^+$LAG-3$^+$ or CD8$^+$CD44$^+$LAG-3$^+$ T cells

Expression levels of Granzyme B in LAG-3$^+$ CD4$^+$ **(A)** or CD8$^+$ **(B)** T cells correlate with IL-10 expression. Similarly, expression of the cytokine IFNγ also increases with IL-10 expression in both CD4$^+$ **(C)** and CD8$^+$ **(D)** T cells. Cells were obtained from the spleens of PbA-infected C57Bl/6 mice 6 dpi and stained after restimulation for 5 h with PMA/Iono. n= 7 from one experiment. Data was analyzed with a paired t-test, p values ≤ 0.001 (***) or ≤ 0.0001 (****) were considered statistically significant.

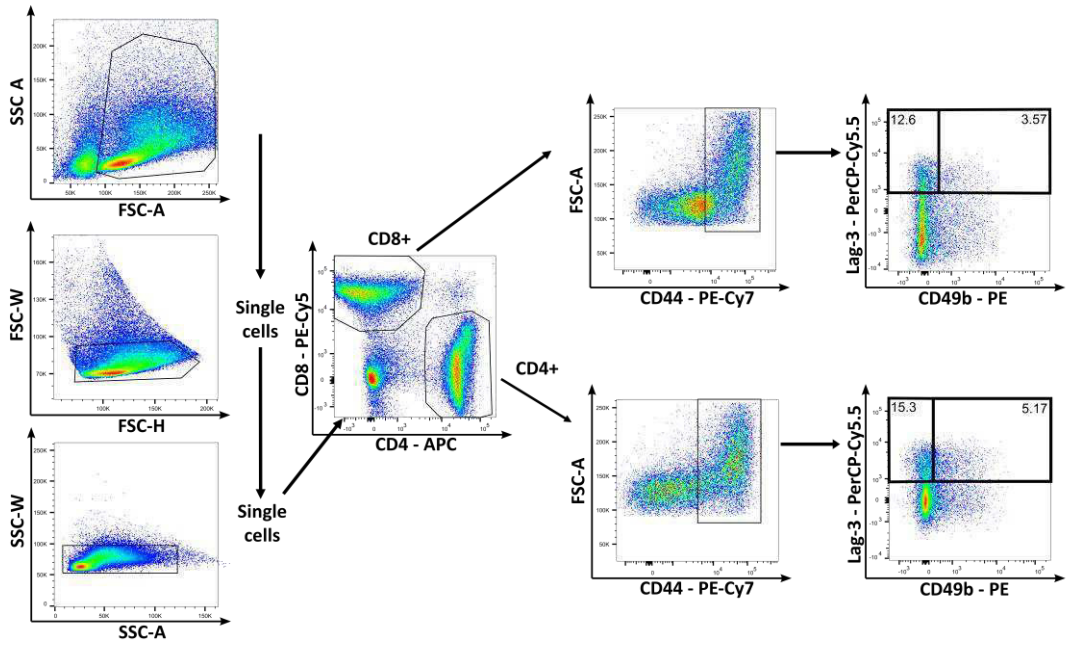
Supplementary figure 2:



Distribution of FoxP3^{+} Tregs in different CD4^{+}T cell subsets

(A) Exemplary staining and gating strategy to analyze **(B)** frequencies of FoxP3^{+} Tregs in LAG3$^{+/-}$ CD49b$^{+/-}$ CD4^{+} T cell subsets. Cells were obtained from the spleens of PbA-infected C57Bl/6 mice 6 dpi. n=10 from two independent experiments. Data was analyzed with Tukeys test for multiple comparisons, p values ≤ 0.001 (***) or ≤ 0.0001 (****) were considered statistically significant.

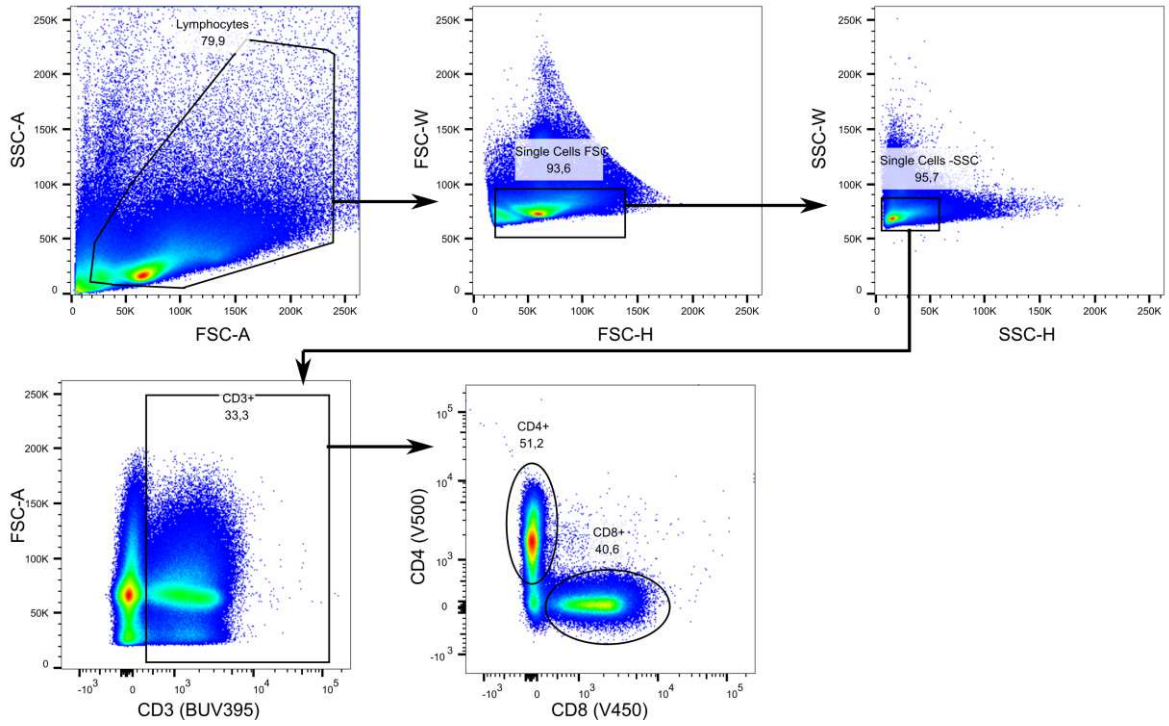
Supplementary figure 3:



Gating strategy for T cell sorting

Flow cytometry gating strategy to sort potential regulatory T cell subsets. Splenocytes of PbA-infected C57Bl/6 mice were isolated and stained 6 dpi. The cells were sorted for $CD4^+$ or $CD8^+$ T cells. Activated T cells were identified by the expression of CD44 and for sorting distinguished fby the expression of LAG-3 or for the co-expression of LAG-3 and CD49b.

Supplementary figure 4



Exemplary gating strategy to analyze CD4⁺ and CD8⁺ T cells.

III. Increased Expression of Multiple Co-Inhibitory Molecules on Malaria-Induced $CD8^+$ T Cells Are Associated With Increased Function Instead of Exhaustion

Johannes Brandi, Mathias Riehn, Alexandros Hadjilaou and Thomas Jacobs

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Increased Expression of Multiple Co-Inhibitory Molecules on Malaria-Induced CD8$^+$ T Cells Are Associated With Increased Function Instead of Exhaustion

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Activated cytotoxic CD8$^+$ T cells can selectively kill target cells in an antigen-specific manner. However, their prolonged activation often has detrimental effects on tissue homeostasis and function. Indeed, overwhelming cytotoxic activity of CD8$^+$ T cells can drive immunopathology, and therefore, the extent and duration of CD8$^+$ T cell effector function needs to be tightly regulated. One way to regulate CD8$^+$ T cell function is their suppression through engagement of co-inhibitory molecules to their cognate ligands (e.g., LAG-3, PD-1, TIM-3, TIGIT and CTLA-4). During chronic antigen exposure, the expression of co-inhibitory molecules is associated with a loss of T cell function, termed T cell exhaustion and blockade of co-inhibitory pathways often restores T cell function. We addressed the effect of co-inhibitory molecule expression on CD8$^+$ T cell function during acute antigen exposure using experimental malaria. To this end, we infected OT-I mice with a transgenic *P. berghei* ANKA strain that expresses ovalbumin (PbTG), which enables the characterization of antigen-specific CD8$^+$ T cell responses. We then compared antigen-specific CD8$^+$ T cell populations expressing different levels of the co-inhibitory molecules. High expression of LAG-3 correlated with high expression of PD-1, TIGIT, TIM-3 and CTLA-4. Contrary to what has been described during chronic antigen exposure, antigen-specific CD8$^+$ T cells with the highest expression of LAG-3 appeared to be fully functional during acute malaria. We evaluated this by measuring IFN-γ, Granzyme B and Perforin production and confirmed the results by employing a newly developed T cell cytotoxicity assay. We found that LAG-3^{high} CD8$^+$ T cells are more cytotoxic than LAG-3^{low} or activated but LAG-3^{neg} CD8$^+$ T cells. In conclusion, our data imply that expression of co-inhibitory molecules in acute malaria is not necessarily associated with functional exhaustion but may be associated with an overwhelming T cell activation. Taken together, our findings shed new light on the induction of co-inhibitory molecules during acute T cell activation with ramifications for immunomodulatory therapies targeting these molecules in acute infectious diseases.

Keywords: malaria, co-inhibitory molecules, CD8, immune regulation, LAG-3

INTRODUCTION

In malaria, following a bite from a mosquito infected with the *Plasmodium* parasite, the sporozoite travels *via* the blood to the liver. After replication in the liver, referred to as liver stage, the infection ensues into the blood stage with intraerythrocytic parasite replication. During the short liver stage, $CD8^+$ T cells recognize and kill infected hepatocytes in an antigen-specific manner through the engagement on MHC class I molecules presenting parasite antigens (1–3). The function of $CD8^+$ T cells during the blood stage is, however, less clear and limited due to the absence of MHC class I molecules on erythrocytes. However, recently it was shown that $CD8^+$ T cells can directly lyse infected MHC class I expressing reticulocytes in *P. vivax* malaria (4, 5). The blood stage is characterized by strong activation of $CD4^+$ and $CD8^+$ T cells, which produce pro-inflammatory cytokines (6, 7) that activate phagocytic cells (8), resulting in clearance of infected erythrocytes (9). Dysregulated production of cytokines and effector molecules, however, can contribute to severe malaria (10–13).

Interestingly, in experimental (14, 15) and human malaria (16, 17), multiple co-inhibitory molecules are upregulated on T cells. Subsets of malaria-specific $CD8^+$ T cells co-express LAG-3 and PD-1 as well as other molecules associated with T cell exhaustion, for instance, T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) (18), T cell immunoreceptor with Ig and ITIM domains (TIGIT) (19), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (20) and CD39 (21, 22). These molecules were shown to be expressed on exhausted T cells in chronic viral infections (23) and in the tumor environment (24–26). Prolonged antigen exposure drives T cell exhaustion (27), which protects the organ's function and integrity from immunopathology, but can favor viral persistence or immune evasion of malignant cells. T cell exhaustion is a functional adjustment mandatory for long-term viral persistence, leading to a T cell activation level that preserves tissue homeostasis but ensures pathogen control (28, 29).

In chronic viral infection and cancer, blockade of co-inhibitory molecules revokes T cells exhaustion and restores functionality, which is already used clinically in cancer immunotherapy (30). In contrast to oncology or chronic infections, in acute infectious diseases, the function of co-inhibitory molecules is less well defined.

Therefore, we addressed whether the expression of co-inhibitory molecules on malaria-specific $CD8^+$ T cell is associated with a decreased function and if the expression level and/or the co-expression of co-inhibitory molecules is associated with a gradual decline in T cell function. We tested this hypothesis using *P. berghei* ANKA (PbA) infection to determine the phenotype and function of activated antigen-specific $CD8^+CD44^+$ T cells not expressing LAG-3 ($LAG\text{-}3^{neg}$), expressing low levels of LAG-3 ($LAG\text{-}3^{low}$) or high levels of LAG-3 ($LAG\text{-}3^{high}$). In conclusion, we have shown that in acute malaria $CD8^+$ T cells expressing high levels of multiple co-inhibitory molecules are highly activated, produce more cytokines and are more cytotoxic compared to their counterparts with less or no co-inhibitory molecules.

MATERIALS AND METHODS

Animal Experiments and Infection

C57BL/6J mice and OT-I mice were bred at the animal facility of the BNITM in Hamburg, Germany. All animal experiments were performed according to German law and registered with the "Behörde für Justiz und Verbraucherschutz" under the license N048/2020. Mice were infected with *P. berghei* ANKA. To obtain consistent infections, pre-experimental mice were first injected i.p. with stabilate solution (blood from previously infected mice with 15% glycerol, 2.1% sorbitol in PBS for long-term liquid nitrogen storage). After 5-7 days, blood was taken from the pre-experimental mice to infect mice for the experiment with $1x10^5$ infected red blood cells (iRBC). *Plasmodium berghei* ANKA expressing OVA (PbTG), which was used as indicated was generated by Lundie et al. (31).

Cell Isolation

Spleens were sieved through a 100 µm cell strainer. Strainers were washed with wash buffer (PBS containing 2% FCS and 1mM EDTA). The cell suspension was centrifuged at 400g for 5 minutes (min) at 4°C. The supernatant was discarded, 20 ml of erythrocyte-lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, H_2O (pH 7.2-7.4)) were added and incubated at room temperature (RT) for 5 min. Afterward, wash buffer was added to stop the lysis reaction and cells were centrifuged. The cell pellet was washed and meshed again through another 100 µm cell strainer.

Phenotypic Analysis

Spleen cells were counted and $1.5x10^6$ cells were seeded in a 96-well round-bottom plate. Cells were centrifuged at 400g for 5min at 4°C and the supernatant was discarded. Cells were resuspended in 50 µl extracellular staining mastermix and incubated at RT for 20 min. Cells were fixed and stained intracellularly using the eBioscience FoxP3/transcription factor staining buffer (Life Technologies, Carlsbad, California) according to the manufacturer's instructions. For sorting, cells were stained extracellularly without any fixation.

For the extracellular staining, we used αLAG-3 (C9B7W, RRID: AB_2871155) BUV737, purchased from BD Biosciences; αLAG-3 (C9B7W, RRID: AB_2572081 & AB_2561517) PE-Dazzle594 & PerCP-Cy5.5, αTIGIT (1G9, RRID: AB_2566573) PE-Dazzle594, αPD-1 (29F.1A12, RRID: AB_2562616) BV605, αTIM-3 (RMT3-23, RRID: AB_2716208) BV711, αCD39 (Duha59, RRID: AB_2563394 & AB_11218603) PE-Cy7 & PE, αCD8α (53-6.7, RRID: AB_2563055 & AB_312745) BV570 & FITC, αCD4 (GK1.5, RRID: AB_2860583) APC/Fire810, αCD44 (IM7, RRID: AB_493713 & AB_830787) Alexa Fluor 700 & PE-Cy7, purchased from BioLegend, San Diego, California. For intracellular staining, αCD3ϵ (145-2C11, RRID: AB_2738278) BUV395 was purchased from BD Biosciences; αGranzyme B (GB11, RRID: AB_2562195) Pacific Blue, αPerforin (S16009A, RRID: AB_2721463) APC, αCTLA-4 (UC10-4B9, RRID: AB_2563063) BV421, αIFN-γ (XMG1.2, RRID: AB_2814432) APC/Fire750 were purchased from BioLegend. Splenocytes of uninfected C57BL/6J mice were used to validate specificity of the

stainings (**Figure S1**). Data were acquired on a BD LSR II, BD LSRFortessa, or Cytek Aurora.

Restimulation Assay

After isolation of spleen cells, $2x10^6$ cells were seeded in a 96-well round-bottom plate and incubated for 5 h with 1 mg/ml Phorbol-myristate-acetate (PMA) and 0.25 mg/ml Ionomycin (Iono) in 200 µl complete RMPI 1640 medium (RPMI 1640 + 5% FCS + 2 mM L-glutamine + 10 mM HEPES + 50 µg/ml Gentamicin) to induce cytokine production. To accumulate cytokines within the cells the medium was also supplemented with Brefeldin A and Monensin (BioLegend) according to the manufacturer's instructions for the whole incubation period. αCD107a APC antibody (clone 1D4B, RRID: AB_2234505, BioLegend) was added 1:100 to the medium to improve the CD107a staining. BD Cytofix/Cytoperm buffer kit (BD Biosciences) was used for fixation and intracellular staining according to the manufacturers instructions for the restimulation assay.

Cytotoxicity Assay

OT-I mice (32) were infected with $1x10^5$ iRBC (PbTG (31) 6 days before the day of the assay (see complete workflow of the assay in **Figure S2**). Spleens of two mice were pooled to isolate sufficient numbers of splenocytes. B cells were depleted to reduce sorting time. 20µl PE-labeled αCD19 antibody (clone 6D5, RRID: AB_313643, BioLegend) was added and incubated for 10 min in the dark at RT. The cells were washed, α-PE magnetic beads (Miltenyi, Bergisch Gladbach, Germany) were added and incubated for 10 min. Afterward, the cell suspension was transferred to a magnetic LS-column (Miltenyi). The column was washed twice, the flow-through (containing the $CD8^+$ T cells) was washed and stained using αLAG-3 (C9B7W, RRID: AB_2561517) PerCP-Cy5.5, αCD8 (53-6.7, RRID: AB_312745) FITC, and αCD44 (IM7, RRID_ AB_830787) PE-Cy7 (all purchased from BioLegend). The stained cells were sorted into the subsets $CD8^+CD44^+$ LAG-3^{neg}, $CD8^+CD44^+$ LAG-3^{low}, $CD8^+CD44^+$ LAG-3^{high} and $CD8^+CD44^-$ (whole $CD44^-$ cells, without dividing into different levels of LAG-3 expression) on a BD FACS Aria IIIu Cell sorter. After sorting, cells were counted manually, concentrations were adjusted to 4 x 10^6 cells/ml, and sorted subsets were re-analyzed to ensure high sort purity (**Figure S3**). For the preparation of target cells, whole splenocytes were obtained from sex- and age-matched C57BL/6J mice as described above. The cells were split in half and resuspended in 1 ml complete RPMI. One-half of splenocytes were pulsed with the target peptide for effector cells, using a final concentration of 10 µg/ml SIINFEKL-peptide. Pulsed and unpulsed samples were incubated at 37°C, 5% CO_2 for 1 h. Subsequently, both samples were washed with PBS, unpulsed cells were stained with eF450, and SIINFEKL-pulsed cells with eF670 tracer dye in PBS (Both Life technologies, Carlsbad, USA). Afterward, cells were washed again, resuspended in complete RPMI, and counted. Cell suspensions were adjusted to 2 x 10^6 cells/ml and then mixed in a ratio of 1:1, resulting in 1 x 10^6 pulsed and 1 x 10^6 unpulsed target cells per ml. Target cells were kept on ice until the effector cells were sorted. Sorted effector cells were washed, counted and adjusted to $4x10^6$ cells/ml. Three different concentrations of effector cells were used in the final experiments: 4 x 10^6 cells/ml (undiluted), 2 x 10^6 cells/ml, and 1 x 10^6 cells/ml. 100 µl (2 x 10^5 cells) of target cell suspension and 100 µl of pre-diluted effector cells were co-cultured in complete RPMI in a V-bottom plate at 37°C, 5% CO_2 for 6 h, creating ratios of effector: target cells of 2:1, 1:1 and 0.5:1. One to two technical replicates of every dilution of each sorted subset of effector cells were analyzed. The correct ratio of pulsed to unpulsed target cells was determined by measuring only target cells without effector cells to mitigate any counting errors during the mixing process. After co-culture, the plate was centrifuged at 500g. The supernatant was collected and stored at -20°C until analyzing IFN-γ release. Surviving target cells were identified by staining with ZombieNIR fixable Viability dye (BioLegend) for 15 min at 4°C in the dark. Cells were washed twice with PBS and then fixed with 50 µl Perm/Fix buffer of the FoxP3 transcription buffer staining kit for 20 min. Cells were washed, resuspended in wash buffer, and stored at 4°C until analysis on a BD LSRFortessa. After data acquisition, the proportion of living SIINFEKL-pulsed cells out of all living target cells from the target cell gate was analyzed by following the gating strategy according to **Figure S4**. This ratio is inversely proportional to the percentage of killed pulsed target cells. The proportion is normalized to the mean proportion of SIINFEKL-pulsed cells in the control wells to mitigate any errors occurring by mixing the target cells.

Cytokine Release

To measure the IFN-γ release, frozen supernatant from co-incubated effector and target cells was analyzed using the DuoSet® ELISA for Mouse IFN-γ (R&D Systems, Minneapolis, Minnesota) according to the manufacturer's instructions. A linear regression curve was used to calculate the concentration based on the included IFN-γ standard.

Software and Statistical Analysis

All data were analyzed using GraphPad Prism Version 9. Data were tested for normal distribution using the Anderson-Darling test with a pass for normality of alpha=0.05. A one-way ANOVA with Tukey test or a Friedmann's test for multiple comparisons was applied, and P-values <0.05 were considered statistically significant. For acquisition of flow cytometry data, we used FACS DIVA (BD LSR II and BD LSRFortessa) and SpectroFlo (Cytek Aurora), respectively. For analysis of flow cytometry data, we used FlowJo Version 10.8. with the UMAP v3.1 (33) for FlowJo Plugin.

RESULTS

Expression Levels of LAG-3 on $CD8^+$ T Cells Correlate With the Expression of Other Co-Inhibitory Molecules

We recently described that blood-stage human and experimental malaria is characterized by a strong induction of co-inhibitory molecules on T cells (14). In chronic viral infections the

induction of co-inhibitory molecules by T cells dampens their effector function (27). T cells with high expression levels of co-inhibitory molecules are considered to be exhausted (27). Using the PbA model of acute experimental malaria, we compared the functionality of antigen-specific $CD8^+$ T cells expressing different levels of co-inhibitory molecules. Whereas $CD8^+CD44^-$ T cells from PbA infected mice lack the expression of any co-inhibitory molecules, PbA-induced $CD8^+CD44^+$ T cells express LAG-3 at variable degree. To further decipher the consequences of variable expression levels of co-inhibitory molecules on T cell function, we distinguish between $CD8^+CD44^+$ T cells expressing no ($LAG\text{-}3^{neg}$), low ($LAG\text{-}3^{low}$) and high levels of LAG-3 ($LAG\text{-}3^{high}$) (**Figure 1A**; **Figure S5**). Interestingly, increasing expression levels of LAG-3 correlates with increasing co-expression of other co-inhibitory molecules. $CD8^+CD44^+LAG\text{-}3^{low}$ T cells express intermediated levels of PD-1, TIGIT, TIM-3 and Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4), whereas $CD8^+CD44^+LAG\text{-}3^{high}$ T cells exhibit statistically significant higher median fluorescence intensities of these co-inhibitory molecules compared to $CD8^+CD44^+LAG\text{-}3^{low}$ and $CD8^+CD44^+LAG\text{-}3^{neg}$ T cells (**Figures 1B–E**). Also, CD39, an ecto-nucleosidase implicated in T cell exhaustion (21, 22), correlates with the expression of LAG-3 (**Figure 1F**).

