**Original Article**

# Expression of PD-1/LAG-3 and cytokine production by $CD4^+$ T cells during infection with *Plasmodium* parasites

Running title: T-cell responses during malaria

Henrietta T. Doe[¶], Daisuke Kimura[¶], Mana Miyakoda[¶], Kazumi Kimura[¶], Masoud Akbari[¶], and Katsuyuki Yui[¶§]

[¶]Division of Immunology, Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University, 1-12-4, Sakamoto, Nagasaki, 852-8523 Japan

[§]Address correspondence to Katsuyuki Yui, Division of Immunology, Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University, 1-12-4 Sakamoto, Nagasaki, 852-8523 Japan.
Phone: 095-819-7070; FAX, 095-819-7073
Email: katsu@nagasaki-u.ac.jp

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**Abstract**

$CD4^+$ T cells play critical roles in protection against the blood-stage of malaria infection, but their uncontrolled activation can be harmful to the host. We compared the expression of inhibitory receptors on activated $CD4^+$ T cells and their cytokine production using rodent models of *Plasmodium* parasites, and compared them with those from a bacterial and another protozoan infection. $CD4^+$ T cells from mice infected with *P. yoelii* 17XL, *P yoelii* 17XNL, *P. chabaudi*, *P. vinckei*, and *P. berghei* expressed the inhibitory receptors, PD-1 and LAG-3 as early as 6 days after infection, while those from either *Listeria monocytogenes* or *Leishmania major*-infected mice did not. In response to T-cell receptor stimulation, $CD4^+$ T cells from mice infected with all the pathogens under study produced high levels of IFN-γ. IL-2 production was reduced in mice infected with *Plasmodium* species, but not with *Listeria* or *Leishmania*. *In vitro* blockade of the interaction between PD-1 and its ligands resulted in increased IFN-γ production in response to *Plasmodium* antigens, implying that PD-1 expressed on activated $CD4^+$ T cells actively inhibit T cell immune responses. Studies using *Myd88*$^{-/-}$, *Trif*$^{-/-}$, and *Irf3*$^{-/-}$ mice showed that the induction of these $CD4^+$ T cells and their ability to produce cytokines was largely independent of toll-like receptor signaling. These studies suggest that the expression of the inhibitory receptors, PD-1 and LAG-3 on $CD4^+$ T cells and their reduced IL-2 production are common characteristic features of *Plasmodium* infection.

**Keywords**: malaria, $CD4^+$ T cells, inhibitory receptor, cytokines

**Introduction**

Malaria remains a major cause of morbidity and mortality both in the tropical and subtropical regions of the world. According to WHO, nearly 214 million cases of malaria occurred globally leading to about 438,000 deaths in 2014 (1). Pathology and mortality from malaria is due to the blood-stage of the parasite's life cycle, and thus understanding host immune responses to this stage is crucial for the development of effective treatment strategies or vaccines. During the blood-stage of *Plasmodium* parasite infection, both $CD4^+$ and $CD8^+$ T cells are activated (2-4). $CD4^+$ T cells are important in mediating protective immunity to eliminate malaria parasites by helping B cells to produce specific antibodies and by activating macrophages and other effector cells (5, 6). However, excessive T-cell responses during malaria infection often damage normal tissue and lead to immunopathologies such as anemia and cerebral malaria, which in some cases is lethal to the host (7). To prevent bystander damages to the host, on-going host immune responses need to be controlled during prolonged infection.

After an acute infection, highly functional memory T cells are generated to protect against re-infection with the same pathogen. In contrast, during chronic infection or cancer, T cells show exhausted phenotypes via the expression of inhibitory receptors such as programmed cell death protein 1 (PD-1) (CD279) (8, 9). PD-1 is induced on T and other immune cells after activation; transduces negative signals and attenuates the activation-signal through T cell receptor (TCR), and thus prevents excessive lymphocyte activation (10, 11). PD-1 has two ligands; PD-L1, which is constitutively expressed on antigen-presenting cells (APCs) as well as other cells, and PD-L2, which is induced

mainly on APCs. It was initially shown that virus specific $CD8^+$ T cells in mice chronically infected with lymphocytic choriomeningitis virus (LCMV) are dysfunctional and constitutively express inhibitory receptors including PD-1 (12). These inhibitory molecules are expressed on exhausted T cells during chronic infection as well as during cancer (8). Furthermore, *in vivo* blockade of the interaction of PD-1 with its ligand enhanced T cell proliferation and effector function, suggesting that signaling of inhibitory receptors is directly linked to limiting T-cell function (10, 12). Subsequent studies have demonstrated that exhausted T cells express multiple inhibitory receptors, whose number and level of expression affect the severity of the dysfunction (8, 13). However, the role of PD-1 in acute phase of infection is less clear (14). In experimental models of infection with rabies virus and *Histoplasma capsulatum*, PD-1 pathway negatively regulated T cell responses and thus inhibited protective immunity (15, 16), while PD-L1 blockade during acute *Listeria monocytogenes* infection reduced protective T cell immune responses (17). PD-1 and PD-L1 can be induced on both lymphocytes and innate immune cells such as dendritic cells during infection, and the consequences of the interaction appear inhibitory or supportive for the protective immune responses depending on the infection. Another inhibitory receptor, LAG-3 is expressed on activated T cells and its structure is homologous to CD4 (9). LAG-3 binds to MHC II molecules, and negatively regulates the activation and proliferation of T cells (18).

T-cell exhaustion occurs in mouse models of malaria as well as in various chronic protozoan infections (19, 20). In mice with prolonged *Plasmodium yoelii* infection, activated $CD4^+$ T cells express PD-1 and LAG-3, and also have a reduced ability to

produce cytokines. *In vivo* blockade of PD-1/PD-L1 as well as LAG-3/MHC II interactions in mice with chronic *Plasmodium* infection restore T-cell function culminating in the rapid clearance of blood-stage parasites (19). These studies established a concept that T-cell exhaustion is maintained in T cells in the chronically infected animals due to negative signaling via inhibitory receptor molecules. We previously reported that $CD4^+$ T cells from mice infected with *P. berghei* ANKA, exhibit enhanced IFN-γ but reduced IL-2 production in response to the anti-TCR monoclonal antibody (mAb) or superantigen stimulation (21). These changes were observed in mice one week after infection. We extended our study and evaluated the expression of the inhibitory receptors, PD-1 and LAG-3, on antigen-specific $CD4^+$ T cells and their ability to produce IFN-γ and IL-2 at an early period after infection with several strains of *Plasmodium* species and compared them with those from mice infected with the protozoan *Leishmania major* as well as the intracellular facultative bacteria, *Listeria monocytogenes*.

