

# Mast cell-mediated immune responses through IgE antibody and Toll-like receptor 4 by malarial peroxiredoxin

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In this study, 2-Cys *Plasmodium berghei* ANKA (PbA) peroxiredoxin (Prx) was identified as an antigenic protein recognized by an anti-PbA IgE antibody using two-dimensional polyacrylamide gel electrophoresis and proteomic analysis. Innate immune responses to PbAPrx were examined using cells from mice deficient in Toll-like receptors (TLR) or related molecules, and it was demonstrated that responses were severely impaired in TLR4<sup>-/-</sup>, MyD88<sup>-/-</sup> and MD-2<sup>-/-</sup> mice, but not in Toll/IL-1 receptor domain-containing adaptor inducing IFN- $\gamma$  (TRIF)<sup>-/-</sup>, TLR2<sup>-/-</sup> or radioprotective 105 (RP105)<sup>-/-</sup> mice. An association between PbAPrx and TLR4 was observed following immunoprecipitation and immunoblotting, suggesting that PbAPrx was associated with TLR4/MD-2. Interactions between Prx and TLR4/MD-2 were also examined by flow cytometry using TLR4/MD-2- or TLR2-expressing cells. NF $\kappa$ B/GFP activity was observed in TLR4/MD-2- but not in TLR2-expressing cells following stimulation with Prx. However, this effect was not observed after treatment with proteinase K, suggesting that PbAPrx is a protein ligand for TLR4 and that the PbAPrx activity observed in this study is not due to contamination with LPS. These findings indicate that malarial Prx induces IgE-mediated protection through Fc $\epsilon$ RI on mast cells and innate immunity through TLR4 with MyD88 and MD-2, suggesting a novel function for malarial Prx in innate and acquired immune responses in malaria.

**Key words:** Immune response · Malaria · Peroxiredoxin

## Introduction

Malaria remains the world's primary parasitic disease. Every year there are approximately 300–500 million episodes of acute malaria, and between 1 and 3 million people die from the disease, most of them being children in Africa under the age of five [1]. Due to its complex life cycle, the rapid changes in its surface antigens

and the interactions between the parasite and the host, innate responses to the parasite are still poorly understood, despite the urgent need for effective immunotherapeutic interventions. Specific immunity to natural infection of malarial parasites is acquired slowly, despite a high degree of repeated exposure to parasite antigens. Malarial parasites have devised many strategies to escape the host's immune response, but the host innate immune system may have evolved the ability to detect parasite-specific molecules.

The pathogenesis of malaria is complex, and it is possible that the immune system may mediate both protection from malaria and

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development of the disease. Many studies [2–4] indicate that elevations in immune mediators such as IL-1, IL-6, IL-8, IL-10, TNF and NO have been associated with disease severity. In particular, high concentrations of TNF have been shown to be associated with disease severity in human *Plasmodium falciparum* infections and several animal malarias [2]. Mast cells can be a major source of TNF [5], suggesting that they may play an important role in mediating protection from malaria and in disease progression. Despite these observations, little is known about the relationship between mast cells and the pathogenesis of malaria.

Recent studies performed by our group suggested an important role for mast cells and mast cell-derived TNF in mediating protection against murine malaria [6]. When mast cell-deficient WBB6F<sub>1</sub>-W/W<sup>v</sup> (W/W<sup>v</sup>) mice and the control littermates WBB6F<sub>1</sub>-+/+ (+/+) were infected with *P. berghei* ANKA (PbA), +/+ mice were found to have lower parasitemia and higher TNF levels compared to W/W<sup>v</sup> mice. The diminished resistance in W/W<sup>v</sup> mice was hypothesized to be due to the deficiency of mast cells and reduced TNF levels. This was confirmed by transferring bone marrow-derived mast cells (BMMC) from +/+ or TNF<sup>-/-</sup> mice into W/W<sup>v</sup> mice. W/W<sup>v</sup> mice with BMMC from +/+ mice exhibited lower parasitemia and mortality in conjunction with significantly higher TNF levels than untreated W/W<sup>v</sup> mice. However, parasitemia was higher in W/W<sup>v</sup> mice with BMMC from TNF<sup>-/-</sup> mice than in +/+ mice. Furthermore, mast cells stimulated by cross-linking FcεRI using an anti-IgE antibody, or by chemical activation using compound 48/80, resulted in the initiation of TNF release and suppressed parasite growth. Thus, IgE-mediated protection through FcεRI on mast cells could be demonstrated in murine malaria and was associated with an increase in serum TNF levels. This suggests that the anti-PbA IgE antibody may play an important role in controlling the host-parasite relationship in malaria. We therefore decided to investigate the immunoreactive proteins recognized by this antibody and hence to examine the possible roles of those molecules in malaria.

We report here that *Plasmodium* 2-Cys peroxiredoxin (Prx), which is an important enzyme involved in the detoxification of H<sub>2</sub>O<sub>2</sub> and NO derived from malarial parasites, induces IgE-mediated protection through FcεRI on mast cells and stimulates Toll-like receptor (TLR)4 on both mast cells and macrophages through a MyD88-dependent pathway to induce TNF production. This is the first reported evidence that Prx from malarial parasites contains a protein ligand for TLR4 and induces an acquired immune response as well as innate immunity.

## Results

### IgE antibody-mediated resistance against PbA infection

To evaluate IgE antibody-mediated protection against PbA infection *in vivo*, PbA-infected mice were treated with the rat anti-murine IgE mAb, 6HD5, to inhibit the function of IgE antibodies. After  $1 \times 10^5$  PbA were inoculated i.v. into mice, anti-IgE antibody-treated mice developed significantly higher para-

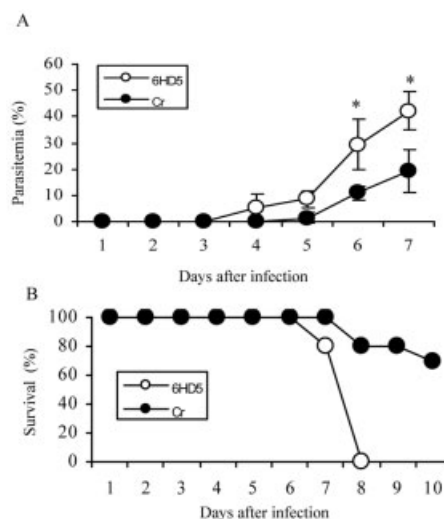


**Figure 1.** Effect of anti-IgE mAb on PbA infection. C57BL/6 mice were injected i.p. with 20 µg anti-IgE mAb (6HD5) or normal rat IgG2a (Cr) after inoculating with  $1 \times 10^5$  PbA-infected erythrocytes. Injection of the antibodies was repeated every third day until the end of the experiment. The percentage parasitemia (A) and survival period (B) were examined following infection. These experiments were repeated three times with similar results;  $n=5$  for each group. \* $p<0.01$ .

sitemia than control mice, as indicated by higher daily parasitemia, and their survival period was shorter than that of control mice (Fig. 1A and B). These findings suggest the presence of IgE antibody-mediated resistance against PbA infection.