High Expression Levels of Co-Inhibitory Molecules Are not Associated With Exhaustion but Rather Increased Function

To verify if effector functions of malaria-induced $CD8^+$ T cells are influenced by the expression of co-inhibitory molecules, we analyzed the intracellular expression of the pro-inflammatory cytokine Interferon γ (IFN-γ) as well as Granzyme B (GrzB) and Perforin, both of which are key effector molecules for the cytotoxic function of $CD8^+$ T cells. Interestingly, IFN-γ, GrzB, and Perforin were found to be higher expressed with increasing expression of co-inhibitory molecules (**Figures 2A–C**). Similarly, the surface expression of the degranulation marker CD107a increases with the expression of co-inhibitory molecules (**Figure 2D**). Thus, malaria-induced $CD8^+$ T cells co-express LAG-3, PD-1, TIGIT, TIM-3, CTLA-4, and CD39 but similarly

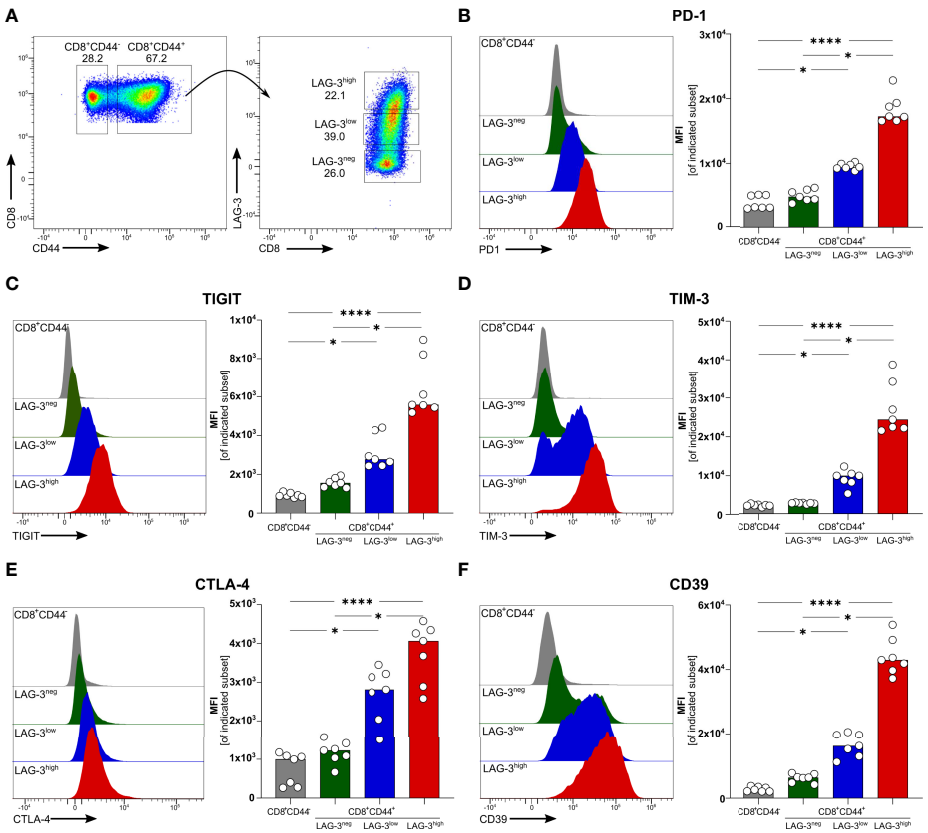

FIGURE 1 | Expression of co-inhibitory molecules and effector molecules correlates with LAG-3 expression. **(A)** Gating strategy and representative examples of dot plots of LAG-3 expression on $CD8^+CD44^+$ T cells. Representative examples of histograms and summarized median fluorescent intensity (MFI) of the co-inhibitory molecules **(B)** PD-1, **(C)** TIGIT, **(D)** TIM-3, **(E)** CTLA-4 and **(F)** CD39 expressed on $CD8^+CD44^+$ T cells expressing different levels of LAG-3 ($CD8^+CD44^+LAG\text{-}3^{neg}$, $CD8^+CD44^+LAG\text{-}3^{low}$, $CD8^+CD44^+LAG\text{-}3^{high}$), compared to expression of those markers on $CD8^+CD44^-$ T cells. Data were analyzed with Friedmann's test for multiple comparisons. Each dot represents an individual mouse, n = 7 from two independent experiments. P values between ≤ 0.05 (*) and ≤ 0.0001 (****) were considered statistically significant.

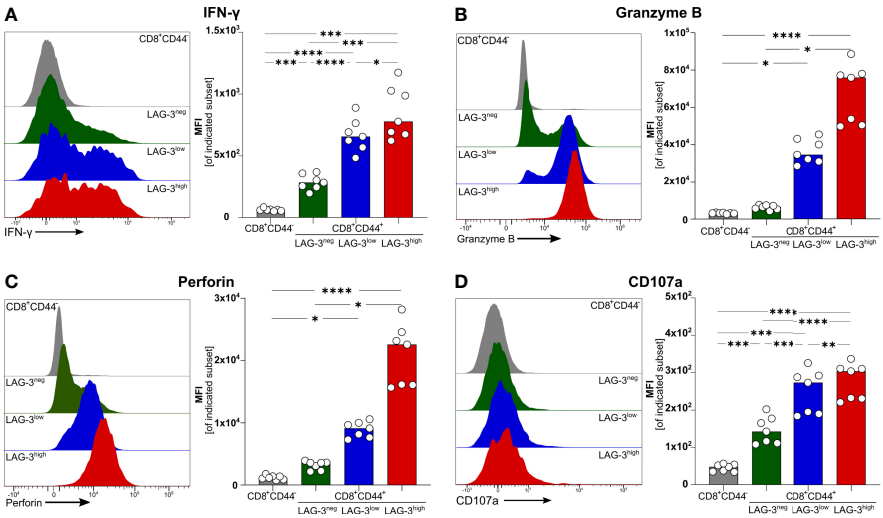

FIGURE 2 | Expression of effector molecules correlates with LAG-3 expression. Exemplary histograms and MFI of **(A)** IFN-γ, **(B)** Granzyme B, **(C)** Perforin and **(D)** CD107a expressed by $CD8^+CD44^+$ cells expressing different levels of LAG-3 ($CD8^+CD44^+LAG\text{-}3^{neg}$, $CD8^+CD44^+LAG\text{-}3^{low}$, $CD8^+CD44^+LAG\text{-}3^{high}$) and by $CD8^+CD44^-$ T cells. Cells were isolated from the spleens of C57BL/6J infected with PbA 6 days post-infection. For B&C, cells were stained directly *ex vivo*. For A&D, cells were stained after 5 h restimulation with PMA/Iono in the presence of Brefeldin A and Monensin. Data were analyzed with Tukey test for multiple comparisons (A&D) or Friedmann's test (B&C). Each dot represents an individual mouse, n=7. P values ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***) or ≤ 0.0001 (****) were considered statistically significant.

express more effector molecules compared to those expressing lower levels of co-inhibitory molecules.

Malaria-Induced $CD8^+$ T Cells Expressing Higher Levels of Co-Inhibitory Molecules Are More Cytotoxic

Next, we determined the cytotoxic capacity of $CD8^+$ T cells rich in co-inhibitory molecules in experimental malaria. Since the function of T cells is not only regulated by co-stimulation and co-inhibition but also by the broad spectrum of affinities of their individual T cell receptor (TCR) to their cognate MHC-class I antigen complex, we used OT-I TCR-TG mice with a transgenic SIINFEKL-specific MHC I on $CD8^+$ T cells (OT-I mice) (32). Infecting these mice with PbA parasites expressing OVA (PbTG) (31) allowed us to induce a strong activation of transgenic SIINFEKL-specific $CD8^+$ T cells, thereby eliminating the influence of the variety of different TCR signaling strength on T cell function. Similar to wild type C57BL/6J mice (**Figure 2**), activated $CD8^+CD44^+$ T cells from OT-I mice express high levels of the co-inhibitory molecules LAG-3, PD-1, TIM-3, CTLA-4 and TIGIT, the ecto-nucleosidase CD39 and the effector molecules Perforin and GrzB (**Figures 3A–H**, **Figure S5**). However, UMAP analysis of $CD8^+CD44^+$ T cells from C57BL/6J mice and OT-I mice revealed that co-expression of co-inhibitory molecules is less pronounced in the later (**Figures 3I, J**). Nevertheless, this analysis confirmed that T cells expressing GrzB and Perforin in the $CD8^+CD44^+LAG\text{-}3^{high}$ compartment do co-express the aforementioned molecules. This finding was corroborated by a correlation analysis showing statistically significant co-expression in $CD8^+$ T cells from C57BL/6J and OT-I mice (**Figure S6**). To investigate the influence of the expression of co-inhibitory molecules on the cytotoxic function of malaria-induced $CD8^+$ T cells, these were sorted according to their LAG-3 expression (**Figure S3A**). Re-analysis of sorted cells revealed distinct populations of $CD8^+CD44^-$, $CD8^+CD44^+LAG\text{-}3^{neg}$, $CD8^+CD44^+LAG\text{-}3^{low}$ and $CD8^+CD44^+LAG\text{-}3^{high}$ (**Figure S3B**), which we then analyzed in a newly developed cytotoxicity assay. To this end, *in vivo* activated, sorted OT-I cells were co-cultured with SIINFEKL-pulsed target cells and specific killing of those target cells was determined by flow cytometry (**Figure S4**; a schematic overview of the workflow is depicted in **Figure S2**).

The proportion of specifically killed target cells increases in $CD8^+CD44^+LAG\text{-}3^{low}$ and $CD8^+CD44^+LAG\text{-}3^{high}$ populations compared to their $CD8^+CD44^-$ and $CD8^+CD44^+LAG\text{-}3^{neg}$ counterparts (**Figure 4A**). Furthermore, the target cell specific killing depends on the dose of effector T cells. Also, $CD8^+CD44^+LAG\text{-}3^{high}$ effector cells diminish significantly more pulsed target cells than the $CD8^+CD44^+LAG\text{-}3^{low}$ effector cell subset, indicating increased cytotoxic capacity with increasing levels of expression of co-inhibitory molecules. This effect is also visible comparing lower effector to target cell ratios.

These data were further confirmed by analyzing the IFN-γ production of the $CD8^+CD44^+$ T cells distinguished on their LAG-3 expression (**Figure 4B**). While $CD8^+CD44^-$ T cells show low amounts of IFN-γ production, increasing levels of LAG-3 expression correlate with increasing levels of IFN-γ production, implying that cells expressing more co-inhibitory molecules show higher cytotoxic capacity.

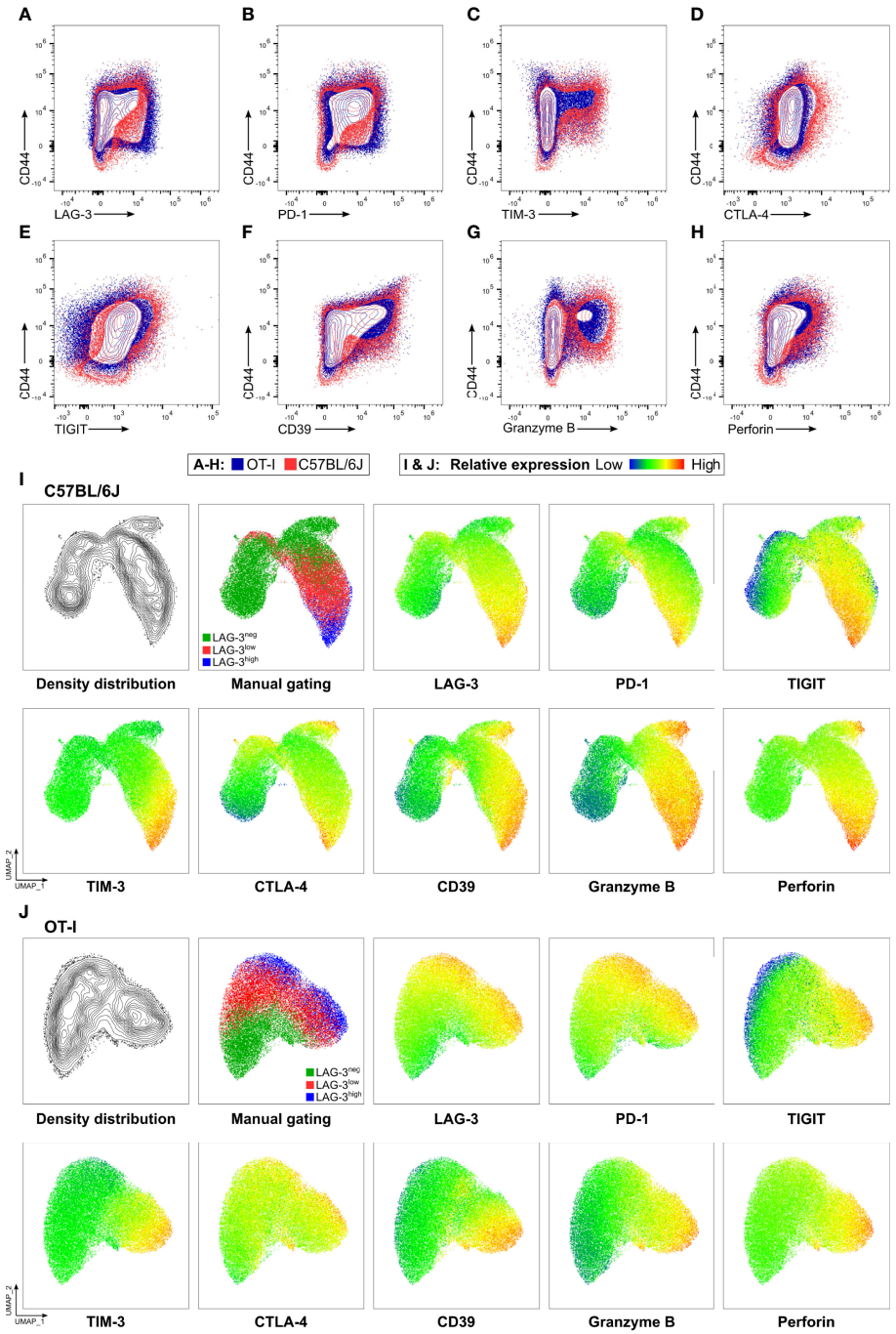

FIGURE 3 | OT-I derived CD8$^+$CD44$^+$ T cells express co-inhibitory and effector molecules in a similar fashion to C57BL/6J derived CD8$^+$CD44$^+$ T cells. Exemplary contour showing expression of **(A)** LAG-3, **(B)** PD-1, **(C)** TIM-3, **(D)** CTLA-4, **(E)** TIGIT, **(F)** CD39, **(G)** GrzB and **(H)** Perforin on CD8$^+$ T cells relative to CD44 expression. Cells were obtained from the spleens of C57BL/6J (red), and OT-I (blue) mice infected with PbTG 6 days past infection. **(I, J)** Representative UMAP clustering of CD44$^+$CD8$^+$ T cells isolated from C57BL/6J and OT-I mice infected with PbTG 6 days post infection showing the density distribution within the plot; classification into CD44$^+$LAG-3^{neg}, CD44$^+$LAG-3^{low} and CD44$^+$LAG-3^{high} as used throughout the manuscript and relative expression of indicated molecules. Sampled were downsampled to 40.000 cells. UMAP parameters: Distance: Euclidean, Nearest Neighbours: 15, Minimum Distance: 0.5, Number of Components: 2, UMAP v3.1 for FlowJo.

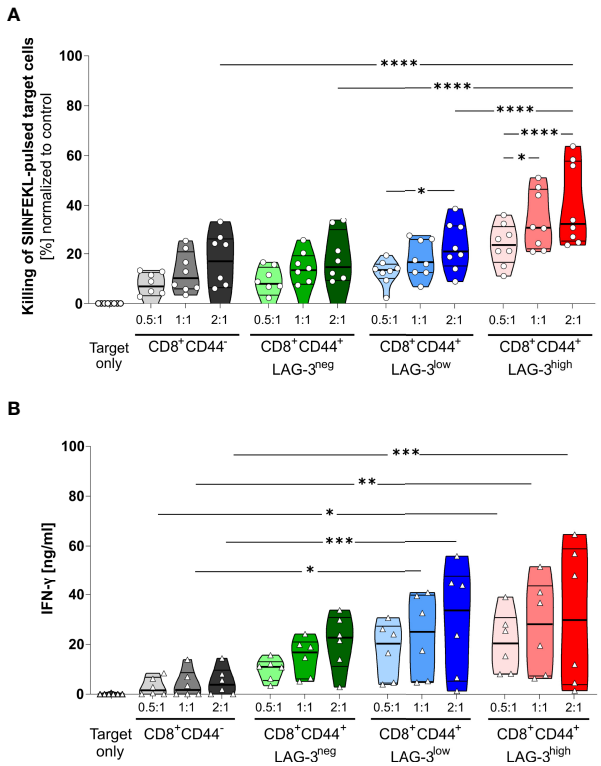

FIGURE 4 | LAG-3 expression correlates with cytotoxic capabilities of $CD8^+$ T cells. **(A)** Killing of SIINFEKL (SII)-pulsed target cells by $CD8^+CD44^+$ T cells expressing different levels of LAG-3 compared to $CD8^+CD44^-$ T cells in a dose dependent manner. SIINFEKL-pulsed target cells obtained from C57BL/6J mice were co-incubated with indicated sorted $CD8^+$ T cells in indicated ratios for 6 hours. $CD8^+$ T cells were obtained from OT-I mice infected with PbTG 6 days prior to T cell isolation. **(B)** Secretion of IFN-γ of the above-mentioned $CD8^+$ T cells subsets from the supernatant of the cytotoxicity assay. Each dot represents the mean of up to two technical replicates. Data were tested for normal distribution with the Anderson-Darling test with a pass for normality of alpha=0.05. ANOVA with Tukey test for multiple comparisons. P= * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001, **(A)** n = 8, **(B)** n = 6. All groups showed significant (P < 0.05) killing compared to the target only group; these significances are not shown to avoid overloading the figure.

DISCUSSION

Blood-stage malaria is characterized by overwhelming activation of $CD4^+$ as well as $CD8^+$ T cells, both of which secrete pro-inflammatory cytokines. These T cells confer protection by activating macrophages capable of clearing infected erythrocytes, especially in the spleen and the liver (6–8). In addition, $CD8^+$ T cells were shown to directly lyse *P. vivax* infected reticulocytes (5). However, they also contribute to activation of endothelial cells which enhances sequestration of infected erythrocytes and cross-presentation of parasitic antigen (34), a hallmark of severe malaria (35–37). Recent data also provide evidence that in experimental and human malaria, $CD8^+$ T cells contribute to the dissolution of the endothelial cell layer and thereby loss of vascular integrity through their cytotoxic capacity (38). Along this line, we have recently shown that GrzB secretion by $CD8^+$ T cells correlates with severity in acute human malaria (11) and $CD8^+Grzb^+$ T cells adhere/accumulate on the brain's vasculature of fatal cerebral malaria (39). These data support the use of the experimental *P. berghei* model for deducing the immunopathology in human malaria. Therefore, tight regulation of T cell function is crucial to ensure efficient parasite clearance and/or control without overwhelming inflammation being a prerequisite for severe malaria. We have recently shown that blood-stage malaria in experimental models but also in human malaria is accompanied by an induction of co-inhibitory molecules like PD-1, LAG-3, and TIM-3 on $CD4^+$ as well as $CD8^+$ T cells.

We showed malaria-specific co-inhibitory rich $CD4^+$ T cells to be capable of suppressing the activation of naïve T cells and might regulate the amount of T cell activation in acute malaria (14, 16, 40). Interestingly, we also found $CD8^+$ T cells with a similar co-inhibitory molecule expression with the same suppressive capacity (14). Therefore, induction of co-inhibitory molecules in acute malaria delineate T cells with functional properties of type 1 regulatory T cells (Tr1 cells) (41). However, expression of co-inhibitory molecules on $CD8^+$ T cells in settings of prolonged antigen exposure like chronic viral infections or cancer was shown to limit T cell function leading to a state often referred to as T cell exhaustion (27). Blocking co-inhibitory molecules by antibodies was shown to, at least partially, revert T cell function, often having a great beneficial effect in cancer therapy (26, 30). The therapeutic effect of blocking co-inhibitory molecules in chronic viral diseases is less well studied. Increasing the function of antigen-specific $CD8^+$ T cells might help to clear persisting virus (42). However, since the viral load in specific organs can be very high and many cells in the respective tissue might be affected, unleashing the function of exhausted $CD8^+$ T cells might have detrimental effects on organ function. Interestingly, it was shown that a blockade of PD-1 in experimental malaria increases T cell memory (43). It was also shown in an LCMV model that $LAG\text{-}3^+CD8^+$ T cells have a disadvantage in memory formation compared to $CD8^+$ T cells from LAG-3-deficient mice (44). Thus, an expression of co-inhibitory molecules on $CD8^+$ T cells as found in acute malaria might restrict memory formation.

In hepatocellular carcinoma, T cell exhaustion is associated with high expression of PD-1 on $CD8^+$ T cells. The extent of exhaustion correlates with increased expression levels of PD-1 and co-expression of other co-inhibitory molecules (45). The reasons mentioned above prompted us to study the regulation of $CD8^+$ T cells in acute malaria in more detail. We hypothesized that $CD8^+$ T cells expressing high levels of multiple co-inhibitory molecules are restricted in their functionality, whereas $CD8^+$ T cells express less co-inhibitory molecules comprising strongly activated T effector cells. However, studying the function of bulk malaria-specific $CD8^+$ T cells in relation to expression with co-inhibitory molecules has several flaws.

Activation of $CD8^+$ T cells is dependent on multiple factors like co-stimulation and TCR affinity to antigen MHC class I complexes. Due to the vast amount of different plasmodial antigens, the resulting $CD8^+$ T cell response is inherently heterogenic. Therefore, a correlation of co-inhibitory molecules

and T effector function remains elusive. To circumvent this, we employed a transgenic system using OVA-expressing PbTG to activate SIINFEKL-specific $CD8^+$ T cells from OT-I mice. This ensures that all activated T cells express an identical T cell receptor. Using this system, we found that also activated transgenic $CD8^+CD44^+$ T cells expressing high levels of LAG-3, also express high levels of other co-inhibitory molecules like PD-1, TIGIT, TIM-3 and CTLA-4. In addition, the strongest expression of CD39, a molecule often associated with exhaustion, was found on $CD8^+CD44^+LAG\text{-}3^{high}$ T cells. $CD8^+CD44^+LAG\text{-}3^{low}$ T cells, in contrast, exhibit a weaker expression of all aforementioned co-inhibitory molecules, whereas activated $CD8^+CD44^+LAG\text{-}3^{neg}$ T cells do express only low levels of indicated co-inhibitory molecules. Using UMAP-clustering and correlation analysis, we showed that the expression-pattern of co-inhibitory molecules is similar between malaria-induced $CD8^+$ T cells from OT-I and C57BL/6J mice. Interestingly, less $LAG\text{-}3^{high}$ OT-I T cells express GrzB and Perforin compared to those T cells from C57BL/6J mice. However, the majority of GrzB and Perforin producing T cells are still within the $LAG\text{-}3^{high}$ compartment and do co-express the other co-inhibitory molecules thereby demonstrating a similar expression pattern to $CD8^+CD44^+$ T cells found in PbA-infected C57BL/6J mice. Those differences might be explained by different TCR-affinities and/or kinetics of antigen-expression of plasmodial antigens and the recombinant expressed model antigen. This allows us to compare the function of activated $CD8^+$ T cells with a uniform expression of low, medium and high expression of multiple co-inhibitory molecules.

