NOTES

Osteopontin Participates in Th1-Mediated Host Resistance against Nonlethal Malaria Parasite *Plasmodium chabaudi chabaudi* Infection in Mice

Yoshimasa Maeno,^{1*} Shusuke Nakazawa,² Naoki Yamamoto,¹ Masanori Shinzato,³ Shigeo Nagashima,¹ Kumiko Tanaka,¹ Jun Sasaki,¹ Susan R. Rittling,⁴ David T. Denhardt,⁵ Toshimitsu Uede,⁶ and Koki Taniguchi¹

Department of Virology and Parasitology, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan¹;

Department of Protozoology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Nagasaki 852-8523, Japan²;

Department of Pathology, Fujita Health University College, Toyoake, Aichi 470-1192, Japan³; The Forsyth Institute,

Boston, Massachusetts 02115⁴; Department of Cell Biology and Neuroscience, Division of Life Sciences,

Rutgers University, Piscataway, New Jersey 08854-8082⁵; and Division of Molecular Immunology,

Institute for Genetic Medicine, Hokkaido University, Sapporo, Hokkaido 060-0815, Japan⁶

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Osteopontin (OPN) knockout mice (OPN-KO mice) died of *Plasmodium chabaudi chabaudi* infection, although wild-type (WT) mice had self-limiting infections. OPN was detected in the WT mice at 2 days postinfection. OPN-KO mice produced significantly smaller amounts of interleukin-12 and gamma interferon than WT mice produced. These results suggested that OPN is involved in Th1-mediated immunity against malaria infection.

Malaria remains a major cause of infant death worldwide; however, neither the acquisition of immunity nor the pathogenesis of the disease is well understood. Studies of the nonlethal murine blood-stage malaria parasite *Plasmodium chabaudi chabaudi* have suggested that the immune responses to this murine malaria parasite are characterized by the production of interleukin-12 (IL-12), tumor necrosis factor alpha, and gamma interferon (IFN- γ) (5, 8, 21, 23). It has been shown that *P. chabaudi chabaudi*-infected mice deficient in IL-12p40 or IFN- γ gene expression have a higher level of mortality with a higher level of parasitemia than wild-type (WT) mice (22, 23).

Osteopontin (OPN), a sialated phosphoprotein, is found in various tissues and is secreted into body fluids (2, 4, 11, 19, 20). OPN plays an active role in immune reactions that support the adhesion and migration of T cells and macrophages, facilitate CD3-mediated T-cell production of IL-2 and T-cell proliferation, and augment CD3-dependent IFN- γ and CD40 ligand expression in T cells (7, 9, 13, 14). The roles of OPN in infectious diseases caused by *Listeria monocytogenes*, herpes simplex virus type 1, and *Mycobacterium bovis* (1, 12) have been described previously. WT mice develop herpes simplex keratitis during herpes simplex virus type 1 infection, while osteopontin knockout (OPN-KO) mice do not readily develop this disease (1). IL-12- and IFN- γ -dependent Th1 responses to *L. monocytogenes* or *M. bovis* Calmette-Guérin are defective in OPN-KO mice (1, 12). These findings suggested that OPN

may polarize the Th1-related cytokine response and contribute to host defense against infectious pathogens.

To our knowledge, however, there have been no reports on the role of OPN in malaria infection. By using OPN-KO mice with a resistant C57BL/6 background, in this study we examined whether OPN is secreted from the beginning of the infection and leads to suppression of *P. chabaudi*. Activated innate and Th1-dependent immunity in C57BL/6 mice has been found to suppress *P. chabaudi chabaudi* infection (5, 8, 21–23).

OPN gene homozygous (OPN^{+/+} C57BL/6, OPN allele a) mice were purchased from Japan SLC (Hamamatsu, Japan). The production of OPN gene-deficient (OPN^{-/-}, OPN-KO) mice (C57BL/6 $\times$ 129, C57BL/6 background, F6) has been described previously (15, 16). Eight-week-old female OPN-KO mice and WT mice were used. The mice were inoculated intraperitoneally with 1×10^6 *P. chabaudi chabaudi*-infected erythrocytes (kindly provided by M. Suzuki, Gunma University School of Medicine, Japan). Parasitemia was monitored daily by examining Giemsa-stained thin blood smears. Blood samples were obtained via cardiac puncture under anesthesia at zero time (before infection) and on days 2, 3, and 7 postinfection. Serum was separated from clotted blood and stored at -80°C until it was used. The mice were then sacrificed, and their spleens were removed. The spleens were processed for flow cytometric, mRNA, and immunohistochemical analyses and stored at -80°C until they were used (except for the flow cytometric analysis). The experiments were carried out using five mice per group for each experimental day and were performed twice independently.