### PbAPrx recognized by anti-PbA IgE antibody

To identify antigens recognized by the anti-PbA IgE antibody, PbA protein was subjected to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and gels were then immunoblotted. Subsequently, spots that appeared on the Coomassie brilliant blue (CBB)-stained gel in a comparable location to those on the immunostained polyvinylidene difluoride (PVDF) membrane were excised for mass spectrometric analysis. In total, 65 spots were identified on 2D gels stained with CBB; however, only one spot on the PVDF membrane was strongly positive when immunoblotting was performed with PbA-infected sera and the anti-mouse IgE antibody (Fig. 2A). Peptide mass fingerprinting revealed that this protein was a Prx family member. To confirm whether an IgE antibody to PbAPrx is produced in PbA-infected mice, we prepared a recombinant PbAPrx protein for use in an ELISA to detect anti-PbAPrx antibody in murine malaria. To exclude the possibility that LPS contamination could influence the experimental data, PbAPrx and *P. falciparum* Prx (PfPrx) samples were pretreated with polymyxin B (PolyB) in all experiments, as described in *Materials and methods* (Fig. 3). When the sera from PbA-infected mice (day 12 after the infection) were reacted with recombinant PbAPrx, the OD was significantly higher than that of the sera from normal mice. However, when these infected sera were preabsorbed with recombinant PbAPrx protein, the OD was

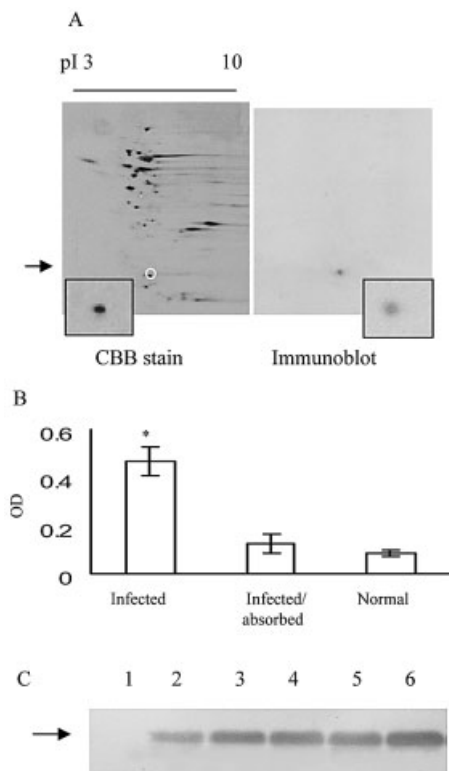


**Figure 2.** Identification of antigenic proteins from PbA protein. (A) Proteins were separated by 2D-PAGE and electroblotted onto PVDF membranes, then probed with anti-PbA mouse sera. Left: PbA protein spots in the CBB staining gel; the spot detected in the corresponding PVDF membrane with immunoblot is indicated by a circle. Right: an antigenic spot detected by immunoblotting. Inset: close-up of the spot in the CBB staining gel or PVDF membrane. The arrow indicates the location of the 25-kDa PbAPrx protein. (B) Recombinant PbAPrx protein was prepared and used in an ELISA to detect IgE anti-PbAPrx antibody in murine malaria. Infected: sera from PbA-infected mice; infected/absorbed: infected sera preabsorbed with PbAPrx; normal: sera from normal C57BL/6 mice;  $n=6$  for each group. These experiments were performed three times with similar results.  $*p<0.01$ . (C) Detection of IgE antibody to PbAPrx by immunoblotting in murine malaria. PbAPrx protein was separated by 10% SDS-PAGE gels and transferred to PVDF membranes for immunoblotting. Lane 1: normal mouse serum; lanes 2–5: sera from separate mice infected with PbA; lane 6: rabbit anti-PbAPrx-immunized serum as a positive control. Arrows indicate the position of the 25-kDa band for PbAPrx.

significantly diminished and comparable to the levels of sera from normal mice (Fig. 2B). In the next studies, we confirmed the presence of an IgE antibody to PbAPrx in murine malaria by SDS-PAGE and immunoblotting. When sera obtained from PbA-infected mice (day 12 after infection) were reacted with recombinant PbAPrx protein, a 25-kDa band was observed with the sera from infected mice but not with control sera. Furthermore, the size of this band corresponded with that of a band reacting with anti-PbAPrx-immunized sera (Fig. 2C). Therefore, the results suggest the production of an IgE antibody against PbAPrx in murine malaria.

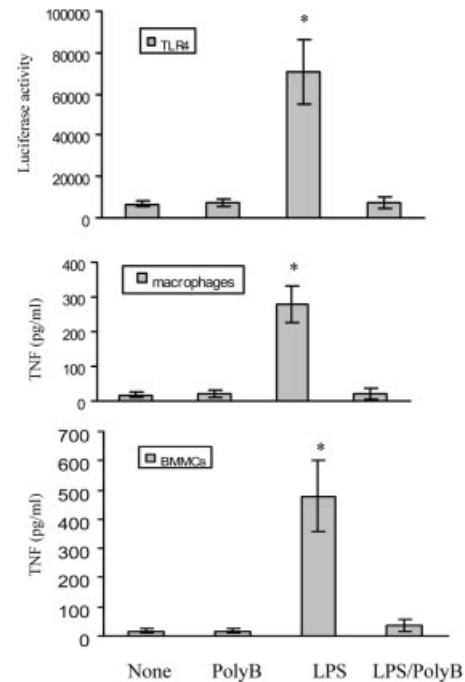


**Figure 3.** Inhibition of LPS-mediated NFκB and TNF by PolyB. TLR4 transfectants, peritoneal Mφ and BMDC were prepared as described in the Materials and methods section, and were treated with LPS in the presence or absence of PolyB. NFκB activity was measured by luciferase assay, and the TNF level in the culture supernatant was measured by ELISA. The same experiments were performed three times and representative results are shown.  $*p<0.01$ .

### TLR4 agonistic activity of PbAPrx

Ba/F3 cells expressing murine (m) TLR2 or mTLR4/mMD-2 and p55Ig κLuc with an NFκB-dependent luciferase reporter construct were stimulated with malarial Prx, and NFκB activity was examined by using the luciferase assay to determine whether Prx activates the signaling via TLR. When these transfectants were stimulated with malarial Prx, increased NFκB activity was observed in mTLR4/mMD-2-expressing cells, and PbAPrx-induced NFκB activation was observed in a dose-dependent manner in transfectants expressing mTLR4/mMD-2 but not mTLR2 (Fig. 4A and B). Since Prx has high homology among *Plasmodium* species, the ability of PfPrx to stimulate transfectants expressing mTLR4/mMD-2 was also examined. The results showed that PfPrx was also able to induce NFκB activation via mTLR4/mMD-2 in a dose-dependent manner (Fig. 4B).