## Materials and Methods

### Mice and parasites

*Myd88*$^{-/-}$ and *Trif*$^{/-}$ mice (22, 23) were provided by Drs. K. Takeda and S. Akira (Osaka University, Japan), and *Irf3*$^{-/-}$ mice (24) by Dr. Taniguchi (The University of Tokyo, Japan). All gene knock-out mice were of C57BL/6 background. C57BL/6 mice were purchased from SLC (Hamamatsu, Japan). Mice were maintained under specific pathogen-free conditions in the Laboratory Animal Center for Animal Research at Nagasaki University and were used at 8–14 weeks of age. All animal experiments reported here were conducted according to the guidelines for Animal Experimentation after approval from the Institutional Animal Care and Use Committee of Nagasaki University.

*P. berghei* ANKA was originally provided by Dr. M. Yuda (Mie University, Tsu, Japan), *P. yoelii* 17XL (lethal strain) and *P. yoelii* 17XNL (non-lethal strain) (25) by Dr. M. Torii (Ehime University, Matsuyama, Japan), *P. chabaudi chabaudi* and *P. vinckei vinckei* by Dr. R. Culleton (Nagasaki University, Nagasaki, Japan). Parasites were passaged through BALB/c (for *P. berghei*) or C57BL/6 (for the rest of parasites) mice before being used to infect experimental animals. Mice were infected intraperitoneally with parasitized Red Blood Cells (pRBCs)($5 \times 10^4$), and the levels of parasitemia was evaluated by microscopic examination of standard thin blood smears stained with Diff-Quik staining kit (Sysmex, Kobe, Japan) from day 4 post-infection. *L. monocytogenes* expressing OVA (LM-OVA) (26, 27) was provided by Dr. Yoshikai

(Kyushu University, Fukuoka, Japan) and Dr. H. Shen (University of Pennsylvania, Philadelphia, USA). Mice were infected *i.p.* with *L. monocytogenes* (LM-OVA, $3 \times 10^5$ CFU). *L. major* (MHOM/SU/73-5-ASKH strain) was provided by Dr. Himeno (Kyushu University), and was cultured in 199 medium supplemented with 10% FCS and penicillin/streptmycin at 26 °C. Six or seven days after culture, stationary phase promastigotes ($5 \times 10^6$) were harvested and subcutaneously injected into the left hind footpads of mice. For all experiments, mice were sacrificed 6 days post-infection unless otherwise stated.

**Flow cytometry**

Spleen cells were prepared and erythrocytes lysed with Gey's solution, and were stained with APCCy7-anti-CD4 (GK1.5), APC-anti-LAG-3 (C9B7W), PECy7-anti-CD3ε (145-2C11), FITC-anti-CD11a (M1714), anti-CD49d (R1-2), APC-anti-PD-1 (J43), or their isotype controls for 30 min at 4 °C. All mAbs were purchased from BioLegend (San Diego, USA), Tonbo Biosciences (San Diego, USA) or eBioscience (San Diego, USA). Cells were analyzed using FACSCanto II or LSRFortessa X-20 cell analyzer (BD Biosciences) and with FlowJo software (Tree Star, Ashland, OR).

**Cell culture and ELISA**

$CD4^+$ T cells (>95%) were enriched from the spleen using anti-CD4 IMag (BD Biosciences) according to the manufacturer's instructions. Cells were suspended in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 2mM glutamine, penicillin/streptomycin, 2-mercaptoethanol ($5 \times 10^{-5}$ M), non-essential amino acids (0.1

mM) and sodium pyruvate (1 mM). Cells ($2 \times 10^5$) were cultured in a 96-well flat-bottomed plate coated with anti-TCR mAb (H57-597, 1 μg/ml) for 48 h. IL-2 and IFN-γ levels in the culture supernatant were determined by sandwich ELISA using anti-IL-2 (JES6-1A12 and biotin-JES6-5H4) and IFN-γ mAbs (R4-6A2 and biotin-XMG1.2)(both from e-Biosciences), respectively.

To examine antigen-specific responses, $CD4^+$ T cells ($2 \times 10^5$) were cultured in the presence of dendritic cells ($1 \times 10^4$) and *P. berghei* crude antigen (freez-thaw lysate of $3 \times 10^6$ infected RBC) for 2 days. Varying concentrations of anti-PD-L1 and anti-LAG-3 mAbs were added to the culture. Hybridoma cell line producing anti-PD-L1 mAb (clone M1H5) (28) was kindly provided by Dr. M. Azuma (Tokyo Medical Dental University, Tokyo, Japan) through RIKEN Bioresource Center, Tsukuba, Japan, and cell line producing anti-LAG-3 mAb (clone C9B7W) (29) was provided by Dr. D.A. Vignali (St. Jude Children's Research Hospital, Memphis, TN, USA). Cells were cultured using BD CELLLine Flask (BD Biosciences), and mAbs were purified from the culture supernatant using HiTrap Protein G columns (GE Health Care).

**Statistical analysis**

Data were analyzed by unpaired 2-tailed Student's t-test using GraphPad Prism6 software (GraphPad Software, La Jolla, CA, USA).

