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MAPK Phosphatase 5 Deficiency Contributes to Protection against Blood-Stage *Plasmodium yoelii* 17XL Infection in Mice

Qianqian Cheng,* Qingfeng Zhang,* Xindong Xu,* Lan Yin,† Lin Sun,† Xin Lin,‡ Chen Dong,§ and Weiqing Pan*†

Cell-mediated immunity plays a crucial role in the development of host resistance to asexual blood-stage malaria infection. However, little is known of the regulatory factors involved in this process. In this study, we investigated the impact of MAPK phosphatase 5 (MKP5) on protective immunity against a lethal *Plasmodium yoelii* 17XL blood-stage infection using MKP5 knockout C57BL/6 mice. Compared with wild-type control mice, MKP5 knockout mice developed significantly lower parasite burdens with prolonged survival times. We found that this phenomenon correlated with a rapid and strong IFN-γ-dependent cellular immune response during the acute phase of infection. Inactivation of IFN-γ by the administration of a neutralizing Ab significantly reduced the protective effects in MKP5 knockout mice. By analyzing IFN-γ production in innate and adaptive lymphocyte subsets, we observed that MKP5 deficiency specifically enhanced the IFN-γ response mediated by CD4+ T cells, which was attributable to the increased stimulatory capacity of splenic CD11c+ dendritic cells. Furthermore, following vaccination with whole blood-stage soluble plasmodial Ag, MKP5 knockout mice acquired strongly enhanced Ag-specific immune responses and a higher level of protection against subsequent *P. yoelii* 17XL challenge. Finally, we found the enhanced response mediated by MKP5 deficiency resulted in a lethal consequence in mice when infected with nonlethal *P. yoelii* 17XNL. Thus, our data indicate that MKP5 is a potential regulator of immune resistance against *Plasmodium* infection in mice, and that an understanding of the role of MKP5 in manipulating anti-malaria immunity may provide valuable information on the development of better control strategies for human malaria. *The Journal of Immunology*, 2014, 192: 3686–3696.

M alaria, which is caused by intracellular protozoan parasites of the genus *Plasmodium*, remains a devastating public health problem resulting in high morbidity and mortality in tropical and subtropical regions (1–3). Of the two phases of the malarial parasite’s life cycle in a human host, the asexual blood stage is pathogenic and responsible for the clinical symptoms and syndromes. Moreover, the severity of the infection depends largely on the interplay between the parasite and the immunological responses of the host (4). Therefore, a better understanding of the mechanisms of the immune responses against the blood-stage parasites will greatly facilitate the development of effective anti-malarial drugs and vaccines. Given the absence of MHC molecules on the surface of erythrocytes, it has been accepted that protective immunity to blood-stage malaria depends mainly on humoral responses (5, 6). However, accumulating evidence from field studies in humans strongly supports the view that protection in the infected host will be enhanced by T cells specific for the malarial parasites (7–11). Furthermore, naïve volunteers immunized with low doses of blood-stage parasites showed proliferative T cell responses and increased resistance to subsequent challenge in the absence of detectable Ab responses (12). These studies highlight the significance of cell-mediated immunity to blood-stage malaria infections.

Experimental rodent malaria models, which display many features in common with human malaria, are widely used to investigate diverse aspects of the immune responses in vivo (13, 14). Moreover, gene knockout mouse models also represent a powerful tool to elucidate the direct role of a given molecule in resistance to malaria (15–17). Extensive studies on murine malaria models have established that CD4+ T cells are the major factor responsible for both the acquisition and maintenance of protective immunity during blood-stage malaria (18–21). Additionally, the existence of Ag-specific CD8+ T cells, the rapid activation of NK cells, NKT cells, macrophages, as well as the cross-talk between innate and adaptive immune systems also appear to be involved in the protection against blood-stage malarial parasites (22–26). In particular, the proinflammatory cytokines, such as IFN-γ and TNF-α, in these cellular responses are implicated as central mediators in limiting parasite replication and promoting parasite clearance during the infections (27–32), which also have to be tightly balanced to avoid immune-mediated pathology (32–35). However, the underlying mechanisms and associated regulators involved in these processes, contributing to the differential resistance to malaria, remain to be fully elucidated.

MAPK phosphatase 5 (MKP5), as an essential regulator of MAPK signaling pathways, has been demonstrated to play im-
portant roles in the regulation of immune responses (36). Previous
in vitro studies have revealed that MKP5 negatively regulates the
activities of the stress-activated MAPKs, JNK and p38 (37, 38).
Owing to the prominent roles of these MAPKs in immune
responses (39), the effects of MKP5 on the modulation of im-
munity have been investigated in Mkp5−/− mice, which were
generated through homologous recombination. In this animal
model, it has been shown that MKP5 is not essential for the de-
velopment of the immune system, but specifically dephosphor-
ylates and inactivates the JNK signaling pathway in vivo (36).
Compared with the wild-type (WT) controls, Mkp5−/− macro-
phages could produce enhanced levels of proinflammatory cyto-
kines in response to LPS. In terms of effector T cell function,
both Th1 and Th2 cells lacking MKP5 could produce significa-
tively higher levels of cytokines after mitogen stimulation (36).
Moreover, investigation of lymphocytic choriomeningitis virus
infection in Mkp5-deficient mice showed that T cells produced
more effector cytokines in response to secondary infection,
which resulted in high rates of immune-mediated mortality, and
therefore implicated MKP5 as a crucial negative regulator of
T cell–mediated immunity during viral infection (36). However,
the importance of MKP5 in the development of host resistance to
other pathogens has rarely been studied.