Interestingly, after restimulation, the effector function of $CD8^+$ T cells positively correlates with the expression of co-inhibitory molecules. $CD8^+CD44^+LAG\text{-}3^{high}$ cells not only produced the highest amount of IFN-γ but produced also the highest amount of the cytotoxic molecules GrzB and Perforin. Furthermore, CD107a, a marker for degranulation, showed the highest expression on $CD8^+CD44^+LAG\text{-}3^{high}$ cells, indicating their increased cytotoxic potential. To further evaluate their cytotoxic capacity, we purified activated $CD8^+CD44^+$ T cells according to their expression level of co-inhibitory molecules. Using a flow cytometry-based cytotoxic assay with differentially labeled SIINFEKL-pulsed and unpulsed target cells, we can corroborate the finding of an increase of cytotoxic function with increased expression of co-inhibitory molecules. This finding clearly demonstrates that even the high expression of multiple co-inhibitory molecules in acute malaria is not associated with decreased effector function or exhaustion. In contrast, it appears that in this acute setting of inflammation, the expression of co-inhibitory molecules delineates $CD8^+$ T cells with the highest effector function. Consistent with this is the recent finding that activation of the PD1-pathway by treatment with programmed death-ligand 1 (PD-L1) fusion proteins may be protective in a model of lethal experimental cerebral malaria (CM) due to inhibition of $CD8^+$ T cells derived cytotoxicity (46). It was recently shown that dampening the function of $CD8^+$ T cells by IL-10-producing NK cells can prevent experimental cerebral malaria (47). However, it remains unclear if the high expression of multiple co-inhibitory molecules on malaria-specific $CD8^+$ T cells might have the potential to dampen their function. We could think of several reasons that might contribute to the absence of exhaustion. The strong inflammatory microenvironment with a vast amount of plasmodial antigens, toll-like receptor (TLR)-agonistic molecules, pro-inflammatory cytokines, high expression of co-stimulatory molecules, and the lack of co-inhibitory ligand interaction might shift the balance to co-stimulation rather than co-inhibition. In addition, several studies have shown that the duration of antigen persistence is critical for establishing exhaustion. Therefore, regarding the highly dynamic kinetics of T cell activation in acute malaria, expression of co-inhibitory molecules might not exert their potential suppressive effects. In conclusion, the high expression of multiple co-inhibitory molecules during acute antigen exposure in blood stage malaria on $CD8^+$ T cells is associated with enhanced cytokine production and cytotoxic activity instead of decreased function. These results are relevant as they provide novel insights into T cell regulation and the function of co-inhibitory molecules in malaria.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Behörde für Justiz und Verbraucherschutz, Hansestadt Hamburg, Germany.

AUTHOR CONTRIBUTIONS

JB, MR and TJ designed the experiments. JB and AH conducted the experiments. JB, MR and TJ analyzed and interpreted the data. JB, MR, AH and TJ wrote the manuscript and prepared the figures. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.878320/full#supplementary-material

Supplementary Figure 1 | Representative stainings demonstrating upregulation of co-inhibitory and effector molecules upon infection. To validate the specificity of the stainings, splenocytes isolated from an uninfected (termed naïve, grey) and an infected C57BL/6J were stained and compared in an overlay. **(A)** *Ex vivo* stainings of LAG-3, PD-1, TIGIT, TIM-3, CTLA-4, CD39, Granzyme B and Perforin. **(B)** IFN-γ and CD107a stainings after restimulation with PMA/Ionomycin.

Supplementary Figure 2 | Workflow of the cytotoxicity assay. OT-I derived splenocytes cells are depleted of B cells and sorted to obtain the subsets of interest. In the meantime, splenocytes from a naïve C57BL/6J are isolated and a portion is pulsed with SIINFEKL. The C57BL/6J derived splenocytes are labelled with their respective tracer dye, mixed and co-incubated with the sorted $CD8^+$ T cells. Killing of SIINFEKL-pulsed target cells is measured *via* flow cytometry and used as a readout to determine cytotoxic capacity of the different $CD8^+$ T cell subsets.

Supplementary Figure 3 | Sorting for $CD8^+$ T cells. **(A)** Gating strategy to sort $CD8^+CD44^+LAG\text{-}3^{neg,low,high}$ and $CD8^+CD44^-$ cells isolated from OT-I mice infected with PbTG 6 days after infection. PE-CD19 labelled cells were removed using α-PE magnetic beads prior to sorting to reduce sorting time. **(B)** Re-analysis of sorted cells, gated on single $CD8^+$ T cells to control the purity of sorted populations.

Supplementary Figure 4 | Analysis of cytotoxicity assay. **(A–D)** Gating strategy to analyse the proportion of living SIINFEKL(SII)-loaded target cells compared to unloaded target cells after incubation with different $CD8^+$ T cell subsets obtained from an OT-I mice infected with PbTG. Effector cells are excluded from the target cells through the lack of either eF450 or eF670 tracer dye. **(E–I)** Representative target cell gates of **(E)**Target cells only and target cells incubated with **(F)** $CD8^+CD44^-$ **(G)** $CD8^+CD44^+LAG\text{-}3^{neg}$, **(H)** $CD8^+CD44^+LAG\text{-}3^{low}$ and **(I)** $CD8^+CD44^+LAG\text{-}3^{high}$ in the highest ratio of effector: target cells of 2:1. A lower percentage of SII-loaded cells relative to the control indicates the higher cytotoxic capacity of the respective subset of $CD8^+$ T cells.

Supplementary Figure 5 | Expression of co-inhibitory molecules and effector molecules correlate with LAG-3 expression in OT-I mice. MFI of the co-inhibitory molecules PD-1, TIGIT, TIM-3, CTLA-4, CD39 and effector molecules CD107a, Granzyme B, Perforin and IFN-γ expressed on $CD8^+CD44^+$ T cells expressing different levels of LAG-3 ($CD8^+CD44^+LAG\text{-}3^{neg}$, $CD8^+CD44^+LAG\text{-}3^{low}$, $CD8^+CD44^+LAG\text{-}3^{high}$) compared to expression of those markers on $CD8^+CD44^-$ T cells isolated from OT-I mice infected with PbTG. Data were analyzed with Friedmann's test for multiple comparisons. Each dot represents an individual mouse, n=8 from two independent experiments. P values between ≤ 0.05 (*) and ≤ 0.0001 (****) were considered statistically significant.

Supplementary Figure 6 | Correlation of analyzed markers on C57BL/6J ad OT-I mice. Correlation of markers analyzed from the *ex vivo* staining on $CD8^+$ T cells isolated from infected C57BL/6J and OT-I mice. **(A, B)** Representative stainings in which all markers are plotted against each other. The accompanying correlation matrix showing **(C, D)** Pearson´s correlation coefficient between the indicated markers was calculated in GraphPad Prism based on 25.000 $CD8^+$ T cells. P values: <0.0001 (****).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

IV. Supplementary material for : Increased Expression of Multiple Co-Inhibitory Molecules on Malaria-Induced $CD8^+$ T Cells Are Associated With Increased Function Instead of Exhaustion

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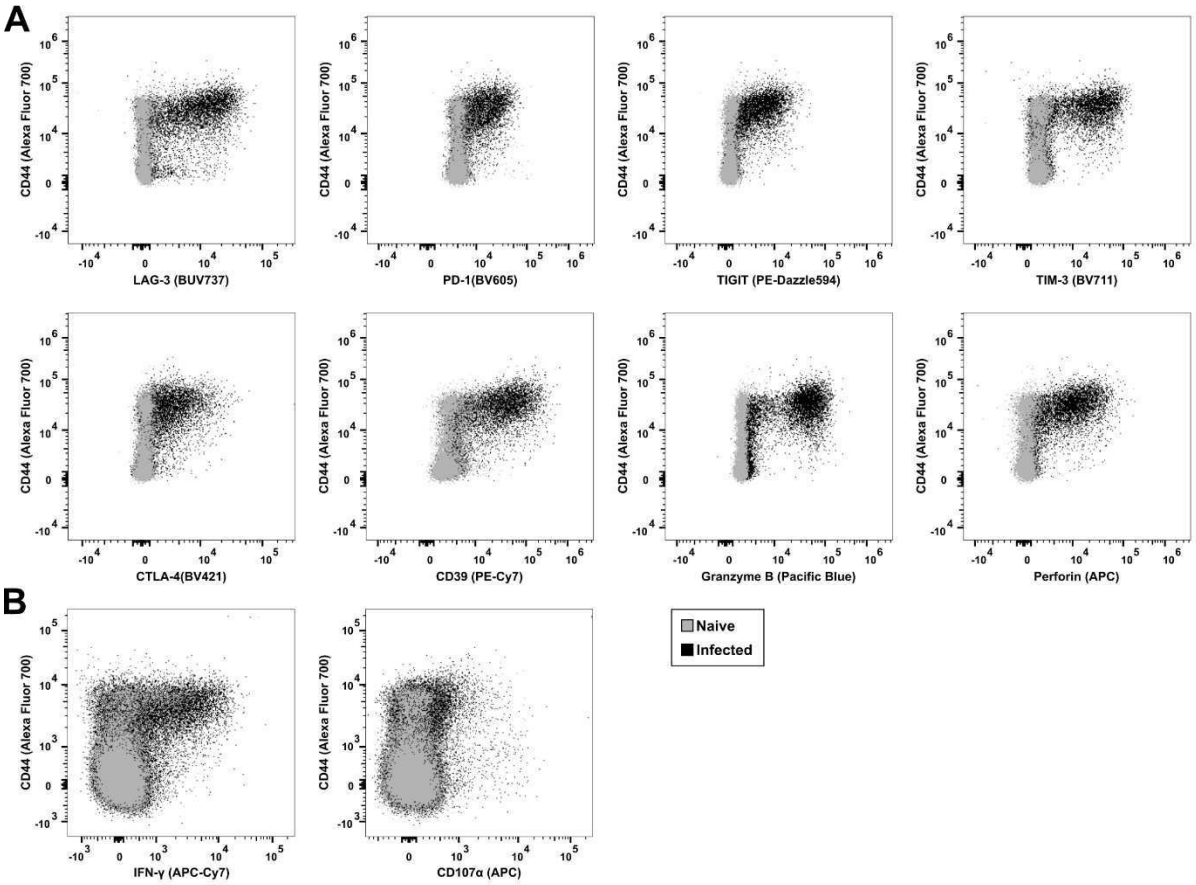
Supplementary figure 1



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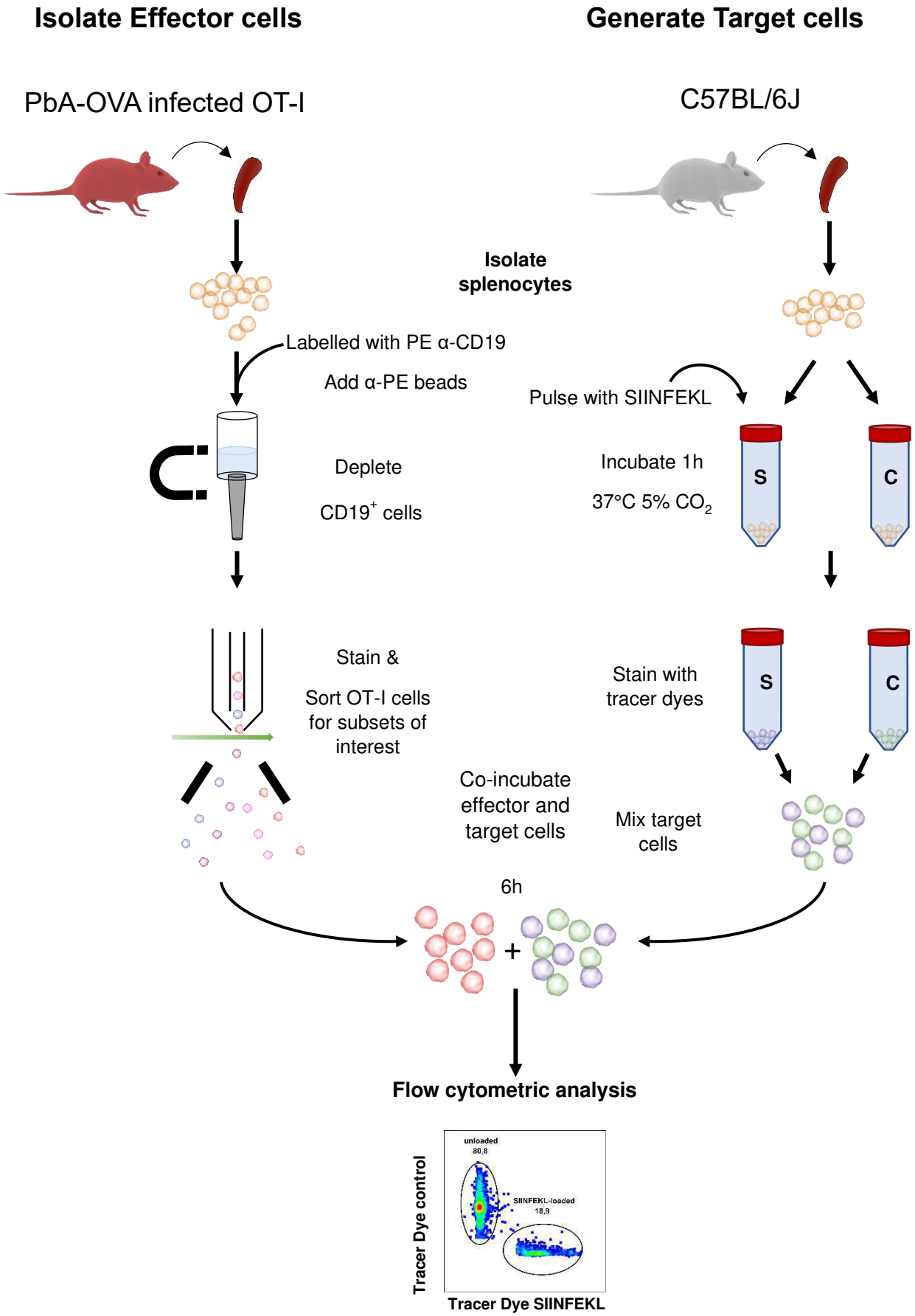
Supplementary figure 2



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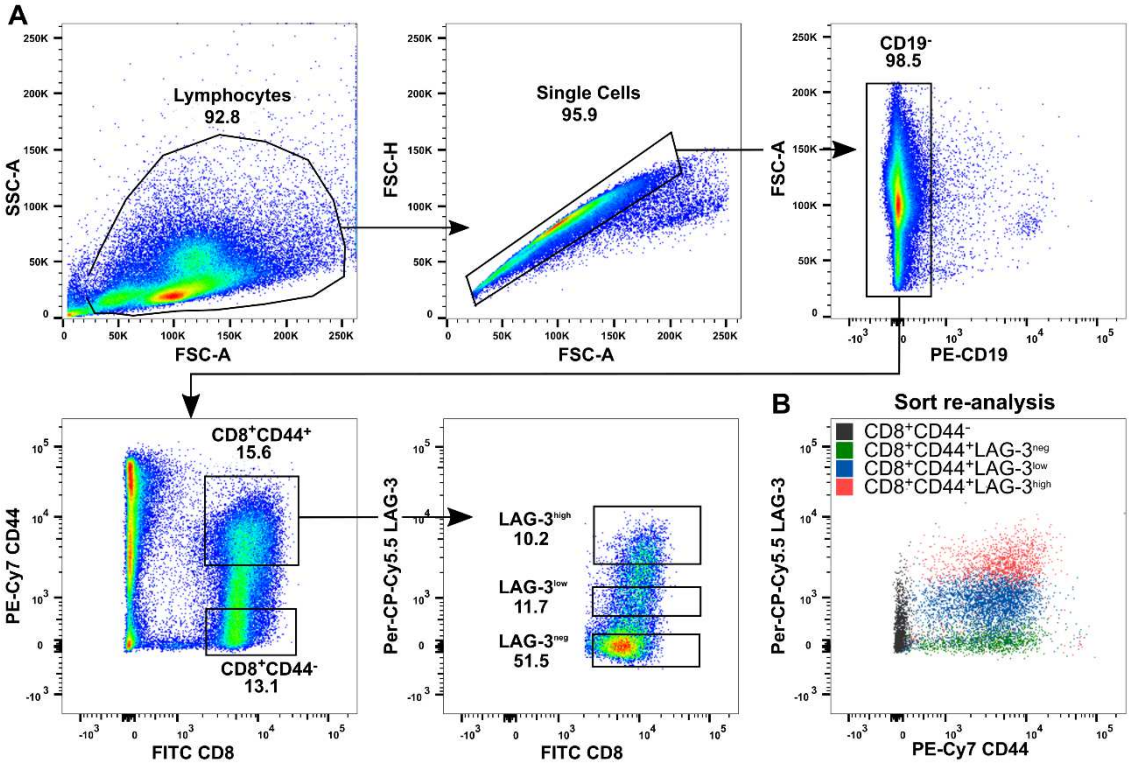
Supplementary figure 3



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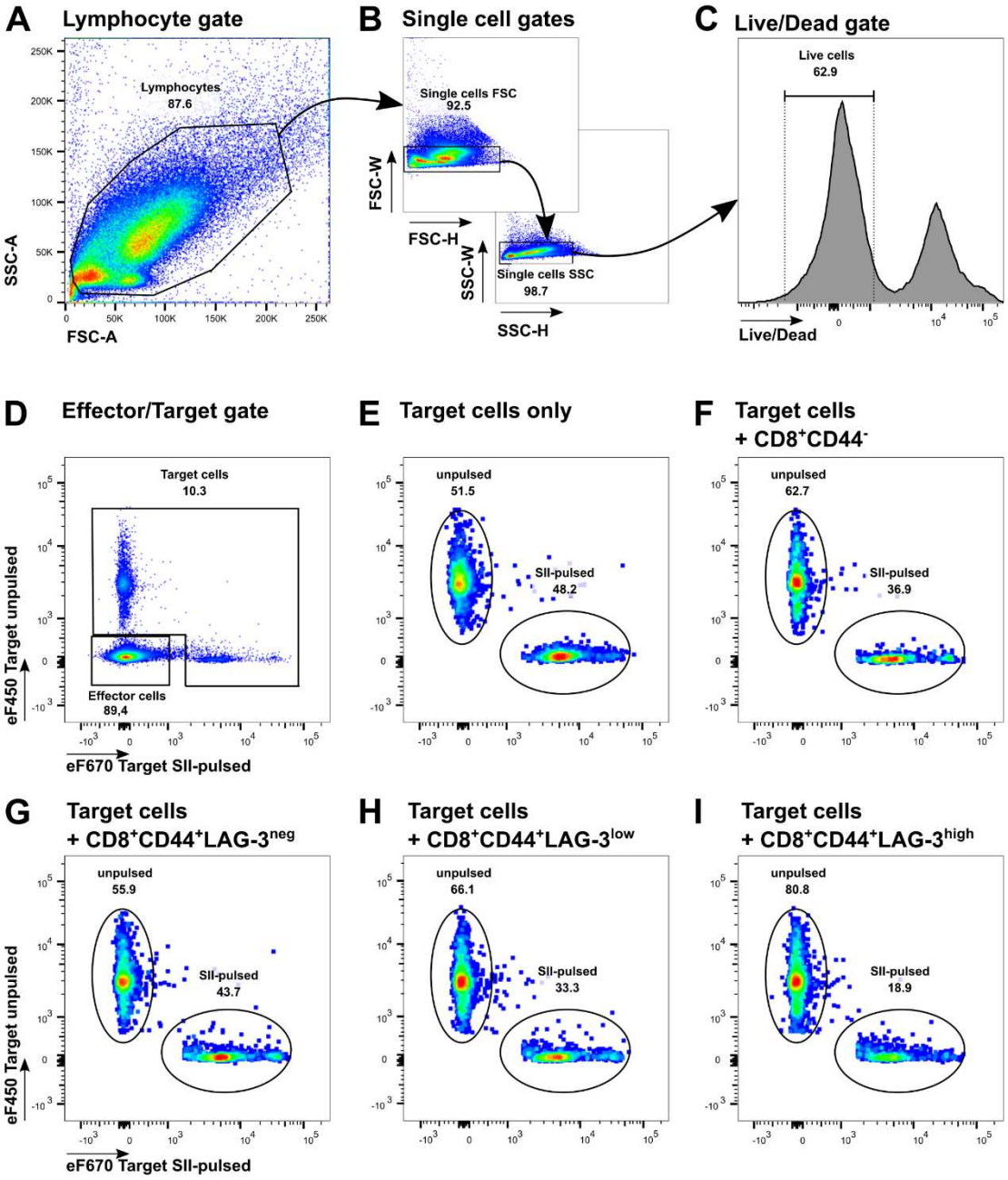
Supplementary figure 4



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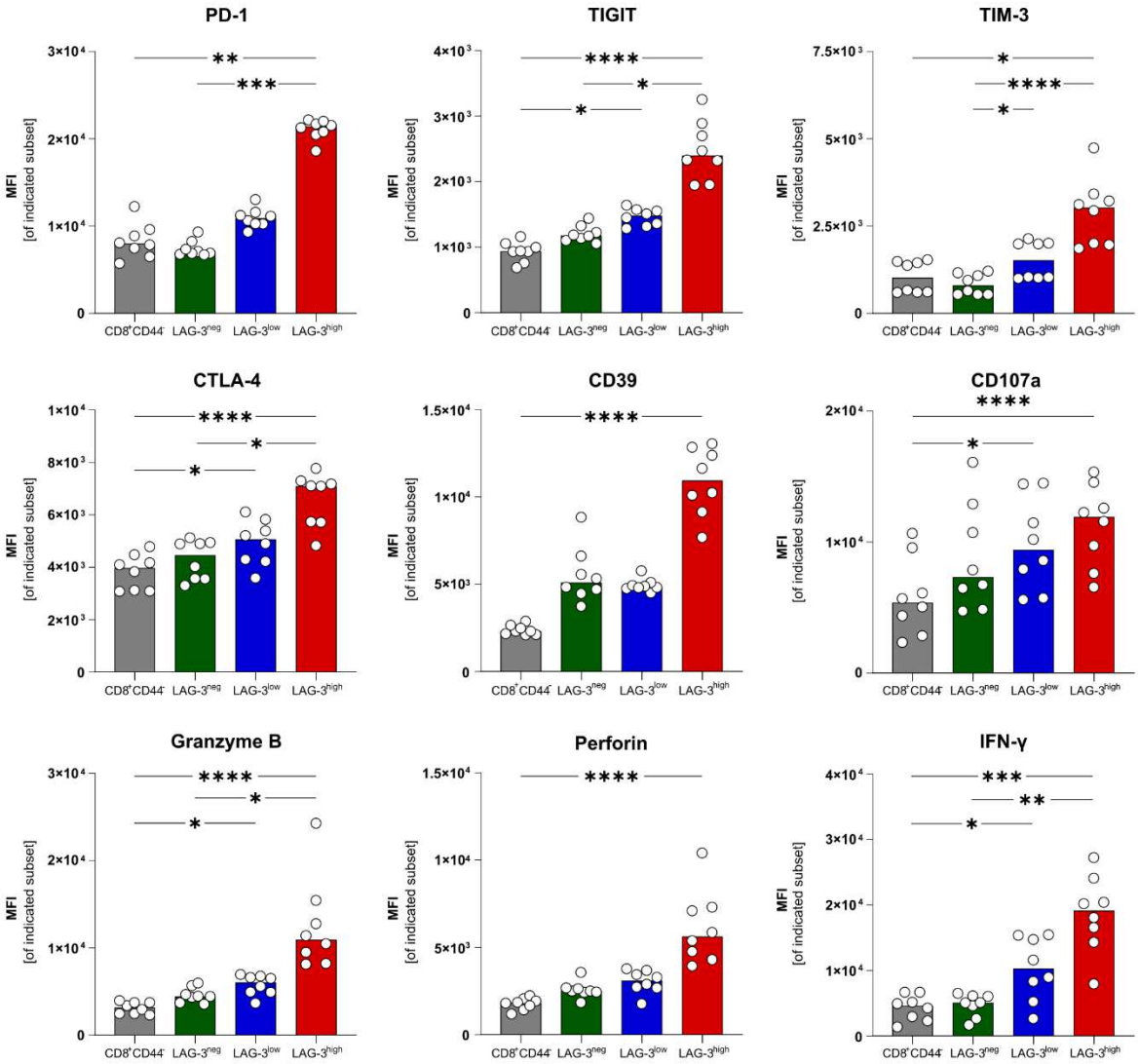
Supplementary figure 5