### PbAPrx-induced activation of cells through TLR4/MD-2- and MyD88-dependent pathways

Further experiments were performed using peritoneal Mφ and bone marrow-derived DC (BMDC) obtained from mice lacking TLR2, TLR4 or MyD88, to investigate the possible roles of TLR in murine malaria. Peritoneal Mφ and BMDC obtained from TLR2<sup>-/-</sup>,

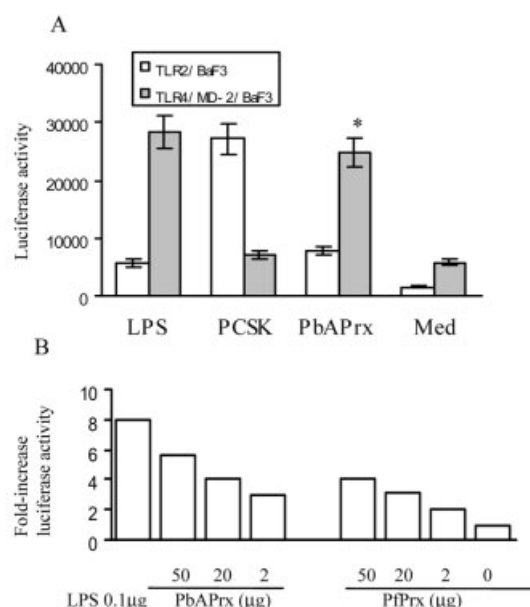


**Figure 4.** TLR4-mediated activation of NFκB by malarial Prx. (A) Ba/F3 cells expressing mTLR2 or mTLR4/MD-2 and p55IgκLuc with an NFκB-dependent luciferase reporter construct were cultured in 96-well plates with or without PbAPrx (1 μg/well). LPS (0.1 μg/well) or PCSK (0.1 μg/well) was used as ligand for TLR4 or TLR2. NFκB activity was examined by luciferase assay. \* $p < 0.01$  compared with TLR2 or medium alone (Med). (B) mTLR4/MD-2 transfectants were stimulated with various doses of PbAPrx or PfPrx. The results are presented as the fold-increase compared to control cultures. These experiments were performed three times with similar results.

TLR4<sup>-/-</sup>, MyD88<sup>-/-</sup> or WT mice were stimulated with PbAPrx to examine cytokine production *in vitro*. Mφ from TLR4<sup>-/-</sup> or MyD88<sup>-/-</sup> mice failed to induce TNF production upon stimulation with PbAPrx as compared with those from TLR2<sup>-/-</sup> or WT mice (Fig. 5A–C). Similarly, BMDC from TLR4<sup>-/-</sup> or MyD88<sup>-/-</sup> mice also could not induce IL-12p40 production following PbAPrx stimulation (Fig. 5D–F). These results suggest that TLR4 and MyD88 are critical for Prx-induced activation of murine Mφ and BMDC.

To confirm that a PbAPrx-induced innate immune response is solely dependent on MyD88, mice lacking Toll/IL-1 receptor domain-containing adaptor inducing IFN-γ (TRIF), an essential adaptor molecule for the MyD88-independent pathway, were used. When peritoneal Mφ from TRIF<sup>-/-</sup> or WT mice were stimulated with PbAPrx to examine cytokine responses, TNF production in Mφ from TRIF<sup>-/-</sup> was found to be slightly lower but not significantly different to that of cells from WT mice (Fig. 5K). These data indicate that MyD88-dependent but TRIF-independent TLR4 signaling pathways are involved in the recognition of PbAPrx.

Mast cells can be activated by molecules involved in innate immunity. Although the mechanisms for the direct activation of mast cells by various microorganisms have not been studied precisely, the activation of mast cells by various bacteria has been shown to be mediated by TLR2 or TLR4. To examine the possibility that PbAPrx-induced cytokine production is mediated by the TLR4

signaling pathway, we stimulated BMMC obtained from TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup>, MyD88<sup>-/-</sup> or WT mice. TNF production was observed for BMMC obtained from TLR2<sup>-/-</sup> and WT mice, but not in cells from TLR4<sup>-/-</sup> or MyD88<sup>-/-</sup> mice (Fig. 5G–J), suggesting that Prx-induced TNF production in BMMC is similarly mediated by TLR4.

In the recognition of LPS by TLR4, TLR4 requires association with MD-2, and a complex of TLR4 with MD-2 binds LPS [7]. Recently, it has been reported that signaling following LPS activation of TLR4/MD-2 is regulated by radioprotective 105 (RP105)/MD-1 expressed on the surface of cells, which acts as a competitive inhibitor of LPS binding to TLR4/MD-2 complexes [8]. To examine the possibility that MD-2 or RP105 is involved in the stimulation of TLR4 by PbAPrx and thus accounts for the biological activity observed with PbAPrx, we stimulated peritoneal Mφ from MD-2<sup>-/-</sup>, RP105<sup>-/-</sup> or WT mice with PbAPrx and compared the levels of TNF produced. Results showed that TNF production in Mφ from MD-2<sup>-/-</sup> mice was significantly lower than that in Mφ from RP105<sup>-/-</sup> or WT mice (Fig. 5L), implying that MD-2 but not RP105 is involved in the recognition of PbAPrx by TLR4.

#### Activation of malarial Prx through the cell surface TLR4/MD-2/CD14 complex

We examined whether Prx activates cells through the TLR4/MD-2/CD14 complex present on the cell surface. Ba/F3 cells expressing TLR4/MD-2/CD14 were stimulated with LPS or malarial Prx, followed by flow cytometric analysis of GFP expression. After stimulation with LPS or PbAPrx, increased GFP expression was found in TLR4/MD-2/CD14-expressing Ba/F3 cells but not in control cells (Fig. 6A; LPS and PbAPrx; arrows 1 and 3). To examine the possibility that LPS contamination of Prx affects cell activation, LPS or malarial Prx was incubated with proteinase K (PK) prior to activation of these cells. After treatment of LPS or Prx with PK, cell activation was observed with PK-treated LPS but not with PK-treated Prx (Fig. 6A; LPS/PK and PbAPrx/PK; arrows 2 and 4). This data suggests that TLR4-mediated innate immune activation by malarial Prx is not due to LPS contamination.

To confirm the association between PbAPrx and TLR4, Ba/F3 cells expressing TLR4/MD-2 or TLR2 that had been tagged with the flag/His6 epitope were incubated with PbAPrx and then lysed with a lysis buffer. PbAPrx bound to cell surface TLR was precipitated with anti-flag (M2) agarose gels and subjected to SDS-PAGE and immunoblotting. The precipitated bands were probed with anti-TLR4 or anti-TLR2 antibody (Fig. 6B, upper panel), or a rabbit anti-PbAPrx antibody (Fig. 6B, lower panel). As shown in Fig. 6B, the precipitated band recognized by the anti-PbAPrx antibody was associated with TLR4/MD-2 but not TLR2 (Fig. 6B, lower panel; lanes 2 and 4), suggesting that PbAPrx associates directly with TLR4/MD-2 and then stimulates MyD88-dependent and TRIF-independent signaling pathways in murine malaria.