In the present study, the role of MKP5 on immune resistance to
blood-stage Plasmodium yoelii infection was evaluated in Mkp5
knockout (KO) and WT control C57BL/6 mice. We demonstrated
that MKP5 deficiency increased host resistance to the lethal
P. yoelii 17XL infection with strongly reduced parasitemia and
prolonged survival, which was associated with the significantly
enhanced protective IFN-γ response during the acute phase of
infection. We also showed an enhanced stimulatory capacity of
splenic CD11c+ dendritic cells (DCs) to induce IFN-γ production
by responding CD4+ T cells in the absence of MKP5. Further-
more, using a prime-boost immunization approach, we observed
that MKP5 deficiency also had a profound effect on immunization-
induced protective efficacy against P. yoelii 17XL challenge.
However, the augmented proinflammatory response mediated by
MKP5 deficiency led to severe mortality in mice infected with the
nonlethal strain, P. yoelii 17XNL. Collectively, these data suggest
that MKP5 has the potential to modulate host immunity against
malaria infection, and the consequence depends largely on the
parasite strains.

Materials and Methods

Mice

Breeding pairs of Mkp5 gene KO mice on the C57BL/6 background,
generated as previously described (36), were bred in the animal care
facility under specific pathogen-free conditions. WT control C57BL/6
mice were purchased from Shanghai Laboratory Animal Center of the
Chinese Academy of Sciences and maintained in the same fa-
cility. Female KO and WT mice (aged 6–8 wk) were used in all
experiments. All procedures conducted on animals in this study were
approved by the Internal Review Board of Tongji University School
of Medicine.

Parasite and experimental challenge

Blood-stage parasites of the highly virulent lethal strain P. yoelii 17XL
and the nonlethal variant P. yoelii 17XNL were originally obtained
from the Department of Tropical Infectious Diseases of Second Mili-
tary Medical University (Shanghai, China). Infections were initiated
by i.p. injection of 5 × 106 P. yoelii 17XL or 1 × 106 P. yoelii
17XNL–parasitized RBCs (pRBCs) obtained from a homologous don-
or, which had been previously infected with a frozen stock of para-
sites. Survival rates were monitored daily and parasitemia was assessed
by microscopic examination of Giemsa-stained thin smears of tail
blood.

Sera collection and spleen cell culture

At the indicated time points of infection, sera of KO and WT mice were
isolated and stored at −20˚C until assayed for cytokine and Ab levels.
For in vitro cytokine production, pRBC lysate used as source of crude Ag
was prepared by Percoll treatment, lysate from uninfected RBCs (uRBCs) used
as control was similarly prepared (40, 41). Single-cell suspensions
of splenocytes were prepared as described previously (27). Briefly, the asep-
tically removed spleens were pressed gently through a sterile fine-wire
mesh into RPMI 1640 supplemented with 10% heat-inactivated FBS (Life
Technologies, Carlsbad, CA) and 1% penicillin/streptomycin (Life Tech-
nologies). RBCs were lysed with NH4Cl lysis buffer (Beyotime Bio-
technology, Jiangsu, China) and the viability of harvested cells, which was
consistently >90%, was determined by trypan blue exclusion. Aliquots
(1 ml) of cell suspensions (5 × 106 live cells/ml) were plated in triplicate
wells of 24-well flat-bottom tissue culture plates (Costar, Corning, NY)
and incubated in the presence of anti-CD3 (145-2C11; BioLegend, San
Diego, CA) plus anti-CD28 (37.51; BioLegend) mAbs (positive control,
both added at 1 μg/ml), pRBC lysate (1 × 106 pRBC equivalents/ml),
uRBC lysate (1 × 106 RBCs/ml), or medium alone for 48 h at 37˚C with
5% CO2. Supernatants were collected and stored at −80˚C until cytokine
measurement was performed.

Cytokine ELISAs and NO detection

Cytokine levels in sera or cell culture supernatants were quantified by
sandwich ELISA. For IFN-γ, TNF-α, and IL-4, rat anti-mouse IFN-γ
(AN-18; BD Pharmingen, San Diego, CA), anti-mouse TNF-α (IF3F3D4;
eBioscience, San Diego, CA), and anti-mouse IL-4 (11B11; BD Phar-
mingen) were used as capture mAbs. Biotinylated rat anti-mouse IFN-γ
(R4-6A2; BD Pharmingen), anti-mouse TNF-α (MP6-XT22 and MP6-
XT3; eBioscience), and anti-mouse IL-4 (BVD6-24G2; BD Pharmingen)
were used as detection mAbs. Reactivity was visualized using 3,3',5,5'–
tetramethylbenzidine substrate, and OD values were read in a microplate
reader at 450 nm with a reference wavelength of 630 nm. The concen-
trations of cytokines were calculated according to the standard curves
generated using murine recombinant cytokines (PeproTech, Rocky Hill,
NJ). Bioactive TGF-β and IL-10 levels were detected by using Platinum
ELISA kits (eBioscience). Serum NO levels were measured with the
Griess reaction kit according to the manufacturer’s instructions (Beyotime
Biotechnology).

In vivo neutralization of IFN-γ

Female KO mice received i.p. injections of 0.1 mg rat anti-mouse IFN-γ
mAb (XM1G12; BioLegend) 1 d prior to P. yoelii 17XL inoculation and
every other day thereafter. Treatments with equivalent volumes of normal
rat IgG (Sigma-Aldrich, St. Louis, MO) and PBS were used as controls.

