Opsonin-Independent Phagocytosis: An Effector Mechanism against Acute Blood-Stage Plasmodium chabaudi AS Infection

Zhong Su, Anny Fortin, Philippe Gros, and Mary M. Stevenson

Opsonin-independent macrophage phagocytosis was investigated as a possible mechanism of controlling early blood-stage Plasmodium chabaudi AS infection. Early during infection, peritoneal macrophages from resistant C57BL/6 (B6) mice exhibited increased phagocytosis of parasitized red blood cells (pRBCs) and free merozoites, which was absent in mice with deficient interferon (IFN)–γ production during infection, including susceptible A/J, interleukin (IL)–12 p40, and IFN-γ gene knockout mice. IFN-γ treatment of macrophages collected from B6 and A/J mice early during infection enhanced phagocytosis of pRBCs, but IL-10 treatment inhibited this function. In vitro and in vivo studies in which type I and II class A scavenger receptor–deficient mice and inhibitors of scavenger and mannose receptors were used revealed that scavenger receptors other than class A type I and II and mannose receptors may play a role in malaria parasite uptake. These results indicate that opsonin-independent phagocytosis contributes to the IFN-γ-dependent control of acute blood-stage malaria infection.

Primary infection with blood-stage Plasmodium chabaudi AS in mice is characterized by acute parasitemia that culminates in peak parasitemia. Susceptible mice, such as A/J, develop a fulminant peak parasitemia and usually die from infection within 2 weeks, whereas resistant mice, such as C57BL/6 (B6) mice, develop less severe parasitemia and control the infection [1]. Previous studies have shown that Th1-associated cytokines, including interleukin (IL)–12, interferon (IFN)–γ, and tumor necrosis factor (TNF)–α, play protective roles in resistance to the acute stage of infection [2–5]. Recent studies that have used IFN-γ gene knockout (GKO) or IL-12 p40 knockout (KO) mice have provided further evidence for the pivotal roles of these cytokines in the control of acute blood-stage P. chabaudi AS infection [6–8].

Although the protective roles of Th1-type cytokines during acute P. chabaudi AS infection have been well established, the immune effector mechanisms that operate during the early phase of infection to control parasitemia are not clear. Blood-stage P. chabaudi AS infection in mice induces increased NK cell cytotoxicity. However, bg/bg mutant mice, which have defective NK cell cytotoxic function but normal IFN-γ production, are able to control the infection as efficiently as control mice [9]. These results, which are consistent with an earlier finding [10], suggest that NK cell cytotoxic activity does not contribute to resistance in blood-stage P. chabaudi AS infection. Antibody-mediated immunity may not contribute to protection during the acute phase of infection, because significant levels of parasite-specific antibody are not produced during this time [6, 8]. Indeed, B cell–deficient and intact control mice develop similar levels of parasitemia during the first 2 weeks of infection [11, 12]. Whereas CD4+ T cells are essential [13, 14], depletion of CD8+ T cells does not diminish the ability of mice to control and resolve blood-stage malaria [13, 15], although cytotoxic CD8+ T cells have been shown to be critical in protection against sporozoites of liver-stage malaria in mice [16]. Infection with blood-stage P. chabaudi AS in resistant mice has been shown to increase production of nitric oxide (NO) [6, 17], and NO has been thought to play a protective role in the control of blood-stage malaria [18]. However, recent studies have shown that NO synthase–2 (NOS2) and NOS3 gene KO mice are able to control and resolve blood-stage P. chabaudi AS infection with a time course similar to that for wild-type mice [19, 20]. It was recently reported that C1q-deficient mice (defective in the classical complement pathway) and B- and C2-deficient mice (defective in both alternative and classical pathways) exhibit an only slightly higher level of parasitemia on days 9–10 after infection [21], which indicates that the complement system plays only a minor role in controlling the acute phase of primary P. chabaudi infection.

An earlier study in our laboratory showed that treatment of
susceptible A/J mice with the immunomodulating agent muramyl dipeptide encapsulated in liposomes to activate macrophages suppressed acute parasitemia and improved the survival of the mice, whereas depletion of mononuclear phagocytes by silica treatment resulted in increased parasitemia and mortality in resistant B6 mice [22]. Those studies suggested that macrophages play a role in controlling early blood parasitemia levels during acute infection. A possible mechanism may be the phagocytosis of malaria parasites, a macrophage function that is up-regulated by the potent macrophage activating factor IFN-γ, which is produced during early acute blood-stage malaria by NK cells and later by CD4+ T cells [2, 4, 23, 24].