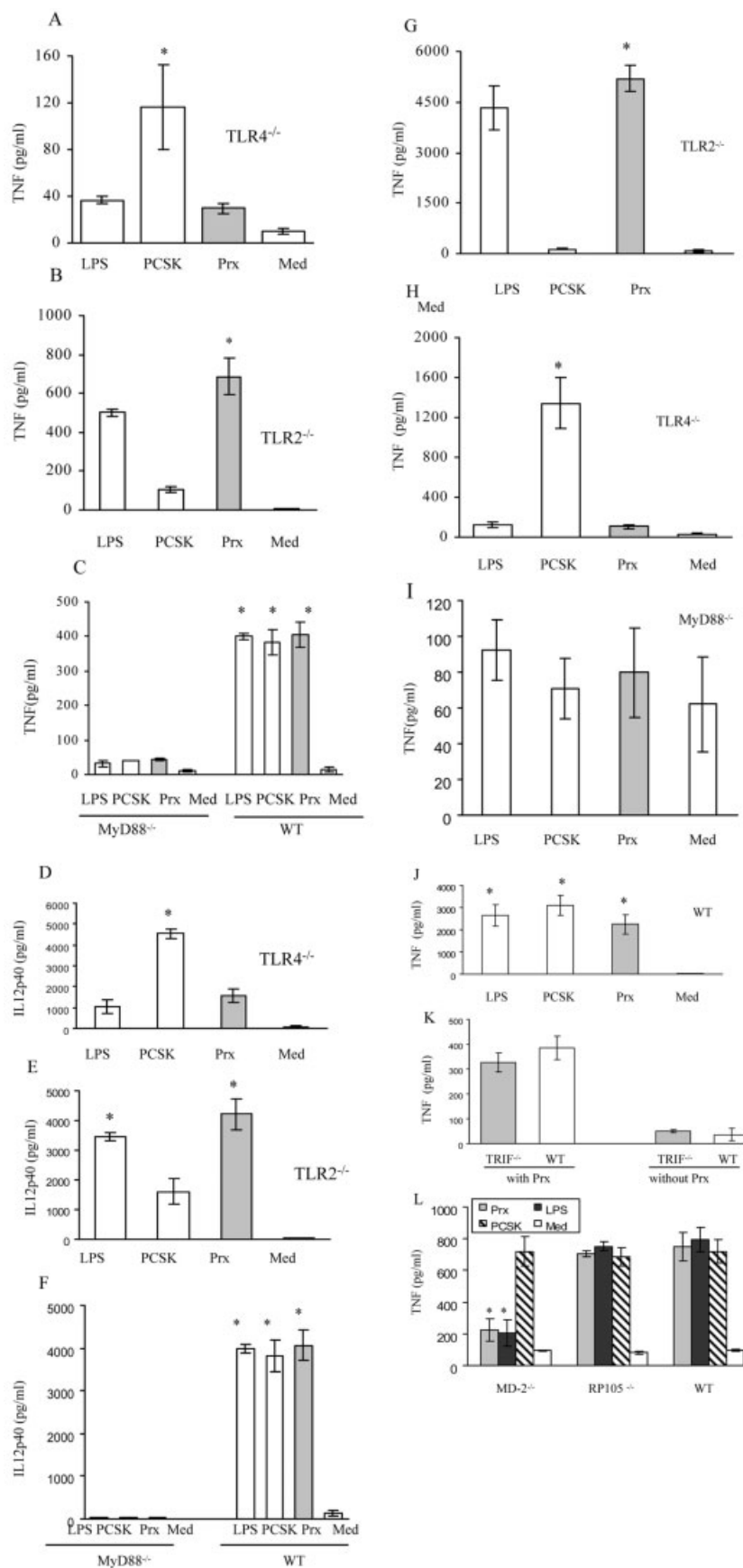


**Figure 5.** Cytokine production by cells stimulated with malarial Prx. Peritoneal M $\phi$  (A–C), BMDC (D–F) or BMMC (G–I) from  $TLR2^{-/-}$ ,  $TLR4^{-/-}$ ,  $MyD88^{-/-}$  or WT mice were cultured in 96-well plates with Prx (1  $\mu$ g/well). LPS or PCSK was used as ligand for TLR4 or TLR2, and Med indicates that cells were cultured with medium alone. Cytokine production was then measured by ELISA. (K) Peritoneal M $\phi$  from  $TRIF^{-/-}$  or WT mice were cultured with or without Prx. (L) Peritoneal M $\phi$  from RP105 $^{-/-}$ , MD-2 $^{-/-}$  or WT mice were cultured with Prx, LPS, PCSK or medium alone. These experiments were performed three times with similar results. The data are expressed as the mean  $\pm$  SD. \* $p < 0.01$ .

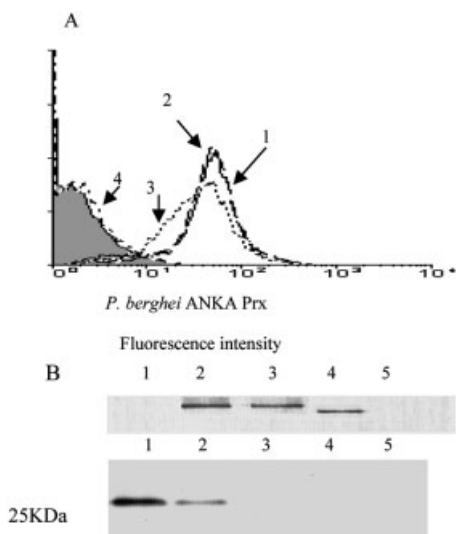


**Figure 6.** (A) Activation of malarial Prx through the TLR4/MD-2/CD14 complex on the cell surface. Ba/F3 cells expressing mTLR4/mMD-2/CD14/NF $\kappa$ B/GFP were cultured in 96-well plates with PbAPrx (1  $\mu$ g/well) or LPS (0.1  $\mu$ g/well). In some experiments PbAPrx and LPS were pretreated with PK, prior to use. Arrow 1: LPS; arrow 2: LPS/PK; arrow 3: Prx; arrow 4: Prx/PK; dark area: medium alone. (B) Direct association of PbAPrx with TLR4. PbAPrx was incubated with Ba/F3 cells stably expressing mTLR2 or mTLR4/mMD-2. The cells were lysed and precipitated with anti-flag (M2) affinity gel. Precipitants were subjected to SDS-PAGE and immunoblotting. The band was probed with anti-TLR4 or -TLR2 antibody (upper panel) or anti-PbAPrx antibody (lower panel). Lane 1: PbAPrx alone; lane 2: TLR4/MD-2 with PbAPrx; lane 3: TLR4/MD-2 alone; lane 4: TLR2 with PbAPrx; lane 5: Ba/F3 (without TLR) with PbAPrx.