Intracellular cytokine staining for flow cytometry

Splenocytes were stimulated in vitro with 50 ng/ml PMA and 1 μg/ml
ionomycin (Sigma-Aldrich) in the presence of 1 μM brefeldin A (eBio-
sience) at 37˚C for 5 h, followed by staining with appropriate combina-
tions of fluorochrome-conjugated Abs. All Abs were purchased from
BioLegend. Phenotypic characterization of cell populations was performed
by surface labeling with anti-mouse CD3 (17A2), anti-mouse CD4
(GK1.5), anti-mouse NK1.1 (PK136), anti-mouse TCRγ/δ (GL3), and
anti-mouse CD8α (53-6.7). Cell populations were defined as CD4+ T cells
(CD3+CD4+), CD8+ T cells (CD3+CD8+), NK cells (NK1.1+CD3–),
NKT cells (NK1.1+CD3+), and γδ T cells (CD3+TCRγδ+). After fixa-
tion with 1% paraformaldehyde, cells were permeabilized with 0.1% sa-
porin (Sigma-Aldrich) and stained intracellularly with anti-mouse IFN-γ
(XMG1.2) or isotype control Ab (RTK2071) before analyzing on a
FACSCalibur (BD Biosciences, San Jose, CA). For detection of other
cytokines produced by CD4+ T cells, anti-mouse IL-4 (11B11), anti-mouse
IL-10 (JES5-16E3), and anti-mouse IL-17A (TC11-18H10.1) were used
for intracellular staining. Data were analyzed using FlowJo software (Tree
Star, Ashland, OR).

Splenic DC/CD4+ T cell cocultures

Splenic DCs from both KO and WT mice were separated 2 d after P. yoelii
17XL infection, according to the methods described previously (40, 42),
and further purified by positive selection using biotinylated anti-CD11c
mAb (N418; BioLegend) in combination with BD IMag streptavidin
particles (BD Biosciences), according to the manufacturers’ instructions.
The purity was routinely ≥85% CD11c+ as confirmed by flow cytometry.
Splenic CD4+ T cells from uninfected KO and WT mice were enriched by
using biotinylated anti-CD4 mAb (RM4-5; BioLegend) in combination

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with the streptavidin-labeled magnetic beads. The purity was routinely >95%. For coculture, 2 × 10^6 purified DCs (designated as KO DCs and WT DCs) were incubated in triplicate with 4 × 10^5 freshly purified CD4^+ T cells (designated as KO CD4^+ T and WT CD4^+ T) in 96-well round-bottom plates (Nunc) at a final volume of 200 μl for 5 d at 37°C. DCs incubated in medium alone were included as controls. The supernatants were collected and analyzed for IFN-γ production by ELISA, as described above.

P. yoelii 17XL soluble Ag preparation and immunization

Whole blood-stage soluble plasmodial Ag (sAg) of P. yoelii 17XL was prepared according to previously described methods with minor modifications (43–45). Briefly, blood (average 40–45% parasitemia) from infected C57BL/6 mice was harvested and lysed by treatment with 0.15% saponin for 10 min at 4°C. Intact parasites were obtained by centrifugation at 10,000 × g, washed extensively, and resuspended in PBS. After 3 cycles of rapid freezing and thawing, samples were briefly sonicated and the sAg fraction was recovered by centrifugation at 10,000 × g for 15 min and frozen at −80°C until used. For immunological and protection studies, groups of KO and WT mice were primed s.c. with 50 μg sAg (equivalent to 1 × 10^8 pRBCs) emulsified in CFA (Sigma-Aldrich), followed by two boost immunizations at 2-wk intervals, with the same doses of Ag in IFA. Control mice were immunized in a similar manner with adjuvant alone. Approximately 2 wk after the final immunization, mice were either challenged with 5 × 10^8 P. yoelii 17XL pRBCs or sacrificed for analysis of cytokine and Ab responses.

Assessment of immune responses induced by immunization

Cytokine production in vitro by spleen cells from P. yoelii 17XL sAg-immunized and control mice was analyzed according to the described protocol. For evaluation of humoral responses, the levels of Ag-specific total IgG, as well as the major isotypes IgG1 and IgG2c, in prechallenge sera were determined by ELISA. The 96-well plates were coated with 100 μl sAg (20 μg/ml) overnight at 4°C and subsequently blocked with 1% BSA in PBS for 1 h. Serum from each animal was assayed at a dilution of 1:100. Ag-specific Abs were detected using HRP-conjugated goat anti-mouse IgG (Promega, Madison, WI), anti-mouse IgG1 (SouthernBiotech, Birmingham, AL), and anti-mouse IgG2c (IgG2a b allotype) (Abcam, Cambridge, MA) with 3,3′,5,5′-tetramethylbenzidine as substrate. Serum samples were run in duplicate and absorbance was read at 450 nm with a reference wavelength of 630 nm. Ab levels in serum are expressed as relative OD values.

Statistical analysis

All analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA). Data are presented as means ± SEM, unless otherwise stated. Statistical significance of differences between two groups (KO versus WT) or two time points was assessed by unpaired Student t tests, except for survival. Kaplan–Meier curves of overall survival were compared by using the log-rank test. A p value <0.05 was considered statistically significant.