Two types of macrophage phagocytosis have been described: opsonin-dependent and opsonin-independent phagocytosis [25]. In opsonin-dependent phagocytosis, antibody or complement opsonizes the pathogen and promotes its phagocytosis via Fc or complement receptors on phagocytes. For opsonin-independent phagocytosis, phagocytes recognize and phagocytose the microorganism via pattern-recognition receptors, such as scavenger and mannose receptors. Opsonin-independent phagocytosis mediated by these pattern-recognition receptors has been shown to contribute to early or innate resistance to a number of bacterial pathogens [26, 27]. Mota and colleagues [28, 29] have demonstrated that peritoneal macrophages from CBA mice show increased phagocytosis of antibody-opsonized P. chabaudi–infected red blood cells (RBCs) and that this antibody-mediated phagocytosis is parasite species specific. Although antibody-opsonized phagocytosis of parasitized RBCs (pRBCs) may represent an important mechanism of acquired immunity to blood-stage malaria, it may not contribute to the control of acute infection, because no significant level of antibody is produced during this time. Therefore, in the present study, we investigated opsonin-independent phagocytosis as a possible defense mechanism for controlling the early phase of blood-stage P. chabaudi AS infection.

Materials and Methods

**Mice, parasites, and experimental infections.** B6 mice were purchased from Charles River Laboratories. A/J mice and breeding pairs of IL-12 p40 KO mice with the B6 background were obtained from Jackson Laboratory. Breeders of GKO mice (originally provided by Genentech) were backcrossed to B6 mice for 8 generations by F. P. Heinzel (Case Western Reserve University School of Medicine, Cleveland) [30] and generously given to us. Class A type I and II macrophage scavenger receptor KO (SR-AI/II KO) mice were kindly provided by T. Kodama (University of Tokyo, Tokyo). The SR-AI/II null mutation was transferred, using the marker-assisted speed congenic strategy, to the B6 genetic background [31] in our laboratory. All KO mice were bred in the animal facility of the Montreal General Hospital Research Institute (Montreal) under specific pathogen–free conditions. The experimental animals were maintained in the same facility, which is lit between 6 A.M. and 6 P.M. Female 8–12-week-old mice were used in all experiments. Blood-stage P. chabaudi AS was maintained in our laboratory as described elsewhere [13]. Infections were initiated by intraperitoneal injection of 1 × 106 P. chabaudi pRBCs. Parasitemia was monitored daily on blood smears stained with Diff-Quik (American Scientific Products).

**Spleen cell culture.** Spleens from normal and infected mice were removed aseptically and pressed through a sterile fine wire mesh into RPMI 1640 (Invitrogen–Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (Hyclone Laboratories), 25 mM HEPES (Invitrogen–Life Technologies), 0.12% gentamicin (Schering), and 2 mM glutamine (Invitrogen–Life Technologies) (complete medium). RBCs were lysed with 0.175 M NH4Cl, and membrane debris was removed by filtering the cell suspensions through sterile gauze. Spleen cells were adjusted to a level of 5 × 106 cells/mL, and aliquots of 1 mL were plated in triplicate in 24-well tissue culture plates in the presence of 106 pRBCs/mL and incubated for 48 h at 37°C in a humidified CO2 incubator. Supernatants were collected and stored at −20°C until assays for cytokine levels were done.

**Cytokine ELISA.** Levels of IFN-γ in cell culture supernatants and IL-12 p70 in serum samples collected 2 days after infection were measured by 2-site sandwich ELISA using paired capturing and detecting antibodies, as described elsewhere [4, 5, 32]. Standard curves for each cytokine were generated using recombinant cytokines (BD Bioscience). Reactivity was revealed using ABTS substrate (Roche), and optical density values were read in a microplate reader at 405 nm with a reference wavelength of 492 nm.