**Results**

***Activated CD4$^+$ T cells from Plasmodium-infected mice express PD-1 and LAG-3***

CD4$^+$ T cells that are specifically activated during infection can be monitored by the up-regulation of surrogate activation markers, CD11a and CD49d (19, 30). We used this approach to identify pathogen-specific CD4$^+$ T cells and examined whether these T cells expressed the inhibitory molecules, PD-1 and LAG-3, during infection of C57BL/6 mice with *P. yoelii* (17XL and 17XNL), *P. chabaudi*, *P. vinckei*, and *P. berghei*. Six days after infection with these parasites, the levels of parasitemia were 38.8 $\pm$ 4.5 % for *P. yoelii* 17XL, 12.7 $\pm$ 1.1 % for *P. yoelii* 17XNL, 10.1 $\pm$ 1.7 % for *P. chabaudi*, 12.3 $\pm$ 1.1 % for *P. vinckeii*, and 9.1 $\pm$ 0.5 % for *P. berghei*. The proportion of CD11a$^{hi}$CD49d$^{hi}$ cells in total CD4$^+$ T cells of uninfected mice was ~ 1-3 %. The proportion of CD11a$^{hi}$CD49d$^{hi}$CD4$^+$ T cells increased to 11.4 $\pm$ 1.0 % in mice infected with all four strains of *Plasmodium* parasites (Fig. 1). We also determined the expression of the inhibitory receptors, PD-1 and LAG-3, on CD11a$^{hi}$CD49d$^{hi}$ and CD11a$^{lo}$CD49d$^{lo}$CD4$^+$ T cells, which are activated and resting cells, respectively. In the infected mice, CD11a$^{hi}$CD49d$^{hi}$CD4$^+$ T cells expressed both PD-1 and LAG-3 on their cell surfaces, while CD11a$^{lo}$CD49d$^{lo}$CD4$^+$ T cells did not. CD11a$^{hi}$CD49d$^{hi}$CD4$^+$ T cells from uninfected mice expressed low levels of PD-1 but did not express LAG-3.

In control groups, C57BL/6 mice were infected with either the facultative intracellular bacteria, *L. monocytogenes* intraperitoneally, or subcutaneously in the left footpad with the protozoan *L. major* (Fig. 2). Six days after infection with *L. monocytogenes*, the proportion of CD11a$^{hi}$CD49d$^{hi}$CD4$^+$ T cells was 15.6 $\pm$ 2.0 % (Fig. 2a). Even though this

population did not express LAG-3, it expressed low levels of PD-1. Since the kinetics of specific T cell responses in *L. monocytogenes*-infected mice is faster than those infected with *P. berghei* (4), we monitored the expression of these molecules at an earlier time period after infection with *L. monocytogenes* (Fig. 2b). On days 2 and 4 post infection, the levels of $CD11a^{hi}CD49d^{hi}CD4^{+}$ T cells were 4.3 $\pm$ 0.7 % and 7.0 $\pm$ 0.3 %, respectively. Although PD-1 expression was highest on day 2 post infection, the expression level was low when compared with that of *Plasmodium* infection. LAG-3 expression was barely detectable on both days (Fig. 2b). In mice infected with *L. major*, we examined $CD4^{+}$ T cells in the draining lymph nodes, where T cells are primed. Under the experimental condition employed, even though we detected a minor increase in the population of $CD11a^{hi}CD49d^{hi}CD4^{+}$ T cells, these cells did not express detectable levels of PD-1 and LAG-3 (Fig. 2c). Also, in *Leishmania*-infected mice, neither PD-1 nor LAG-3 expression were detected 2 to 3 weeks post infection (data not shown). Taken together, activated $CD4^{+}$ T cell in *L. monocytogenes*-infected mice expressed a low level of PD-1 during an early period of infection, but barely expressed LAG-3 throughout the infection period under examination. The expression of PD-1 and LAG-3 was undetectable in mice infected with *L. major*. In both infections, the expression of PD-1 and LAG-3 on activated $CD4^{+}$ T cells was low when compared with that of *Plasmodium* parasites.

**Reduced expression of IL-2 by $CD4^{+}$ T cells in *Plasmodium*-infected mice**

We next examined cytokine production by $CD4^{+}$ T cells in response to anti-TCR mAb (Fig. 3). $CD4^{+}$ T cells from mice infected with the *Plasmodium* species produced high levels of IFN-$\gamma$ but reduced levels of IL-2 when compared with $CD4^{+}$ T cells from

uninfected mice (Fig. 3a-d). Although $CD4^+$ T cells from *L. monocytogenes*-infected mice produced IFN-γ at a high level similar to those from *Plasmodium*-infected mice, they produced IL-2 at a level comparable to that of uninfected mice in response to anti-TCR mAb (Fig. 3e). Also, lymph node $CD4^+$ T cells from mice infected with *L. major* produced IFN-γ and IL-2 at levels higher than those from uninfected mice (Fig. 3f). Taken together, $CD4^+$ T cells from mice infected with *Plasmodium* species, *L. monocytogenes*, or *L. major* produced higher levels of IFN-γ in response to TCR-stimulation, but only those from *Plasmodium*-infected mice exhibited a reduced production of IL-2 when compared with those from uninfected mice.

**Blockade of PD-1/LAG-3 interactions *in vitro***

Ligands of PD-1 and LAG-3, PD-L1 and MHC II, respectively, are mainly expressed on antigen-presenting cells. To determine whether PD-1 and/or LAG-3 expressed on activated $CD4^+$ T cells are actively engaged in the inhibition of T cell responses, we cultured $CD4^+$ T cells from *P. berghei*-infected mice in the presence of dendritic cells and *Plasmodium* antigen, and blocked the interactions of PD-1/LAG-3 and their ligands with varying doses of anti-PD-L1 and anti-LAG-3 mAbs (Fig. 4a). Specific IFN-γ production by $CD4^+$ T cells was enhanced in the presence of anti-PD-L1 mAb but not in the presence of anti-LAG-3 mAb. Dual blockade of PD-L1 and LAG-3 also increased IFN-γ production but the amount produced was similar to that of only PD-L1 blockade (data not shown). In the absence of *P. berghei* antigen, $CD4^+$ T cells did not show enhanced IFN-γ production indicating that anti-PD-L1 mAb specifically augmented antigen-specific responses (Fig. 4a). Also, IL-2 production by these $CD4^+$ T cells was undetectable in

both blocked and unblocked culture supernatants. As a control group, $CD4^+$ T cells were stimulated with plate-coated anti-TCR mAb without antigen-presenting cells and the effect of these mAbs was evaluated (Fig. 4b). $CD4^+$ T cells from infected mice did not show enhanced IFN-γ or IL-2 production when stimulated with plate-coated anti-TCR mAb after blockade (Fig. 4b). These results imply that antigen-specific activation of $CD4^+$ T cells by dendritic cells is inhibited by PD-1/PD-L1 signaling in mice infected with *P. berghei*.