For detection of OPN mRNA by reverse transcriptase PCR

* Corresponding author. Mailing address: Department of Virology and Parasitology, Fujita Health University School of Medicine, 1-98 Kutsukake, Toyoake, Aichi 470-1192, Japan. Phone: 81-562-93-2486. Fax: 81-562-93-4008. E-mail: ymaeno@fujita-hu.ac.jp.

(RT-PCR), total RNA was extracted from mouse spleens using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The isolated total RNA was reverse transcribed to synthesize the first strand of cDNA using ReverTra Ace- α (Toyobo, Osaka, Japan). The cDNA was subjected to PCR using specific oligonucleotide primers to amplify cDNAs encoding β -actin and OPN. The reaction mixtures (25 μ l) contained 0.8 μ g of cDNA as a template, each primer at a concentration of 0.5 μ M, each deoxynucleoside triphosphate at a concentration of 200 μ M, 0.625 U of *Taq* DNA polymerase (QIAGEN, Tokyo, Japan), and 1 $\times$ PCR buffer (containing 1.5 mM MgCl₂). The target cDNA was amplified using a PCR protocol consisting of denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, the optimal annealing temperature for the target primer for 30 s, and 72°C for 1 min. The final cycle was followed by extension at 72°C for 7 min. This protocol was confirmed to be optimal for amplifying mouse OPN mRNA and β -actin mRNA by preliminary tests. The primer sequences used and optimal annealing temperatures were as follows: for β -actin, sense primer 5'-CCA GAG CAA GAG AGG TAT CC-3', antisense primer 5'-AGT CTA GAG CAA CAT AGC ACA G-3', and 55°C; and for OPN, sense primer 5'-ATG AGA TTG GCA GTG ATT TG-3', antisense primer 5'-GTT GAC CTC AGA AGA TGA AC-3', and 54°C. A GeneAmp 2400 PCR system (Perkin-Elmer, Norwalk, CT) was used for all PCR. The PCR products were separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. The densities of the DNA bands were determined with the Lane & Spot Analyzer software (Atto, Tokyo, Japan). The results of OPN mRNA expression were standardized by quantification of β -actin mRNA, which was used as an internal control.

The serum levels of IL-12 (IL-12 + p40) and IFN- γ were determined with sandwich enzyme-linked immunosorbent assay kits (BioSource International, Camarillo, CA). The minimum detection levels for IL-12 and IFN- γ were 2 pg/ml and 1 pg/ml, respectively.

An indirect immunofluorescence assay of spleen tissue was performed in a standard manner. Rabbit anti-mouse OPN (O-17) antibody (IBL, Gunma, Japan) and rat anti-macrophage (F4/80 specific, A3-1) monoclonal antibody (BMA Biomedicals, Augst, Switzerland) were used as primary antibodies, and labeled goat anti-rabbit immunoglobulin G (IgG) (Alexa Fluor 568; Molecular Probes, Eugene, OR) and fluorescein isothiocyanate-conjugated anti-rat IgG (MBL, Nagoya, Japan) antibodies were used as secondary antibodies. The sections were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI).

