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T cell Ig and mucin-1 and -3 in *Plasmodium berghei* ANKA infection

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Abstract

Cerebral malaria (CM) is a serious and often fatal complication of *Plasmodium falciparum* infections; however, the precise mechanisms leading to CM is poorly understood. Mouse malaria models have provided insight into the key events in pathogenesis of CM. T cell immune response is known to play an important role in malaria infection, and members of the T-cell immunoglobulin- and mucin-domain-containing molecule (TIM) family have roles in T-cell-mediated immune responses. Tim-1 and Tim-3 are expressed on terminally differentiated Th2 and Th1 cells, respectively, and participate in the regulation of Th immune response. Until now, the role of Tim family proteins in *Plasmodium* infection remains unclear. In the present study, the mRNA levels of Tim-1, Tim-3, and some key Th1 and Th2 cytokines in the spleen of female Kunming outbred mice infected with *P. berghei* ANKA (*PbANKA*) were determined using real-time polymerase chain reaction (qRT-PCR). Tim-1 expression was significantly decreased at day 10 postinfection (p.i.) in infected mice with CM, and significantly increased at day 22 p.i. in infected mice with non-CM, compared with uninfected controls ($P < 0.01$); in contrast, Tim-3 expression was significantly increased in both CM and non-CM infected mice at days 10 and 22 p.i., respectively. Furthermore, the expression of Tim-1 and Tim-3 mRNA in spleen was significantly positively correlated with the level of Th2 and Th1 cytokine mRNA in the spleens, respectively. *PbANKA* infection could inhibit the differentiation of T lymphocytes toward Th2 cells, promote the Th1 cell differentiation, and induce Th1-biased immune response in the early infective stage in infected mice with CM; whereas the infection could promote Th2 cell

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differentiation, and induce Th2-biased immune response in the late infective stage, in infected mice with non-CM. Our data suggest that both Tim-1 and Tim-3 may play an important role in the pathogenesis of *P. berghei* infection, which may represent a potential therapeutic target.

Keywords

Cerebral malaria; *Plasmodium berghei*; rodent; immune response; Tim gene expression

Introduction

Malaria continues to be one of the leading causes of morbidity and mortality in the world, with an estimated 655,000 malaria deaths in 2010, of which 91% were in Africa; approximately 86% of malaria deaths globally were of children under 5 years of age (WHO. 2011). Malaria infection causes a life-threatening complex multisystem disorder, a wide variety of clinical symptoms, ranging from mild unspecific signs to severe forms marked by severe anemia, respiratory distress syndrome, cerebral malaria (CM), and death in a wide range of mammalian hosts (Gómez et al. 2011). However, the pathogenic processes leading to CM are incompletely understood.

T cell immunoglobulin domain and the mucin domain (Tim) family, a new gene family that is expressed on the surface of T cells, play a critical role in regulation of T cell response (McIntire et al. 2004). Tim protein was initially identified through a screen for T helper 1 (Th1)-specific and Th2-specific markers. Tim-1 and Tim-3 are both identified in mice and humans. Tim-1 is preferentially expressed in Th2 cells, but not in Th1 cells, and is involved in the development and regulation of Th2-biased immune responses; in contrast, Tim-3 is expressed on terminally differentiated Th1 cells, but not on Th2 cells, and functions to inhibit aggressive Th1-mediated immune responses (Sánchez-Fueyo et al. 2003). It has been reported that Tim-1 and Tim-3 are involved in the immune mechanisms for several pathogens causing acute and chronic infections (Rennert 2011; Zhu et al. 2011). However, the roles of Tim genes in *Plasmodium* infection remain to be investigated. Since murine CM caused by *P. berghei* ANKA (*PbANKA*) has been used as a valuable model of human disease (Jennings et al. 1997; Helegbe et al. 2011), in the present study, we used a lethal murine malaria model with *PbANKA* to investigate the immune regulation during the course of *P. berghei* infection in Kunming mice, focusing on the effects of Tim-1 and Tim-3 expression on *PbANKA* infection *in vivo*, thus providing theoretical and experimental evidence for the prevention and treatment of *Plasmodium* infection.