### Activation of pro-inflammatory cytokines by PbAPrx is MyD88 and TLR4 dependent *in vivo*

To further confirm that PbAPrx activation is mediated by TLR4 and is dependent upon MyD88 and TRIF *in vivo*, PbAPrx was injected i.p. into WT, MyD88 $^{-/-}$ , TRIF $^{-/-}$  and TLR4 $^{-/-}$  mice, and the serum cytokine levels were examined by ELISA. PbAPrx injection led to a significantly increase in serum TNF and IL-12p40 levels in TRIF $^{-/-}$  and WT mice at 2 and 4 h after injection. In contrast, such increases were completely abrogated in MyD88 $^{-/-}$  and TLR4 $^{-/-}$  mice (Fig. 7). These data clearly demonstrate that PbAPrx-induced pro-inflammatory responses were mediated by MyD88 and TLR4 both *in vitro* and *in vivo*.

### Discussion

In the present study, we defined *Plasmodium* 2-Cys Prx as a molecule recognized by an anti-PbA specific IgE antibody in murine malaria. This Prx molecule stimulated TLR4 on both mast cells and macrophages through a MyD88-dependent pathway to induce TNF production, suggesting that PbAPrx has an agonistic activity for TLR4 and induced an innate immune response. This is the first reported evidence that *Plasmodium* 2-Cys Prx functions as

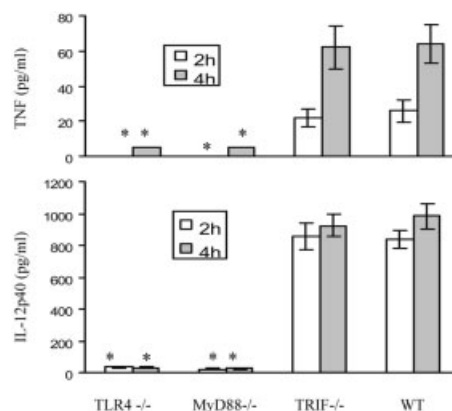


**Figure 7.** Cytokine production by PbAPrx *in vivo* is dependent on TLR4 with MyD88. Five TLR4 $^{-/-}$ , MyD88 $^{-/-}$ , TRIF $^{-/-}$  or WT mice were injected i.p. with 500  $\mu$ g PbAPrx. The serum levels of TNF and IL-12p40 were measured by ELISA at 2 and 4 h after injection. The experiment was performed three times with similar results;  $n=5$  for each group. \* $p<0.01$ .

a protein ligand for TLR4 and induces an acquired immune response as well as innate immunity. Direct association between PbAPrx and TLR4 was confirmed by SDS-PAGE and immunoblotting after the precipitation, suggesting that malarial Prx was directly recognized by TLR4/MD-2. Association of Prx with the TLR4/MD-2 complex was also confirmed using TLR4/MD-2/CD14/GFP- or TLR2/GFP-expressing cells by flow cytometry. A significant amount of GFP activity was observed in TLR4/MD-2/CD14/GFP but not TLR2/GFP cells when stimulated with PbAPrx. However, these activities disappeared after treatment with PK, which is a serine protease that exhibits broad cleavage specificity for the digestion of proteins in biological samples, suggesting that malarial Prx is a protein ligand for TLR4. These results provide the first evidence that malarial Prx associates directly with TLR4. Recently, glycosyl phosphatidylinositol (GPI) [9] and hemozoin derived from *Plasmodium* parasites [10, 11] have been identified as the ligands for TLR2 and TLR9, respectively. However, regarding TLR4 ligands in malarial studies, so far, there is no reported evidence.

We suggested that the immunoreactive PbA protein recognized by the anti-PbA IgE antibody was PbAPrx. This protein was recognized by sera from mice infected with PbA but not by those from uninfected mice (Fig. 2B and C), implying that Prx is expressed during infection and stimulates IgE antibody production, which has protective roles in murine malaria (Fig. 1) [6]. In the present study, we observed one spot with clearly strong positive signal on immunoblots using PbA-infected sera. However, when 2D-PAGE and immunoblot are carried out with different conditions of 2D-PAGE, different preparations of parasite lysates or different sources of IgE antibodies (PbA-infected or parasite antigen-immunized sera) for detecting positive spots in immunoblots, more spots might be observed. To examine the possibility that malarial Prx induces both IgG and IgE antibody production, the level of anti-PbAPrx IgG antibody in murine malaria was measured by ELISA. Since the levels of IgG antibody were highly

increased 12 days after infection (data not shown), it is thought that PbAPrx induces IgE and IgG antibodies, which both mediate protection *in vivo*.

*P. falciparum* infection increases the serum levels of IgM and IgG antibodies, and also IgE in individuals living in endemic areas [12]. IgE antibodies may protect against, or participate in, the pathogenesis of malaria. The association of high anti-*P. falciparum* IgE levels with a reduced risk of developing clinical malaria suggests a protective role [13], whereas the observation that circulating levels of IgE most often correlate with severe rather than uncomplicated disease suggests a pathogenic role [14]. Recently, Duarte *et al.* [15] have reported that circulating levels of total IgE do not appear to correlate with protection or pathology during malaria. On the contrary, the *P. falciparum*-specific IgE response seems to contribute to the control of parasites, since the functional activity was higher in asymptomatic and uncomplicated malaria patients than in severe or cerebral malaria groups. Since the major IgE effector cell is the mast cell, these results correlate with our previously reported findings that mast cells and mast cell-derived TNF have protective roles in murine malaria [6].

In the present study, we showed that PbAPrx has an agonistic activity for TLR4 and induces an innate immune response. To examine the importance of PbAPrx for the protection in murine malaria, TLR4<sup>-/-</sup> and WT mice were infected with PbA to compare their parasite growth. However, we could not observe significant differences in parasitemia between TLR4<sup>-/-</sup> and WT (data not shown). Since recent reported evidences indicate that glycosyl phosphatidylinositol (GPI) [9] and hemozoin derived from *Plasmodium* parasites [10, 11] participate in innate immune responses in murine malaria, these findings might suggest that other malarial molecules also will be involved in the induction of innate resistance in murine malaria. To clarify whether malarial Prx induces a complete protective immunity in malaria or not, further experiments will be needed.

Prx protein from *Plasmodium* constitutes about 0.25% of the total protein in late-stage trophozoites and is constitutively expressed during asexual development as well as in the gametocyte, both in *P. falciparum* [16] and rodent *Plasmodium* parasites [17, 18]. Since this protein is present in the cytoplasm of malarial parasites but is not associated with the parasite membrane, it is likely that it might be released from the parasites when schizonts are ruptured to release merozoites, or when erythrocytes infected with malarial parasites are destroyed in the spleen. In the present study, since malarial Prx caused a significant induction of TNF – a critical factor in both the protection against and the pathogenesis of malaria – in mast cells and macrophages, malarial Prx seems to be one of the important factors contributing to protection and pathogenesis in malaria.

The studies presented here represent the first time that a role for malarial Prx in the host-parasite relationship in PbA-mediated infection has been defined. Furthermore, it appears that this molecule has an important protection role and may serve as a diagnostic tool, as well as being a possible target in the development of effective therapeutic agents and vaccines for malaria. It is hoped that these findings may improve our

understanding of IgE antibodies involved in host-parasite interactions in malaria.