**PD-1/LAG-3 expression and reduced IL-2-production are independent of TLR signaling**

Innate immune responses orchestrate the development of adaptive immune responses during infection with various pathogens (31), and toll-like receptors (TLRs) mediate host immune responses to blood-stage infection with *Plasmodium* species (32). Activation modes of antigen-presenting cells may affect unique $CD4^+$ T cell responses during *Plasmodium* infection. We therefore assessed the requirement of the TLR signaling pathway for $CD4^+$ T-cell responses using gene knock-out mice lacking Myd88 or Trif adaptor molecules, which are essential for TLR signaling. We infected *Myd88*$^{-/-}$, *Trif*$^{-/-}$, and C57BL/6 mice with *P. berghei*, and analyzed the expression of PD-1 and LAG-3 as well as cytokine production by $CD4^+$ T cells. The proportions of $CD11a^{hi}CD49d^{hi}CD4^+$ T cells and their expression of PD-1 and LAG-3 were similar between infected *Myd88*$^{-/-}$ and C57BL/6 mice (Fig 5a). These $CD4^+$ T cells produced IFN-γ and IL-2 at levels comparable to that of C57BL/6 mice (Fig. 5d). $CD4^+$ T cells from infected *Trif*$^{-/-}$ mice also up-regulated PD-1 and LAG-3, and produced high levels of IFN-γ and reduced levels

of IL-2 (Fig 5b and e). We did not find significant difference in $CD4^+$ T cell responses between C57BL/6 and *Trif*$^{-/-}$ mice. We also assessed the involvement of IRF3, which is a major transcription factor that induces type I interferon in response to TLR4 as well as other innate immune receptors (33). While the proportions of $CD11a^{hi}CD49d^{hi}CD4^+$ T cells in *Irf3*$^{-/-}$ mice was lower than that of C57BL/6 mice, there were no significant differences in their expressions of PD-1 and LAG-3 (Fig 5c). Also, the amounts of IFN-γ and IL-2 produced by $CD4^+$ T cells from infected *Irf3*$^{-/-}$ mice were comparable to that from infected C57BL/6 mice (Fig 5f). Of note, the level of parasitemia was slightly higher in infected *Irf3*$^{-/-}$ mice than in C57BL/6 mice. Also, no significant difference in parasitemia was observed between infected *Myd88*$^{-/-}$, *Trif*$^{-/-}$ and C57BL/6 mice (Fig. 5 legend). These results indicate that the expression of PD-1/LAG-3 on $CD4^+$ T cells, enhanced production of IFN-γ and the reduction in IL-2 production are induced in a manner independent of TLR signaling during infection with *Plasmodium* parasites; also, type I interferon signaling pathway might be involved in the induction of specific $CD4^+$ T cells in *P. berghei* infection.

**Discussion**

We used five different rodent models of malaria parasites, *P. yoelii* 17XL, *P. yoelii* 17XNL, *P. chabaudi*, *P. berghei*, and *P. vinckei*, and showed that activated $CD4^+$ T cells from mice, marked by the $CD11a^{hi}CD49d^{hi}$ phenotype, expressed the inhibitory receptors PD-1 and LAG-3 as early as 6 days after infection, when host T cell responses are induced. These $CD4^+$ T cells exhibited increased IFN-γ production and reduced IL-2 production in response to TCR stimulation as we previously reported for those in *P. berghei*-infected mice (21). In contrast, $CD4^+$ T cells did not express LAG-3 but expressed only low levels of PD-1 in mice infected with *L. monocytogenes*, a facultative intracellular Gram-positive bacteria. Similarly, induction of these molecules was undetectable on $CD4^+$ T cells from the draining lymph nodes of *L. major*-infected mice. These results imply that expression of PD-1 and LAG-3 is induced during infection with *Plasmodium* parasites, but barely during infection with *L. monocytogenes* or *L. major*. Previous studies in which LCMV was used, showed that PD-1 is transiently expressed on activated $CD8^+$ T cells in acute infection, while it is constitutively expressed on specific $CD8^+$ T cells during chronic infection (12). This and other studies lead a concept that T cell inhibitory receptors including PD-1 are active inhibitory molecules expressed on exhausted T cells during chronic infection as well as cancer. Our study suggest that the expression of these inhibitory receptors during chronic infection, may not be simply due to the prolonged presence of antigen *in vivo,* but rather may be due to qualitative differences in T cell activation during infection with *Plasmodium* species from that of *L. monocytogenes* or *L. major*. One possibility is that the antigen-presenting cells that activate $CD4^+$ T cells are different among these pathogens. Alternatively, the local

environment for T cell activation including the available cytokines may affect the induction of inhibitory receptors in responding T cells. Although the precise mechanisms are not clear, these differences during the induction of immune responses against different pathogens may play a key role in the expression of PD-1 and LAG-3, which restrict effective protective immunity. This leads to failure to eliminate pathogens resulting in chronic infection.

To examine whether PD-1 and/or LAG-3 expressed on the surface of $CD4^+$ T cells of malaria-infected mice are actively engaged in the inhibition of T cell responses, we blocked PD-1/LAG-3 signaling *in vitro* using mAbs in the presence of dendritic cells and malaria antigen. $CD4^+$ T cells produced higher levels of IFN-γ upon PD-1 blockade, implying that the activation of $CD4^+$ T cells is actively inhibited through the engagement of PD-1 and its ligands during *Plasmodium* infection. This result is consistent with a previous study, which showed that *in vivo* blockade of PD-1/LAG-3 starting at 2 weeks after infection with *P. yoelii* or *P. chabaudi* infection restored T cell function and enhanced protective immunity (19). These studies suggest that the inhibition of $CD4^+$ T cell responses by PD-1/LAG-3 inhibitory receptors is a common mechanism of immune response inhibition during *Plasmodium* infection, and that the inhibition of the immune responses via these receptors may occur right after the onset of T cell activation. These mechanisms might be required to prevent the damage of host tissue by the excessive activation of immune system. Our current data and previous report (21) have shown that IL-2 production by $CD4^+$ T cells is reduced regardless of the strain of rodent *Plasmodium*. However, we did not observe any enhancing effect of anti-PD-L1 mAb on the IL-2

production in response to plate-coated anti-TCR mAb. Therefore, the direct correlation between the expression of the inhibitory receptors and the reduction of IL-2 production is unclear, although both are commonly observed in all mice infected with *Plasmodium* species.