Materials and methods

Mice and experimental infections

Female Kunming (KM) mice (6–8-weeks old), an outbred strain, were used throughout the study. *P. berghei* ANKA (*PbANKA*) were maintained in our lab. Forty-five mice were injected intraperitoneally (i.p.) with 10^6 *PbANKA*-infected red blood cells (RBCs). Mortality was monitored daily. Mice were considered suffering from cerebral malaria (CM) if they displayed neurological signs such as ataxia, loss of reflex, or hemiplegia, and died

between days 8 and 10 postinfection (p.i.) with higher parasitaemia (n=9). The remaining mice did not exhibit obvious symptoms of CM (non-CM) and died between days 14–22 of other malaria-related pathologies (n=14). The mice were sacrificed by CO₂ asphyxiation for further examination when they became moribund after *PbANKA* infection. All experiments were performed in compliance with the requirements of the Animal Ethics Committee at Sun Yat-sen University.

Parasitaemia

For assessment of parasite multiplication in *PbANKA*-infected mice, malaria parasitaemia was monitored daily by Giemsa-stained thin blood smears of tail blood. Erythrocyte counts were performed with a hematocytometer, and more than 1,000 RBCs were counted by microscopy (100×) to determine the percentage of parasitized cells.

Histopathology

For histopathological analysis, brains from *PbANKA*-infected mice were fixed in 10% neutral-buffered formalin and then embedded in paraffin, sectioned (4 µm), and stained with hematoxylin and eosin (H&E) for evaluation of pathologic changes.

Measurement of mRNA expression using quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from about 100 mg of mouse spleen tissue using a RNA Extraction Kit (TaKaRa) according to the manufacturer's protocol. The quality of total RNA was analyzed by running 5 µl of each RNA sample on a 1.0% agarose gel stained with ethidium bromide. The quantity of total RNA was estimated by measuring the ratio of absorbance at 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). First-strand cDNA was constructed from 1.0 µg of total RNA with oligo (dT) as primers using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa) following the manufacturer's protocol. cDNA was stored at –80°C until use.

To determine interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), interleukin (IL)-12p40, IL-4, IL-10, transforming growth factor- β (TGF- β), and Tim-1 and Tim-3 mRNA levels of spleen tissues, qRT-PCR was performed using SYBR Green QPCR Master Mix (TaKaRa) according to manufacturer's instructions. Primers are listed in Table 1. Briefly, a total of 10 µl reaction mixture contained 5.0 µl of SYBR[®] Premix Ex Taq[™] (2×), 0.5 µl of each primer (10 pM), 3.0 µl of dH₂O, and 1.0 µl of cDNA (0.2 µg/µl). Amplification was pre-denatured for 30 s at 95°C followed by 43 cycles of 5 s at 95°C and 20 s at 60°C with a LightCycler[®] 480 instrument (Roche Diagnostics). Specific mRNA expression levels were normalized to that of the housekeeping gene, β -actin, and the results are expressed as fold change compared to uninfected controls.

Statistical analysis

Results of experimental studies are reported as mean $\pm$ standard deviation. However, the survival rate was expressed as the percentage of live animals. Differences were analyzed by using the Wilcoxon rank sum test and the Student *t* test, and a value of *P* < 0.05 was considered statistically significant.

Results

Survival rate of mice infected with PbANKA parasite

KM mice were infected with *PbANKA* after injection of 10^6 iRBCs, and the parasitaemia and symptoms were monitored daily. Approximately 40% (9/23) of the mice infected with *PbANKA* developed severe malaria with neurological signs and became moribund between 8 and 10 days p.i. (Fig. 1). Mice showing neurological signs (CM) usually died within a few hours. The remaining mice did not exhibit symptoms of CM (non-CM) and died between day 14–22 mainly due to other malaria-related pathologies such as hyper-parasitaemia or severe anemia without showing obvious neurological signs.