## Materials and methods

### Experimental animals and PbA infection

TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup>, MyD88<sup>-/-</sup>, TRIF<sup>-/-</sup>, RP105<sup>-/-</sup> and MD-2<sup>-/-</sup> mice of the strain C57BL/6 [7, 19–22] were bred and maintained under specific pathogen-free conditions at the Animal Center, Institute of Medical Science, University of Tokyo. C57BL/6 (WT) mice were purchased from Japan SLC (Hamamatsu, Japan). Mice of both sexes between 5 and 6 wk old were used for these experiments. The animal experiments were approved by the Committee for Animal Experiments of the Institute of Medical Science, University of Tokyo. C57BL/6 mice were inoculated i.v. with  $1 \times 10^5$  PbA-infected erythrocytes to examine parasite growth [6].

### The effect of an anti-IgE antibody on PbA infection

PbA-infected C57BL/6 mice were injected i.p. with 20 µg of a rat IgG<sub>2a</sub> anti-IgE mAb (6HD5) or normal rat IgG<sub>2a</sub> after infection with PbA. Immunization with the antibodies was repeated every third day until the end of the experiment [6, 23]. The percentage parasitemia in the blood was assessed daily following infection.

### Preparation of PbA protein

Unless otherwise specified, reagents were obtained from Sigma (St. Louis, MO).

PbA protein was prepared as follows: PbA-infected erythrocytes were collected from infected mice and purified by centrifugation through 72% Percoll [24]. They were then washed with PBS and lysed by ten cycles of freezing and thawing in PBS buffer containing 1 mM PMSF. The lysate was centrifuged at  $14\,000 \times g$  for 15 min at 4°C to remove insoluble material, and the proteins in the resulting supernatant were precipitated with 10% trichloroacetic acid. The pellet was resuspended in isoelectric focusing (IEF) buffer (9 M urea, 4% CHAPS, 40 mM DTT and 0.5% ampholytes 3–10). Proteins were divided into single-use samples and stored at –80°C until analysis.

### 2D SDS-PAGE, immunoblotting and protein identification

Two-dimensional gel electrophoresis was performed as described elsewhere [25]. Briefly, the extracted protein samples were loaded onto 7-cm Immobiline DryStrips (pH range 3–10; Amersham Biosciences, Uppsala, Sweden) at room temperature overnight. Up to 400 µg of the extracted proteins was applied onto the DryStrip

gels for immunoblotting, and up to 600 µg for analysis by mass spectrometry. Isoelectric focusing (IEF) was performed using MultiPhor II (Amersham Biosciences). The second electrophoresis was then performed using 10% SDS-PAGE gels. The gels were either stained with CBB or used for protein transfer onto PVDF membranes (Millipore). Blotted membranes were blocked with 3% skimmed milk and then incubated with the PbA-infected sera (harvested on day 12 after the infection; 1:100 dilution). The bound antibody was reacted with either AP-conjugated anti-IgE antibody (specific for the  $\epsilon$  chain, 1:1000 dilution; KPL, Gaithersburg, MD) or AP-conjugated anti-rabbit IgG (1:2000 dilution; KPL) for 1 h. Membranes were then washed with TBST again and developed with NBT/BCIP according to the manufacturer's instructions. Protein spots on the gel stained with CBB that corresponded to positive spots on the immunoblot were recovered, digested with trypsin and finally identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF; Applied Biosystems, Farmingham, MA) using the Mascot software program (Matrix Science, London, UK) as described previously [25, 26].

### Production of recombinant malarial Prx

Recombinant 2-Cys PbAPrx or PfPrx was produced in *E. coli* and purified as described previously [16, 17]. The purity of the recombinant protein was confirmed by SDS-PAGE (data not shown). The protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL) with BSA as standard. To estimate LPS contamination in PbAPrx or PfPrx samples, a limulus assay (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was performed, which showed that the LPS concentration was below 21 pg/mL. The appropriate dose of PolyB (Wako Pure Chemical Industries) to use for blocking LPS activity was checked by luciferase assay using the TLR4 transfectant or by measuring TNF induction using BMMC and peritoneal M $\phi$ , as described below. It was found that treatment with 15 µg/mL PolyB at 37°C for 2 h was sufficient to neutralize the effect of 100 ng/mL LPS, both in the luciferase assay and in terms of inducing TNF production *in vitro* (Fig. 3). Therefore, PbAPrx and PfPrx samples were treated with 15 µg/mL PolyB in all experiments to exclude the possibility that LPS contamination could influence experimental data. To detect IgE antibodies to malarial Prx by immunoblotting, PbAPrx protein (5 µg/well) was separated by 10% SDS-PAGE gels and blotted onto PVDF membranes, as described above. For immunostaining, PbA-infected mouse serum (harvested on day 12 after infection; 1 : 100 dilution) was used as the primary antibody and AP-conjugated anti-mouse IgE antibody (specific for the  $\epsilon$  chain, 1:1000 dilution) or goat anti-rabbit IgG (1:2000 dilution) as secondary antibody. As a positive control, a rabbit anti-PbAPrx serum (1:500 dilution) was employed.

### Detection of IgE antibody against PbAPrx in murine malaria

The production of IgE antibody against PbAPrx in murine malaria was measured using a standard ELISA with slight modifications [27]. Briefly, 96-well ELISA plates were coated with 50 µL recombinant PbAPrx (2.5 µg/well in PBS) overnight and then blocked with 5% skimmed milk in 50 mM TBS for 2 h. Of each test serum (day 12 after the infection), 50 µL diluted 1:50 with TBS was added to each well and then incubated for 2 h. In some experiments, test sera (50 µL) were preabsorbed with PbAPrx protein (10 µg/mL) at 37°C for 2 h before use. After washing with TBST, 50 µL of HRP-labeled goat anti-mouse IgE antibody ( $\epsilon$  chain specific, 1:1000 dilution; KPL) was added to each well and incubated for 2 h. After washing again with TBST, 100 µL of tetramethylbenzidine liquid substrate was added to each well, and the reaction was then stopped with 50 µL of 2 N sulfuric acid. Each sample was assayed in triplicate under identical conditions.

### Preparation of peritoneal M $\phi$ , BMMC and BMDC

Mice were injected i.p. with 2 mL 4% thioglycolate medium. After 3 days, peritoneal exudate cells were isolated from the peritoneal cavity by washing with ice-cold HBSS. Cells were cultured for 2 h, then washed with HBSS to remove nonadherent cells. Adherent monolayer cells were used as peritoneal M $\phi$ . To prepare BMMC, BM cells were harvested from both femurs of the mice and cultured in 10-cm plastic plates with complete RPMI 1640 medium supplemented with 10% FBS, 50 U/mL penicillin, 50 mg/mL streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 50 mM 2- $\beta$ -ME and 4 ng/mL IL-3 [28]. BMMC were used at >99% purity, as determined by flow cytometric analysis (Fc $\epsilon$ RI<sup>+</sup> c-kit<sup>high</sup>) and normal granular staining with metachromatic dyes such as toluidine blue [6]. BMDC were obtained by culturing BM cells at  $1.3 \times 10^6$ /mL in 24-well plates with RPMI 1640 supplemented with 10% FBS and 10 ng/mL murine GM-CSF. At day 10, loosely adherent cells were harvested by gentle pipetting and used as BMDC [29].