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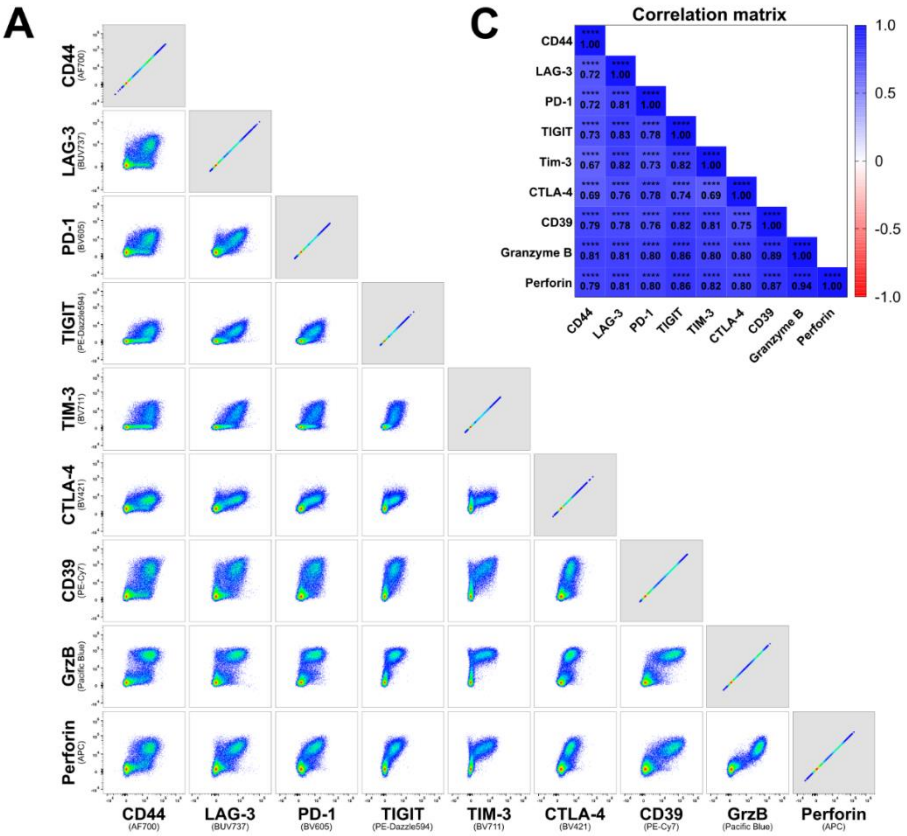
Supplementary figure 6

C57BL/6J



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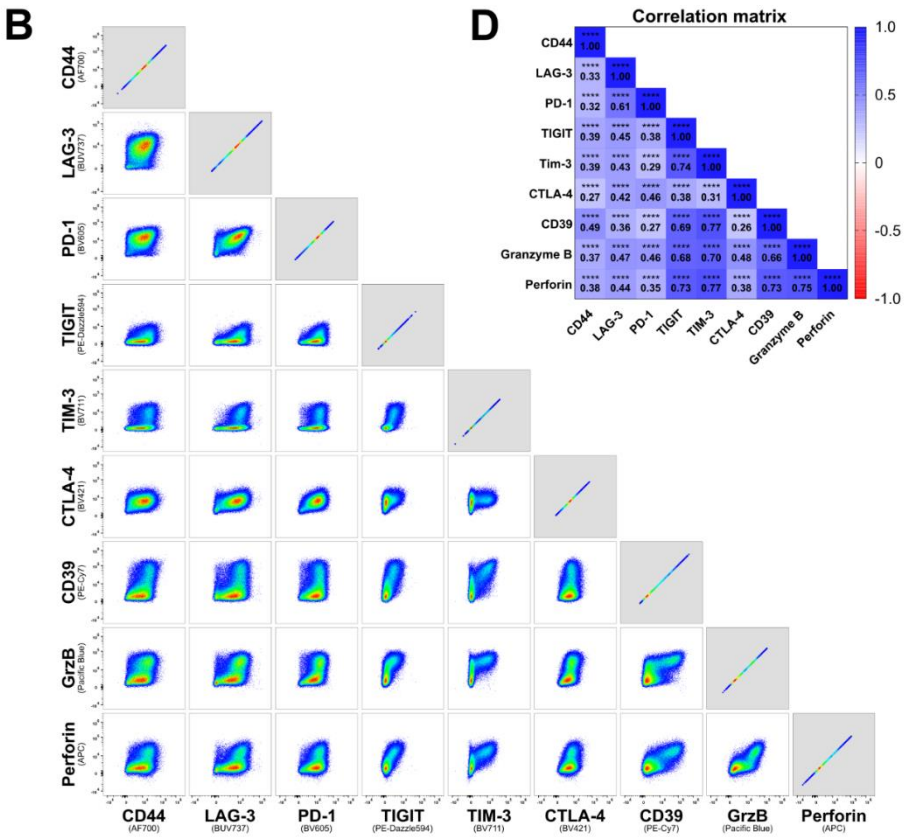


2. List of abbreviations

APC	Antigen presenting cell
AR	Additional results
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BTLA	B- and T-lymphocyte attenuator (CD272)
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
CM	Cerebral malaria
CNS	Central nervous system
CTLA-4	Cytotoxic T-lymphocyte-associated Protein 4 (CD152)
CXCL9/10	CXC-ligand9/10
CXCR3	CXC-motif-chemokine receptor 3 (CD183)
DAMP	Damage-Associated Molecular Pattern
DC	Dendritic cell
ECM	experimental cerebral malaria
EJI	European Journal of Immunology
ELISA	Enzyme-linked Immunosorbent assay
FGL1	Fibrinogen-like protein 1
FiI	Frontiers in Immunology
FoxP3	Forkhead-Box-Protein P3
HCV	Hepatitis C Virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule 1
ICOS	Inducible T cell co-stimulator (CD278)
Ig	Immunoglobulin
IL-10	Interleukin-10
IL-15C	Interleukin-15 Complex
IL-2	Interleukin-2
IL-2Rα	IL-2 Receptor α-chain (CD25)
INF-γ	Interferon-γ
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
iRBC	infected red blood cells
Ki-67	Kiel-67
KLRG-1	Killer cell lectin-like receptor subfamily G member 1
LAG-3	Lymphocyte-activation gene 3 (CD223)
LCMV	Lymphocytic choriomeningitis virus
MHC	Major histocompatibility complex
MST	Minimum-spanning tree
NK cell	Natural killer cell
NOD	Non-obese diabetic
OMIP	Optimized multicolor immunofluorescence Panel
P. falciparum	*Plasmodium falciparum*

PbA	*Plasmodium berghei* ANKA
PbTG	Transgenic PbA strain (expressing Ovalbumin)
PD-1	Programmed Cell Death Protein-1 (CD279)
PD-1KO	PD-1 knockout mice
PD-L1/L2	PD-1 Ligand 1/2 (CD274/ CD273)
PI3K	Phosphoinositide 3-kinase
PRR	Pattern Recognition Receptor
PVR	Poliovirus receptor (CD155)
SCID	Severe combined immunodeficiency
SIINFEKL	Peptide with the sequence SIINFEKL
SOM	Self-organizing maps
T. cruzi	*Trypanosoma cruzi*
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper cell
TIGIT	T-cell immunoreceptor with immunoglobulin and ITIM domains
TIM-3	T-cell immunoglobulin and mucin-domain containing-3 (CD366)
TNF-α	Tumor necrosis factor α
Tr1	Type 1 regulatory T cells (FoxP3 negative)
Treg	Regulatory T cell (FoxP3 positive)
UKE	University Medical Centre Hamburg Eppendorf, Germany
UMAP	Uniform Manifold Approximation and Projection for Dimension Reduction
V(D)J	Variable, joining and diversity regions
WHO	World Health Organization
wt	wildtype
ZAP70	Zeta-chain-associated protein kinase 70

3. Introduction

Malaria is one of the most prevalent infectious diseases with around ~230.000.000 annual cases, resulting in more than 600.000 deaths globally (WHO, 2021). The majority of these deaths are accounted to children under the age of 5, making malaria a pediatric emergency. The disease is caused by parasites belonging to the *Plasmodium* group, which are transmitted through the bite of an infected *Anopheles* mosquito. Malaria is endemic in tropical regions (Asia, South America, Africa) due to the habitat of the vector, with 95% of cases and 96% of deaths occurring in the African region (WHO, 2021). Whilst *A. gambia* is the principal vector of malaria in Africa, other species of *Anopheles* capable of transmitting malaria exist worldwide, for example *A. freeborni* in the western United States (McHugh, 1989) or *A. maculipennis* in Europe (Kuhn, Campbell-Lendrum and Davies, 2002; Arends *et al.*, 2013). Though eradicated in Europe in the 1970s (WHO Europe, 2016), rising temperatures in light of climate change, combined with increased international travel and migration, could lead to a re-emergence of malaria in Europe (Fischer *et al.*, 2020). Competent vectors are present in Europe and locally transmitted cases have been reported in recent years (Krüger *et al.*, 2001; ECDC, 2021).

The five species of *Plasmodium* (*P.*) known to cause malaria in humans are *P. falciparum*, *P. vivax, P. ovale, P. malariae* and *P. knowlesi*. Whilst *P. vivax* and *P. falciparum* are the most widespread strains worldwide, *P. falciparum* is the most prevalent strain in Africa and is responsible for most of the deaths caused by Malaria (Menkin-Smith and Winders, 2021). Symptoms of Malaria generally include characteristic periodically re-occurring fever episodes, and, among others, headaches, chills, nausea, vomiting and diarrhea (Crutcher and Hoffman, 1996). This work will mostly focus on *P. falciparum* and *Plasmodium berghei* ANKA (PbA), which is the strain most commonly used in the mouse model of experimental malaria to imitate *P. falciparum* infections in humans.

The life cycle of *Plasmodium* parasites consists of multiple stages across different species; the vector, an *Anopheles* mosquito transmitting the parasite, and the host which ultimately falls ill of the disease (Figure 1). When an infected female *Anopheles* mosquito bites the host, it releases so called *sporozoites* from its salivary glands which travel through the bloodstream into the liver. Here, the sporozoites infect hepatocytes,

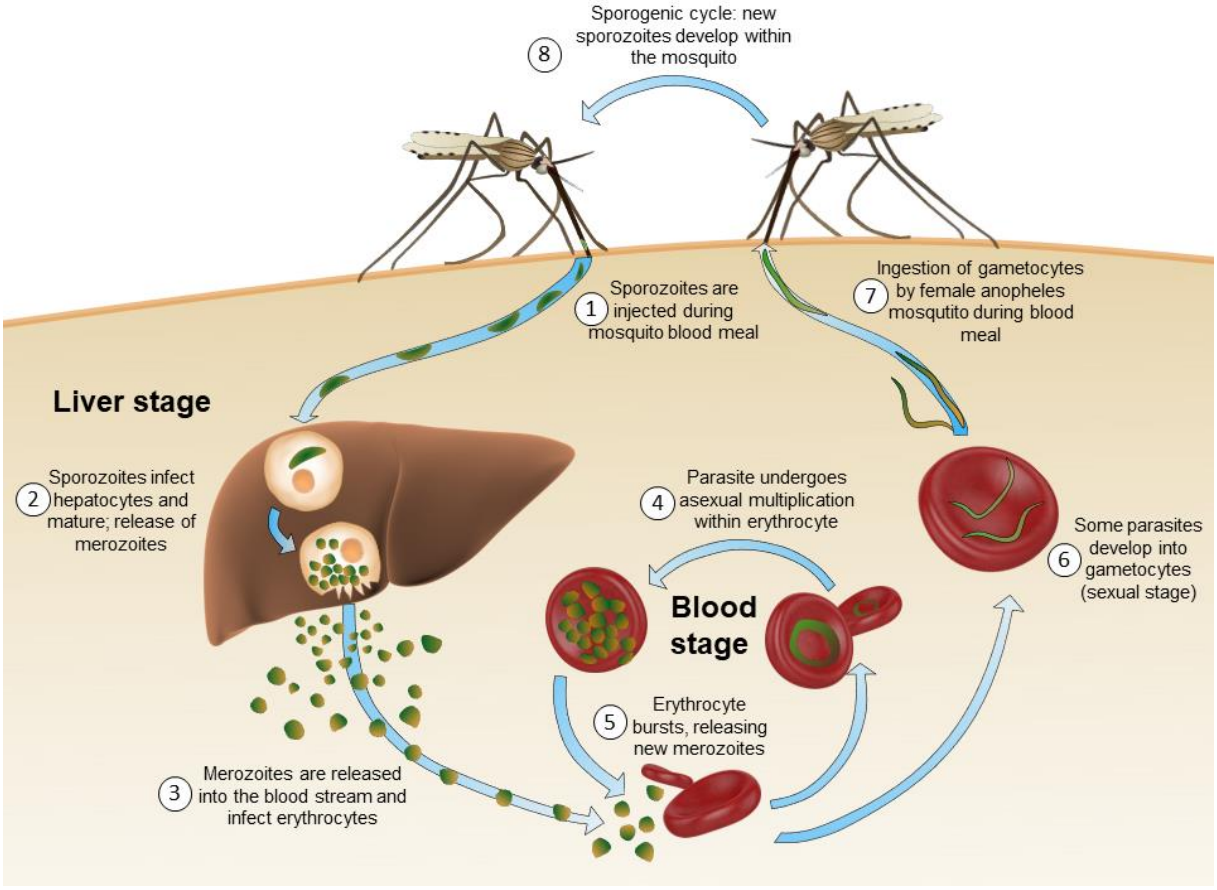


Figure 1: Schematic life cycle of malaria-causing Plasmodium parasites

marking the beginning of the liver stage of the disease. During the liver stage, the sporozoites mature and develop into *merozoites*. After roughly 7 days in humans (~2 days in mice) (Crompton *et al.*, 2014; Cowman *et al.*, 2016) the hepatocytes rupture and release the merozoites into the blood stream. Merozoites then infect erythrocytes, manifesting the beginning of the blood stage of the disease. Some parasites can develop into the sexual stage of the parasite, the *gametocytes.* If a female Anopheles mosquito takes a blood meal from an infected individual during the blood stage, it may ingest the gametocytes*,* which can differentiate into male *microgametes* and female *macrogametes* within the mosquitos gut (Bennink, Kiesow and Pradel, 2016) to start the *sporogenic cycle* of the parasite. Here, during this sexual stage of the parasites' life cycle, the gametocytes combine to generate new sporozoites. However, most of the parasites undergo asexual multiplication within the erythrocyte, generating more merozoites. When the number of parasites exceeds the tolerable amount for the erythrocyte, the erythrocyte ruptures and the merozoites are once again released into the blood stream. Here, the free parasites infect new erythrocytes, maintaining the

blood stage. This process is consistent in duration (~48h for *P. falciparum*, dependent on the strain) and thus, infection of erythrocytes occurs in cycles (Rouzine and McKenzie, 2003; Su, Ruan and Wei, 2011). Consequently, the simultaneous rupture of multiple erythrocytes may lead to periodic fever episodes as various immune cells react and produce pro-inflammatory cytokines (Kluger *et al.*, 1996; LH *et al.*, 2002).

The immune system consists of different layers of defense, ranging from the skin and mucous membrane, which prevent pathogens from entering the body, to highly specialized cells recognizing specific pathogenic epitopes. These players of the immune system can be categorized into two main groups, the *innate* and the *adaptive* immune response. Beyond a physical barrier, the innate immune response consists of different cells with specific tasks. Typically, cells of the innate immune system recognize a broad range of pathogens, usually within minutes to hours, to provide a fast-acting defense. Innate immune cells have various tasks; natural killer cells (NK) cells recognize and kill virus-infected cells. Dendritic cells (DC) capture, process and present antigen to T cells. Neutrophils and monocytes are phagocytic cells, which are able to detect pathogens using their Pattern Recognition Receptors (PRRs). PRRs recognize molecules frequently found in pathogens. Furthermore, PRRs can recognize certain molecules that may be released by damaged or destroyed cells, the so-called Damage-Associated Molecular Patterns (DAMPs). Monocytes can further differentiate and also present antigen from phagocytosed pathogens to T cells. Because the innate immune response reacts broadly to various germs, pathogens, and foreign substances, it is sometimes referred to as “nonspecific”. This is contrasted by the adaptive immune response; Along with the bone-marrow-derived B lymphocytes, which are responsible for the humoral immunity, thymus-derived T lymphocytes make up the adaptive immune response. Unlike cells of the innate immune system, B and T cells require complex activation, they are clonally unique and recognize a very precise pathogenic protein. It takes several days for them to proliferate, but they are then able to attack the pathogen with highly specific receptors and generate a long-lived immune memory to protect against future re-infection. During maturation, both B and T cells use a mechanism of somatic recombination, called V(D)J recombination, in which part of the coding sequence (namely the variable (V), joining (J) and the diversity (D) regions) of B cell-derived antibodies (also called immunoglobulins (Ig)) and T cell receptors (TCR) are randomly rearranged. This process leads to a broad repertoire of different B cell and T cell clones, enabling the recognition of an abundance of antigens.

Maturation in the thymus ensures the recognition of the major histocompatibility complex (MHC) by the TCR and simultaneously prevents recognition of autoantigen through a process called positive and negative selection, respectively. Upon leaving the thymus, naïve T cells roam through secondary lymphoid organs, which include the spleen, lymph nodes, Peyer´s patches and mucosal- and nasal tissues. Each T cell clone carries a unique rearranged TCR which is only able to identify antigen when the antigen is presented by other cells via their MHC molecules on the cell surface. In order to become fully activated, a T cell requires co-stimulatory signals from the antigen-presenting cells (APC). The interaction between a T cell and an APC is called the immunological synapse. Binding of Cluster of differentiation (CD)80/CD86 on the surface of the APC to the T cells' CD28 leads to activation of the T cell, which induces rapid proliferation and clonal expansion. Classical T cells, the $CD4^+$ and $CD8^+$ T cells, differ in function. $CD8^+$ T cells are known for their cytotoxic capabilities and recognize peptides presented by MHC class I molecules, which are expressed on almost every cell type of the body. $CD4^+$ T cells have a range of functions and can polarize into different subsets. Importantly, they recognize peptides presented on MHC class II molecules, which are expressed on so-called professional APCs such as macrophages, DCs and B cells. Originally described as *T helper* cells, the $CD4^+$ T cells play a critical role in the regulation of the immune response through their ability to differentiate into several distinct subsets. Through their release of specific cytokines, they activate cells of the innate immune system like macrophages, are fundamental in the activation of B lymphocytes and $CD8^+$ T cells, suppress the immune response and even interact with non-immune cells.

The immune response towards malaria is multifaceted, not alone due to the large number of antigens produced by a complex organism such as *Plasmodium*, but also due to the parasite's complex life cycle (Figure 1), as the parasite develops throughout the liver- and the blood stage. Whilst the blood stage is responsible for disease-associated symptoms like fever, the liver stage is the only promising target for T cell-mediated immunity against *P. falciparum*. The *sporozoites* infect hepatocytes, which are able to present pathogen-derived peptides over their MHC I molecules, enabling the detection and potential $CD8^+$ T cell-mediated killing of infected hepatocytes. Erythrocytes, which do not express MHC molecules, cannot present parasitic peptides and thus, infected erythrocytes cannot be directly recognized by $CD8^+$ T cells (Lima-Junior and Pratt-Riccio, 2016).

Plasmodium-specific $CD8^+$ T cells are found both in humans living in endemic areas (Sedegah *et al.*, 1992; Doolan *et al.*, 1997; Doolan and Hoffman, 2000) and after malaria-specific vaccination (Epstein *et al.*, 2011), indicating the establishment of immune memory against the parasite. However, the relevance of these cells in the context of a disease-limiting or even sterilizing immunity is not yet completely understood. Humans living in endemic regions experience multiple infections with malaria, but usually develop fewer symptoms over time (Cowman *et al.*, 2016). However, they do not necessarily elicit a sterilizing immunity, and several attempts to induce long-lasting immunity with vaccines have failed (Bejon *et al.*, 2007). A major obstacle may be the limited number of sporozoites delivered by a single mosquito. Usually, only a few hundred sporozoites are transferred into the dermis (Medica and Sinnis, 2005) resulting in even fewer *Plasmodium*-infected hepatocytes. Given the fact that the liver-stage in malaria is limited in time, there are only sparse opportunities for $CD8^+$ T cells to detect *Plasmodium*-antigen-presenting hepatocytes and develop a long-term immune memory, let alone identify and terminate all infected hepatocytes during a future re-infection. One possibility to overcome this issue and to provide larger amounts of antigen was discovered in 1967 by immunization with large doses of radiation-attenuated sporozoites (Nussenzweig *et al.*, 1967). Due to the radiation, these sporozoites would not be able to transform into merozoites and leave the hepatocytes, thereby providing ample opportunities to generate a protective immune response (Clyde *et al.*, 1973, 1975). Though effective in theory, the generation of large numbers of sporozoites is difficult to scale up, since they require mosquitoes as part of the sporogenic cycle, making this attempt – at least for now – not feasible for cost-effective large-scale vaccine production. With advances in genetic engineering, it is possible to synthesize specific proteins or peptides. With this, advances in vaccine development have been made, enabling immunization against liver-stage specific antigens (Duffy and Patrick Gorres, 2020). The RTS,S vaccine (also known as Mosquirix™) was developed by GlaxoSmithKline and targets sporozoite epitopes. Results of a Phase 3 study showed an efficacy against malaria of 36% in children aged 5-17 months and 26 % in infants 6-12 weeks of age who received 4 doses of the vaccine (RTSS Clinical Trials Partnership, 2015). Although a meaningful step, more effective vaccines and vaccination strategies are required to reduce the global health burden originating from malaria.

A major contributor to the death toll of malaria is its most severe complication, cerebral malaria (CM). During CM, fluid accumulates within the brain parenchyma which subsequently leads to swelling of the brain (Jerusalem *et al.*, 1984). The exact mechanisms of this process are still not completely understood. Initially, it was thought that a stroke-like pathogenesis was the main mechanism for CM. During the blood stage, infected erythrocytes have increased cytoadhesive properties, perhaps to avoid clearance in the spleen (de Koning-Ward et al., 2016). Infected red blood cells (iRBCs) adhere to endothelial cells in a process called sequestration, which, in the brain, can cause an obstruction of the blood flow with ensuing ischemia (White et al., 2013), similar to the mechanism of a stroke (Khoshnam et al., 2017). However, this explanation has been called into question in recent years. Beyond obstruction of the vessel's lumen, theories of an immune cell-mediated disruption of the blood brain barrier (BBB), the interface between intravascular space and central nervous system (CNS), gained attraction in recent years. It has been shown that lack of $CD8^+$ T cells (Yañez *et al.*, 1996; Belnoue *et al.*, 2002) or their effector molecules Granzyme B (Haque *et al.*, 2011) and Perforin (Nitcheu *et al.*, 2003; Potter *et al.*, 2006) in mice is protective against experimental CM (ECM) in PbA infection of C57BL/6 mice, the most common animal model to study CM. $CD8^+$ T cells are highly antigen-specific and it remained unclear how exactly they would damage endothelial cells, which are not known to be infected by the parasite.

During CM, both the sequestration of iRBC to the brain's endothelium and the proinflammatory milieu leads to a widespread activation of the brain's endothelium (Mai *et al.*, 2013). In the murine model, it was demonstrated that, upon Interferon-γ (IFN-γ) induced activation, endothelial cells cross-present PbA-derived antigenic epitopes (Howland *et al.*, 2013; Howland, Poh and Rénia, 2015). Furthermore, it was shown that antigen from the non-lethal *P. yoelii 17X* strain is cross-presented less efficiently than PbA antigen (Howland *et al.*, 2013). Experiments utilizing intravital microscopy revealed that PbA-antigen-specific $CD8^+$ T cells crawl along and interact with the luminal surface of CNS blood vessels, inducing fatal vascular breakdown (Shaw *et al.*, 2015; Swanson *et al.*, 2016). This has been further supported by showing internalization of iRBC into brain endothelial cells in an intercellular adhesion molecule 1 (ICAM-1)-depending manner in *in vitro* culture, as well as identifying internalized iRBC in postmortem samples from CM patients (Adams *et al.*, 2021).