**Phagocytosis of parasitized RBCs.** Peritoneal cells were collected from uninfected and infected B6, A/J, GKO, IL-12 p40 KO, and SR-AI/II KO mice by peritoneal lavage with 10 mL of complete medium. The cells were washed twice, and cytosin slides were made and stained with Diff-Quik to determine the percentage of macrophages in individual samples. Cell suspensions were adjusted to 5 × 106 macrophages/mL. Aliquots of 0.5 mL of the cell suspension were added to an 8-well chamber slide (Nunc) and incubated at 37°C for 2–3 h. After incubation, the cells were washed with warm complete medium to remove nonadherent cells. In some experiments, to test the effects of cytokines on phagocytosis, murine-recombinant IFN-γ (100 U/mL), TNF-α (100 U/mL), IL-4 (200 U/mL), or IL-10 (200 U/mL) (all from R&D Systems) was added (0.5 mL/well) to the cells, incubated at 37°C for 12 h, and washed with warm complete medium. Blood samples were collected from P. chabaudi AS–infected B6 mice with ~30% parasitemia at ~6 P.M. on the day of the experiment, to obtain parasites at the trophozoite stage. Blood was washed 3 times and resuspended at a level of 109 RBCs/mL in complete medium. Aliquots of 0.2 mL of pRBC suspension were added to the macrophage monolayers and incubated at 37°C for 40–45 min. The macrophage monolayer was washed with warm complete medium 3 times, air dried, and stained with Diff-Quik. Phagocytosis of pRBCs by macrophages was scored by microscopy and expressed as phagocytic index (number of pRBCs phagocytosed by 100 macrophages).

**Phagocytosis of free merozoites.** To quantitate macrophage phagocytosis of free merozoites, pRBCs were cultured in the presence of [3H]-hypoxanthine to label the merozoites, as described elsewhere [33, 34]. In brief, blood samples were collected from infected B6 mice (day 7–8 after infection, with ~30% parasitemia) between 9 and 10 P.M. on the day of the experiment, to collect parasites at
the mature trophozoite stage. The heparinized blood was passed through a CF-11 cellulose column (Whatman) to remove leukocytes and platelets. After being washed with medium, RBCs were loaded on 74% Percoll (Sigma-Aldrich) and centrifuged at 5000 g for 20 min at room temperature. The top band, which contained >99% pRBCs, was collected, washed with medium, and resuspended at 3 × 10^8 pRBCs/mL in RPMI 1640 medium supplemented with 10% heat-inactivated rat serum, 2 mM glutamine, 10 µg/mL gentamicin, 0.2% glucose, and 10 µL/mL ^3H-hypoxanthine (42 Ci/mmol or 1.15 mCi/mL) (Sigma). The pRBC were cultured in a 12-well tissue culture plate in a candle jar at 37°C for 18 h [35]. At the end of the culture period, the majority (>98%) of pRBCs were ruptured, and the majority of ^3H-hypoxanthine-labeled merozoites had been released. The merozoites were collected by centrifugation at 5000 g for 15 min and washed 5 times with complete medium. The merozoite pellet was resuspended with complete medium to the original culture volume. Peritoneal macrophages from uninfected and infected mice were prepared as described above, and cell suspensions were adjusted to 3 × 10^6 macrophages/mL. Aliquots of 0.2 mL of cell suspensions were incubated in triplicate in 48-well culture plates at 37°C for 2 h. Macrophage monolayers were then washed with medium, and 200 µL of the ^3H-hypoxanthine-labeled merozoite suspension (equivalent to 6 × 10^7 pRBCs) were added to each well. The plate was briefly spun and incubated for 1 h at 37°C. Noningested merozoites were removed by 3 washes with warm PBS, and 100 µL of 1% SDS was added to lyse the cells. The lysate was collected for scintillation counting. Data are presented as mean counts per minute ± SE.