During infection with microbes, dendritic cells are activated via innate recognition receptors by pathogen-associated molecular patterns expressed on microbes. One of the key receptors is Toll-like receptors that recognize glycosylphosphatidylinositol (GPI) and the hemozoin of malaria parasites (32). We considered that activation of innate immunity in *Plasmodium* infection might be linked to the unique T cell activation modes, and thus examined the possible role of Myd88/Trif in the induction of PD-1/LAG-3 during infection with *P. berghei*, since Myd88 and Trif are critical adaptor molecules for the signal transduction of TLRs as well as IL-1 and IL-18 (22, 23). However, PD-1 and LAG-3 were induced on $CD4^+$ T cells and their IL-2 production was reduced in mice lacking Myd88 or Trif/IRF3, suggesting that these phenomena occur independent of TLR signaling. It is, however, possible that activation of the innate immune system through other receptors is required for the induction of co-inhibitory molecules on the surface of activated $CD4^+$ T cells.

Functional exhaustion of *Plasmodium*-specific $CD4^+$ T cells play critical roles in the regulation of immune responses during the blood-stage of chronic malaria infection (19), but the mechanisms underlying the induction and maintenance of T cell exhaustion are not clearly understood. Our study imply that, during *Plasmodium* infection,

antigen-specific $CD4^+$ T cells express PD-1 and LAG-3 as soon as they are activated *in vivo,* and $CD4^+$ T cell activation is constitutively suppressed by the negative signaling through these receptors. Further studies on the mechanisms underlying the induction and maintenance of these inhibitory receptors on $CD4^+$ T cells and their role in immune modulation in mice infected with *Plasmodium* species may reveal a novel therapeutic strategy for this devastating infectious disease.

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**Disclosure**

All of authors have no financial conflicts of interest.

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**Figure legends**

**Figure 1**

$CD11a^{hi}CD49d^{hi}CD4^{+}$ T cells expressed PD-1 and LAG-3 during infection with *Plasmodium* parasites.

C57BL/6 mice were infected with *P. yoelii*17XL, *P. yoelii*17XNL (a), *P. chabaudi* (b), *P. vinckei* (c), or *P. berghei* (d). Six days after infection, spleen cells were stained for cell surface molecules. Contour plots indicate the gating strategies for $CD49d^{lo}CD11a^{lo}$ (white gate) and $CD11a^{hi}CD49d^{hi}$ (black gate) $CD4^{+}$ T cells. Numbers in the plots indicate the proportions of gated $CD11a^{hi}CD49d^{hi}$ cells. Histograms indicate the expression of LAG-3 and PD-1 (black line) and isotype control (gray shadow) of cells in the square-gates in the contour plots. Numbers in the histograms indicate the proportions of cells expressing PD-1 or LAG-3. Graphs are summaries of the proportions of $CD11a^{hi}CD49d^{hi}$ cells within $CD4^{+}$ T cells in the infected (Py 17XL, Py17XNL, Inf) and uninfected (Uninf) mice (left); and the differences in median florescence intensities (ΔMFI) between anti-LAG-3/anti-PD-L1 mAbs and isotype control groups in $CD11a^{lo}CD49d^{lo}$ (Low) and $CD11a^{hi}CD49d^{hi}$ (Hi/High) $CD4^{+}$ T cells (right). Data are representative of two independent experiments with 3-5 mice per group. *:$p<0.05$, **:$p<0.01$,

**Figure 2**

$CD11a^{hi}CD49d^{hi}CD4^{+}$ T cells did not express PD-1 and LAG-3 during infection with *L. monocytogenes* or *L. major*.

C57BL/6 mice were infected intraperitoneally with *L. monocytogenes* (a, b) or subcutaneously in the left footpad with *L. major* (c). For *Listeria* experiments, spleen cells were prepared from mice uninfected (Uninf) or infected for 2, 4 (b) and 6 days (a) after infection; while for *Leishmania*, popliteal lymph node cells from both the right (Uninf) and left (Inf) hind footpads were prepared 6 days after infection (c), and were stained for cell surface molecules. Contour plots indicate the gating strategies for $CD11a^{lo}CD49d^{lo}$ (white gate) and $CD11a^{hi}CD49d^{hi}$ (black gate) $CD4^{+}$ T cells. Numbers in the plots indicate the proportions of cells in the gated $CD11a^{hi}CD49d^{hi}$ $CD4^{+}$ T cells. Histograms indicate the expression of PD-1 and LAG-3 (black line) and isotype control (gray shadow) of cells in the square-gates in the contour plots. Numbers in the histograms indicate the proportions of cells expressing PD-1 or LAG-3. Graphs are summaries of the proportions of $CD11a^{hi}CD49d^{hi}$ cells in $CD4^{+}$ T cells of the infected (Inf) and uninfected (Uninf) mice (3 mice/group) (left), and the differences of median florescence intensities (ΔMFI) between anti-LAG-3/anti-PD-1mAbs and isotype control groups in $CD11a^{lo}CD49d^{lo}$ (Low) and $CD11a^{hi}CD49d^{hi}$ (Hi) $CD4^{+}$ T cells (right). *:$p<0.05$, **:$p<0.01$,

**Figure 3**

$CD4^+$ T cells from *Plasmodium*-infected mice produced higher levels of IFN-γ and reduced levels of IL-2 in response to TCR stimulation.

$CD4^+$ T cells were prepared from the spleen of mice uninfected (shaded bar) and infected (open bar) with *P. yoelii* 17XL (Py 17XL), *P. yoelii* 17XNL (Py 17XNL) (a), *P. chabaudi* (b), *P. vinckei* (c), *P. berghei* (d), and *L. monocytogenes* (e), and the draining lymph nodes of mice uninfected and infected with *L. major* (f). Cells were cultured in anti-TCR-coated plates for 2 days. The levels of IFN-γ and IL-2 in the culture supernatant were determined by ELISA. Data are the means for 3-4 mice per group and are representative of two independent experiments. Error bars represent the standard deviation. *:$p<0.05$, **:$p<0.01$, ns: not significant

**Figure 4**

Blockade of PD-L1 and LAG-3 *in vitro* enhanced production of IFN-γ by $CD4^+$ T cells.