Course of parasitaemia

As shown in Fig. 2, parasites were first detected 2 days after inoculation with infection of 10^6 *PbANKA* and grew rapidly in all the infected mice up to day 4 p.i. Parasitaemia in CM mice were significantly higher than those of non-CM mice on days 7–10 p.i. ($P < 0.01$ in all the time points), and parasitaemia reached 56.9% when deaths occurred in CM mice, and parasitaemia reached 37.1% to 68.8% when deaths occurred in the non-CM mice 18–22 days p.i.

Histopathological study

To characterize *PbANKA*-induced lesion in KM mice, their brains were examined for pathological changes after infection with 10^6 *PbANKA*-infected RBCs at 8–22 days p.i. It was observed that the brain tissues of the uninfected (naive) control mice did not show any sign of RBC sequestration, leukocyte infiltration, or hemorrhage (Fig. 3A). Infected mice on days 8–10 p.i. showed moderate intravascular infiltrates consisting primarily of mononuclear cells and highly parasitized RBCs (Fig. 3B), and multifocal hemorrhages (Fig. 3C). In some instances, cerebral edema was apparent. Mice dying acutely frequently developed neurological signs including hemiplegia, ataxia, convulsions, and coma. Accumulations of mononuclear cells with highly parasitized RBCs in cerebral blood vessels were also observed in the brain tissues on day 18–22 p.i. (data not shown).

Tim-1 and Tim-3 mRNA expressions in spleens

Tim-1 and Tim-3 are expressed on terminally differentiated Th2 and Th1 cells, respectively, and participate in the regulation of Th immune response. The expressions of Tim-1 and Tim-3 on the mRNA level in spleen were determined using qRT-PCR (Fig. 4). Compared with uninfected controls, Tim-1 mRNA expression was significantly lower ($P < 0.01$) and Tim-3 expression was significantly higher ($P < 0.01$) in CM mice on day 10 p.i. However, both Tim-1 and Tim-3 mRNA expressions were significantly increased ($P < 0.01$) in non-CM mice on day 22 p.i. compared with those in uninfected controls.

Cytokine responses in spleens

It is established that cytokines are involved in the development of experimental CM. Thus, the cytokine responses were evaluated by measuring IFN- γ , TNF- α , IL-12p40, IL-4, IL-10, and TGF- β mRNA expressions in the spleens of *PbANKA*-infected mice. Compared with

naive control mice, levels of all the Th1 cytokines (IFN- γ , TNF- α , and IL-12p40) and IL-10 were significantly increased, while Th2 cytokine (IL-4) was significantly decreased in spleens in CM mice on day 10 p.i. ($P < 0.01$). In contrast, only the mRNA level of IFN- γ was significantly increased ($P < 0.01$) but levels of both TNF- α and IL-12p40 were significantly decreased ($P < 0.01$), while mRNA levels of Th2 cytokines (IL-4, IL-10, and TGF- β) were significantly increased ($P < 0.01$) in non-CM mice on day 22 p.i. compared with those in uninfected controls (Fig. 5). The change of Th2 cytokine had the similar tendency as that of Tim-1 expression; alternatively, the change of Th1 cytokine had the similar tendency as that of Tim-3 expression in *PbANKA*-infected mice.

Discussion

CM is one of the most significant complications and a primary cause of death, from *P. falciparum* (John et al. 2008) and is characterized by coma, presence of peripheral asexual *P. falciparum* parasites, and exclusion of other causes of encephalopathy. Although inflammatory cytokines have been demonstrated to be critical in experimental CM, it is unclear if inflammatory cytokines have a causal role in human CM due to the difficulty of conducting mechanistic studies in humans (Serghides et al. 2011). Experimental CM caused by *PbANKA* infection in mice displays key features of human CM (Engwerda et al. 2005). In the present study, parasitaemia was assessed from Giemsa-stained thin smears of tail blood prepared every day p.i., and the parasitaemia levels in CM mice were significantly higher as compared with those in non-CM mice on days 7–10 p.i. ($P < 0.01$).