**In vitro and in vivo treatment with scavenger and mannose receptor ligands.** Peritoneal macrophages were collected from uninfected B6 mice and from infected B6 mice at day 7 after infection and incubated in chamber slides or tissue culture plates as described above. To block scavenger receptors, cells were preincubated at 37°C for 30 min with 100 µg/mL polysinosine acid (poly[I]), a synthetic ligand for scavenger receptors [36, 37], or 100 µg/mL polycytidylic acid (poly[C]) as a cognate nonligand control for poly(I) (Sigma). Cytochalasin D (8 µM; Sigma) was used as a positive control. To block mannose receptors, cells were preincubated with 5 mg/mL mannan or 5 mg/mL β-glucan as a control [38]. The inhibitors and control chemicals were retained in the subsequent incubation with pRBCs or ^3H-hypoxanthine-labeled free merozoites. After incubation with pRBCs or free merozoites, the phagocytes in chamber slides or in tissue culture plates were processed as described above. To determine the role of scavenger receptor-mediated phagocytosis in vivo, mice were treated with poly(I) to block scavenger receptors, as described elsewhere [39]. Groups of B6 mice were infected by intravenous injection of 1 × 10^8 pRBCs and treated intraperitoneally with PBS or 100 µg of poly(I) or poly(C) on the day of infection and then daily for 14 days. To determine the role of mannose receptors in vivo, B6 mice were treated intraperitoneally with 50 mg of mannan or β-glucan per day for 14 days, beginning on the day of infection (1 × 10^8 pRBCs administered intravenously). Parasitemia was monitored as described above.

**Statistical analysis.** Data are presented as mean ± SE. The statistical significance of differences in phagocytosis between groups was analyzed by Student’s t test. Repeated-measures analysis of variance was performed to test the significance of differences in parasitemia levels between mouse groups. All analyses were performed using SAS/STAT software (SAS Institute), and P < .05 was considered to be significant.

**Results**

As observed in our previous studies [3–6, 8], after inoculation with blood-stage *P. chabaudi* parasites, B6 mice developed moderate levels of parasitemia, and infection resolved by 3–4 weeks after inoculation (figure 1A). Susceptible A/J mice showed fulminant primary parasitemia, and all mice died by day 10 after infection. In comparison with wild-type B6 mice, GKO and IL-12 p40 KO mice developed rapidly increasing parasitemia and reached significantly higher peak parasitemia levels at days 8–9 after infection. GKO mice had 40%–50% mortality during days 10–14 after infection, whereas all B6 and IL-12 p40 KO mice survived the infection (figure 1B). It should be noted that these studies were performed using female mice. Previous studies done in our laboratory [6, 8] demonstrated that the mortality rate is higher among male GKO (100%) and IL-12 p40 KO (40%) mice after blood-stage *P. chabaudi* AS infection. Infection with blood-stage *P. chabaudi* AS in resistant B6 mice induced a strong IFN-γ response, as demonstrated by the high level of IFN-γ produced in vitro by spleen cells in response to stimulation with parasite antigen (figure 1C). This strong IFN-γ response was absent in susceptible A/J and IL-12 p40 KO mice (figure 1C).

To understand the effector mechanism of IFN-γ-dependent resistance, we analyzed the phagocytic function of peritoneal macrophages from the 4 groups of mice before and during infection. Peritoneal macrophages from uninfected mice had low levels of opsonin-independent phagocytosis of pRBCs (figure 2A). However, macrophages from B6 mice at 7 days after infection showed significantly increased phagocytic activity. As the infection progressed, the phagocytic activity of macrophages from B6 mice was found to be decreased at day 10 (figure 2A), compared with the high level of phagocytosis seen at day 7 after infection. In contrast, the phagocytosis of macrophages from infected GKO, IL-12 p40 KO, and A/J mice remained at low levels during the first 10 days after infection, similar to levels seen in uninfected mice (figure 2A). Treatment of pRBCs with heat-inactivated normal mouse serum from uninfected B6 mice or with serum collected during the acute stage of infection (day 7) did not increase levels of phagocytosis by macrophages collected from infected B6 mice at day 7 after inoculation. Macrophages collected from infected B6 mice at day 7 after inoculation showed only a low level (2%–3%) of phagocytosis of normal RBCs from uninfected B6 mice.

During the blood-stage cycle, merozoites of the malaria parasite infect RBCs and develop inside the RBCs, progressing from ring forms to the trophozoite stage and then into mature schizonts that contain newly produced merozoites that are re-
Course of blood-stage *Plasmodium chabaudi* AS infection and interferon (IFN)-γ production in vitro by spleen cells of C57BL/6 (B6), interleukin (IL)-12 p40 knockout (KO), IFN-γ gene KO (GKO), and A/J mice. A, Mice were infected intraperitoneally with $10^6$ parasitized red blood cells (pRBCs), and levels of parasitemia were determined. Data are mean parasitemia levels for 5–6 mice/group from 1 of 3 replicate experiments. SEs (not shown) are $\pm 15\%$ of mean parasitemia levels. * and **, for comparison of peak parasitemia levels with those of B6 mice.