$CD4^+$ T cells from mice infected with *P. berghei* for 7 days, and were cultured with dendritic cells and *P. berghei* antigen (a) or in plates coated with anti-TCR mAb (b) in the presence or absence of anti-PD-L1 (open circle) and anti-LAG-3 (open square) mAbs for 2 days. $CD4^+$ T cells were also cultured with dendritic cells without antigen in the presence of anti-PD-L1 (shaded circle) or anti-LAG-3 (shaded square) mAbs (a). $CD4^+$ T cells from uninfected mice were cultured in plates coated with anti-TCR mAb (closed triangle)(b). The levels of IFN-γ and IL-2 in the supernatant were determined by ELISA. NS: not significant ND: not detected; *:$p<0.05$, **:$p<0.01$

**Figure 5**

Activation status and cytokine production of $CD4^+$ T cells from *P. berghei*-infected mice are independent of TLR signaling.

*Myd88*$^{-/-}$ (a, d), *Trif*$^{/-}$ (b, e) and *Irf3*$^{-/-}$ (c, f) mice as well as control C57BL/6 mice (WT) were infected with *P. berghei*, and spleen cells were analyzed 6 days later.

(a-c) Contour plots indicate CD49d/CD11a staining of $CD4^+$ spleen cells. The numbers in the plots indicate the proportion of gated $CD11a^{hi}CD49d^{hi}$ $CD4^+$ T cells. Histograms indicate PD-1 and LAG-3 staining (black line) and isotype control (gray shadow) of cells that were gated as shown in the squares in the contour plots. Numbers in the histograms indicate proportions (%) of positive cells as indicated by the bar. Graphs represent the proportions of $CD11a^{hi}CD49d^{hi}$ cells within $CD4^+$ T cells (3 mice/group)(left), and ΔMFI between anti-LAG-3/anti-PD-1 mAbs and isotype control in $CD11a^{lo}CD49d^{lo}$ (Low) and $CD11a^{hi}CD49d^{hi}$ (Hi) $CD4^+$ T cells (right).

(d-f) $CD4^+$ T cells were prepared from uninfected (shaded bar) and *P. berghei*-infected (open bar) mice, and were cultured for 2 days in plates coated with anti-TCR mAb. The levels of IFN-γ and IL-2 in the culture supernatant were determined by ELISA. Parasitemia was 10.1 ± 1.3% in *Myd88*$^{-/-}$ and 8.8 ± 0.5% in C57BL/6; 9.4 ± 0.4% in *Trif*$^{/-}$ and 8.0 ± 0.6% in C57BL/6; 10.3 ± 0.7% in *Irf3*$^{-/-}$ and 7.2 ± 0.7% in C57BL/6 mice. Data are pooled from 2-3 experiments with 3-4 mice per group in each experiment. ND: not detected, Comparisons were made between Infected WT and infected KO mice by unpaired 2-tailed Student's t-test. *:$p<0.05$, **:$p<0.01$ by , NS: not significant

**Abbreviations**

LAG-3: Lymphocyte activated gene-3; LM-OVA: *Listeria monocytogenes* expressing OVA; LCMV: lymphocytic choriomeningitis virus; mAb: monoclonal antibody; PD-1: Programmed cell death protein 1; TLR: toll-like receptor

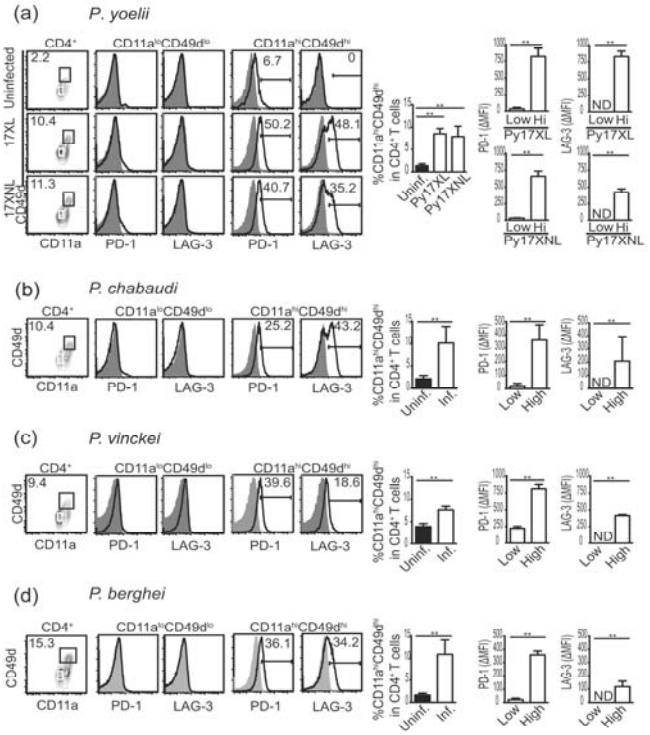
(a)
P. yoelii
CD4+
CD11a^lo CD49d^lo
CD11a^hi CD49d^hi
Uninfected
17XL
17XNL CD49d
2.2
6.7
0
10.4
50.2
48.1
11.3
40.7
35.2
CD11a
PD-1
LAG-3
PD-1
LAG-3
%CD11a^hi CD49d^hi in CD4+ T cells
Uninf.
Py17XL
Py17XNL
PD-1 (ΔMFI)
LAG-3 (ΔMFI)
ND
Low Hi
Py17XL
Py17XNL
(b)
P. chabaudi
10.4
25.2
43.2
CD49d
CD11a
PD-1
LAG-3
PD-1
LAG-3
Uninf.
Inf.
Low
High
(c)
P. vinckei
9.4
39.6
18.6
(d)
P. berghei
15.3
36.1
34.2