Histopathological changes in brains of KM mice infected with *PbANKA* were analyzed to further define the disease. Our data showed that accumulation of mononuclear cells with highly parasitized RBCs in cerebral blood vessels in both CM and non-CM mice; cerebral edema and hemorrhages were observed in the brains of CM mice infected with *PbANKA*. Jennings et al. (1997) reported that murine CM is characterized by widespread damage to the microvasculature in the brain with focal infiltration of inflammatory cells. Endothelial-cell damage in addition to obstruction of the microcirculation by parasitized RBCs has been reported in human *falciparum* malaria (Porta et al. 1993). Some investigators report that the murine model can duplicate the pathologic changes of the human disease rather well (Polder et al. 1991; Patnaik et al. 1994). KM mice are the most widely used outbred colony in China, started from Swiss mice brought to Kunming of China, from the Indian Haffkine Institute in 1944 (Shang et al. 2009). In this paper, we showed there was a high mortality and typical murine histopathology of CM in KM mice infected with *PbANKA*, suggesting that KM mice may be a good alternative animal model for the study of lethal murine malaria.

Tim-1 and Tim-3 are proteins belonging to the T cell immunoglobulin and mucin domain family, which are being studied in the context of immune regulation. Tim gene family encoding type I membrane glycoproteins have been shown to be expressed on T cells that are involved in the differentiation of CD4⁺ T cells and the regulation of Th1 and Th2 cell mediated immunity. McIntire et al. (2004) reported that the Tim proteins are expressed by activated spleen cells and T cells, and Tim family members are important in regulation of T cell activation; Tim-1 is expressed by T cells during Th2 differentiation, and Tim-3 is specifically expressed by Th1 cell lines, but not Th2 cell lines. Although initially identified as regulators of T helper cell differentiation, these proteins seem also involved in the

regulation of regulatory T cells (Tim-1), are expressed in cells other than T cells (Tim-3, in dendritic cells) and may also be potentially involved in the regulation of monocytes and other cell types (Su et al. 2008). Fatal murine CM is an immunopathological process mediated by proinflammatory cytokines, which has been characterized by inflammation in the central nervous system, with monocyte adherence to the endothelium of the microvasculature (Ma et al. 1997). Although CM is a major life-threatening complication of *P. falciparum* infection, its pathophysiology is not well understood. So far no references exist in respect to their involvement in malaria pathology. In the present study, the expressions of Tim-1 and Tim-3 in spleen lymphocytes from *PbANKA*-infected mice were examined. We demonstrated that Tim-1 in spleens of *PbANKA*-infected mice was under-expressed and Tim-3 was over-expressed in CM mice at 10 days p.i. compared with uninfected controls; however, both Tim-1 and Tim-3 were over expressed in non-CM mice at 22 days p.i. Therefore, reduced Tim-1 and increased Tim-3 expression may explain the expansion of pathogenic subset of T cells in murine CM.