B, Cumulative survival of infected B6, IL-12 p40 KO, GKO, and A/J mice was determined from 15–20 mice pooled from 3 experiments. Note that 100% of female B6 and IL-12 p40 KO mice survived.

C, Spleen cells from uninfected mice and infected mice at day 7 after inoculation were cultured in the presence of pRBCs ($10^6$ cells/mL), and IFN-γ levels in the cell culture supernatants were determined by ELISA. GKO mice do not produce biologically active IFN-γ. Data are for 4 mice/group from 1/2 experiments. ***, vs. uninfected control mice.

Released after rupture of the RBCs. Merozoites have a brief free-living stage before invasion into RBCs [40]; during this time, they may be subjected to surveillance mechanisms in the circulation. Therefore, we also analyzed opsonin-independent phagocytosis of free merozoites as a possible defense mechanism during early infection. Macrophages from uninfected B6, A/J, GKO, and IL-12 p40 KO mice showed similar baseline levels of phagocytosis of free merozoites (figure 2B). Seven days after infection, a significant increase in merozoite phagocytosis was observed in macrophages from B6 but not from A/J, GKO, or IL-12 p40 KO mice. As observed with phagocytosis of pRBCs, macrophages harvested from B6 mice 10 days after infection showed reduced levels of phagocytosis, compared with macrophages harvested at 7 days after infection. Microscopic examination of merozoite phagocytosis on chamber slides showed that >90% of macrophages from uninfected mice were able to phagocyte at least 1 merozoite, but the majority of macrophages collected from infected B6 mice at day 7 after infection phagocytosed as many as 10 merozoites/cell.

To determine the effects of Th1 and Th2 cytokines on opsonin-independent phagocytosis, peritoneal macrophages from uninfected and malaria-infected B6 and A/J mice were treated with IFN-γ, TNF-α, IL-10, or IL-4 before the addition of pRBCs. In comparison with the medium control in each group, IFN-γ treatment of macrophages from uninfected B6 mice and from infected B6 mice and infected A/J mice at day 7 after inoculation significantly increased the phagocytosis of pRBCs.
macrophages harvested from B6 mice at day 7 after infection (figure 3
A and B), and slightly increased phagocytosis by macrophages collected from infected A/J mice at day 7 after infection (figure 3D). Of interest, as described above, macrophages harvested from B6 mice 15 days after infection had low levels of phagocytosis, compared with cells harvested 7 days after infection (figure 3C), and none of the cytokines had significant effects on the phagocytosis of pRBCs by macrophages obtained at this time (figure 3C).

Macrophage scavenger receptors have been shown to mediate opsonin-independent phagocytosis and contribute to early resistance to some bacterial pathogens [26]. These receptors may also be involved in mediating phagocytosis of blood-stage malaria parasites. To examine this possibility, we infected SR-AI/KO and wild-type control B6 mice intraperitoneally with 10^6 pRBCs and monitored parasitemia. SR-AI/KO mice and wild-type B6 mice developed similar levels of parasitemia, and all mice in both groups survived infection (figure 4). Peritoneal macrophages collected from infected SR-AI/KO mice at day 7 after inoculation showed levels of phagocytosis of both pRBCs and free merozoites similar to those seen in B6 mice (data not shown).

To determine whether other members of the scavenger receptor family mediate opsonin-independent phagocytosis, poly(I) was used to block these receptors in vitro and in vivo. In vitro treatment with poly(I) of macrophages collected from infected B6 mice at day 7 after incubation only slightly inhibited phagocytosis of pRBCs (13% ± 1.1%; P > .05), compared with the levels in medium (17% ± 1.4%) and poly(C) (15% ± 1.8%) controls. However, it significantly (P < .01) reduced the phagocytosis of free merozoites (figure 5A). Treatment of the macrophages from infected mice with cytochalasin D, but not poly(C), also significantly inhibited phagocytosis of merozoites (figure 5A).