It has been shown that $CD8^+$ T cells express CXC-motif-chemokine receptor 3 (CXCR3), a chemokine receptor which recognizes – among others – the chemokines CXC-ligand 9 (CXCL9) and CXCL10. CXCL9 and CXCL10 are upregulated during *P. falciparum* infection in humans (Jain *et al.*, 2008) and PbA infection in mice (Campanella *et al.*, 2008). Both chemokines are produced by endothelial cells (Campanella *et al.*, 2008; Miu *et al.*, 2008). Knockout of CXCR3 hinders development of ECM, and interestingly, its expression is upregulated on both $CD4^+$ and $CD8^+$ T cells upon infection in ECM-susceptible C57BL/6 mice, but not ECM-resistant Balb/c mice (Van den Steen *et al.*, 2008). Accordingly, malaria induced T cells of C57BL/6 readily migrate in response to CXCL10, explaining how $CD8^+$ T cells might be recruited to the brain, which does not happen in Balb/c mice (Belnoue *et al.*, 2008; Miu *et al.*, 2008; Van den Steen *et al.*, 2008; Nie *et al.*, 2009). These findings deliver an explanation for CM beyond the concept of a stroke-like pathology and paint $CD8^+$ T cells as a, if not the major contributor to ECM in mice. With most of these mechanistic findings based on the murine model of CM, recent data indicate that $CD8^+$ T cells are also a major contributor to the pathogenesis in human CM. Though it should be noted that some studies did not find $CD8^+$ T cells in post mortem studies of brains of infected children (Dorovini-Zis *et al.*, 2011), others found that numbers of $CD8^+$ T cells are not only increased in the brain of PbA infected mice (Belnoue *et al.*, 2002), but also in children infected with *P. falciparum* (Barrera *et al.*, 2019; Riggle *et al.*, 2020). Furthermore, increased levels of the $CD8^+$ T cell effector molecules Granzyme A and B were found in plasma of children infected with *P. falciparum* (Hermsen *et al.*, 2003) and Kaminski et al. demonstrated a correlation between disease severity and Granzyme B expression in $CD8^+$ T cells (Kaminski *et al.*, 2019). Taken together, these findings indicate an important role of $CD8^+$ T cells in the pathogenesis of CM.

There are several mechanisms in place to balance the severity of the immune response and to prevent the associated damage that may be caused by an immense immune response. Many immune cells display a large amount of plasticity, allowing them to both induce or suppress further immune responses through various mechanisms. The $CD4^+$ T cell compartment consists of different populations with distinct functions, with more defined, specialized subsets being identified throughout the years. Being originally divided into T helper (Th) 1 (inducing cellular immunity) and Th2 (inducing humoral immunity), several other subsets have been identified as of now. Each of them fulfills different functions in modulating the immune response.

Among them are the classical Forkhead-Box-Protein P3 (FoxP3)$^{+}$CD25^{+}CD4^{+} regulatory T cells (Tregs). As their name suggests, Tregs are a subset of CD4^{+} T cells capable of regulating, or more accurately, suppressing, the activation of other immune cells, among them other T cells. To do this, Tregs are equipped with a number of mechanisms to mediate suppression of activation, expansion, functionality or homeostasis of other cells (Schmidt, Oberle and Krammer, 2012). Tregs are capable of producing the anti-inflammatory cytokine Interleukin-10 (IL-10) (Asseman *et al.*, 1999; Tang *et al.*, 2004). IL-10 is a multifunctional cytokine, capable of inhibiting activation and effector function of T cells, but also has a suppressing effect on other immune cells such as macrophages and monocytes, in which it downregulates the production of pro-inflammatory cytokines (Fiorentino, Bond and Mosmann, 1989; Moore *et al.*, 2001). Furthermore, IL-10 is able to downregulate expression of MHC class II molecules (de Waal Malefyt *et al.*, 1991) and co-stimulatory molecules CD80 and CD86 on APCs (McBride *et al.*, 2002), dampening the response of effector T cells. Additionally, Tregs have the capacity of secreting Transforming growth factor ß (TGF-ß) (Tang *et al.*, 2004), another immunosuppressive cytokine, which is, among other things, capable of limiting CD8^{+} T cell and NK cell function (Yang, Pang and Moses, 2010). Tregs may also suppress the immune response through the induction of apoptosis in immune cells in a granzyme-mediated manner, sometimes (Grossman *et al.*, 2004), but not necessarily (Gondek *et al.*, 2008), involving the secretion of Perforin. Other immune modulating mechanisms include the consumption of Interleukin-2 (IL-2), an essential cytokine in T cell activation and homeostasis (Ross and Cantrell, 2018) through high expression of CD25, also named IL-2 Receptor α-chain (IL-2Rα), which may limit IL-2 availability for other T cells (Pandiyan *et al.*, 2007). Another molecule often found on murine and human Tregs is the ectonucleoside triphosphate diphosphohydrolase-1, also known as CD39. This enzyme, in conjunction with CD73, is able to catalyze the hydrolysis of extracellular Adenosine triphosphate (ATP) to adenosine (Antonioli *et al.*, 2013). Extracellular ATP can originate from several sources, for example cell lysis (Grygorczyk *et al.*, 2021), and thereby acts as a pro-inflammatory signal (Cauwels *et al.*, 2014). Accordingly, degradation of ATP through CD39 has an immuno-suppressive effect. Tregs can be divided into “natural” and “induced” Tregs; the former being developed in the thymus, whilst the later emerge from naive FoxP3^{-}CD4^{+} cells in the periphery (Thorstenson and Khoruts, 2001). Tregs´ crucial role in controlling the immune response was identified when mutations in the

human *FOXP3* gene, coding for the transcription factor FoxP3, which is almost exclusively expressed in Tregs, were linked to IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), an autoimmune disease affecting multiple endocrine organs (Chatila *et al.*, 2000; Wildin *et al.*, 2001). Since then, Tregs – or the lack thereof – have been shown to be involved in several other immune-mediated diseases. It is therefore conceivable that Tregs play an important role in regulating the immune response towards malaria. However, several studies indicate that the role of Tregs during malaria pathogenesis seems to be limited at most (Couper *et al.*, 2008; Randall *et al.*, 2008; Steeg *et al.*, 2009). Steeg et al. have shown that Treg numbers expand only marginally during PbA infection in mice and that depletion of Tregs has only minor influence on T cell activation and no influence on the clinical outcome (Steeg *et al.*, 2009).

In addition to classical FoxP3$^+$ Tregs, other CD4$^+$ T cells with immuno-suppressive capabilities have been identified. Type 1 regulatory T (Tr1) cells were first described when a patient suffering from severe combined immunodeficiency (SCID), a disease in which patients are deficient in T cells, was successfully treated with fetal liver hematopoietic stem cells (Roncarolo *et al.*, 1988). Interestingly, though the human leukocyte antigen (HLA) was mismatched, which can lead to severe Graft vs Host disease (Barnes and Loutit, 1957; Shlomchik, 2007), the transferred T cells in this patient were able to proliferate, indicating a mechanism of immune tolerance *in vivo*. It was demonstrated that in this patient, as well as in a second tolerant SCID patient who received a hematopoietic stem cell transfer, the tolerance was associated with increased IL-10 levels and induction of IL-10-producing CD4$^+$ T cells (Bacchetta *et al.*, 1994). This led to the discovery of another population of suppressive CD4$^+$ T cells. Crucially, whilst a temporary expression has been reported in humans (Brun *et al.*, 2009), Tr1 cells do not permanently express the transcription factor FoxP3, distinguishing them from classical Tregs. Tregs can clearly be defined by their expression of FoxP3. However, the definition of Tr1 cells is somewhat diffuse. Initially, Tr1 cells have been defined as a subset of CD4$^+$ T cells producing large amounts of anti-inflammatory cytokines like IL-10 (Roncarolo *et al.*, 1988). Aside from production of IL-10, which is not always easy to detect, different properties have been identified, leading to a broader terminology. In 2013, Gagliani *et al.* were able to detect co-expression of the co-inhibitory molecule lymphocyte-activation gene 3 (LAG-3) and the cell adhesion molecule Integrin α-2, also known as CD49b (Gagliani *et al.*, 2013) on

the cell surface, simplifying the definition of the Tr1 subset. Since then, these markers have also been found to be co-expressed on other cells, including B cells and $CD8^+$ T cells. Thus, it is suggested that ideally, additional markers like Programmed Cell Death Protein-1 (PD-1), cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4), T-cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) and other co-inhibitory molecules, as well as the expression – or lack thereof – of the transcription factor FoxP3 should be analyzed when researching Tr1 cells (Roncarolo *et al.*, 2018). Nonetheless, Tr1 cells are a very plastic subset of $CD4^+$ T cells. Tr1 cells, like Tregs, have suppressive capacities and use some similar mechanisms. Even though there are differences in transcriptomic profiles (Gagliani *et al.*, 2015; Kunicki *et al.*, 2018) and in metabolomic analysis (Mascanfroni *et al.*, 2015) between Tregs and Tr1 cells, the regulatory mechanisms employed often overlap. First of all, secretion of the anti-inflammatory molecule IL-10 is the hallmark function of Tr1 cells (Roncarolo *et al.*, 1988, 2018; Gagliani *et al.*, 2013). Additionally, Tr1 cells secrete TGF-ß, which inhibits cytokine production, proliferation and differentiation of Th1 cells and suppressing APC function (Levings *et al.*, 2001). Furthermore, Tr1 cells are able to induce granzyme B and perforin-dependent apoptosis in APCs, thus limiting activation in both antigen-specific and bystander $CD4^+$ and $CD8^+$ T cells (Tree *et al.*, 2010; Magnani *et al.*, 2011). Like Tregs, Tr1 cells are capable of expressing the ectonucleotidase CD39 (Mascanfroni *et al.*, 2015). Expression of certain co-inhibitory molecules like CTLA-4 and PD-1 have been shown to be involved in downregulation of effector T cells via cell-cell contact dependent mechanisms (Akdis *et al.*, 2004; Read *et al.*, 2006). However, the role of other co-inhibitory receptors, including LAG-3, TIGIT, or T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), remains elusive.

Expression of co-inhibitory receptors on T cells is usually associated with a state called *T cell exhaustion* (Blank *et al.*, 2019). The definition of “T cell exhaustion” is broad, as the term is employed to describe a T cell in a state of dysfunction. However, the cause, extend, permanence and the resulting magnitude of said dysfunction may vary drastically between different diseases and situations, resulting in a wide range of conditions in which a T cell may be considered “exhausted” (Blank *et al.*, 2019). Generally speaking, the term refers to effector T cells with reduced capacity to secrete cytokines and/ or kill specific target cells. However, this does not necessarily mean a complete absence of functionality, but may just mean decreased functionality, as some

exhausted T cells may still proliferate *in vivo* and produce effector molecules such as granzymes and cytokines (Jin et al., 1999; Wherry, 2011).

The hallmark characteristic of exhausted T cells is the expression of co-inhibitory molecules. These molecules are generally associated with a suppressive effect in the cell they are expressed on or its immunological environment. They are frequently co-expressed, hence earning them the categorization as co-inhibitory molecules (Wherry, 2011; Kuchroo, Anderson and Petrovas, 2014; Pauken and Wherry, 2015; Wherry and Kurachi, 2015). There are several co-inhibitory molecules known to date which function in various ways. PD-1, one of the most renowned co-inhibitory receptors, acts as a "brake" and reduces T cell function upon recognition of its ligands PD-1 Ligand 1 or 2 (PD-L1 or PD-L2) (Freeman *et al.*, 2000; Latchman *et al.*, 2001; Arasanz *et al.*, 2017). PD-1/ PD-L1 interactions are antagonists of CD28/CD80 co-stimulation, counteracting the TCR signal transduction. PD-1 is capable of terminating Zeta-chain-associated protein kinase 70 (ZAP70), a molecule critical in T cell activation and development (Wang *et al.*, 2010). PD-1 also terminates Phosphoinositide 3-kinase (PI3K) phosphorylation (Sheppard *et al.*, 2004; Hui *et al.*, 2017), which, as part of the PI3K/Akt pathway, is responsible for multiple different processes including cell survival, differentiation, proliferation, migration and metabolism (Ashcroft *et al.*, 1999; Hemmings and Restuccia, 2012). Critically, TCR signaling also employs the PI3K/Akt pathway (Okkenhaug and Vanhaesebroeck, 2003). Furthermore, prolonged PD-1/PD-L1 engagement can induce TCR internalization, resulting in impaired antigen-recognition (Nurieva *et al.*, 2006; Karwacz *et al.*, 2011).

Alongside PD-1, a range of other co-inhibitory molecules exist. CTLA-4 is able to suppress T cell function in multiple ways. Most notably, CTLA-4 is a homolog to CD28 with a much higher binding affinity (Linsley *et al.*, 1994; Greene *et al.*, 1996) to CD80/CD86, but does not provide the co-stimulatory signal usually provided by CD28/CD80/CD86 binding during T cell interaction with APCs (Chambers *et al.*, 2001; Egen, Kuhns and Allison, 2002). Additionally, it has recently been shown that CTLA-4 is capable of capturing CD80/CD86 from APCs and increasing free PD-L1 by disrupting cis-CD80/PD-L1 heterodimers, implicating that CTLA-4-expressing cells may have a suppressive effect on other cells through depletion of CD80/CD86 co-stimulatory molecules and releasing PD-L1 (Tekguc *et al.*, 2021).

TIM-3 is another co-inhibitory receptor associated with T cell exhaustion (Wolf, Anderson and Kuchroo, 2020). TIM-3 has several ligands, the most prominent being Galectin-9 (Kandel *et al.*, 2021). The exact molecular mechanisms by which TIM-3 acts are still not fully understood, but is has been suggested that TIM-3 is involved in the TCR signaling pathway (Lee *et al.*, 2011).

Furthermore, expression of TIGIT has been associated with exhaustion. TIGIT mainly recognizes the molecule poliovirus receptor (PVR, CD155), which may be expressed on myeloid cells or cancer cells (Ge *et al.*, 2021). Additionally, it was shown that TIGIT is able to compete with the co-stimulatory receptor CD226 expressed on T cells for binding with CD155 (Tahara-Hanaoka *et al.*, 2004; Yu *et al.*, 2009), in a similar manner to CTLA-4 and CD28.

LAG-3 is another co-inhibitory molecule able to modulate T cell function. Similarly in mechanism to CTLA-4, which is homologous to CD28, LAG-3 is structurally homologous to CD4. Thus, it may be able to negatively regulate TCR signaling by competing for the binding of MHC II expressed on APCs, for which it has a higher affinity than CD4 (Huard *et al.*, 1994, 1995). However, LAG-3 expression is also associated with exhaustion in $CD8^+$ T cells, which do not interact with MHC II. Recently, Fibrinogen-like protein 1 (FGL1), a proliferation- and metabolism-related factor secreted by the liver, has been identified as a ligand to LAG-3 (Wang, Sanmamed, *et al.*, 2019). Though the exact mechanisms are still unknown, knockout of LAG-3 showed its regulatory role in *in vitro* and *in vivo* expansion of both $CD4^+$ and $CD8^+$ T cells, indicating a suppressive role beyond limiting CD4-MHC II interaction (Workman and Vignali, 2003).

The exact reason why a T cell becomes exhausted is difficult to pinpoint due to the broad range of conditions in which the term is applicable. Often, it is described as a mechanism to protect tissues against severe immunopathology from an overwhelming T cell response (Cornberg et al., 2013). It is likely an evolutionary conserved mechanism to adapt to chronic antigen exposure, which, in turn, leads to permanent TCR stimulation upon which T cells become gradually silenced (Wherry and Kurachi, 2015).

Whilst a protective mechanism to prevent severe immunopathology certainly has its merits, the mechanism of exhaustion can also be hijacked to impede clearance of hostile actors (Collier *et al.*, 2021). As such, T cell exhaustion accompanied by co-

expression of multiple co-inhibitors can be observed in multiple forms of cancer (Fourcade *et al.*, 2010; Baitsch *et al.*, 2011; Gros *et al.*, 2014; Jiang, Li and Zhu, 2015). Inducing T cell exhaustion in the tumor microenvironment is favorable for the tumor as it hinders clearance of the mutated cells. Another situation yielding exhausted T cells are chronic viral infections, for example in chronic infection with lymphocytic choriomeningitis virus (LCMV), where the principle of exhaustion was first identified (Zajac et al., 1998). Again, the dysfunction of T cells prevents efficient elimination of infected cells. Similarly, exhausted T cells have been identified in chronic parasitic infections such as with *Trypanosoma cruzi* (*T. cruzi*), which, in its chronic form, can cause Chagas disease (Gálvez and Jacobs, 2022). Here, $CD8^+$ T cells isolated from mice infected with *T. cruzi* expressing multiple co-inhibitory molecules are impaired in function, as indicated by reduced IFN-γ, Tumor necrosis factor (TNF)-α or Granzyme B production. Other parasitic infections in which T cell exhaustion has been reported include toxoplasmosis (Bhadra *et al.*, 2011) or visceral leishmaniosis (Gautam *et al.*, 2014).

Antibody-mediated blockade of these co-inhibitory molecules, preventing interaction with their respective ligands and thus allowing T cells to regain their function, has been a breakthrough in oncology in recent years and led to the award of a nobel prize (Nobel Prize Outreach, 2018). Compared to most chemotherapeutic agents, monoclonal antibodies provoke fewer side effects due to their targeted nature and are generally less toxic. Monoclonal therapies targeting PD-1 and PD-L1 have been approved as treatment for different tumors (Swart, Verbrugge and Beltman, 2016; Syed, 2017; Zhang *et al.*, 2020). Consequently, other co-inhibitory molecules are now considered as a potential target for monoclonal antibody therapy (Ge *et al.*, 2021), putting these molecules and their role in the immune response in the spotlight of future research.

Expression of various co-inhibitory molecules undeniably plays an important role in the regulation of the T cell response towards a given thread or stimulus. In this thesis, I set out to determine the role of LAG-3 expressing $CD4^+$ T cells, with a focus on their Tr1-like properties, in the model of experimental malaria. Their induction throughout different stages of the disease, their phenotypic characteristics and suppressive properties were analyzed. The function of a similar subset of LAG-3^+CD8^+ T cells was evaluated and the results were compared to data obtained from human samples of patients suffering from *P. falciparum* infection, collected at the University Medical

Centre Hamburg Eppendorf, Germany (UKE). This data resulted in the publication *T cells expressing multiple co-inhibitory molecules in acute malaria are not exhausted but exert a suppressive function in mice* (DOI: 10.1002/eji.202149424) in the journal *European Journal of Immunology* (Brandi *et al.*, 2021).

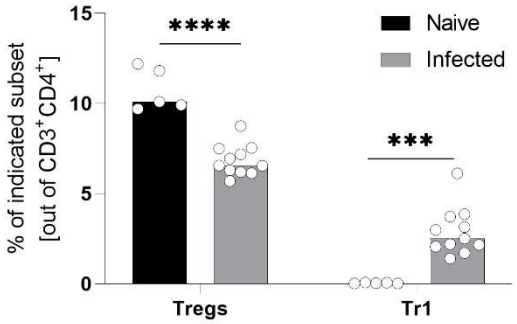
Subsequently, I focused on the cytotoxic capabilities of $CD8^+$ T cells expressing multiple co-inhibitory molecules during the infection. Here, the co-expression of these markers was characterized in detail. Infected C57BL/6 mice were compared to OT-I mice, which were mice infected with a transgenic PbA strain expressing Ovalbumin (PbTG) to avoid TCR-affinity bias during the analysis in future steps. Next, I developed, established and applied an *in vitro* $CD8^+$ T cell cytotoxicity assay to compare phenotypic properties with *in vitro* cytotoxic capacities of $CD8^+$ T cells expressing different amounts of multiple co-inhibitory molecules. The results from these experiments were published in the journal *Frontiers in Immunology* under the title *Increased expression of multiple co-inhibitory molecules on malaria-induced $CD8^+$ T cells are associated with increased function instead of exhaustion* (DOI: 10.3389/fimmu.2022.878320) (Brandi et al., 2022).

To recapitulate, in the first paper, these co-inhibitor expressing cells were identified. Their kinetic was elucidated and a suppressive function was recognized, whilst we found no decrease in functionality in $CD8^+$ T cells. Furthermore, phenotypic similarities were found in malaria patient blood samples. The second paper continued with the expression of co-inhibitory molecules with a focus on $CD8^+$ T cells. Substantial similarities between the different mouse strains used were shown to further support the validity of the cytotoxicity assay. Furthermore, it was demonstrated that expression of co-inhibitory molecules correlates with increased functionality of $CD8^+$ T cell in acute experimental malaria, which is contrary to previous findings from chronic diseases. Lastly, I will present data obtained beyond the aforementioned articles that were generated during my PhD, that aim to continue the project presented in this thesis. This will include further supplementary data to the published experiments, as well as data describing an additional project, the establishment of a 41-color high parameter panel focusing on analyzing co-inhibitory molecules and their ligands on T cells and various other immune cells via flow cytometry. A panel describes the selection of fluorochrome-conjugated antibodies used to analyze samples in flow cytometry. The panel has been rigorously optimized and submitted (submitted on 22.06.2022) as a

methodological paper in a special format tailored to optimized panels in the journal C*ytometry Part A*. This article has not yet passed through the peer-review process and subsequently has not been published, yet. However, as it was an integral part of my doctorate program and will lay the groundwork for future works to thoroughly analyze and compare the role of co-inhibitory molecules in the immune response towards malaria and other diseases, it will be included in the discussion. The unpublished data will be found in the following section *Additional results* where it will briefly be described. Throughout the following text of this doctorate thesis, I will refer to the first published paper as “EJI” (***E****uropean* ***J****ournal of* ***I****mmunology)*, the second paper will be referred to as “FiI” (***F****rontiers* ***i****n* ***I****mmunology)* and “AR” will refer to the **a**dditional **r**esults. For example, “EJI Fig. 3” will refer to figure 3 in the first published paper. Unpublished figures presented in this thesis will contain the abbreviation “AR” from this point to avoid confusion.