**Figure 1**

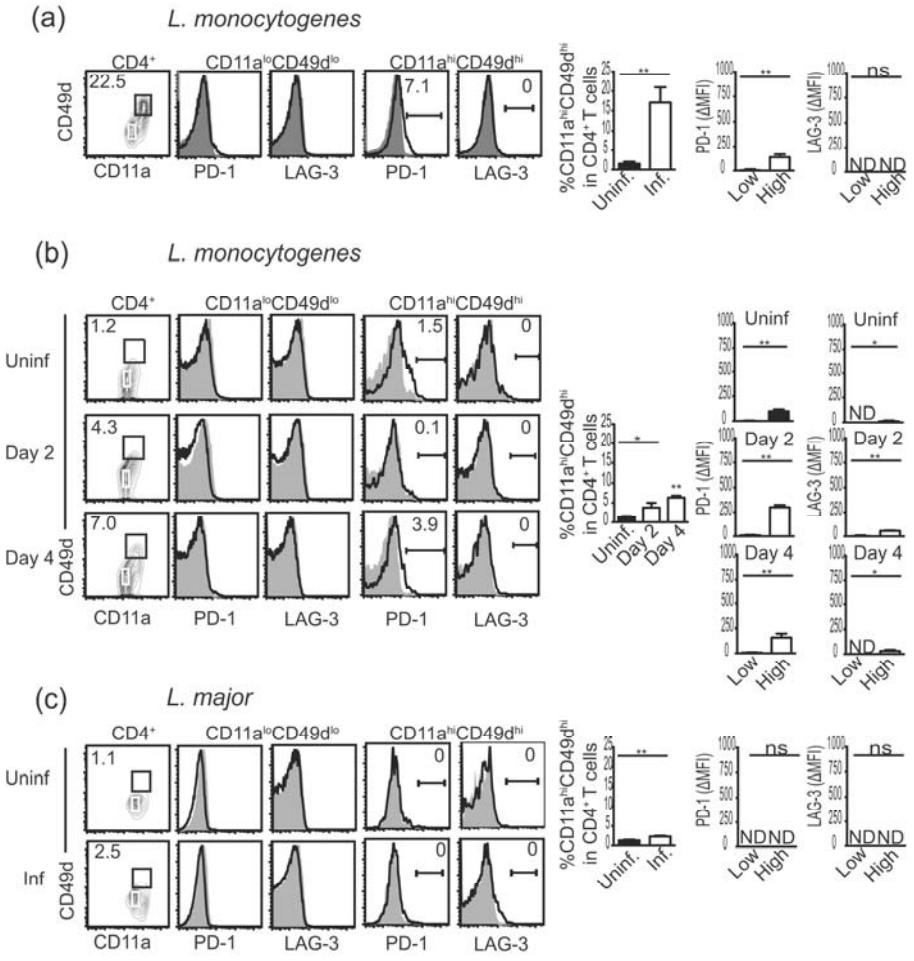
(a)
L. monocytogenes
CD4+
CD11alo CD49dlo
CD11ahi CD49dhi
22.5
7.1
0
CD49d
CD11a
PD-1
LAG-3
PD-1
LAG-3
%CD11ahiCD49dhi in CD4+ T cells
Uninf.
Inf.
PD-1 (ΔMFI)
LAG-3 (ΔMFI)
Low
High
ND
ns
(b)
L. monocytogenes
Uninf
Day 2
Day 4
1.2
1.5
0
4.3
0.1
7.0
3.9
(c)
L. major
Inf
1.1
2.5

**Figure 2**

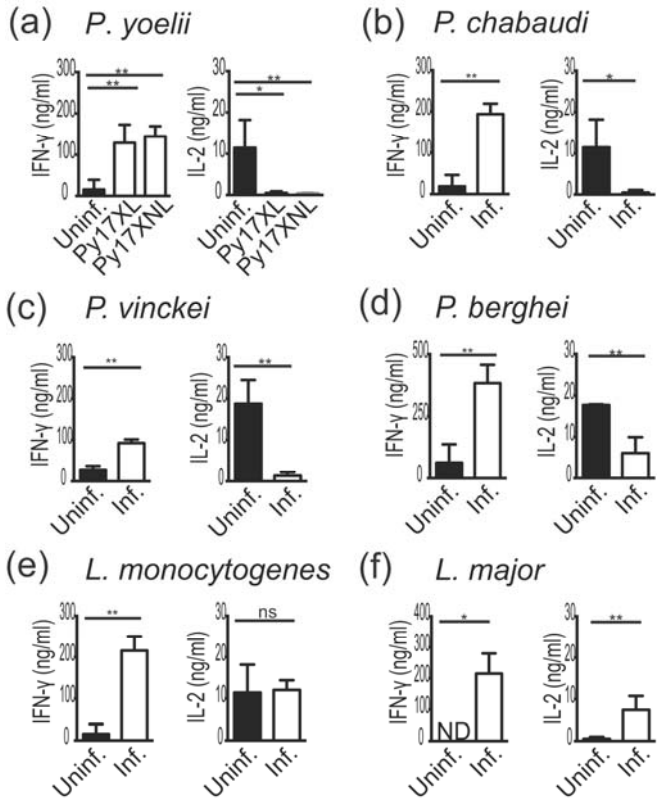
(a) P. yoelii
(b) P. chabaudi
(c) P. vinckei
(d) P. berghei
(e) L. monocytogenes
(f) L. major
IFN-γ (ng/ml)
IL-2 (ng/ml)
Uninf.
Py17XL
Py17XNL
Inf.
ns
ND