To evaluate the in vivo effect of blocking scavenger receptors, B6 mice were treated with poly(I) or poly(C) during the first 2 weeks of malaria infection. Mice treated with poly(I) developed a rapidly ascending parasitemia from days 4 to 8 after infection (figure 5B). The overall levels of parasitemia in poly(I)-treated mice during this period were significantly higher than those of PBS- and poly(C)-treated control groups (P < .01, by analysis of variance). After the peak of parasitemia on day 8 after infection, poly(I)-treated mice had levels of parasitemia similar to those of the control mice until the infection was resolved at 4 weeks. All mice in the 3 groups survived the infection. To confirm that poly(I) treatment did not alter the development of protective immune responses, blood was collected from the 3 groups of mice 2 days after infection, and the levels of IL-12 p70 in serum were determined by ELISA [6, 32]. Similar levels of serum IL-12 p70 were detected in PBS-treated control (3.9 ± 0.14 ng/mL), poly(I)-treated (3.7 ± 0.11 ng/mL), and poly(C)-treated (3.6 ± 0.13 ng/mL) mice during infection.

We also examined the in vitro and in vivo role of mannose receptors in the opsonin-independent phagocytosis of pRBCs and merozoites. Treatment with mannan of peritoneal macrophages collected from infected B6 mice at day 7 after infection to block mannose receptors significantly inhibited the phagocytosis of pRBCs (figure 6A) and free merozoites (figure 6B),
by 30% and 42%, respectively, compared with medium controls. However, treatment of P. chabaudi AS–infected B6 mice with mannan for the first 14 days of infection did not alter the levels of parasitemia in comparison with PBS and β-glucan–treated control groups (data not shown).

Discussion

In the present study, we demonstrate that peritoneal macrophages from resistant B6 mice exhibit increased levels of opsonin-independent phagocytosis of both pRBCs and free merozoites during the acute phase of P. chabaudi AS infection, whereas increased phagocytosis was absent in macrophages from susceptible A/J, IL-12 p40 KO, and GKO mice. Previous studies have shown that Th1-associated cytokine responses, in particular IFN-γ, early during infection are critical for control of acute blood-stage P. chabaudi AS infection and survival of the host. Resistant B6 mice, which mount strong IL-12 and IFN-γ responses, are able to control and survive acute blood-stage P. chabaudi AS infection [4, 6, 8, 32]. A/J and IL-12 p40 KO mice, which have defective IFN-γ responses [4, 8], as well as GKO mice, developed higher levels of parasitemia after infection than did resistant B6 mice. GKO and A/J mice also had high mortality rates. Taken together, these results provide evidence that opsonin-independent phagocytosis of pRBCs and free merozoites is an important effector mechanism of IFN-γ–dependent control of acute blood-stage P. chabaudi AS infection. In addition, we observed that treatment of P. chabaudi AS–infected susceptible A/J mice with oligodeoxynucleotides containing CpG motifs, which are known to induce Th1 cytokine production [41], significantly suppressed acute fulminant parasitemia and protected these mice from death (data not shown).

Moreover, peritoneal macrophages recovered at 7 days after infection from CpG-treated A/J mice showed significantly increased opsonin-independent phagocytosis of pRBCs (17%–20%), compared with the levels of 3%–4% seen in A/J mice that were infected and received no treatment (figure 2A).

It was also observed that macrophages from resistant B6 mice initially showed increased phagocytic activity during acute infection but that the phagocytic activity declined to baseline levels after peak parasitemia during the chronic stage of infection. The kinetics of phagocytic activity closely coincided with the level of IFN-γ production observed in B6 mice during P. chabaudi AS infection [8]. These results suggest that opsonin-independent phagocytosis may not play a protective role as the infection progresses to the chronic phase. Indeed, mice treated with poly(I) developed higher levels of acute parasitemia than did control mice. But poly(I) treatment had no effect on parasitemia during the chronic phase of infection. The reduction in the level of phagocytosis of parasites by macrophages during the late stage of infection may be due to decreased levels of IFN-γ and increased production of IL-10 [8], which may down-regulate the function of macrophages [42]. Indeed, we observed

Figure 4. Course of blood-stage Plasmodium chabaudi AS infection in C57BL/6 (B6) and class A type I and II macrophage scavenger receptor knockout (SR-AI/II KO) mice. Mice were infected intraperitoneally with 10⁷ parasitized red blood cells, and the course of parasitemia was determined. Data are mean for 5–6 mice/group from 1 of 3 replicate experiments. SEs (not shown) are <10% of mean level of parasitemia.