Results

Primary infection of MKP5 KO and WT C57BL/6 mice with P. yoelii 17XL

Female KO mice and sex-matched WT littermates were infected i.p. with the lethal rodent malarial parasite P. yoelii 17XL as described in Materials and Methods. Survival rates and parasitemia were monitored throughout the course of the infection. In both groups of mice, regardless of genetic background, parasites were detected in peripheral blood 2 d after inoculation. However, WT controls exhibited a more severe course of infection (Fig. 1). Whereas mice in the WT group suffered 100% mortality within 1 wk postinfection (p.i.), none of the KO mice died during this period and most (five of six) survived the infection for >20 d (p < 0.01) (Fig. 1A). Infection in WT mice resulted in rapidly ascending parasitemia, which peaked at 5 d p.i. with the levels in excess of 60%. Nevertheless, it was notable that KO mice developed significantly reduced parasite burdens (days 3–6 p.i.) than did their WT counterparts (p < 0.001), with the peak parasitemia reaching only 40% at day 5 p.i. (Fig. 1B). Somewhat unexpectedly, all KO mice that survived acute infection displayed a recrudescence parasitemia in the chronic stage, followed by a decline in parasite burden, although the associated and irreversible severe anemia led to the gradual death of mice. These results indicate that MKP5 deficiency contributes, at least in part, to protection against P. yoelii 17XL infection in C57BL/6 mice.

Enhanced IFN-γ response in MKP5 KO mice during the early course of P. yoelii 17XL infection

The ability of KO mice to develop effective resistance to acute P. yoelii 17XL infection was consistent with the major biological function of MKP5 in regulating immune responses. To test the hypothesis, a comparative analysis of the mediators that have been found to be essential for elimination of malarial parasites, as well as the major anti-inflammatory cytokines that are known to be important components of the regulatory responses, was performed in KO and WT mice.

First, circulating levels of IFN-γ, TNF-α, and NO, as well as TGF-β and IL-10, were quantified following the parasite inoculation (Fig. 2). As shown in Fig. 2A, the most pronounced effect of MKP5 deficiency observed was the large burst of serum IFN-γ as
early as day 3 p.i. (p < 0.05 versus day 0), reaching a maximum at day 5 p.i., in parallel with the time of peak parasitemia. In accordance with the previous observation on infection of C57BL/6 mice with *P. yoelii* 17XL parasites (32), WT mice also displayed gradually and moderately increased IFN-γ production in vivo. However, the levels were significantly lower than those detected in KO mice (0.587 ± 0.099 versus 0.287 ± 0.064 ng/ml at day 3, p < 0.05; 1.343 ± 0.097 versus 0.721 ± 0.097 ng/ml at day 5 p.i., p < 0.01). Because a previous report implied that macrophages were involved in the responses to primary malaria infections (41), and although our analysis also revealed slightly increased TNF-α, there were no apparent differences in TNF-α or NO levels between KO and WT mice up to day 5 p.i. (Fig. 2B, 2C). Owing to the 100% mortality of WT mice within 1 wk of infection, we were unable to further compare this response, although the peak TNF-α and NO levels were noted at day 10 p.i. in the KO group (data not shown). Additionally, consistent with the previous report (32), an extremely early TGF-β production within the first 2 d of infection was evident in both KO and WT mice (p < 0.05), and IL-10 levels were detectable at day 3 and increased at day 5 p.i. However, the amounts of the two cytokines were comparable between the two groups of mice (Fig. 2D, 2E).

Next, we assessed IFN-γ and IL-4 production by spleen cells following in vitro stimulation (Fig. 3). Although the responses of naïve cells were extremely low and similar to those of WT mice, cells harvested from infected KO mice were hyperresponsive in the presence of pRBC lysate, with remarkably elevated IFN-γ production at day 3 p.i. (p < 0.05) and peaked at day 5. Again, in agreement with the serum cytokine profiles, spleen cells from KO mice produced significantly greater amounts of IFN-γ than did those from their WT counterparts at the early time points (3.178 ± 0.6895 versus 0.6163 ± 0.2066 ng/ml at day 3, p < 0.05; 5.015 ± 0.6904 versus 2.020 ± 0.4707 ng/ml at day 5, p < 0.05) (Fig. 3A). In contrast, no significant IL-4 secretion was detected in either KO or WT mice during the early phase (Fig. 3B).

These results clearly suggest that, in the absence of MKP5, the malaria-specific IFN-γ response is significantly increased during the early stage of *P. yoelii* 17XL infection, which might correlate with the limitation of parasite replication in KO mice.

**Protective role of IFN-γ in the resistance of MKP5 KO mice to primary *P. yoelii* 17XL infection**

KO mice were injected either with normal rat IgG or with XMG1.2, a rat anti-mouse IgG1 that has been found to block IFN-γ activity in vivo (46, 47), prior to and during the early course of infection with *5 × 10^6* pRBCs of *P. yoelii* 17XL. Negative control animals were injected with PBS at the same time points. As expected, the course of infection in KO mice treated with rat IgG was similar to that resulting from PBS treatment (Fig. 4). In contrast, treatment of KO mice with anti–IFN-γ mAb greatly exacerbated the infection, with 75% mortality (three of four) as early as that observed in WT mice treated with PBS. All mice succumbed to the infection by day 9 (Fig. 4A). Thus, neutralization of IFN-γ significantly increased the susceptibility of KO mice to infection (p < 0.05 for anti–IFN-γ treatment versus rat IgG treatment in KO mice). Moreover, mice treated with anti–IFN-γ had significantly higher parasitemia at day 4 (p < 0.05) and peak parasitemia at day 5 p.i. (p < 0.01) compared with rat IgG-treated mice, while the overall course of parasitemia was not significantly different from that observed in WT mice administered with PBS (Fig. 4B). We obtained essentially the same results in two successive experiments. These results clearly demonstrate the essential role of IFN-γ in the development of early defense against *P. yoelii* 17XL infection in KO mice.
MKP5 deficiency enhances IFN-γ response mediated by CD4+ T cells during acute P. yoelii 17XL infection