For the flow cytometric analysis, macrophages and monocytes were harvested from splenocytes by adhesion to a plate (MSP-P; JIMRO, Gunma, Japan), and the cells from five mice were pooled. Cells were first incubated with rat anti-macrophage (F4/80 specific, A3-1) monoclonal antibody and were then postfixed and permeabilized with 100% methanol. These cells were incubated with rabbit anti-mouse OPN (O-17) antibody. The cells were then treated with phycoerythrin-Cy5-conjugated anti-rabbit IgG (Santa Cruz, Santa Cruz, CA) and labeled goat anti-rat IgG (Alexa Fluor 488; Molecular Probes) antibodies. Samples were analyzed with a flow cytometer (Vantage SE fluo-

FIG. 1. Kinetics of parasitemia (a) and expression of OPN mRNA (b) in WT and OPN-KO mice infected with *P. chabaudi chabaudi*. The parasitemia was monitored by examining Giemsa-stained thin blood smears, and OPN mRNA was measured by a semiquantitative RT-PCR. The level of OPN mRNA was expressed the ratio of OPN mRNA to β -actin mRNA, which was used as an internal control. Each data point and bar indicate the mean of the results for 10 mice per group in two independent experiments in which five mice per group were used. An asterisk indicates that the *P* value is <0.001 . "d" indicates death of mice.

rescence-activated cell sorter; Becton Dickinson, San Jose, CA); at least 10,000 gated macrophages were collected.

Data were expressed as means $\pm$ standard errors of the means. The statistical evaluation was performed with Student's unpaired *t* test (two tailed). All analyses were performed using SPSS software (SPSS Japan, Tokyo, Japan), and a *P* value of <0.05 was considered significant.

OPN-KO mice showed increasing parasitemia and died on days 7 and 8 postinfection (Fig. 1a), while WT mice also showed increasing parasitemia but suppressed it after day 8. Thus, the results indicated that OPN was essential for resolution of the infection; likewise, OPN has been shown to be an important factor for pathogen control in *L. monocytogenes* and *M. bovis* BCG infections (1, 12).

The levels of OPN mRNA expression in the spleens of OPN-KO and WT mice were examined by a semiquantitative RT-PCR at zero time and on 2, 3, and 7 postinfection (Fig. 1b). The spleen was enlarged in all the WT and OPN-KO mice. OPN mRNA was not detected in the spleens of OPN-KO mice throughout the infection. On the other hand, expression of

FIG. 2. Fluorescence-activated cell sorting analysis of splenic macrophages from WT mice infected or not infected with *P. chabaudi chabaudi*. (a) The splenic macrophages were stained with anti-OPN antibody. The thin line indicates the results for uninfected WT mice, and the thick line indicates the results for infected WT mice 7 days after infection. (b) The cells from WT mice on 7 day after infection were stained with anti-OPN and anti-F4/80 antibody. PE, phycoerythrin.

OPN mRNA in WT mice was detected on day 2 and increased until it reached the highest level on day 7. To confirm that increased mRNA levels correlated with increased protein production and identification of OPN-producing cells, we analyzed OPN expression using flow cytometry and immunohistochemistry. We found that OPN expression in splenic macrophages from WT mice was upregulated after infection, and 82% of the splenic macrophages showed both OPN expression and F4/80 antigen expression (Fig. 2). By using immunohistochemistry, OPN-positive cells were observed in the

red pulp of the spleen tissue from WT mice (Fig. 3a), and they multiplied in the original area (Fig. 3d). A number of OPN-positive cells were also positive for F4/80 antigen, as observed by the fluorescence-activated cell sorting analysis (Fig. 3f). These data indicated that splenic macrophages produced OPN soon after *P. chabaudi chabaudi* infection, and they are consistent with previous reports that OPN is produced by macrophages, which are additional major producers in infectious diseases (1, 10, 12).

Recent studies suggested that OPN polarizes the immune

FIG. 3. Fluorescence micrographs showing the localization of OPN-positive cells (a, d, and g) and F4/80 antigen (macrophage)-positive cells (b, e, and h) and merged images for OPN- and F4/80 antigen-positive cells (c, f, and i) in spleen tissue from WT mice (a to f) and OPN-KO mice (g to i) on days 2 (D2) and 7 (D7) after *P. chabaudi chabaudi* infection. Insets show part of the plate at higher magnification. Bars, 100 μ m.