4. Additional results

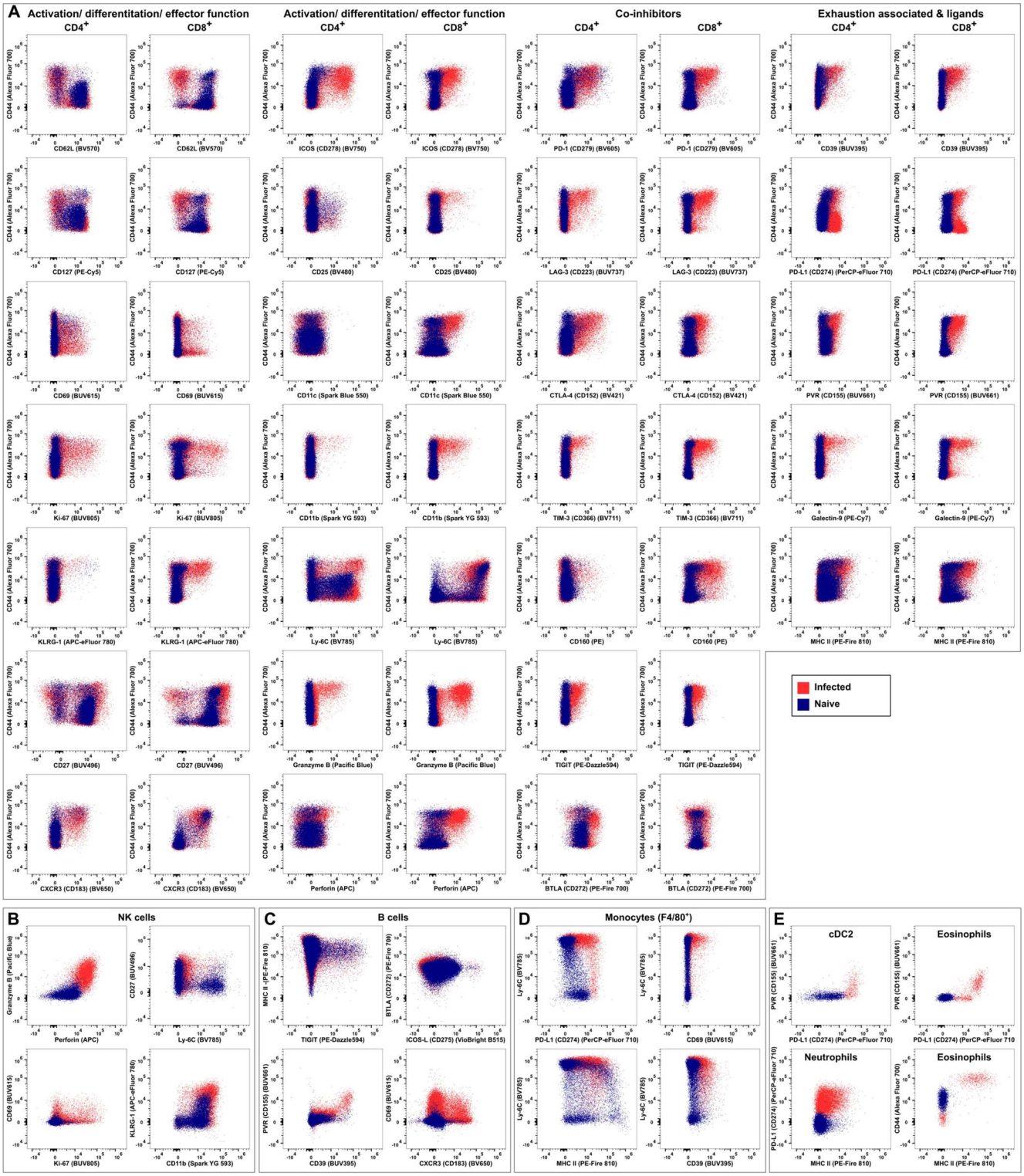
When analyzing Tr1 cells, it is important to differentiate them from FoxP3$^+$ Tregs. For this purpose, FoxP3 and CD25 were stained. In accordance with previous results, it was shown that Tr1 cells, but not Tregs, are induced upon infection (AR Figure 2) (Steeg *et al.*, 2009; Brockmann *et al.*, 2018).



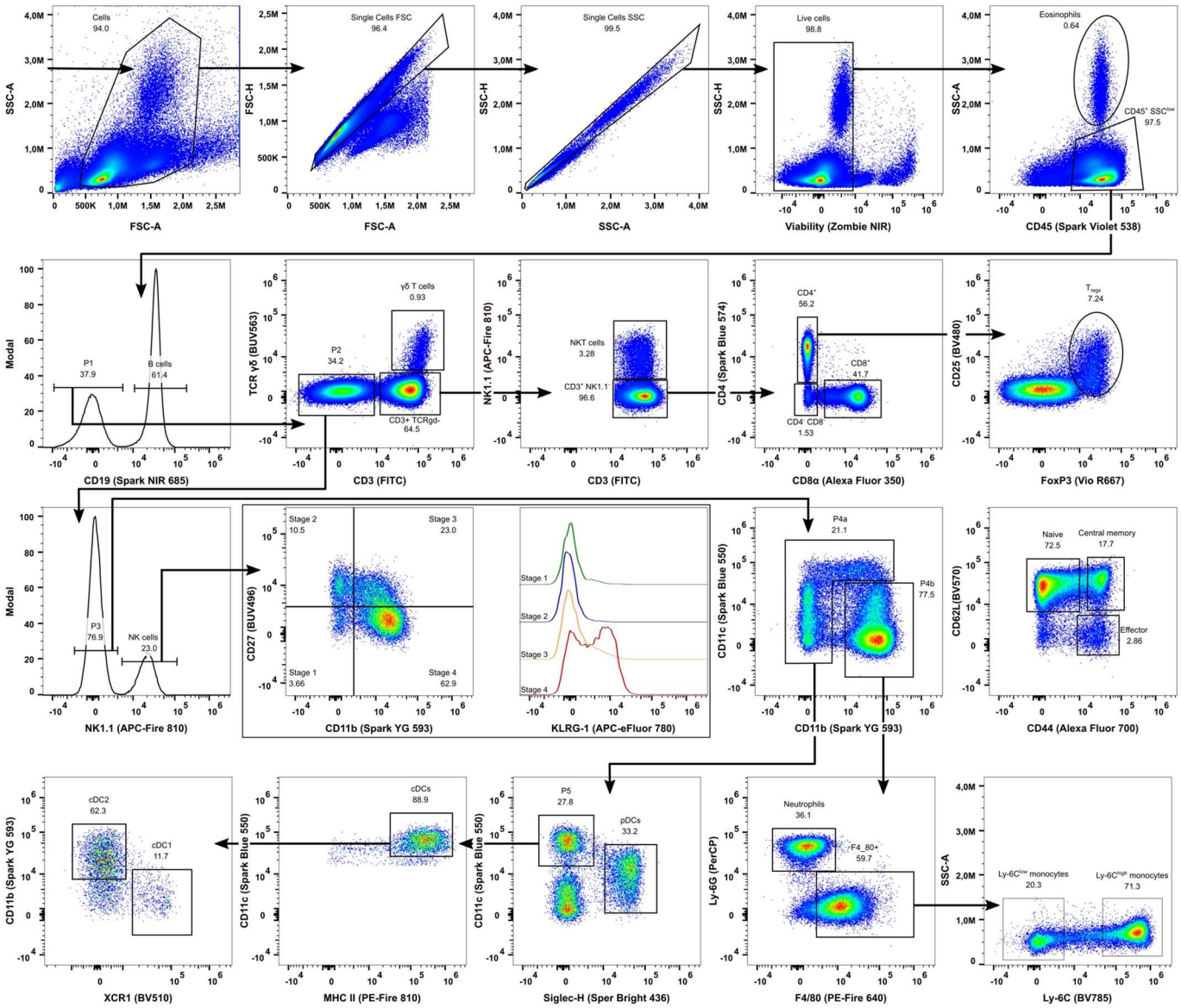
AR Figure 2: Expansion of regulatory CD4$^+$ T cells subsets upon infection. Proportion of classical Tregs (FoxP3$^+$ CD25$^-$) and Tr1 (FoxP3$^-$LAG-3$^+$CD49b$^+$) cells out of bulk CD4$^+$ T cells isolated from the spleen of naive and PbA infected C57BL/6J mice 6 days post infection. *** p<0.001, **** p<0.00001, unpaired, two-sided t-test, n=5-11 from two independent experiments.

In an attempt to better characterize the immune response towards malaria in mice, I developed a 41-color high parameter panel. The panel focuses on the T cell mediated immune response and their expression of co-inhibitory molecules (AR Figure 3). Besides conventional CD4$^+$ and CD8$^+$ T cells, the panel is able to identify γδ T cells, NK cells, NKT cells, B cells, neutrophils, monocytes, DCs and Eosinophils. (AR Figure 4). The co- inhibitory molecules analyzed in this panel are PD-1, LAG-3, TIM-3, TIGT, CTLA-4, B- and T-lymphocyte attenuator (BTLA), CD160 and the exhaustion-associated molecule CD39 (AR Figure 3). In order to better characterize the activation and differentiation state of the cells, this panel also allows analysis of CD62L, which, in combination with CD44, can be used to differentiate T cells into naïve (CD62L^{hi}CD44^{lo}), central memory (CD62L^{hi}CD44^{hi}), and effector (CD62L^{lo}CD44^{hi}) T cells (AR Figure 4). Additionally, the panel includes analysis of CD69, an activation

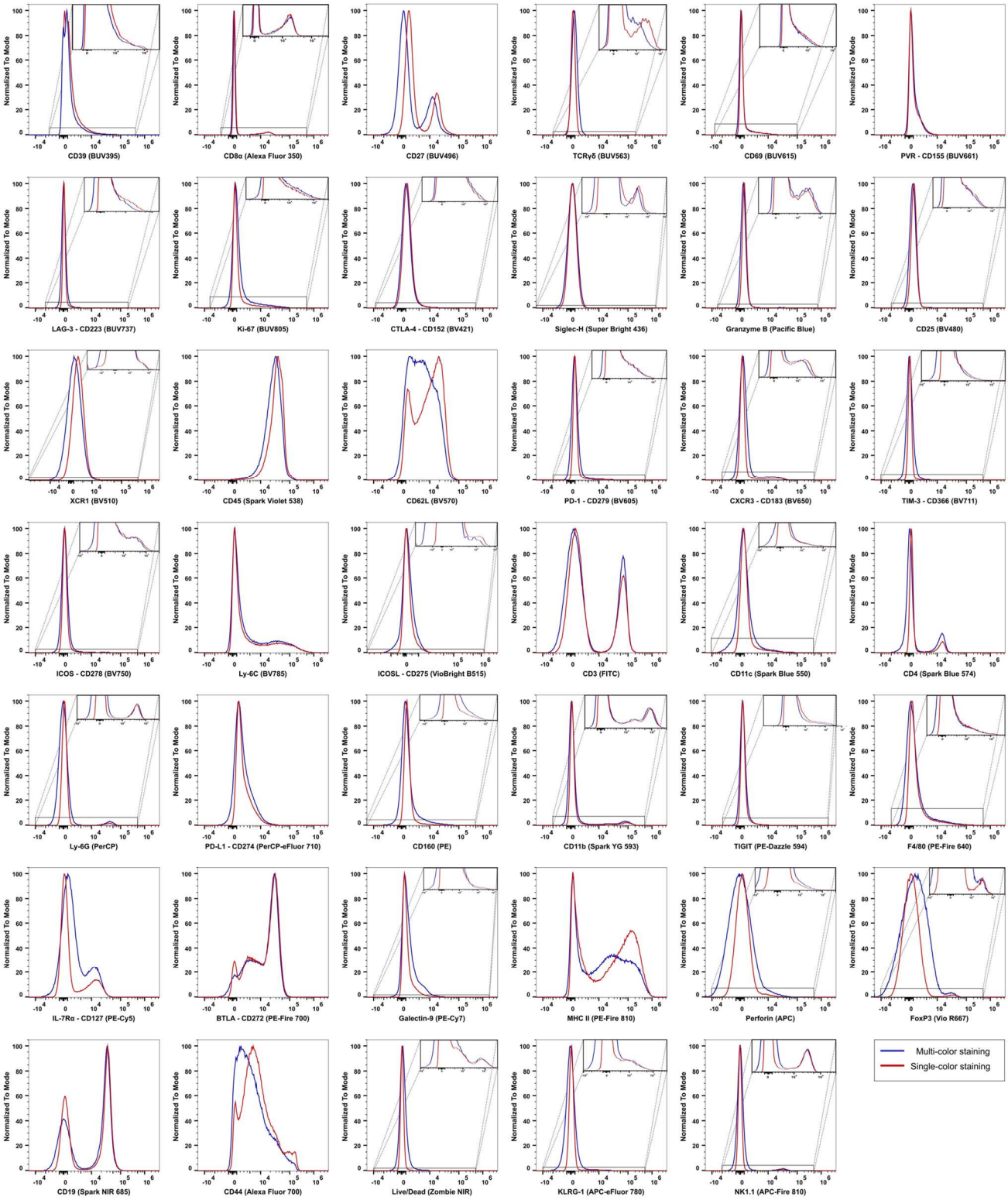
marker for B, NK and T cells (Zimmermann *et al.*, 2019), CD27, a promotor of cell survival (Hendriks, Xiao and Borst, 2003), and Killer cell lectin-like receptor subfamily G member 1 (KLRG-1), a marker traditionally used to identify terminally differentiated $CD8^+$ T cells (Hand, Morre and Kaech, 2007). Furthermore, the panel includes Kiel (Ki)-67, a proliferation marker, CD127, another memory differentiation marker, CXCR3, a cytokine receptor important for the development of ECM, and the effector molecules Granzyme B and Perforin (Voskoboinik, Whisstock and Trapani, 2015). The molecule Inducible T cell co-stimulator (ICOS, CD278), a co-stimulatory molecule similar to CD28 that is upregulated during T cell activation (Hutloff *et al.*, 1999; Wikenheiser and Stumhofer, 2016), can also be analyzed. Furthermore, the panel can be used to investigate the expression of ligands to these co-inhibitory molecules. These ligands are PD-L1 (the ligand to PD-1), PVR (ligand to TIGIT), Galectin-9 (ligand to TIM-3), MHC II (ligand to LAG-3) and ICOS-L (ligand to ICOS). The ligands' expression can be analyzed on T cells as well as on the other aforementioned immune cells, enabling analysis of both the T cells as well as the APCs. Furthermore, molecules whos' expression is traditionally associated with the myeloid compartment may also be expressed on T cells, which was also taken into consideration during panel design. The panel was established on C57BL/6 mice infected with PbA. Comparing T cells isolated from infected mice and comparing them to uninfected mice (AR Figure 3 red and blue, respectively) demonstrated that unspecific binding of antibodies was kept to a minimum and induction of the analytes was clearly detectable. Furthermore, I compared single stain controls to the multicolor staining (AR Fig. 5) to ensure that no antibody-antibody interactions or signal spill-over affected the analysis. Besides PbA infection, this panel may be used to analyze T cell responses in other diseases, including other infectious diseases as well as tumors.



AR Figure 3: Representative overlays of multicolor stainings of PbA-infected (red) and naïve (blue) C57BL/6J mice. Stainings of (A) activation, differentiation and effector markers, co-inhibitory molecules and their ligands are shown on CD4$^+$ and CD8$^+$ T cells in relation to CD44. Exemplary stainings of distinct markers are shown for (B) NK cells (C) B cells, (D) Monocytes (gated on F4/80+), (E) cDC2s, Neutrophils and Eosinophils.



AR Figure 4: **Gating strategy to identify Eosinophils, B cells, T cells, NK cells, Neutrophils, Macrophages and Dendritic cells**. Within the T cell compartment, CD4+ T cells, T_{regs}, CD8+ T cells, NKT cells, and γδ T cells can be identified. Differentiation state can be determined by examining CD44 and CD62L. NK cells are identified via NK1.1 and their maturation can be determined by analyzing CD11b and CD27 expression. Monocytes can be divided into Ly-6C^{low}, Ly-6C^{int} and Ly-6C^{high} populations. Dendritic cells can be subdivided into different populations.



AR Figure 5: Validation of staining. To validate the integrity of the staining, signal intensity of each marker in the multicolor staining (blue) was compared to single stain controls (red). Cells obtained from naïve and infected animals were mixed in an equal proportion to best represent all possible populations. For markers expressed only on a small subset of cells (for example Siglec-H SuperBright 436), multicolor staining and single cell controls were downsampled to an equal number of cells, and the Y-axis was adjusted manually to better represent these rare subsets (magnification). All stainings are gated on single cells as determined by scatter gating.

5. Discussion

The regulation of the immune system is a complex process, especially in a multifaceted infection such as one caused by *Plasmodium*. On one side, an effective T cell response and establishment of an effective memory response is important to protect the individual from re-occurring infections, especially in endemic regions where contact with the parasite is unavoidable. On the other side, this immune response can lead to serious complications when an overarching reaction causes severe damage, ending in death at the worst. Though the exact mechanism is still disputed, data collected from the murine model implicate an overwhelming $CD8^+$ T cell response as a major contributing factor to CM (Yañez *et al.*, 1996; Belnoue *et al.*, 2002), the most severe complication of malaria, and solidifying evidence suggests a similar mechanism in humans as well (Barrera *et al.*, 2019; Kaminski *et al.*, 2019; Riggle *et al.*, 2020).

Fortunately, not every case is fatal and especially after multiple infections, the disease usually has a mild progression (Cowman *et al.*, 2016). This implicates a better regulation of the immune reaction, preventing an overwhelming T cell response. Interestingly, there are several reports demonstrating that upon infection with *Plasmodium*, T cells express co-inhibitory receptors in both humans (Illingworth *et al.*, 2013; Mackroth *et al.*, 2016; Abel *et al.*, 2018) and mice (Jacobs *et al.*, 2002; Khandare *et al.*, 2017; Muscate *et al.*, 2018). It was shown that blockade of PD-1 and LAG-3 leads to better control of parasitemia in a chronic model of malaria (Butler *et al.*, 2012). This is in accordance with the notion that these molecules are associated with a state of dysfunction of $CD8^+$ T cells. Is has been demonstrated that a T cells' function can be restored through antibody-mediated blockade of these co-inhibitory molecules, preventing them from recognizing their respective ligands, which has led to breakthroughs in tumor therapies in recent years (Zhang *et al.*, 2020). Conversely, blockade of CTLA-4 would exacerbate the disease (Lepenies *et al.*, 2007). Even though thoroughly studied in chronic diseases, the function of these molecules, especially on $CD4^+$ T cells, is not well characterized in acute infections. It is conceivable that the expression of co-inhibitory molecules is a mechanism employed by *Plasmodium* to limit parasite clearance. Similar mechanisms have been described to exist in other pathogens; PD-L1 expression can be induced by human immunodeficiency virus (HIV) Tat protein on DCs (Planès *et al.*, 2014), or on Kupfer cells and monocytes by the Hepatitis C Virus (HCV) core protein (Tu *et al.*, 2010; Zhai

et al., 2017). Furthermore, multiple pathogens are able to induce production of cytokines like IL-10, which in turn induce upregulation of co-inhibitory molecules (Schönrich and Raftery, 2019). On the other side, it is possible that expression of co-inhibitory molecules on T cells is a protective mechanism employed during the infection to counteract the immense inflammation, acting as a brake to the immune system and preventing T cell mediated damage of tissues. For example, complete knockout of CTLA-4 in C57BL/6 mice lead to the development of a lymphoproliferative disease, resulting in lymphocytic infiltration in several organs, ultimately leading to tissue destruction. The mice suffered from severe myocarditis and pancreatitis, resulting in death only 3-4 weeks after birth (Tivol *et al.*, 1995). Similar results were reported recently in a conditional CTLA-4 knockout mouse model, where mice developed , among others, gastritis, insulitis and generation of organ-specific autoantibodies (Klocke *et al.*, 2016).

Several co-inhibitors have been identified over the recent years, among them PD-1, LAG-3, TIM-3, CTLA-4 and TIGIT, as well as the ectonucleotidase CD39, which is also associated with T cell exhaustion and immune suppression (Barber *et al.*, 2006; Blackburn *et al.*, 2009; Joller *et al.*, 2011; Fourcade *et al.*, 2012; Jiang, Li and Zhu, 2015; Canale *et al.*, 2018).

The first goal was to analyze the expression of these markers on T cells during acute malaria. To this end, C57BL/6 mice were infected with PbA, the most widely used murine model to study ECM. Additionally, The expression of CD27, CD69, ICOS, CD44, CD62L, KLRG-1 and Granzyme B was analyzed in order to identify activation, final differentiation and effector function of T cells (Hutloff *et al.*, 1999; Hendriks, Xiao and Borst, 2003; Voskoboinik, Whisstock and Trapani, 2015; Wikenheiser and Stumhofer, 2016; Walsh *et al.*, 2019). We first were interested in analyzing which molecules are upregulated upon acute infection on T cells and how these markers would be distributed among the different types of T cells. To avoid missing distinct subsets of T cells, which can be difficult to identify using classical gating, we employed an unbiased clustering algorithm based on self-organizing maps (SOM) called FlowSOM (Van Gassen *et al.*, 2015). This algorithm groups cells into clusters, according to their expression of the aforementioned molecules and displays them in a minimum-spanning tree (MST). Simply put, the algorithm clusters cells with a similar phenotype together and thereby allows identification of distinct cell subsets which are

induced upon infection when compared to an uninfected control. To achieve consistent results, the algorithm was trained by combining T cells isolated from spleens of PbA infected and naïve mice to create the MST. $CD4^+$ T cells were investigated first, as they are known for their wide variety in function, including suppression of the immune response. Consequently, the FlowSOM algorithm was applied to $CD4^+$ T cells found in the spleen, an important lymphatic tissue, and the liver, the first stage within the parasites host-sided life cycle (EJI Fig.1). The focus was set on activated cells as indicated by expression of CD44. Interestingly, distinct clusters of activated $CD4^+$ T cells co-expressing high levels of the co-inhibitory molecules LAG-3, TIM-3, TIGIT, PD-1 and the ectonucleotidase CD39 were identified. However, these subsets were negative for FoxP3 and were therefore not considered classical regulatory T cells. Interestingly, LAG-3 can be used in conjunction with CD49b to identify Tr1 cells, another subset of $CD4^+$ T cells with suppressive capacity, as $LAG\text{-}3^+CD49b^+CD4^+$ T cells (Roncarolo *et al.*, 1988, 2018; Gagliani *et al.*, 2013). Furthermore, besides expression of many co-inhibitory molecules, these clusters also expressed ICOS, a marker for T cell activation with co-stimulatory properties (Hutloff *et al.*, 1999). ICOS-expression, though not exclusively specific to Tr1 cells (Maynard *et al.*, 2007), has been associated with Tr1-like cells (Häringer *et al.*, 2009). Additionally, the effector molecule Granzyme B, which is typically expressed by $CD8^+$ T cells to induce apoptosis in target cells (Voskoboinik, Whisstock and Trapani, 2015), has been found to be expressed on Tr1 cells (Schmetterer *et al.*, 2015; Yan *et al.*, 2017) and is also expressed in the here-described cluster. Whilst the proportion of Tr1 cells out of all $CD4^+$ T cells increased, we were able to confirm previous results, showing a lower proportion of Tregs upon infection (AR Fig. 2) (Steeg *et al.*, 2009). Therefore, the induced Tr1 cells could be an alternative population to Tregs to regulate the immune response. Next, the $CD8^+$ T cell compartment was analyzed by using the FlowSOM algorithm. Again, bulk $CD8^+$ T cells from infected mice were analyzed and compared to the ones from naïve mice. Whilst we found induction of classical activated $CD44^+CD8^+$ T cells, distinct clusters co-expressing the co-inhibitory molecules LAG-3, PD-1, TIM-3, TIGIT and CD39 were identified as well. Again, these $CD8^+$ T cells express high levels of Granzyme B. Furthermore, some $CD8^+$ T cells express LAG-3 and CD49b in a similar fashion to the aforementioned potential Tr1 cells in the $CD4^+$ T cell compartment. We were intrigued by the possibility that subsets of immunomodulatory T cells are induced by the infection.

However, the course of an infection consists of several stages and induction of these immunomodulatory cells may have different implications depending on the timepoint of their induction. A pro-inflammatory environment might be of advantage during the liver stage, helping to establish a persistent immune response. However, upon induction of the blood stage, anti-inflammatory processes might suppress an overarching immune response. It is worth remembering that $CD8^+$ T cells are not able to target parasites during the blood stage, as they require MHC I mediated presentation of parasitic peptides. Since erythrocytes do not express MHC I molecules, pathogens can essentially hide from a T cell mediated immune response within the erythrocytes. Subsequently, we wanted to study the induction of these cells during the different stages of the disease. An experiment was designed to distinguish between immune responses to liver or blood stage. During infection with PbA, the transition from liver- to blood stage takes place after around three days. After demonstrating a strong co-expression with multiple other co-inhibitory molecules (EJI Fig 1&2), LAG-3 was used as a representative marker for expression of multiple co-inhibitory molecules as it may also be used to identify Tr1 cells in conjunction with CD49b (Gagliani *et al.*, 2013). Our results show that the co-inhibitor-rich cells are induced by the blood-, not the liver stage of the disease (EJI Fig 3). After withdrawal of the antigen through treatment, the induced LAG-3^+ cells disappeared rapidly and were undetectable five days later. Analyzing the $CD8^+$ T cell compartment paints a similar picture. LAG-3 positive (and accordingly, co-inhibitor-rich) cells are induced by the blood-, not the liver stage (EJI, Fig 4). This rapid induction and disappearance shows that these cells are active only during a strong inflammatory process and indicates that the presence of these cells expressing multiple co-inhibitory molecules might be involved in the immune regulation in an inflammatory setting.