In previous studies it has been demonstrated that many lymphocyte subsets, including αβ T, NK, NKT, and γδ T cells contribute to the IFN-γ response in malaria (28, 29, 48–51). In this study, we used FACS analysis to assess IFN-γ production by these cell subsets during the early acute stage of P. yoelii 17XL infection. To take into account the increase in total spleen cells that occurs after infection, we compared both the proportions and total numbers of IFN-γ+ cells in each of the gated subsets. As shown in Fig. 5, IFN-γ production by the various cell subsets was simultaneously elevated in both KO and WT mice at day 4 p.i., 1 d earlier than the appearance of peak parasitemia. However, of all the lymphocyte subsets concerned, only splenic CD4+ T cells exhibited significant differences between the two groups, that is, 11.61- and 4.80-fold higher levels of IFN-γ than those of uninfected mice in the KO and WT groups, respectively. Furthermore, both the frequency and total number of IFN-γ–producing cells were significantly higher in KO mice than in WT mice (p < 0.01), whereas no significant differences were observed for the IFN-γ+ CD8+ T, NK, NKT, and γδ T cells between the two groups (Fig. 5A, 5B). Additionally, analysis of the relative contribution to the overall IFN-γ response showed that αβ T cells (CD4+ T plus CD8+ T) were the dominating producer of IFN-γ during acute P. yoelii 17XL infection, accounting for nearly 81.13 and 70.81% of total IFN-γ–secreting cells at day 4 p.i., with $5 \times 10^6$ and $5.4 \times 10^6$ cells per spleen in KO and WT mice, respectively (Fig. 5C). Therefore, the comparative analysis suggests that deficiency of MKP5 increases CD4+ T cell IFN-γ production during the early phase of infection, which may account for the phenotypic differences between KO and WT mice. Because the importance of immune responses generated by CD4+ T lymphocytes in protection against blood-stage malaria had been demonstrated previously (20, 21), other cytokines produced by CD4+ T cells were also analyzed. In contrast to the IFN-γ response, the proportions of IL-4–, IL-10–, and IL-17A–producing CD4+ T cells remained at basal or only slightly increased levels, with no statistical differences detected between KO and WT groups (Fig. 5D), suggesting that strong Th2 and Th17 responses were not induced during the early course of P. yoelii 17XL infection. Taken together, these data reaffirm the pivotal role of CD4+ T cell–mediated responses in immune defense against blood-stage malaria infection, and that the absence of MKP5 enhanced Th1 response in mice during the early stages (36).
Splenic DCs from KO mice support increased IFN-γ production by CD4+ T cells

Given that APCs play a key role in shaping adaptive immune responses, and several studies on mouse models of malaria have provided evidence that splenic CD11c+ DCs activated in vitro or in vivo are potent stimulators of IFN-γ production by naive CD4+ T cells (40, 52–55), we therefore used an in vitro priming approach to further investigate whether the phenotype observed in P. yoelii 17XL infection was linked to the impact of MKP5 deficiency on the stimulatory capacity of DCs, or a result of the direct regulation on T cell function. IFN-γ levels in the single cultures of purified DCs from infected mice were nominal and comparable, excluding the possibility that the increased IFN-γ was directly produced by KO DCs or the small population of contaminating cells copurified. In contrast, it was notable to observe that KO DCs cultured with either KO CD4+ T cells or WT CD4+ T cells produced significantly greater extent of IFN-γ (> 5-fold) than did the WT DCs with either KO CD4+ T cells or WT CD4+ T cells (KO DCs plus KO CD4+ T versus WT DCs plus KO CD4+ T cells, p < 0.05; KO DCs plus WT CD4+ T cells versus WT DCs plus WT CD4+ T cells, p < 0.01), whereas the levels were not significantly affected by CD4+ T cells from either KO or WT mice (Fig. 6), indicating that DCs from KO mice are associated with the substantial increase in IFN-γ production.

Thus, these data support the view that the enhanced IFN-γ–producing CD4+ T cell response in KO mice lies primarily on the increased ability of DCs to activate malaria-specific T cells early after infection.

**FIGURE 5.** Enhanced IFN-γ response mediated by CD4+ T cells during the early phase of P. yoelii 17XL infection in MKP5 KO mice. (A) Spleen cells isolated from KO and WT mice at days 0 (D0) and 4 (D4) of infection were stained for surface markers and intracellular IFN-γ following in vitro stimulation with PMA plus ionomycin and analyzed by FACS. Representative dot plots show the frequencies of IFN-γ–producing cells contained in each gated subset. (B) Total numbers of splenic IFN-γ–producing CD4+ T cells, IFN-γ–producing CD8+ T cells, IFN-γ–producing NK cells, IFN-γ–producing NKT cells, and IFN-γ–producing γδ T cells are compared between KO and WT mice. Data shown are means ± SEM (n = 3 mice/group) from one of two replicate experiments. **p < 0.01. (C) Pie charts showing the relative contributions of αβ T (CD4+ T plus CD8+ T cells), NK, NKT, γδ T, and other subsets to the total number of IFN-γ–producing cells in KO and WT mice at day 4. (D) Representative dot plots showing the proportions of IL-4–, IL-10–, and IL-17A–producing cells in gated splenic CD4+ T cells.