### Measurement of cytokine production *in vitro*

For measurement of *in vitro* cytokine production,  $1.5 \times 10^5$  peritoneal M $\phi$ ,  $1.5 \times 10^5$  BMMC, or  $1.5 \times 10^5$  BMDC were cultured at 37°C overnight in 96-well plates containing RPMI 1640 supplemented with 10% FBS and antibiotics, in the presence or absence of PbAPrx or PfPrx (1 µg/well). LPS (0.1 µg/well) or Pam<sub>3</sub>CSK<sub>4</sub> (PCSK, 0.1 µg/well; ECM PG Microcollections) was used as ligand for TLR4 and TLR2. Culture supernatants were collected 24 h later, and the levels of TNF or IL-12p40 in the supernatants were measured by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Each sample was assayed in triplicate under identical conditions.

## Luciferase assay

The luciferase assay was performed using Ba/F3 cells stably expressing mTLR2 or mTLR4/mMD-2 and p55Ig  $\kappa$ Luc with an NF $\kappa$ B-dependent luciferase reporter construct [30]. For the assay, the transfectants were washed and cultured in RPMI 1640 supplemented with 10% FBS and IL-3. Individual wells of a 96-well plate containing  $1 \times 10^5$  cells were stimulated with PbAPrx or PfPrx, as indicated in Fig. 4. LPS (0.1  $\mu$ g/well) or PCSK (0.1  $\mu$ g/well) was used as ligand for TLR4 or TLR2. After 4 h of stimulation with either ligand or malarial Prx, cells were harvested, washed and then lysed in 100  $\mu$ L lysis buffer. Luciferase activity was measured using 10  $\mu$ L lysate and 50  $\mu$ L luciferase substrate (Nippon Gene, Toyama, Japan), quantified as relative light units in a luminometer (Berthold, Tokyo, Japan). Each sample was assayed in triplicate under identical conditions.

## Cell staining and flow cytometric analysis

Ba/F3 cells stably expressing mTLR4/mMD-2/CD14/NF $\kappa$ B/GFP or NF $\kappa$ B/GFP were kindly provided by Dr. S. Takamura-Akashi, University of Tokyo [31]. The mouse cDNA encoding TLR4, MD-2, and CD14 were cloned, and Ba/F3 cells were transfected with expression vectors of pMX-puromycin encoding mTLR4, pEFBOS encoding mMD-2, and pcDNA3 encoding mCD14. An NF $\kappa$ B/GFP reporter construct was also introduced. The cells ( $1 \times 10^5$ ) were cultured in 96-well plates overnight at 37°C with PbAPrx (1  $\mu$ g/well) or LPS (0.1  $\mu$ g/well) as a positive control. In some experiments, PbAPrx (1  $\mu$ g/mL) or LPS (0.1  $\mu$ g/mL) was pre-treated with PK (20  $\mu$ g/mL) at 37°C for 2 h prior to use. Cells were washed with staining buffer (PBS containing 2.5% FBS and 0.01% Na $_3$ ), and analyzed by flow cytometry using a FACSCalibur (Becton Dickinson, Mountain View, CA).

## Immunoprecipitation and immunoblotting

Ba/F3 cells stably expressing mTLR2 or mTLR4/mMD-2 were established by transduction with the retroviral vector pMX-puromycin encoding these molecules, which were tagged with the flag epitope followed by His6 at the C terminus, as previously reported [31]. Cells ( $1 \times 10^8$  in 10 mL medium) were incubated with PbAPrx (20  $\mu$ g/mL) at 37°C for 2 h. After washing, cells were lysed on ice for 30 min in a lysis buffer containing 150 mM NaCl, 50 mM Tris/HCl (pH 7.6), 2 mM EDTA, 10  $\mu$ g/mL aprotinin, 10  $\mu$ g/mL leupeptin, 1 mM PMSF, and 1% Triton X-100. PbAPrx bound to the cell surface TLR with the flag epitope tag were precipitated using anti-flag (M2) agarose gels overnight at 4°C. Gels were washed three times and then boiled at 99°C for 5 min in the sample buffer. Samples were subjected to SDS-PAGE and immunoblotted as described above [32]. PVDF membranes were probed with an anti-rabbit PbAPrx antibody to detect malarial Prx, or anti-mouse TLR4 or TLR2 antibody to detect TLR4/MD-2 or TLR2 [31]. Binding was detected using AP-conjugated anti-mouse

IgG or anti-rabbit IgG antibodies (2:1000 dilution; KPL), and bands were visualized using NTB and BCIP reagents.

## Cytokine production by PbAPrx in mice

Five each of TLR4<sup>-/-</sup>, MyD88<sup>-/-</sup>, TRIF<sup>-/-</sup> or WT mice were injected i.p. with 500  $\mu$ g PbAPrx. Sera were collected at 2 and 4 h for cytokine assays.

## Statistical analyses

Statistical significance was established using an unpaired, two-tailed Student's *t*-test. Statistically significant data are indicated in the figures by an asterisk, and the corresponding *p* values are listed in the figure legends.

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## References

- 1 Breman, J. G., The ears of the hippopotamus: Manifestations, determinants and estimates of the malaria burden. *Am. J. Trop. Med. Hyg.* 2001. **64** (Suppl): 1–11.
- 2 Grau, G. E., Taylor, T. E., Molyneux, M. E., Wirima, J. J., Vassalli, P., Hommel, M. and Lambert, P. H., Tumor necrosis factor and disease severity in children with *falciparum* malaria. *N. Engl. J. Med.* 1989. **320**: 1586–1591.
- 3 Clark, I. A. and Rockett, K. A., Nitric oxide and parasitic disease. *Adv. Parasitol.* 1996. **37**: 1–56.
- 4 Friedland, J. S., Ho, M., Remick, D. G., Bunnag, D., White, N. J. and Griffin, G. E., Interleukin-8 and *Plasmodium falciparum* malaria in Thailand. *Trans. R. Soc. Trop. Med. Hyg.* 1993. **87**: 54–55.
- 5 Zhang, Y., Ramos, B. F. and Jakschik, B. A., Neutrophil recruitment by tumor necrosis factor from mast cells in immune complex peritonitis. *Science* 1992. **258**: 1957–1978.
- 6 Furuta, T., Kikuchi, T., Iwakura, Y. and Watanabe, N., Protective roles of mast cells and mast cell-derived TNF in murine malaria. *J. Immunol.* 2006. **177**: 3294–3302.
- 7 Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., Kitamura, T. et al., Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat. Immunol.* 2002. **3**: 667–672.