TABLE 1. Serum levels of cytokines and expression of osteopontin mRNA in spleens of WT and OPN-KO mice infected with *P. chabaudi chabaudi*^a

Cytokine or mRNA	WT mice				OPN-KO mice			
	Day 0	Day 2	Day 3	Day 7	Day 0	Day 2	Day 3	Day 7
IL-12 ^b	100 ± 2	150 ± 3	260 ± 9 ^d	121 ± 7 ^c	90 ± 4	110 ± 6	150 ± 7	75 ± 5
IFN-γ ^b	0 ± 0	0 ± 0	19 ± 1 ^d	64 ± 6 ^d	0 ± 0	0 ± 0	5 ± 1	28 ± 3
Osteopontin mRNA ^c	0.0 ± 0.0	0.03 ± 0.01	0.05 ± 0.01	0.21 ± 0.05	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

^a The serum levels of IL-12 (IL-12 + p40) and IFN-γ were determined by a sandwich enzyme-linked immunosorbent assay. Osteopontin mRNA was measured by a semiquantitative RT-PCR. The results are means ± standard errors of the means for 10 mice per group in two independent experiments using five mice per group.

^b IL-12 and IFN-γ levels are expressed in pg/ml.

^c The level of OPN mRNA is expressed as the ratio of OPN mRNA to β-actin mRNA, which was used as an internal control.

^d The difference between WT and OPN-KO mice is statistically significant ($P < 0.001$).

^e The difference between WT and OPN-KO mice is statistically significant ($P < 0.05$).

response toward a Th1-dominant condition rather than a Th2-dominant condition through induction of IL-12 production by macrophages in mice and that it is involved at an early time in establishment of Th1 immunity (1, 12). As shown in Fig. 2 and 3, OPN-positive cells should be activated by the antigen-presenting cells and might interact with T cells in the red pulp of the spleen to produce IL-12. IL-12 production by dendritic cells is generally considered to be T cell dependent and is induced by ligation of CD40 on dendritic cells and by CD40 ligands on activated T cells, as well as through direct signaling by major histocompatibility complex class II molecules cross-linked by T-cell receptors (3). OPN plays a role in increasing CD3-mediated T-cell production of IFN-γ and CD40 ligands, which augments the IL-12 production by monocytes (14). In order to evaluate the influence of OPN on Th1-related cytokine production in malaria, we measured the serum levels of IL-12 and IFN-γ in mice with and without the OPN gene. WT mice had significantly increased levels of IL-12 (IL-12 + p40) on days 2 and 3, while the level of IL-12 was low in OPN-KO mice (Table 1). Serum levels of IFN-γ were detected in both WT and OPN-KO mice on day 3 and increased thereafter. The levels of IL-12 and IFN-γ in OPN-KO mice were significantly lower than the levels in WT mice on days 3 and 7 (Table 1). These results suggest that OPN should facilitate IL-12 and IFN-γ production and secretion. It has been shown that the serum level of IL-12 is elevated early in malaria infection in mice and humans (17, 18, 22). The malaria parasite-susceptible A/J mouse strain treated with recombinant IL-12 was protected from *P. chabaudi* infection through upregulation of IFN-γ, tumor necrosis factor alpha, and nitric oxide production (21). IFN-γ gene- or IFN-γ receptor gene-deficient mice with the resistant C57BL/6 background showed susceptibility to *P. chabaudi* infection (6, 22, 24). These findings suggest that IL-12 contributes to the protection via IFN-γ. In our study, OPN-KO mice produced significantly smaller amounts of IL-12 and IFN-γ than WT mice produced, which may have been responsible for the increased parasitemia and mortality in OPN-KO mice. The impairment of protective immune responses against *P. chabaudi chabaudi* in OPN-KO mice suggests that OPN may play a critical role in the modulation of Th1 immune responses.

In WT mice, the level of OPN mRNA expression increased continuously, as did the level of IFN-γ until day 7, while the level of IL-12 decreased to almost the baseline level (Table 1). Similar behavior of OPN and IFN-γ was reported in a recent study in which THP-1 cells (human monocytes) or primary

human monocytes treated with IFN-γ were found to express OPN mRNA and protein in a time- and dose-dependent fashion (10). These findings, including our results, suggest that OPN may function in a positive feedback loop in Th1 immune responses; that is, OPN itself may upregulate the OPN gene via IFN-γ expression.

In conclusion, our results revealed that infection with the nonlethal murine malaria parasite *P. chabaudi chabaudi* induces OPN production and that OPN is involved in the clearance of the malaria parasites through Th1 immune responses at an early stage of *P. chabaudi chabaudi* infection.

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