**FIGURE 6.** IFN-γ production in cocultures of splenic CD11c+ DCs and CD4+ T cells. Splenic DCs from day 2–infected KO and WT mice were purified by magnetic sorting and were cultured in medium alone or cocultured with naive CD4+ T cells enriched from spleens of uninfected KO and WT mice, respectively. After 5 d of incubation, supernatants were assayed for IFN-γ production by ELISA. Data are presented as means ± SEM (n = 4–5 mice/group) from one of two replicate experiments. *p < 0.05, **p < 0.01.
Immunization with malaria Ag enhances protection against P. yoelii 17XL challenge in KO mice

In an attempt to investigate the modulatory effect of MKP5 on vaccination-induced protection, KO and WT mice were immunized with soluble P. yoelii 17XL Ag and subsequently challenged with pRBCs obtained from a homologous donor. The courses of infection were monitored for 5 wk in Ag-immunized and adjuvant control mice of both genotypes (Fig. 7A, 7B). Similar to our previous results, both groups of control mice suffered 100% mortality, although KO mice developed significantly lower levels of peak parasitemia than did their WT counterparts at day 5 p.i. ($p < 0.01$) and survived longer ($p < 0.05$). In marked contrast, strong protection was conferred in terms of highly reduced parasitemia and significantly increased survival in both KO and WT vaccinated mice (both $p < 0.01$ for comparison of survival rates). Interestingly, although the difference was not statistically significant, only 80% (four of five) of the immunized WT mice survived the challenge whereas 100% of KO mice recovered completely (Fig. 7A). More importantly, it was noted that following immunization, the parasitemia in WT mice rose slowly to between 20 and 30%, peaked approximately at day 14, and subsided by day 32. In contrast, almost complete protection was achieved in the KO mice, all of which developed extremely low-grade parasite burdens, with the peak parasitemia reaching only 2.64% (2.420 ± 1.288% versus 26.47 ± 2.806%, $p < 0.001$), and all animals in this group were found to be virtually free from parasites as early as day 18 after challenge (Fig. 7B). These results raise the possibility that KO mice acquired more effective immune protection in response to immunization with malaria Ag than did the WT mice.

Enhanced protective immune responses induced by immunization in KO mice

Because a stronger IFN-γ response is associated with increased resistance to acute blood-stage P. yoelii 17XL during a primary infection, cellular immunity induced by anti-malaria vaccination was evaluated in the four groups of mice. Following immunization with P. yoelii 17XL Ag, spleen cells from either KO or WT mice produced elevated levels of IFN-γ in response to pRBC lysate compared with the responses of the control groups. However, following immunization with Ag, KO mice exhibited significantly increased (>7-fold) parasite-specific IFN-γ production compared with WT mice (36.10 ± 7.225 versus 4.915 ± 1.172 ng/ml, $p < 0.05$) (Fig. 7C). Immunization with Ag also resulted in higher expression of IL-4 in the KO group (0.8646 ± 0.0546 versus 0.3688 ± 0.0542 ng/ml, $p < 0.01$) (Fig. 7D). Furthermore, we observed that the total malaria-specific Ab production, as well as that of IgG1 and IgG2c, was significantly increased in immunized animals compared with the levels detected in control mice prior to challenge. These data indicated the induction of humoral immune responses by immunization, and similar to the cellular responses observed, the Ab levels were significantly higher in immunized KO mice than in their WT counterparts ($p < 0.001$ for total IgG, $p < 0.001$ for IgG1, and $p < 0.01$ for IgG2c) (Fig. 7E). Collectively, these findings indicate that in the absence of MKP5, immunization with the parasite Ag confers significantly enhanced protective immunity against blood-stage P. yoelii 17XL challenge.

![Figure 7](http://www.jimmunol.org/) Course of blood-stage P. yoelii 17XL challenge and protective responses elicited by immunization of MKP5 KO and WT mice. Groups of KO and WT mice were immunized and boosted twice with 50 μg soluble P. yoelii 17XL Ag emulsified in Freund’s adjuvant. Control mice were immunized with adjuvant alone. Two weeks after the last immunization, mice were either challenged i.p. with 5 × 10⁶ pRBCs obtained from a homologous donor or sacrificed for detection of protective immunity induced by vaccination. (A) Survival rates and (B) parasitemia of Ag-immunized and adjuvant control mice of both genotypes were monitored after challenge. Spleen cells from groups of immunized and control mice were cultured in presence of anti-CD3 plus anti-CD28 (+), pRBC lysate (Ag), or medium alone (m). Levels of (C) IFN-γ and (D) IL-4 in supernatants were quantified by ELISA. (E) Levels of total IgG, IgG1, and IgG2c specific for parasite Ag present in prechallenge sera of KO and WT mice were determined by ELISA. The levels of IgG isotypes were expressed as absorbance (OD₄₅₀). All the results are expressed as means ± SEM ($n = 5$ mice/group) from one of two replicate experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for comparison between Ag-immunized KO and WT mice. *$p < 0.05$, **$p < 0.01$ for comparison between adjuvant control KO and WT mice.
Infection of MKP5 KO and WT mice with a nonlethal strain P. yoelii 17XNL