**Figure 3**

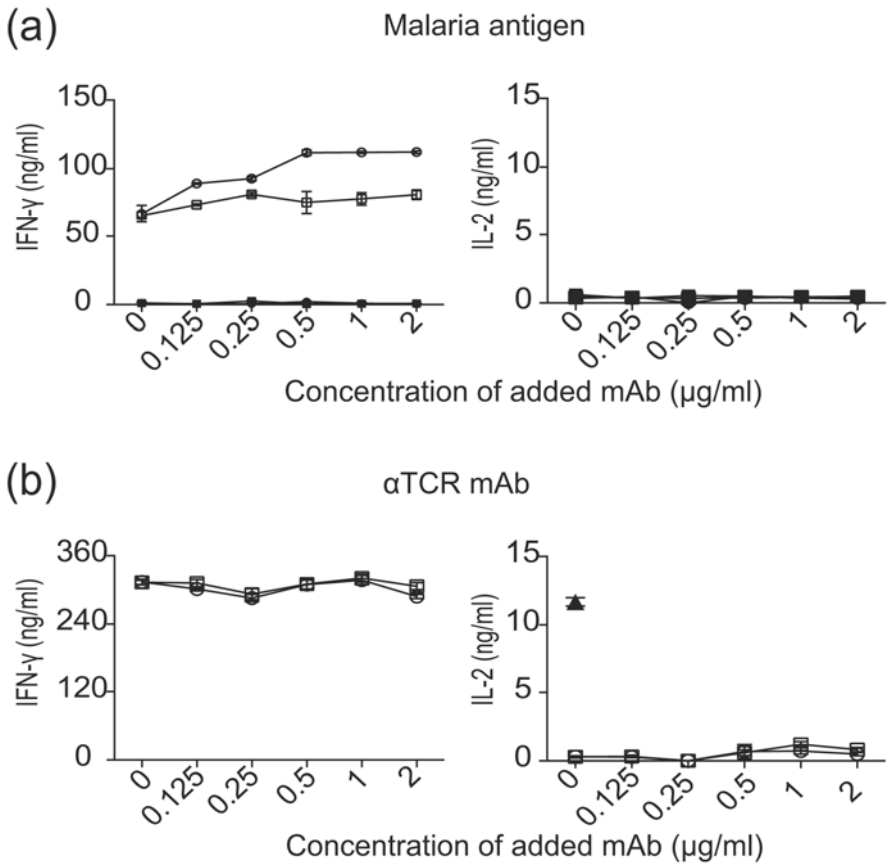
(a)
Malaria antigen
IFN-γ (ng/ml)
150
100
50
0
IL-2 (ng/ml)
15
10
5
0
0
0.125
0.25
0.5
1
2
Concentration of added mAb (µg/ml)
(b)
αTCR mAb
IFN-γ (ng/ml)
360
240
120
0
IL-2 (ng/ml)
15
10
5
0
0
0.125
0.25
0.5
1
2
Concentration of added mAb (µg/ml)

**Figure 4**

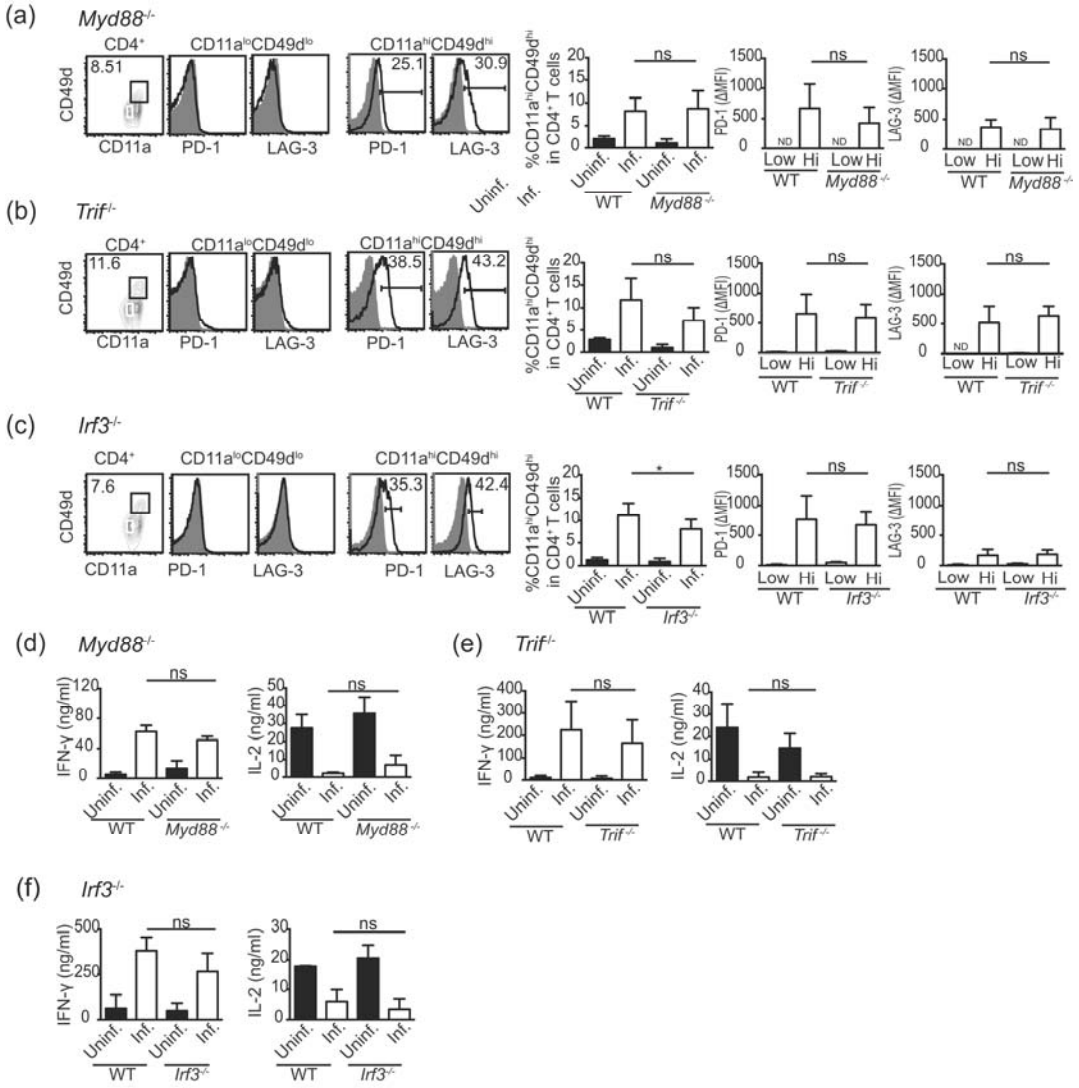
(a) Myd88-/-
CD4+
CD11aloCD49dlo
CD11ahiCD49dhi
CD49d
CD11a
PD-1
LAG-3
8.51
25.1
30.9
Uninf.
Inf.
%CD11ahiCD49dhi in CD4+ T cells
ns
PD-1 (ΔMFI)
LAG-3 (ΔMFI)
ND
Low Hi Low Hi
WT
Myd88-/-
(b) Trif-/-
11.6
38.5
43.2
Trif-/-
(c) Irf3-/-
7.6
35.3
42.4
*
Irf3-/-
(d) Myd88-/-
IFN-γ (ng/ml)
IL-2 (ng/ml)
(e) Trif-/-
(f) Irf3-/-

**Figure 5**