- 8 Divanovic, S., Trompette, A., Atabani, S. F., Madan, R., Golenbock, D. T., Visintin, A., Finberg, R. W. *et al.*, Negative regulation of Toll-like receptor 4 signaling by the Toll-like receptor homolog RP105. *Nat. Immunol.* 2005. **6**: 571–578.
- 9 Krishnegowda, G., Hajjar, A. M., Zhu, J., Erika, J., Douglass, E. J., Uematsu, S., Akira, S. *et al.*, Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of *Plasmodium falciparum*: Cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity. *J. Biol. Chem.* 2005. **280**: 8606–8616.
- 10 Pichyangkul, S., Yongvanitchit, K., Kum-arb, U., Hemmi, H., Akira, S., Krieg, A. M., Heppner, D. J. *et al.*, Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a Toll-like receptor 9-dependent pathway. *J. Immunol.* 2004. **172**: 4926–4933.
- 11 Coban, C., Ishii, K.-J., Kawai, T., Hemmi, H., Sato, S., Uematsu, S., Yamamoto, M. *et al.*, Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J. Exp. Med.* 2005. **201**: 19–25.
- 12 Miller, L. H., Good, M. F. and Milon, G., Malaria pathogenesis. *Science* 1994. **264**: 1878–1883.
- 13 Berczky, S., Montgomery, S. M., Troye-Blomberg, M., Rooth, I., Shaw, M. A. and Farnert, A., Elevated anti-malarial IgE in asymptomatic individuals is associated with reduced risk for subsequent clinical malaria. *Int. J. Parasitol.* 2004. **34**: 935–942.
- 14 Perlmann, H., Helmsby, H., Hagstedt, M., Carlson, J., Larsson, P. H., Troye-Blomberg, M. and Perlmann, P., IgE elevation and IgE anti-malarial antibodies in *P. falciparum* malaria: Association of high IgE levels with cerebral malaria. *Clin. Exp. Immunol.* 1994. **97**: 284–292.
- 15 Duarte, J., Deshpande, P., Guiedy, V., Mécheri, S., Fescl, C., Cazenave, P.-A., Mishra, G.C. *et al.*, Total and functional parasite specific IgE responses in *Plasmodium falciparum*-infected patients exhibiting different clinical status. *Malaria J.* 2007. **6**: 1–13.
- 16 Kawazu, S.-I., Komaki, K., Tsuji, N., Kawai, S., Ikenoue, N., Hatabu, T., Ishikawa, H. *et al.*, Molecular characterization of a 2-Cys peroxiredoxin from the human malaria parasite *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 2001. **116**: 73–79.
- 17 Kawazu, S.-I., Tsuji, N., Hatabu, T., Kawai, S., Matsumoto, Y. and Kano, S., Molecular cloning and characterization of a peroxiredoxin from the human malaria parasite *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 2000. **109**: 165–169.
- 18 Kawazua, S.-I., Nozaki, T., Tsuboi, T., Nakano, Y., Komaki-Yasuda, K., Ikenoue, N., Torii, M. *et al.*, Expression profiles of peroxiredoxin proteins of the rodent malaria parasite *Plasmodium yoelii*. *Int. J. Parasitol.* 2003. **33**: 1455–1461.
- 19 Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. *et al.*, TLR4-deficient mice are hyporesponsive to LPS: Evidence for TLR4 as the *lps* gene product. *J. Immunol.* 1999. **162**: 3749–3752.
- 20 Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K. *et al.*, Differential rules for TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 1999. **11**: 443–451.
- 21 Ogata, H., Su, I.-H., Miyake, K., Nagai, Y., Akashi, S., Mecklenbräuer, I., Rajewsky, K. *et al.*, The Toll-like receptor protein RP105 regulates lipopolysaccharide signaling in B cells. *J. Exp. Med.* 2000. **192**: 23–29.
- 22 Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O. *et al.*, Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science* 2003. **301**: 640–643.
- 23 Watanabe, N., Hayashi, Y., Kobayashi, A. and Ohtomo, H., *Brugia malayi* infection in mice with selective suppression of IgE production. *Int. Arch. Allergy Immunol.* 1996. **109**: 192–196.
- 24 Omer, F. M., De Souza, J. B., Corran, P. H., Sultan, A. A. and Riley, E. M., Activation of transforming growth factor- $\beta$  by malaria parasite-derived metalloproteinases and a thrombospondin-like molecule. *J. Exp. Med.* 2003. **198**: 1817–1827.
- 25 Xiang, Y., Sekine, T., Nakamura, H., Imajoh-Ohmi, S., Fukuda, H., Nishioka, K. and Kato, T., Proteomic surveillance of autoimmunity in osteoarthritis identification of triosephosphate isomerase as an autoantigen in patients with osteoarthritis. *Arthritis Rheum.* 2004. **50**: 1511–1521.
- 26 Perkins, D. N., Pappin, D. J., Creasy, D. M. and Cottrell, J. S., Probability based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999. **20**: 3551–3567.
- 27 Kikuchi, T., Furuta, T. and Kojima, K., Kinetics of nucleoside triphosphate hydrolase of *Toxoplasma gondii* in mice with acute and chronic toxoplasmosis. *Ann. Trop. Med. Parasitol.* 2002. **96**: 35–41.
- 28 Razin, E., Ihle, J. N., Seldin, D., Mencia-Huerta, J.-M., Katz, H. R., Leblanc, P. A., Hein, A. *et al.*, Interleukin 3: A differentiation and growth factor for the mouse mast cell that contains chondroitin sulfate E proteoglycan. *J. Immunol.* 1984. **132**: 1479–1486.
- 29 Inaba, K., Pack, M., Inaba, M., Sakuta, H., Isdell, F. and Steinman, R. M., High levels of a major histocompatibility complex II-self peptide complex on dendritic cells from the T cell areas of lymph nodes. *J. Exp. Med.* 1997. **186**: 665–672.
- 30 Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K. and Kimoto, M., MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J. Exp. Med.* 1999. **189**: 1777–1782.
- 31 Akashi, A., Saitoh, S.-I., Wakabayashi, Y., Kikuchi, T., Takamura, T., Nagai, Y., Kusumoto, Y. *et al.*, Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: Higher affinity than that with MD-2 or CD14. *J. Exp. Med.* 2003. **198**: 1035–1042.
- 32 Konno, K., Wakabayashi, Y., Akashi-Takamura, S., Ishii, T., Kobayashi, M., Takahashi, K., Kusumoto, Y. *et al.*, A molecule that is associated with Toll-like receptor 4 and regulates its cell surface expression. *Biochem. Biophys. Res. Commun.* 2006. **339**: 1076–1082.

**Abbreviations:** BMDC: bone marrow-derived DC · BMMC: bone marrow-derived mast cell · CBB: Coomassie brilliant blue · m: murine · PbA: Plasmodium berghei ANKA · PCSK: Pam<sub>3</sub>CSK<sub>4</sub> · PfPrx: P. falciparum Prx · PK: proteinase K · PolyB: polymyxin B · Prx: peroxiredoxin · PVDF: polyvinylidene difluoride · RP105: radioprotective 105 · TRIF: Toll/IL-1 receptor domain-containing adaptor inducing IFN- $\gamma$

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