Previous study demonstrated that an early proinflammatory response is sufficient for the resolution of infection with nonlethal P. yoelii in C57BL/6 mice (32), which raised the hypothesis that MKP5 deficiency could induce exaggerated inflammation in this process. Therefore, we infected KO and WT mice with the non-lethal strain, P. yoelii 17XNL. As expected, most WT mice (75%) developed moderate parasitemia and recovered completely within 4 wk. In contrast, although KO mice developed lower parasite burdens over the entire course, all succumbed to the infection between days 15 and 21 p.i. (Fig. 8A, 8B). Then, we detected serum cytokine levels during the infection. Unlike the lethal P. yoelii 17XL challenge, both KO and WT mice showed a marked increase in IFN-γ production as early as day 3 p.i. (p < 0.001), but the level dramatically increased at day 5 p.i. and peaked earlier in KO mice, compared with WT controls (p < 0.05) (Fig. 8C). Additionally, serum TNF-α levels in KO mice were greater than in WT mice at days 8 and 11 p.i. (p < 0.05) (Fig. 8D). No differences of any anti-inflammatory cytokines were observed between infected KO and WT mice (Fig. 8E, 8F). These data suggest that, despite an accelerated clearance of parasites, the enhanced responses mediated by MKP5 deficiency might cause immunopathology and consequently a lethal effect in mice when challenged with the nonlethal P. yoelii 17XNL.

Discussion

MKP5 is recognized as an essential negative regulator of the JNK signaling pathway that plays an important role in both innate and adaptive immune responses (36). Apart from the correlation with T cell–mediated immunity against secondary infection with lymphocytic choriomeningitis virus, little is known of the pathogenic or protective roles of MKP5 in protozoan parasite infections.

In the present study, we analyzed the influence of MKP5 on the course of blood-stage P. yoelii 17XL (lethal) infection in an Mkp5−/− mouse model. As expected, our data provided in vivo evidence that MKP5 is involved in the regulation of host resistance to the parasite challenge. Both MKP5 KO and WT B6 mice

**FIGURE 8.** Course of infection and cytokine production in MKP5 KO and WT C57BL/6 mice infected with nonlethal P. yoelii 17XNL. Mice were infected i.p. with $1 \times 10^5$ pRBCs and the course of infection was followed by monitoring (A) percentage survival and (B) parasitemia. Serum levels of (C) IFN-γ, (D) TNF-α, (E) TGF-β, and (F) IL-10 were determined by ELISA. Data are presented as means ± SEM (n = 4 mice/group) from one of two replicate experiments. *p < 0.05, **p < 0.001.
were found to be susceptible to primary infection. However, in contrast to the WT controls, which succumbed rapidly to hyperparasitemia and acute anemia, KO mice displayed slowly increasing and significantly lower parasite burdens, with most exhibiting relatively prolonged survival after the initial acute phase. These observations indicate that MKP5 deficiency has a significant effect on the early control of *P. yoelii* 17XL infection. Additionally, during the chronic stage, the parasite burdens in surviving KO mice fluctuated at relatively low levels (4.83–15.20%). However, the observation that these mice failed to recover from the infection in the long term was somewhat unexpected, and the underlying cause for the irreversible anemia in this model remains to be clarified. Given the potent immunoregulatory properties of MKP5, it is reasonable to speculate that immunopathology may be linked to the inability to resolve the chronic infection in KO mice. Taken together, these results demonstrate that anti-*P. yoelii* 17XL immunity is partially enhanced in the absence of MKP5, thus indicating that the MKP5-dependent pathway represents a potential regulator of protection against malaria infection.

The ability to establish a precise balance between the proinflammatory and regulatory immune responses is critical to guarantee the parasite clearance and successful resolution of malaria (32, 33). Therefore, to elucidate the immunological mechanism underlying the different resistance between KO and WT mice, we analyzed the effects of MKP5 deficiency on the key molecules that might participate in the responses of the effective protection against *Plasmodium* infection (20, 21, 32, 35, 56). Interestingly, we found that IFN-γ production during the early stage of primary *P. yoelii* 17XL infection was significantly elevated in KO mice compared with their WT counterparts. Conclusive evidence for the pivotal role of IFN-γ in controlling acute parasitemia and survival of mice is well established in *Plasmodium chabaudi* or *Plasmodium berghei* infections (27, 29, 57, 58), and studies in human malaria also demonstrate that IFN-γ is associated with protection (49, 56, 59). However, the importance of this cytokine in *P. yoelii* infection remains a matter of controversy, which is likely to be attributable to the differences in parasite virulence and technical approaches (30–32, 41). To address this controversy, we tested the definitive role of the increased IFN-γ in protection by administering a neutralizing mAb to KO mice prior to and during the early course of *P. yoelii* 17XL infection (22, 58). Our results clearly showed that anti–IFN-γ treatment resulted in substantially higher parasitemia in these mice and fatalities occurred much earlier than those treated with irrelevant Ab. Thus, our findings strongly support the conclusion that optimal levels of proinflammatory cytokines are essential for the protective immunity against the initial phase of primary *P. yoelii* 17XL challenge, and most importantly, they point to an IFN-γ–mediated regulatory role of MKP5 in the immune resistance to this infection.