$CD4^+$ T cells are known for their immunomodulatory properties through secretion of cytokines. With the kinetic of the induction now established, the next step was to further analyze the functional capacity of these cells. To analyze their secretory properties, a restimulation experiment was performed by incubating cells with a PbA peptide mix in the presence of Monensin and Brefeldin A. The peptide mix provides antigen and stimulates antigen-specific T cells (Poh *et al.*, 2014), but the secretion apparatus of the cells is impaired, enriching cytokines within the cells which are otherwise difficult to detect (Jung *et al.*, 1993). LAG-3^+CD4^+ T cells expressed more of the effector molecule Granzyme B and the proinflammatory cytokine IFN-γ, compared to their LAG-3^-

counterparts, indicating full functionality. This is in contrast to their expression of co-inhibitory molecules, which is usually considered a sign of T cell exhaustion (Faleiro *et al.*, 2018; Wykes and Lewin, 2018; Blank *et al.*, 2019). Interestingly, LAG-3$^+$ cells also express more of the anti-inflammatory cytokine IL-10 than LAG-3$^-$ cells. The co-expression of IL-10 and IFN-γ is a hallmark characteristic of Tr1 cells, further hinting at an induction of these cells as an immune-modulating mechanism upon infection. CD4$^+$ T cells, especially naïve ones, are known for their plasticity. They can differentiate into different subsets upon receiving distinct signals. Importantly, this differentiation is not necessarily set in stone. Tr1 cells can be induced from a pool of naïve CD4$^+$ T cells upon stimulation with IL-27 and/ or IL-10 in both humans and mice (Bacchetta *et al.*, 2010; Roncarolo *et al.*, 2014). However, they can also be differentiated from non-naïve CD4$^+$ T cells. This has been demonstrated for effector memory T cell precursors (Bollyky *et al.*, 2011), but also for other CD4$^+$ T cell subsets. For example, it has been shown that Tr1 cells were differentiated from Th17 cells (Gagliani *et al.*, 2015) and Th2 cells (Pacciani *et al.*, 2010). Importantly, RNA sequencing has revealed that Th1 cells are capable of differentiating into Tr1 cells upon malaria infection in humans (Lönnberg *et al.*, 2017). This plasticity of CD4$^+$ T cells is an important property of the immune response allowing to rapidly adapt to upcoming immune challenges. CD4$^+$ T cells confer multiple tasks and their differentiation into various subsets has been described in depth. However, CD8$^+$ T cells are lesser-known for differentiating into different subsets but are usually defined by their cytotoxic capacity. That is not to say that CD8$^+$ T cells can´t produce certain cytokines which affect other immune cells, but generally, CD8$^+$ T cells are not categorized into different subsets as is the case for CD4$^+$ T cells. However, following the discovery in the FlowSOM analysis that showed substantial similarities in expression patterns between CD4$^+$ and CD8$^+$ T cells, we also analyzed expression and induction patterns of the same markers on CD8$^+$ T cells. Again, very similar characteristics to the CD4$^+$ T cells were identified. LAG-3$^+$ expression on CD8$^+$ T cells is induced by the blood-, not the liver-stage and similarly, LAG-3 expressing CD8$^+$ T cells disappear after antigen withdrawal. CD8$^+$ T cells were also analyzed in the restimulation assay. The restimulation assay demonstrated that LAG-3$^+$CD8$^+$ T cells express higher levels of Granzyme B, IFN-γ and, interestingly, also IL-10, compared to their LAG-3$^-$ counterparts. Expression of IL-10 has been described to be a feature of regulatory CD8$^+$ T cells (Noble, Giorgini and Leggat, 2006). Taken together, these data indicate that the T cell response shifts towards developing

immunosuppressive properties during the course of the infection. An *in vitro* suppression assay was employed to determine if the observed expression of these anti-inflammatory molecules translates to an actual suppressive capacity. Whilst LAG-3$^+$CD49b$^+$ CD4$^+$ T cells, which express both defining markers for Tr1 cells, show suppressive capacity, we found that LAG-3 single positive CD4$^+$ T cells feature a similar, if not higher suppressive capacity. Both subsets are able to inhibit proliferation of both naïve CD4$^+$ and CD8$^+$ T cells in a dose-dependent manner. In contrast, LAG-3$^-$ CD49b$^-$ cells are not able to significantly alter proliferation of the reporter cells. This stands in contrast to previous reports, in which suppression-capable Tr1 cells required expression of both LAG-3 and CD49b (Gagliani *et al.*, 2013). There are several reasons which could explain why expression of CD49b did not play a significant role to define a suppressive subset, compared to the original publication (Gagliani *et al.*, 2013). First of all, the mouse models that were used differ, which alone explains a vastly different immune environment. In the original study defining Tr1 cells as LAG-3$^+$CD49b$^+$CD4$^+$ T cells, cells were isolated from healthy human volunteers and, more comparable to our mouse model, intestinal inflammation and helminth infection were analyzed. The CD4$^+$ T cells used in those suppression assays were isolated from the colon of mice treated with αCD3 antibody (Gagliani *et al.*, 2013), whilst the cells used in our experiments were isolated from the spleen. CD49b is an Integrin that is responsible for cell-cell contacts and adhesion (Carter *et al.*, 1990). It is conceivable that upregulation of this molecule is organ-specific and its significance is simply a byproduct of the location of the cells in the intestine, whilst splenocytes used in our experiments did not require CD49b. Interestingly, it was recently reported that Tr1 cells (defined as LAG-3$^+$Cd49b$^+$ CD4$^+$ T cells) accumulated in the gut compared to peripheral blood of HIV-1 patients (Augustin *et al.*, 2018), implicating an accumulation of CD4$^+$ T cells with this phenotype in this organ. It should be considered that this data was acquired in humans and should not be overinterpreted in this context. However, considering the broad definition of Tr1 cells that was described above, it seems in accordance with our data that CD49b is not necessarily required to describe suppressive CD4$^+$ T cells in acute experimental malaria. Therefore, it shows the importance of analyzing multiple other co-inhibitory and/ or activating molecules acting as surrogate markers, as was done with PD-1, TIM-3, TIGIT and ICOS in addition to LAG-3 and CD49b (Roncarolo *et al.*, 2018). On the other side, it was recently shown that co-expression of LAG-3 and CD49b not only identifies Tr1 cells, but also other IL-

10 producing cells (Huang *et al.*, 2018), implicating a role for CD49b in identifying IL-10-producing cells in some cases. Surprisingly, yet in agreement with this data and our previously generated data, a similar picture was found with the PbA-induced $CD8^+$ T cells. Whilst the $CD8^+$ T cells identified in the FlowSOM analysis display a similar phenotype to $CD4^+$ T cells, they also behave alike in terms of their suppressive capacity. Similar to the findings in $CD4^+$ T cells, LAG-3 expressing $CD8^+$ T cells are able to suppress proliferation in the reporter cells and again, CD49b expression is not required to identify suppressive $CD8^+$ T cells (EJI Fig 5 C&D).

After analyzing the suppressive capacities of the $CD8^+$ T cells, we wanted to analyze their functionality in terms of cytolytic performance. In order to do so, I developed an *in vitro* cytotoxicity assay to determine the $CD8^+$ T cells' cytolytic capacity. Individual $CD8^+$ T cell clones recognize a specific peptide presented to them via MHC I molecules by other cells. Due to the vast heterogeneity in $CD8^+$ T cell clones found in a wildtype (wt) C57BL/6 mouse, a different mouse strain was chosen for this experiment. OT-I mice are bred based on the C57BL/6 background, however, their $CD8^+$ T cell repertoire has been severely altered. $CD8^+$ T cells in OT-I mice originate from a single clone, meaning all $CD8^+$ T cells within these mice can only recognize one single peptide (Hogquist *et al.*, 1994). The peptide in question has the sequence SIINFEKL and is part of the ovalbulmin protein. Infecting these mice with the transgenic PbTG strain expressing ovalbumin (Lundie *et al.*, 2008) induces a $CD8^+$ T cell immune response specific against a single peptide, which makes analysis of these T cells far easier. For once, the affinity of all $CD8^+$ T cells TCRs is homogeneous, preventing bias in the recognition of target cells independent of marker expression. Furthermore, it increases sensitivity of the assay as a pre-defined peptide (SIINFEKL) can be used as opposed to a peptide pool which may not be recognized by all isolated T cells.

After OT-I mice were infected with PbTG, $CD8^+$ T cells were isolated and sorted based on LAG-3 and CD49b expression, to compare the results to the suppression assay. Additionally, $CD8^+CD44^-$ cells were sorted, which depict naïve $CD8^+$ T cells isolated from the same infected mouse as a negative control. Target cells were obtained from spleens of naïve, uninfected C57BL/6 mice. Half of the target cells were pulsed with SIINFEKL-peptide, the other half was not (FiI, Supp Fig. 2), to obtain both cells recognizable by the $CD8^+$ T cells and a reference population. Both target cells were mixed and then co-incubated with the sorted OT-I derived $CD8^+$ T cells, to determine

the cytolytic capacity of the respective $CD8^+$ T cell subset towards the peptide-pulsed target cells (EJI Fig. 6 A-D). To our surprise, the LAG-3^+ expressing cells displayed full functionality (EJI Fig. 6 D). Similarly, CD49b expressing cells also show cytolytic capacity. Interestingly, LAG-3^+CD49b$^+$CD8$^+$ T cells show the highest cytolytic capacity compared to LAG-3^-CD49b$^-$CD8$^+$ T cells, which, despite expressing the activation marker CD44, do not show significantly higher cytolytic capacity relative to CD44$^-$CD8$^+$ T cells from the same mouse. Taken together, the here presented data indicates that malaria-induced T cells are able to engage an ambiguous phenotype, performing their pro-inflammatory tasks whilst simultaneously suppressing further immune responses.

Malaria, both in humans as well as in the murine model can cause an immense inflammatory response. Mice infected with PbA usually succumb to the disease within 4-8 days after onset of the blood stage, due to ECM, a severe form of immunopathology. Expression of multiple co-inhibitory molecules on T cells, especially $CD8^+$ T cells, is associated with exhaustion (Blackburn *et al.*, 2009; Wherry, 2011; Blank *et al.*, 2019; Panetti, Kao and Joller, 2021). Although the mice are not able to survive the disease, it is tempting to assume that the expression of these co-inhibitory molecules serves as a last effort of the immune system to counteract the immunopathologic mechanisms by limiting the T cell response. The expression of some co-inhibitory receptors like CTLA-4 can be directly linked to suppressive effects towards other cells (Tekguc *et al.*, 2021) and the production of anti-inflammatory cytokines like IL-10 gives a foundation to explain the regulatory capacity found in these cells. Whilst the LAG-3^+ T cells are highly active, producing pro-inflammatory cytokines and recognizing and killing target cells, they are also able to engage in mechanisms to limit further immunopathology.

To determine if these findings have an equivalent relevance in human malaria, expression of co-inhibitory receptors was analyzed on T cells isolated from the blood of patients infected with *P. falciparum,* the most deadly form of malaria (WHO, 2021). Similar to the murine cells, an induction of activation markers like the C-C chemokine receptor type 5 (CCR5) (Contento *et al.*, 2008) was identified on both $CD4^+$ and $CD8^+$ T cells. Furthermore, expression of LAG-3 was detected on both $CD4^+$ and $CD8^+$ T cells in malaria patients, whilst this marker is virtually absent on T cells of healthy volunteers (EJI Fig. 7 A&B). Some of these cells also express the marker CD49b, which, in case of the $CD4^+$ T cells, could identify them as classical Tr1 cells (Gagliani

et al., 2013). Similar to the mouse model, co-expression of PD-1 and TIGIT with LAG-3 was detected on both $CD4^+$ and $CD8^+$ T cells. TIM-3 and CD39 were especially upregulated on $CD4^+$ and $CD8^+$ T cells co-expressing both LAG-3 and CD49b, in contrast to the mouse-model, where CD49b seemed to play less of a role. Previous publications showed co-expression of PD-1 and CTLA-4 on $CD4^+$ T cells during acute *P. falciparum* infection and demonstrated that these cells have a suppressive capacity similar to $FoxP3^+$ Tregs (Mackroth *et al.*, 2016).

In conclusion, the induction of co-inhibitory rich $CD4^+$ and $CD8^+$ T cells upon malaria infection was identified. These cells are induced by the immunopathogenic blood stage of the disease and share characteristics with Tr1 cells, displaying suppressive capacity towards other T cells. Surprisingly, despite expression of co-inhibitory molecules, $CD8^+$ T cells are still able to lyse target cells efficiently. Following this publication, we were intrigued by this seeming contradiction. Expression of co-inhibitory molecules should decrease a T cells' function as they enter a state called exhaustion. In tumors or chronic infections, $CD8^+$ T cells expressing multiple co-inhibitory receptors are dysfunctional, producing fewer cytokines and cytolytic effector molecules (Fourcade *et al.*, 2010; Wherry, 2011; Gálvez and Jacobs, 2022) To further follow up on this, I focused on the influence of expression of multiple co-inhibitory molecules on a $CD8^+$ T cells capacity to kill target cells. Up-regulation of co-inhibitory receptors like PD-1 (Kinter *et al.*, 2008) or LAG-3 (Annunziato *et al.*, 1993) has been demonstrated on activated T cells, though it is usually only described for a single marker at a time. High levels of (co-)expression of these markers are associated with dysfunctional T cells, though usually only described in a chronic disease setting.

Deeper understanding the role of $CD8^+$ T cells could be of crucial importance to better recognize their impact on disease progression. Whilst not able to kill infected erythrocytes due to their lack of MHC I molecules, $CD8^+$ T cells have recently been shown to be able of lysing *P. vivax* infected reticulocytes (Junqueira *et al.*, 2018). Reticulocytes are immature red blood cells and retain the protein translation machinery (Ney, 2011), enabling them to process and present *P. vivax*-derived peptides via MHC I. Additionally, $CD8^+$ T cells help pathogen clearance through activation of monocytes, which in turn are able to phagocytose infected erythrocytes (Imai *et al.*, 2015; King and Lamb, 2015; Kurup, Butler and Harty, 2019). However, data obtained from the murine model (Belnoue *et al.*, 2002, 2008; Nitcheu *et al.*, 2003; Haque *et al.*,

2011) implicate $CD8^+$ T cells as a main driver of immunopathology and recent studies support a similar role in human patients (Hafalla, Cockburn and Zavala, 2006; Kaminski *et al.*, 2019; Riggle *et al.*, 2020), manifesting the use of experimental PbA infections to study the immunopathology of malaria. Accordingly, understanding the immune regulation could help to improve vaccination strategies or prevent immunopathology.

To this end, the expression of the co-inhibitory molecules PD-1, TIGIT, TIM-3, CTLA-4 and the exhaustion-associated molecule CD39 was analyzed on $CD8^+$ T cells. Additionally, the expression of the pro-inflammatory cytokine IFN-γ, the effector molecules Granzyme B and Perforin and the degranulation marker CD107a was investigated. Instead of focusing on the co-expression of LAG-3 and CD49b, which is how cells were categorized in the previous publication in remembrance of Tr1 cells, I wanted to compare cells expressing different levels of co-inhibitory molecules. It is conceivable that truly cytolytic $CD8^+$ T cells, categorized as LAG-3 positive in the previous publication, express just enough LAG-3 to reach the threshold set to reach this category, whilst truly exhausted cells express much higher levels of these markers. It is also plausible that distinct subsets exist within the LAG-3^+ compartment, expressing different constellations of co-inhibitory molecules. To circumvent these issues whilst staying comparable to the first publication, LAG-3 was used as a guideline marker to categorize activated ($CD44^+$) $CD8^+$ T cells into different levels of expression as LAG-3^{neg}, LAG-3^{low} and LAG-3^{high} cells whilst using $CD8^+CD44^-$ cells as a comparison, representing naïve cells (FiI, Fig. 1). Interestingly, expression strength of the co-inhibitory molecules PD-1, TIM-3, TIGIT, CTLA-4 and CD39 (FiI, Fig. 1), as well as pro-inflammatory (FiI, Fig. 2) molecules IFN-γ, CD107a, Granzyme B and Perforin was found to positively correlate with LAG-3 expression. To ensure this was not an artifact unique to LAG-3, a correlation matrix was generated based on the single cell expression of each marker. This way, co-expression of all markers was both visualized and mathematically quantified (FiI, Supp. Fig 6). The correlation matrix confirmed that expression of the described markers not only positively correlated with LAG-3, but indeed also among each other. Interestingly, it seemed as if expression of co-inhibitory molecules is not a sign of dysfunction but rather a surrogate readout for a T cells' activity. To confirm these *ex vivo* findings, the same cytotoxicity assay employed in the first publication was used. The assay relies on the use of OT-I mice, which has both advantages and disadvantages. OT-I mice produce only a single $CD8^+$ T cell clone with a defined target peptide, which, from a methodical point of view, is relatively easy

to provide to the target cells. The vast heterogeneity of plasmodial antigens will elicit a T cell response consisting of a multitude of T cell clones in wt mice. However, compared to $CD8^+$ T cells obtained from wt mice, a single peptide will be recognized by all OT-I derived T cells, whilst a mix of peptides designed to engage wt $CD8^+$ will inevitably miss peptides recognized by certain clones or evoke unspecific killing (Ream, Sun and Braciale, 2010), in both cases lowering the resolution of the assay. On the downside, a mouse bearing only a single $CD8^+$ T cell clone is an artificial system that does not exist outside a laboratory. The absence of a diverse T cell repertoire could have implications on the overall immune response. However, it could be argued that any laboratory experiment being performed in a controlled environment does not represent the real world. Compared to cell line experiments, the immune cells in this transgenic mouse still undergo a very similar infection progression and interact with a variety of unmodified immune cells. Crucially though, we were particularly interested in analyzing the influence of co-inhibitor expression on a $CD8^+$ T cells' potency. TCR affinity can have a significant impact on a T cells function (Border *et al*., 2019). To remove this variable, working with a homogenous population in terms of TCR affinity was required. To ensure a similar $CD8^+$ T cell activation and marker-expression profile in *Pb*TG-infected OT-I mice compared to $CD8^+$ T cells found in wt mice upon infection, the same phenotyping analysis was performed on infected OT-I mice, analyzing expression strength in relation to LAG-3 (Fil Fig S5). Additionally, the correlation matrix was calculated to quantify co-expression. Lastly, a Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) analysis was performed of both the wt and the OT-I $CD8^+$ dataset. UMAP is a visualization tool designed to display complex datasets containing multiple parameters (McInnes, Healy and Melville, 2018), which, in this case, were the $CD8^+$ T cells expressing multiple co-inhibitory as well as effector molecules. Whilst there are differences noticeable between the two datasets, they are minor and $CD8^+$ T cells display a similar expression pattern in both mice. In general, the correlation between the marker's expression is not as strong in the OT-I mice as in the wt mice. This is surprising, considering the homogenous TCR clonality should display a more gradual activation than cells with heterogenous TCRs. It is possible that distinct clones assert dominance in wt mice, displaying the strongest potency, whilst less affine TCR clones gradually possess less potency. In OT-I mice, the homogeneity might lead to a more "average" expression level between all cells, resulting in a less defined co-expression overall. It is also possible that the amount of

antigen available to each T cell is a limiting factor to OT-I derived $CD8^+$ T cells, given the fact that parasitemia is usually relatively low at the time of sacrifice, resulting in insufficient activation of some $CD8^+$ T cells in infected OT-I mice. Furthermore, it should be noted that the significant ($p>0.001$) correlation between all markers is also positive in OT-I mice.

Another apparent difference is that fewer LAG-3^{high} cells express Perforin and Granzyme in OT-I mice than in wt mice. However, the majority of the Perforin- and Granzyme B-producing $CD8^+$ T cells is still found in the group defined as LAG-3^{high} $CD8^+$ T cells, similar to the wt mice. In the cytotoxicity assay, this should only dilute their proportion within the LAG-3^{high} population, decreasing the resolution of the assay but not change the meaning of the results obtained.

Based on these results, the cytotoxicity assay was performed by sorting activated $CD8^+$ T cells according to their LAG-3 expression. An enzyme-linked Immunosorbent assay (ELISA) was employed to determine IFN-γ secretion, a method previously used to determine cytotoxic capacity of $CD8^+$ T cells. Both the cytotoxicity assay and the subsequent ELISA (FiI Fig 4) confirmed the phenotypic characterization, demonstrating that, in acute PbA-infection, cytolytic potential of $CD8^+$ T cells increases with expression of co-inhibitory molecules. Considering that the LAG-3^{high} population was still detected to be the compartment with the highest cytolytic capabilities despite the aforementioned dilution reinforces the finding of their immense cytolytic potential.

This is contradictory to the currently dominating concept which associates expression of high levels of co-inhibitory molecules on T cells with a dysfunctional state termed exhaustion. For example, $CD8^+$ T cells isolated from hepatocellular carcinoma patients were analyzed with a similar methodology; they were compartmentalized into PD-1^{high}, PD-$1^{intermediate}$ and PD-$1^{negative}$ cells. Similarly, expression of co-inhibitory molecules TIM-3 and LAG-3 correlated with expression of PD-1; however, conversely, production of INF-γ and TNF-α decreased with higher PD-1 expression (Kim *et al.*, 2018). Similar findings have been made in colorectal carcinomas, in which co-expression of PD-1 and TIM-3 was also associated with lower expression of pro-inflammatory cytokines (Klapholz *et al.*, 2022) which is in accordance with the current view on T cell exhaustion in chronic tumors (Jiang, Li and Zhu, 2015). However, during acute malaria, it seems that expression of co-inhibitory molecules delineates $CD8^+$ T cells with the highest cytolytic potential. Here, T cells co-expressing PD-1, LAG-3, TIM-3, TIGIT and CTLA-

4 produce higher amounts of IFN-γ and, crucially, display a higher cytolytic capacity *in vitro*. Of note, it is very difficult to directly compare between expression levels of a given marker measured via flow cytometry between two different experiments, let alone between two different laboratories. There are a multitude of factors that influence the measured signal, ranging from cell isolation and staining protocol, the antibody clones and fluorochrome combinations used to the actual flow cytometer and how it is set up. Comparing markers that are "highly" expressed between two different studies should not be considered as a direct comparison in a quantifiable expression strength, but should always be set in relation to, for example an uninfected control within the same experiment (FiI Supp Fig. 1). Besides these technical limitations, it should be noted that infection with PbA elicits an acute immune response, whilst T cell exhaustion is usually described in chronic infections or tumors. Comparing these diseases should be done with care, considering the various factors at play. All things considered, in this work, focusing on an acute PbA-infection, rapid induction of co-inhibitory molecules on T cells was identified, which also normalizes rapidly after antigen withdrawal. This is similar to recent findings in acute Covid-19 infection, in which co-inhibitory molecules on T cells were induced, but also disappeared swiftly during recovery of the patients (Herrmann *et al.*, 2020).