Cytokines are important immune mediators and regulators with both protective and pathogenic functions in malaria (Engwerda et al. 2005; Good et al. 2005). IFN- γ and Lymphotoxin α (LT α) are critical mediators of *PbANKA* tissue sequestration, and IL-12 plays an important role in the adaptive immune response to malaria (Malaguarnera and Musumeci. 2002; Amante et al. 2010). TNF- α has both beneficial and detrimental effects in malaria infections. It has been reported that elevated serum concentrations of TNF- α during malaria correlate strongly with increasing severity of malaria in both humans and mice (Grau et al. 1987; Othoro et al. 1999). In the present study, our data showed that cytokine mRNA expression of IFN- γ , TNF- α , and IL-12p40 were significantly increased in the spleens of *PbANKA*-infected KM mice with CM at 10 days p.i., while TNF- α and IL-12p40 were significantly decreased in infected mice with non-CM at 22 days p.i. It has been reported that the difference between lethal and nonlethal infections can be explained in part by the ability of the mice to mount an early IFN- γ , TNF- α , or IL-12 response; however, overproduction of IFN- γ or TNF- α predisposes to severe pathology (Shear et al. 1989; Jacobs et al. 1996). Prolonged activation of Th1 response characterized by the production of pro-inflammatory cytokines such as IFN- γ and TNF- α has been suggested to be responsible for immunopathological process leading to cerebral malaria unless they are downregulated by the anti-inflammatory cytokines produced by the Th2 response (Nuchnoi et al. 2008). Taken together, some Th1 cytokines (e.g., IFN- γ , lymphotoxin, and TNF- α) have been implicated in driving the immunopathological process leading to CM, whereas some Th2 cytokines (e.g., IL-10 and TGF- β) appear to oppose this process (Hunt and Grau. 2003). Our data showed that IL-4 decreased in mice with CM at 10 days p.i., while IL-4 and TGF- β increased in mice with non-CM at 22 days p.i., and IL-10 increased in both CM and non-CM mice on days 10 and 22 p.i., respectively. Elevated levels of anti-inflammatory IL-10 in severe malaria have been reported (Sarhou et al. 1997). In Vietnamese adults with severe malaria, plasma IL-10 levels were higher in those who died than in those who survived but CM victims had lower levels than the others (Hunt and Grau. 2003). IL-10 seems to have a host-protective role in murine malaria (Hunt and Grau. 2003) and is important for preventing and limiting pathology associated with different experimental malaria models (Amante et al. 2010). TGF- β is an important regulator of inflammation, being

proinflammatory at low concentrations and anti-inflammatory at high concentrations (Omer et al. 2000). Susceptibility to *P. berghei* ANKA infection shows reduced expression of TGF- β mRNA compared with resistant strains of mice, indicating that TGF- β may be important in maintaining the balance between protection and pathology (de Kossodo and Grau. 1993). Experimental mouse models of CM point to an important role for the balance between Th1 and Th2 cytokines in the complications associated with CM (Omer et al. 2000). Thus, cytokines can determine the outcome of CM, and it is possible that a relative overproduction of proinflammatory cytokines or underproductions of anti-inflammatory cytokines are factors that explain the variation seen in susceptibility to CM malaria in human populations and mouse strains (Hunt and Grau. 2003). The Tim family of proteins is cell surface proteins involved in regulating Th1 and Th2 immune responses. Therefore, engagement with Tim-1 in combination with T cell receptor stimulation induces anti-inflammatory Th2 cytokine production, which can protect the development of CM caused by overproduction of pro-inflammatory Th1 cytokines; higher Tim-1 expression associated with the protective Tim-1 promoter haplotype confers protection against CM (Nuchnoi et al. 2008).

In conclusion, rodent experimental models have been helpful in the study of severe malaria. In the present investigation, *Pb*ANKA downregulates the expression of Tim-1 and upregulates the expression of Tim-3 *in vivo* in the early infective stage in mice with CM; upregulates both Tim-1 and Tim-3 in the late infective stage in mice with non-CM, indicating that *Pb*ANKA could inhibit the differentiation of T lymphocytes toward Th2 cells, promote the Th1 cell differentiation, and induce Th1-biased immune response in CM mice, while promoting the Th2 cell differentiation and inducing Th2-biased immune response in non-CM mice. The expression of Tim-1 and Tim-3 could reflect Th2 and Th1 immune response, respectively, which provides evidence for the prevention and treatment of *Pb*ANKA infection and correlation of diseases through regulation of Tim-1 and Tim-3. The precise molecular pathways by which Tim-1 and Tim-3 control immunity require further elucidation.

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Fig. 1.