Meanwhile, by phenotypic characterization of the cellular sources of IFN-γ, we noted in both mouse strains that NK cells contribute significantly to the earliest IFN-γ response (within 2 d; data not shown), whereas αβ T lymphocytes represent the predominant contributors at later time points (e.g., day 4 p.i.). These data are consistent with the previous observations on other malaria models (28, 48) and suggest that there is little direct effect of MKP5 on the kinetics of IFN-γ production during the acute phase of infection. However, of all the lymphocyte subsets analyzed in this study, we found a specific and significant increase in the number of IFN-γ–producing CD4+ T cells in infected KO mice compared with WT mice, whereas other cytokine responses in these cells and IFN-γ production by other cell types were minimally affected, which strongly suggest that the augmented IFN-γ response mediated by CD4+ T cells may confer, at least partially, more efficient control of parasitemia in MKP5-deficient mice, leading to the prolongation of survival during the early stage of infection. On the basis of the stimulatory role of activated DCs in IFN-γ production by naïve CD4+ T cells in murine malaria models (40, 53–55), as well as the demonstration that APCs from KO mice are hyperresponsive to LPS and exhibit increased priming capabilities (36), two possible mechanisms may account for the differing IFN-γ response derived from CD4+ T cells in the two groups of mice: one is the direct influence of MKP5 deficiency on T cell activity, the other is the indirect modulation on DC function. By using an in vitro coculture approach to mimic the initial interaction between the parasite-activated DCs and naïve CD4+ T cells, we found that splenic DCs from infected KO mice incubated with CD4+ T cells could produce higher levels of IFN-γ, whereas the different sources of CD4+ T cells did not affect the response levels, suggesting that the ability of DCs to induce CD4+ T cell IFN-γ production is significantly enhanced in the absence of MKP5 early after *P. yoelii* 17XL infection. Additionally, the activation of parasite-specific CD8+ T cells observed in both KO and WT mice implied the existence of cross-presentation of malaria Ags by DCs (60, 61).

On the basis of the potential to induce a stronger protective response to primary blood-stage *P. yoelii* 17XL infection, we hypothesized that MKP5 deficiency may enhance immunization-induced protection against blood-stage malaria (62). To investigate this possibility, we used soluble *P. yoelii* 17XL Ag emulsified in Freund’s adjuvant as an experimental malaria vaccine. Using a prime-boost approach, we observed a significantly less severe course of *Plasmodium* challenge infection in KO mice, as demonstrated by greatly reduced parasitemia and accelerated clearance of parasites in comparison with WT immunized mice. As previously reported, *Mkp5−/−* mice showed increased production of IFN-γ and IL-4 in response to vaccination with keyhole limpet hemocyanin in CFA (36). Similarly, we observed that spleen cells from immunized KO mice produced significantly higher levels of IFN-γ as well as Th2-polarized IL-4 in response to malaria Ag than did their WT counterparts. Despite these differences, significantly higher levels of Ag-specific total IgG as well as Th1-associated IgG2c and Th2-associated IgG1 responses were detected in immunized KO mice than WT mice prior to infection. These observations indicate that, in the absence of MKP5, malaria-specific cellular as well as humoral responses were significantly augmented by anti-*P. yoelii* 17XL immunization. As a consequence of this, the protective efficacy against subsequent challenge was enhanced in the murine host. Overall, these results have important implications not only for the regulation of MKP5 in T cell–mediated immunity elicited by immunization, but also in extending its immunoregulatory role to Ab-mediated immunity. Because selection of potent adjuvants capable of improving the immune efficacy is an important aspect of malaria vaccine development, this study provides a piece of information that MKP5 may be a potential target in such application. However, there are many limitations of animal models for testing malaria vaccines, and there is still much to be learned about the biological effects of MKP5 signaling pathway in humans, the safety issues must be carefully addressed. If possible, by reducing the likelihood of adverse effects, the transient downregulation approach performed by MKP5 antagonists at proper times might serve as part of the vaccination strategies to alleviate the burden of malaria.

MKP5 deficiency greatly enhances IFN-γ-dependent early cell-mediated protective response as well as vaccination-induced protection against the lethal *Plasmodium yoelii* 17XL infection. However, considering the effect of the immunological balance on infection outcomes in hosts as demonstrated previously (32), we...
further evaluated the influence of MKP5 deficiency on the non-lethal strain \textit{P. yoelli} 17XNL. Consistent with the finding that an early and strongly protective response is sufficient for controlling infection in WT mice, we found the lack of MKP5 resulted in an excessive inflammatory process that could increase the severity of infection by inducing immunopathology. Thus, the enhanced proinflammatory response mediated by MKP5 deficiency can confer either beneficial or deleterious effects to the murine host, depending largely on the parasite strains and pathogenesis of infection. Gaining a comprehensive understanding of protection versus pathology on MKP5 deficiency would provide valuable information for the control of human malarial parasites with different virulence such as \textit{Plasmodium falciparum} or \textit{Plasmodium vivax}.

In summary, the present study investigates the immunoregulatory role of MKP5 in blood-stage malaria, which not only adds significantly to our understanding of anti-malarial immunity, but also furthers our understanding of the detailed regulatory pathway and molecular mechanisms of MKP5 in immune responses to \textit{Plasmodium} parasites. Most importantly, the data suggest that MKP5 may represent a potential target for the regulation of host resistance to blood-stage malaria.

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Disclosures

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 ROLE OF MKP5 IN MALARIA INFECTION