It is tempting to challenge the collective term of "co-inhibitory" molecules for markers like PD-1, LAG-3 etc., given the contradiction that their expression is associated with a higher cytokine and effector molecule production and strong cytolytic properties in this study. However, the influence of time may be an important factor to consider, which may explain the difference in phenotype of co-inhibitory molecules expressing T cells in acute and chronic infections. Tumors usually develop over several months to years, leading to persistent antigen exposure and similarly, chronic viral infections also expose T cells to permanent activation (Wherry and Kurachi, 2015). Furthermore, the expression of co-inhibitory molecules could have a profound impact on the generation of an effective memory response. This does not become apparent in an acute infection that is lethal within days, as is the case in the model of experimental malaria, where mice succumb to the disease within 6-12 days post infection (Lou, Lucas and Grau, 2001; Bagot *et al.*, 2002). Interestingly, it was found that in the experimental model of chronic malaria, in which C57BL/6 mice are infected with *P. chabaudi*, PD-1 expression is actually associated with a dysfunctional T cell response (Horne-Debets *et al.*, 2013). In this study, PD-1 knockout mice (PD-1KO) generated $CD8^+$ T cells produced more

Granzyme B compared to their wt counterparts. Upon re-infection, PD-1KO mice were able to generate stable immunity, whilst wt mice showed detectable parasitemia (Horne-Debets *et al.*, 2016). Interestingly, it was recently discovered that exhausted $CD8^+$ T cells in HCV and HIV-patients acquire a distinct epigenetic phenotype, defined through chromatin accessibility, especially near super-enhancers of exhaustion-related genes, that is largely irreversible (Yates *et al.*, 2021). This phenotype is described as “epigenetically scarred”. It is conceivable that a similar mechanism is engaged in T cells during acute malaria, both in mice and humans. Considering this mechanism in the context of constant exposure to multiple pathogens and in malaria-endemic regions, T cell exhaustion and expression of co-inhibitory molecules could have significant impact on both memory generation and regulation of the immune response.

Usually, co-inhibitory molecules are targeted in therapy by using monoclonal antibodies to block PD-1/PD-L1 interactions or CTLA-4 binding. The use of antibodies has been successfully established in tumor therapy and lead to multiple new treatment options (Callahan, Postow and Wolchok, 2016). By preventing interaction of PD-1 with its natural ligand, the transmission of an inhibitory signal to a T cell is interrupted. Subsequently, the T cell can re-acquire their pro-inflammatory and cytolytic capabilities to help defend against malicious tumor cells. The opposite idea of this concept might be used during acute infections. Instead of preventing co-inhibitor-ligand interaction to restore a T cells function, it might be advantageous to *increase* the interaction between co-inhibitor and its respective ligand, either through admission of soluble ligands or agonistic antibodies, mirroring said ligands properties to decrease a T cells pathogenic potential. In view of our results, which show T cells expressing multiple co-inhibitory molecules are the most active, it is probable that these are the specific T cells that are most responsible for the immense immunopathology. The most common cause of death in malaria is the complication of CM, which, in mice, can be averted through either depletion (Yañez *et al.*, 1996; Hermsen *et al.*, 1997) of $CD8^+$ T cells or knockout of their crucial effector molecules Granzyme B or Perforin (Nitcheu *et al.*, 2003; Potter *et al.*, 2006; Huggins *et al.*, 2017). Neither of these options are feasible or possible in humans today. However, using co-inhibitory molecules as a surrogate marker to identify the specific cells which are responsible for the immunopathology might be a possible solution to decrease the damage caused by these $CD8^+$ T cells. In accordance with this assumption, treatment of PbA-infected mice with soluble PD-L1-fusion

molecules was able to prolong survival compared to untreated infected mice (Wang, Li, *et al.*, 2019). Similarly, stable peptide-MHC II, capable of binding to LAG-3, was used to convey T cell suppression *in vitro* and to treat autoimmune responses in non-obese diabetic (NOD) mice (Maruhashi *et al.*, 2022). Other reports focused on cytokine-facilitated immuno-suppressive interventions. For example, treatment of mice with an artificial IL-15 Complex (IL-15C) lead to induction of IL-10 producing NK cells, which were able to suppress the immune response and to prevent development of ECM (Burrack *et al.*, 2018).

These results fit well with the previously discussed notion that expression of co-inhibitory molecules might be a regulatory mechanism of the immune response. An increase in activation and cytotoxic potential also induces a mechanism to prevent further immunopathology and to counteract the pro-inflammatory reaction. The T cells are activated and, in an attempt to self-regulate, express co-inhibitory molecules. They are able to recognize suppressive signals through their co-inhibitory molecules (Wang, Li, *et al.*, 2019). Nonetheless, perhaps due to the rapid nature of the infection, these counteractive mechanisms are not sufficient to prevent immense immunopathology in this acute infection.

T cell regulation in malaria is a complicated topic; a balance is required that takes into account the need for an effective memory development, whilst also preventing an overarching immune response. T cells are not the only players contributing to an immune response and they interact with several different immune compartments. Cells from these compartments may produce co-stimulatory or suppressive signals in the form of cytokines or ligands to interact with the T cells. Increased expression of co-inhibitory molecules was observed upon infection, but this, in contrast to observations in other diseases, did not lead to a decreased functionality in T cells. However, with datasets of different mouse models often obtained from different laboratories, it is difficult to compare the number of cells expressing a certain molecule and the expression strength of said molecule via flow cytometry, due to vastly different experimental setups, protocols, antibody clones and conjugates used between different groups. It is therefore possible that there are differences in expression patterns of co-inhibitory molecules induced between acute PbA-infection and a chronic tumor model, but these differences get lost in the incompatibility of the datasets. To this end, the journal *Cytometry Part A* developed a platform on which peer-reviewed,

optimized multicolor immunofluorescence Panels (OMIP) can be published, aiming to both reduce the time to optimize the panel for a given analysis and provide a precise protocol to better compare between different laboratories (Roederer and Tárnok, 2010). I developed a 41-color high parameter OMIP which aims to accurately depict expression of multiple co-inhibitory molecules on T cells in mice (AR Fig. 3). Crucially, the panel also allows for the analysis of other immune cells like APCs, as well as the expression of several ligands to the co-inhibitory molecules. The list of ligands includes PD-L1 (ligand to PD-1), MHC II (ligand to LAG-3), Galectin-9 (ligand to TIM-3) and PVR (ligand to TIGIT). Accurately quantifying the expression strength of both a receptor and its ligand is important, as both can have an impact on a cells response towards the signal (Zheng, Fang and Li, 2019). In total, the panel is designed to analyze 41 fluorescent parameters from a single sample. This panel will therefore aid in identifying and characterizing key players in the immune response, specifically the T cell response and its regulation, in experimental malaria. Furthermore, applying this panel to other disease models would allow us to accurately compare expression patterns of co-inhibitory molecules between acute PbA infection and chronic diseases. It will be interesting to see if there are major differences between T cell responses or if, for example, a larger array of inhibiting ligands is present in chronic infections.

In conclusion, this thesis investigated the role of co-inhibitory rich T cells in experimental malaria. In the first publication (EJI) we discovered that T cells induced in experimental malaria develop a dual phenotype; whilst expressing multiple pro-inflammatory cytokines and effector molecules, representing a classical Th1 ($CD4^+$ T cells) and cytotoxic ($CD8^+$) T cell phenotype, they also produce the anti-inflammatory cytokine IL-10 and are able to suppress proliferation of other T cells. The $CD8^+$ T cells are fully able to perform their cytolytic ability at the same time. T cells with a similar phenotype were found in malaria patients, indicating that the results found in the murine model may be transferred to the infection process in humans. Interestingly, these activated T cells are characterized by expression of multiple co-inhibitory molecules, a phenotype usually associated with “T cell exhaustion”, a dysfunctional state usually found in chronic diseases. However, the second paper (FiI) further concentrated on the $CD8^+$ T cells and found expression of co-inhibitory molecules to positively correlate with a $CD8^+$ T cells functional capabilities. To deeper analyze the interaction between T cells and other immune cells and to identify potential regulatory interconnections, I developed a 41-color high parameter panel capable of displaying

the interplay of co-inhibitory molecules and their ligands. This will not only help to further characterize T cells in malaria, but also provide a tool that may be applied to a wide range of diseases. Knowledge gained from these experiments could aid in development of both vaccination strategies and potential treatment schemes, in order to identify a balance between an ineffective and overreaching immune response.

6. References

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7. Information and contribution to papers

I. T cells expressing multiple co-inhibitory molecules in acute malaria are not exhausted but exert a suppressive function in mice

Johannes Brandi, Cari Lehmann, Lea-Christina Kaminski, Julian Schulze zur Wiesch, Marylyn Addo, Michael Ramharter, Maria Mackroth, Thomas Jacobs and Mathias Riehn

For the clustering analysis of $CD4^+$ and $CD8^+$ T cells, I designed and optimized the panel and planned the experiment. I performed the experimental work together with Mathias Riehn and I generated the data used in figure 1 and 2. I visualized the gating strategy and thus created supplementary figure 4.

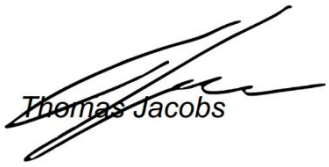
To determine the kinetics of the induction of the cells, I and Mathias Riehn developed the experiment and categorized the different groups and we performed the experiments together. Data for figure 3A-D and 4A-B were generated, analyzed and processed by me. This included data processing and statistical analysis, as well as preparation of the figures. The restimulation assay (3 E-G, 4 C-E) was performed by Mathias Riehn.

Furthermore, I established and performed the cytotoxicity assay of PbTG-induced $CD8^+$ T cells. Hence, the data for figure 6 was generated, analyzed and processed by me. Additionally, I performed the statistical analysis and created the figure.

Additionally, I designed the experiment, generated the data and analyzed it to determine the proportion of $FoxP3^+$ Tregs within the different $CD4^+$ T cell subsets in supplementary figure 2.

I wrote large parts of the methods section of the manuscript, participated in the editing and contributed with literature research.

I hereby confirm the accuracy of these contributions



II. Increased expression of multiple co-inhibitory molecules on malaria-induced CD8$^+$ T cells are associated with increased function instead of exhaustion

Johannes Brandi, Mathias Riehn, Alexandros Hadjilaou, Thomas Jacobs

I designed and planned the experiment to analyze the expression of co-inhibitory molecules on CD8$^+$ T cells. I generated the experimental data, performed statistical analysis and visualized the results as figure 1. The same goes for figure 2, which deals with the expression of effector molecules. I also established the stainings and performed quality control experiments which resulted in supplementary figure 1. Furthermore, I performed the same experiments on OT-I mice to check the expression of these molecules in this strain. The results from this analysis are shown in supplementary figure 5. I also did the planning, statistical analysis and visualization for this experiment.

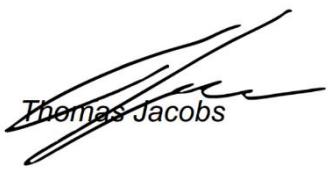
For figure 3, I designed and planned the comparison between infected OT-I and C57BL/6J mice. I generated the experimental data and performed the UMAP clustering analysis to create this figure.

I planned and designed the experiment to determine the cytotoxic capabilities of CD8$^+$ T cells with the help of Mathias Riehn. I performed the experiment with the help of Alexandros Hadjilaou to generate the experimental data. I performed statistical analysis and visualized the results as figure 4. As I established the cytotoxicity assay, I also visualized the workflow, gating strategy and analysis, which can be seen in supplementary figure 2, 3 and 4.

I planned the experiment and generated the data used for the correlation matrices seen in supplementary figure 6. I also performed all statistical analysis for this experiment and created this figure to visualize positive correlation among the markers analyzed.

I wrote the first version of the manuscript together with Thomas Jacobs. Furthermore, I edited following versions and aided in communication with the reviewers and editors.

I hereby confirm the accuracy of these contributions



8. Abstract

Malaria is among the deadliest infectious diseases worldwide, causing over half a million fatalities annually. The disease is caused by a Plasmodium parasite which is transmitted through the bite of an infected Anopheles mosquito and undergoes a liver and a blood stage, the latter being responsible for the majority of symptoms. The most severe complication of malaria is cerebral malaria, which often leads to a fatal outcome. The exact mechanisms of cerebral malaria are not completely elucidated in humans, but accumulating data is in line with findings from the mouse model of cerebral malaria, infection of C57BL/6 mice with *Plasmodium berghei* ANKA, identifying $CD8^+$ T cells as main drivers of cerebral malaria pathology. T cells have a range of functions and certain T cells can regulate the immune response by suppressing proliferation of other immune cells, including other T cells. One mechanism to regulate the immune response or, more specifically, suppress T cell activity during a persisting antigen-exposure, is the expression of multiple co-inhibitory molecules such as PD-1 or LAG-3 on the T cell. Upon contact to the cognate ligands to these molecules, this activates inhibitory pathways within the T cell, rendering the T cell dysfunctional. This state is called T cell exhaustion; whilst exhaustion may be a protective mechanism to prevent overwhelming immunopathology, it may also be abused as an immune evasion mechanism of tumor cells or virus-infected cells. However, the exhausted state may be reverted through antibody-mediated blockade of the co-inhibitory molecules, which has led to advances in anti-tumor therapies. Interestingly, increased expression of co-inhibitory molecules on T cells was recently found in blood samples from malaria patients. The aim of this work was to identify the role of these T cells in experimental malaria. Expression of co-inhibitory molecules on T cells is induced by the blood-, not the liver-stage of the disease. Interestingly, both $CD4^+$ and $CD8^+$ T cells expressing multiple co-inhibitory molecules shared characteristics with a subpopulation of $CD4^+$ T cells called type 1 regulatory (Tr1) T cells which co-express LAG-3 and CD49b. Both $CD4^+$ and $CD8^+$ T cells expressing the molecule LAG-3 are capable of suppressing proliferation of other T cells, indicating regulatory capacity. Expression of CD49b, a key marker previously associated with Tr1 cells, does not seem to be relevant for their suppressive capacity in malaria. In line with this, LAG-3^+ T cells produce more of the anti-inflammatory cytokine IL-10 than their LAG-3^- counterparts. A similar expression pattern of co-inhibitory molecules can

be identified on T cells isolated from the blood of malaria patients, indicating a similar function in humans. Next, a flow based CD8+ T cell cytotoxicity assay was employed to evaluate their killing capacity in vitro. Accordingly, OT-I mice were infected with a transgenic *Plasmodium berghei* ANKA (PbTG) strain. This allowed us to analyze the antigen-specific $CD8^+$ T cell response in experimental malaria. Surprisingly, LAG-3^+CD8^+ T cells had a higher cytotoxic capacity than CD8+ T cells not expressing LAG-3^+, which is in contrast to previous findings, in which expression of co-inhibitory molecules is associated with dysfunction. Accordingly, the expression of multiple co-inhibitory molecules and $CD8^+$ T cell effector molecules Granzyme B and Perforin was analyzed. Expression of the co-inhibitory molecules LAG-3, PD-1, TIM-3, TIGIT, CTLA-4 and CD39 all correlated positively with each other but crucially, also correlated with expression of Granzyme B and Perforin. The correlation of expression was similar in both C57BL/6 mice and OT-I mice. Consequently, $CD8^+$ T cells were divided in groups expressing no, low and high levels of the co-inhibitory molecule LAG-3. Cells from these groups were used in the cytotoxicity assay which showed that, with an increased expression of co-inhibitory molecules, the cytotoxic capabilities of $CD8^+$ T cells increased. These findings were in line with IFN-γ secretion during the assay, a measure previously used to characterize cytotoxicity. Demonstrating increased effector function with increased expression of co-inhibitory molecules during acute malaria is in contrast to previous findings from chronic diseases, in which expression of co-inhibitory molecules is associated with a loss of T cell function. In conclusion, $CD4^+$ and $CD8^+$ T cells were demonstrated to express co-inhibitory molecules during acute malaria and acquire suppressive capacity. $CD8^+$ T cells expressing co-inhibitory molecules have increased cytotoxic capacity, demonstrating bilateral capabilities in terms of immune regulation. Analysis of blood from malaria patients revealed a similar phenotype in T cells as was found in mice. Taken together, these findings reveal new insights into the expression of co-inhibitory molecules during acute infection. This information improves our understanding of T cell regulation in acute settings and could lead to immunomodulating therapy approaches targeting co-inhibitory molecules.

9. Zusammenfassung

Malaria ist einer der tödlichsten Infektionskrankheiten weltweit und führt jährlich zu über einer halben Millionen Todesopfern. Die Krankheit wird durch einen Parasiten der Gattung Plasmodium verursacht, der über den Biss einer infizierten Anopheles Mücke übertragen wird. Der Parasit durchgeht die Leber und die Blutphase, wovon die letztere verantwortlich für den Großteil der Symptomatik ist. Die schwerste Komplikation ist die zerebrale Malaria, welche oft zum Tod führen kann. Der exakte Mechanismus der zerebralen Malaria im Menschen ist noch nicht bekannt, aber Daten deuten auf starke Ähnlichkeiten zum Mausmodell der zerebralen Malaria, der Infektion von C57BL/6 Mäusen mit *Plasmodium berghei* ANKA, hin, in dem $CD8^+$ T Zellen als Hauptverursacher der zerebralen Malaria identifiziert wurden. T Zellen haben eine Reihe an Funktionen, und bestimmte T Zellen können durch Suppression der Proliferation anderer Immunzellen, unter anderem anderer T Zellen, die Immunantwort regulieren. Ein Mechanismus, um die Immunantwort zu regulieren, bzw. um T Zell Aktivität während einer anhaltenden Antigen-exposition zu supprimieren, ist die Expression mehrerer ko-inhibitorischer Moleküle wie PD-1 oder LAG-3 auf der T Zelle. Sobald die entsprechenden Liganden diese ko-Inhibitoren auf der T Zellen binden, werden inhibitorische Kaskaden innerhalb der T Zelle in die Wege geleitet, sodass sie in einen dysfunktionalen Zustand verfällt. Diesen Zustand nennt man T Zell *exhaustion* („T Zell Erschöpfung“). T Zell Erschöpfung kann ein schützender Mechanismus sein, der eine überwältigende Immunpathologie verhindert soll, jedoch kann der Mechanismus auch als ein Mittel zur Immune *evasion* („Immunflucht“) von Tumorzellen und virusinfizierten Zellen genutzt werden. Allerdings ist es inzwischen möglich, durch antikörper-vermittelte Blockade der ko-inhibitorischen Moleküle die Funktion der Zellen wieder herzustellen, was zu erheblichen Fortschritten in der Krebstherapie geführt hat. Interessanterweise wurde Expression von ko-inhibitorischen Molekülen auf T Zellen von Malaria Patienten entdeckt. Das Ziel dieser Arbeit war es, die Rolle dieser T Zellen in experimenteller Malaria zu identifizieren. Zunächst konnte gezeigt werden, dass die Expression von ko-inhibitorischen Molekülen auf T Zellen durch die Blutphase, aber nicht durch die Leberphase induziert wird. Interessanterweise teilen sowohl $CD4^+$ als auch $CD8^+$ T Zellen, die mehrere ko-inhibitorische Moleküle exprimieren, bestimmte Eigenschaften mit einer Subpopulation der CD4+ T Zellen, den sogenannten Typ 1 regulatorischen T Zellen (Tr1 Zellen), welche sich durch die Ko-Expression von LAG-

3 und CD49b auszeichnen. Sowohl $CD4^+$ als auch $CD8^+$ Zellen, die das Molekül LAG-3 exprimieren, sind in der Lage, die Proliferation anderer T Zellen zu supprimieren, was auf regulatorische Eigenschaften der Zellen hindeutet. Expression von CD49b, einem Schlüsselmarker zur bisherigen Identifikation von Tr1 Zellen, schien nicht relevant für die regulatorischen Eigenschaften der T Zellen zu sein, zumindest nicht in diesem akuten Model der Malaria. Passend zu diesen Ergebnissen produzieren LAG-3^+ T Zellen mehr IL-10, einem anti-inflammatorischen Zytokin, als LAG-3^- Zellen aus der gleichen Maus. Darüber hinaus wurde ein ähnliches Expressionsmuster von ko-inhibitorischen Molekülen auf humanen T Zellen, isoliert aus dem Blut von Malaria Patienten, identifiziert, was auf ähnliche Funktionen dieser Zellen im Menschen hindeutet. Als nächstes wurde ein Durchflusszytometrie-basierter CD8+ T Zell Zytotoxizitäts-Assay entwickelt, um die $CD8^+$ T Zell Effektor Funktion *in vitro* untersuchen zu können. Hierfür wurden OT-I Mäuse mit einem transgenen *Plasmodium berghei* ANKA (PbTG) Stamm infiziert, was uns erlaubte, die antigen-spezifische CD8+ T Zell Antwort in experimenteller Malaria zu untersuchen. Überraschenderweise hatten LAG-3^+CD8^+ T Zellen eine höhere zytotoxische Kapazität als LAG-3^- Zellen. Dies ist gegenteilig zum klassischen Kenntnisstand, nach dem die Expression von ko-inhibitorischen Molekülen mit einer T Zell-Dysfunktion assoziiert ist. Um dies weiter zu untersuchen wurde die Expression mehrerer ko-inhibitorischer Moleküle und den CD8+ Effektor-Molekülen Granzym B und Perforin untersucht. Expression der ko-inhibitorischen Moleküle LAG-3, PD-1, TIM-3, TIGIT, CTLA-4 und CD39 (einer Ektonukleotidase, deren Expression auch mit T Zell Erschöpfung assoziiert ist) korrelierte sowohl miteinander positiv, als auch mit der Expression der Effektor Moleküle Granzym B und Perforin. Dabei ähnelten sich die Korrelations- und Expressionsmuster in C57BL/6 und OT-I Mäusen. Daraufhin wurde die CD8+ T Zellen anhand ihrer Expression des ko-inhibitorischen Moleküls LAG-3 in verschiedene Gruppen unterteilt: „Keine“, „niedrige“ und „Hohe“ LAG-3 Expression. Die Zellen dieser Gruppen wurden anschließend mit dem Zytotoxizitätsassay getestet. Interessanterweise ist die erhöhte zytotoxische Kapazität der $CD8^+$ T Zellen mit der Expression von ko-inhibitorischen Molekülen assoziiert. Diese Ergebnisse wurden mit der Messung von erhöhter IFN-γ Freisetzung durch ko-inhibitor-reiche Zellen bestätigt, einer Methode, die früher zur Messung der Zytotoxizität genutzt wurde. Das eine erhöhte Effektor Funktion mit erhöhter Expression von ko-inhibitorischen Molekülen korreliert ist im Kontrast zu vorherigen Ergebnissen, die allerdings auf Basis

chronischer Krankheiten gewonnen wurden. Hier war Expression ko-inhibitorischer Moleküle mit einer verminderten T Zell Funktion assoziiert. Zusammengefasst konnte gezeigt werden, dass die $CD4^+$ und $CD8^+$ T Zellen, die während der akuten Malaria ko-inhibitorische Moleküle exprimieren, einen suppressiven Phänotyp erlangen. Darüber hinaus wurde bewiesen, dass die $CD8^+$ T Zellen, die ko-inhibitorische Moleküle exprimieren, erhöhte zytotoxische Kapazitäten aufweisen, was auf sehr gegenseitige Eigenschaften im Kontext der Immunregulation hinweist. Analyse von Blutproben von Malaria Patienten zeigte, dass die T Zellen einen ähnlichen Phänotyp aufwiesen. Diese Ergebnisse werfen ein neues Licht auf die Expression von ko-inhibitorischen Molekülen und auf die T Zell Regulation während einer akuten Infektion. Die Ergebnisse könnten zu neuen immunmodulierenden Therapieansätzen führen, die gezielt auf Zellen, die ko-inhibitorische Moleküle exprimieren, gerichtet sind.

10. Acknowledgements

First, I would like to express my gratitude to my doctorate supervisor PD Dr. Thomas Jacobs for giving me the opportunity to work on this interesting thesis in his laboratory, which provided me a wide range of scientific opportunities. I am grateful for his advice and discussion on experiments, whilst also giving me the liberty to follow my own thoughts, perform and design experiments on my own and take responsibilities for projects.

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Lastly, I would like to thank my friends and family, who supported me during the whole process of this dissertation. Without them, this would not have been possible.

11. Statutory declaration/ Eidesstattliche Erklärung

I hereby declare upon oath that I have written the present dissertation independently and have not used further resources and aids than those stated in the dissertation.

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

Johannes Brandi

Hamburg, 21.07.2022