Time course of survival of KM mice infected with *PbANKA*-parasitized RBCs (n=23). The mice were monitored daily for morbidity or mortality until the termination of the experiment. The experiment was performed twice with similar results.

Fig. 2.

Time course of parasitaemia in infected mice. Peripheral parasitaemia was assessed at the indicated times from Giemsa-stained blood smears. KM mice were infected with 10^6 *PbANKA*-parasitized RBCs. CM = mice that developed CM on day 8–10 after infection (n=9); non-CM = mice that did not develop CM and died on day 14–22 after infection (n=14). Each point of parasitaemia is expressed as mean $\pm$ SEM. The data presented represent one of these infections; however, the two infections provided similar results. *, $P < 0.01$ (compared to control).

Fig. 3.

Histological examination of KM mice infected with *PbANKA*. KM mice were injected i.p. with 10^6 *PbANKA*-parasitized RBCs. Histological examination of the brains of *PbANKA*-infected mice (n=4) were dissected at day 8–10 p.i., and sections of brains were stained with H&E and analyzed with light microscopy. Section of normal mouse brain showing a healthy unaffected blood vessel (A), cerebral blood vessels were packed with numerous mononuclear cells and parasitized RBCs at day 9 p.i. (B), and multifocal hemorrhages with parasitized RBCs in the brain at day 8 p.i. (C). Magnification, $\times 100$.

Fig. 4.

Tim-1 and Tim-3 mRNA expressions in spleens were analyzed using real-time quantitative polymerase chain reaction. Mice were at days 10 and 22 post i.p. injection of 10^6 *PbANKA*. The values were shown as a fold change to the non-infected control. There were four mice per group, and data are representative of two separate experiments. Values are means from triplicate measurements. *, $P < 0.01$ (compared to control).

Fig. 5. mRNA cytokine expressions in spleens were analyzed using real-time quantitative polymerase chain reaction. Mice were at days 10 and 22 post i.p. injection of 10^6 *PbANKA*. The values were shown as a fold change to the non-infected control. There were four mice per group, and data are representative of two separate experiments. Values are means from triplicate measurements. *, $P < 0.01$ (compared to control).

Table 1

Primer sequences of mouse target cytokines and housekeeping genes used for quantitative real-time polymerase chain reaction (qRT-PCR) assays

Genes	Primer sequence (5'→3')		References
IFN- γ	Forward primer	GGAAGTGGCAAAAGGATGGTGAC	Jones et al. (2010)
	Reverse primer	GCTGGACCTGTGGGTGTTGAC	
TNF- α	Forward primer	CCCTCACACTCAGATCATCTTCT	Zhao et al. (2011)
	Reverse primer	GCTACGACGTGGGCTACAG	
IL-4	Forward primer	ACAGGAGAAGGGACGCCAT	Jash et al. (2011)
	Reverse primer	GAAGCCCTACAGACGAGCTCA	
IL-10	Forward primer	AGCCGGAAGACAATAACTG	Jones et al. (2010)
	Reverse primer	CATTTCCGATAAGGCTTGG	
IL-12p40	Forward primer	CCTGGTTTGCCATCGTTTTG	Jones et al. (2010)
	Reverse primer	TCAGAGTCTCGCCTCCTTTGTG	
TGF- β	Forward primer	AACTATTGCTTCAGCTCCACAG	Siddiqui et al. (2012)
	Reverse primer	AGTTGGCATGGTAGCCCTTG	
Tim 1	Forward primer	TTCTCCCAGGCACTGTGGAT	Xu et al. (2008)
	Reverse primer	CAGGAATCTCCACTCGACAA	
Tim 3	Forward primer	CCACGGAGAGAAATGGTTC	Geng et al. (2006)
	Reverse primer	CATCAGCCCATGTGGAAAT	
β -actin	Forward primer	TGGAATCCTGTGGCATCCATGAAAC	Jones et al. (2010)
	Reverse primer	TAAACGCAGCTCAGTAACAGTCCG	