Figure 5. Inhibition of phagocytosis of free merozoites by polynosinic acid (poly[I]) and in vivo effect of poly(I) treatment on parasitemia after blood-stage Plasmodium chabaudi AS infection. A, Adherent peritoneal macrophages collected from uninfected mice and from infected C57BL/6 (B6) mice at day 7 after inoculation were incubated with poly(I) (100 µg/mL), polycytidylic acid (poly[C]; 100 µg/mL), or cytochalasin D (Cytocha-D; 8 µM), followed by the addition of [3H]hypoxanthine–labeled free merozoites. The subsequent steps of the assay were the same as described in the legend to figure 2B. Phagocytic activity is expressed as cpm. Data are mean ± SE for 4 mice/group from 1 of 3 experiments. ***P < .001, vs. medium control. B, Groups of B6 mice were treated intraperitoneally with 100 µg of poly(I), poly(C), or PBS on the day of infection with 10⁷ parasitized red blood cells (intravenous) and daily for 14 days after infection. Parasitemia was determined at the indicated times. Data are mean for 5 mice/group from 1 of 2 experiments. SEs (not shown) are <10% of the mean parasitemia. *P < .05 and **P < .01, vs. PBS control.
that the treatment with IL-10, but not IL-4, of peritoneal macrophages collected from infected B6 mice at day 7 after infection significantly inhibited the phagocytosis of pRBCs. In addition, peritoneal macrophages collected from B6 mice after peak parasitemia and peak IFN-γ production—that is, after postinfection day 10—had reduced phagocytic activity for pRBCs and free merozoites. The subsequent steps of the assay were the same as described in the legend to figure 2B. Data are mean ± SE for 4 mice/group from 1 of 3 experiments. *P < .05 and **P < .01, vs. medium control.

Figure 6. Inhibitory effect of blocking mannose receptor on phagocytosis of parasitized red blood cells (pRBCs; A) and free merozoites (B). Adherent peritoneal macrophages collected from uninfected mice and from infected C57BL/6 (B6) mice at day 7 after infection were incubated with mannan (5 mg/mL) or β-glucan (5 mg/mL) before the addition of pRBCs or 3H-hypoxanthine–labeled free merozoites. The subsequent steps of the assay were the same as described in the legend to figure 2B. Data are mean ± SE for 4 mice/group from 1 of 3 experiments. *P < .05 and **P < .01, vs. medium control.

levels of parasitemia was not significantly different, compared with those in wild-type control mice. We observed that SR-AI/II KO mice had a course of P. chabaudi AS infection similar to that seen in wild-type B6 mice. Macrophages from P. chabaudi AS–infected SR-AI/II KO and B6 mice showed similar levels of opsonin-independent phagocytosis of pRBCs and free merozoites. Of interest, studies from our laboratory [46] and others [47] demonstrated sequence polymorphisms at 9 positions in SR-AI/II expressed on macrophages from B6 mice. These polymorphisms affect recognition by the rat anti–mouse SR-AI/II monoclonal antibody 2F8 but do not affect function with respect to the internalization and degradation of 125I-labeled acetylated low-density lipoprotein [46].

However, blocking total macrophage scavenger receptors with poly(I) in vitro inhibited phagocytosis of free merozoites and, to a lesser extent, pRBCs. In vivo treatment of B6 mice with this inhibitor significantly increased the levels of ascending parasitemia. These results indicate that members of the scavenger receptor family other than SR-AI/II may mediate opsonin-independent phagocytosis of pRBCs and free merozoites. CD36, a class B scavenger receptor, has been shown to be involved in the cytoadherence of P. falciparum–infected RBCs to endothelial cells [48]. Blocking CD36 on human monocytes with antibody significantly inhibits nonopsonic phagocytosis of pRBCs [49]. In the present study, poly(I) treatment blocked CD36, as well as other scavenger receptors, such as MARCO (macrophage receptor with a collagenous structure), macroslalin, and Lox-1, and inhibited phagocytosis of merozoites and pRBCs, leading to increased acute parasitemia seen in poly(I)-treated mice. Furthermore, these results suggest that the increased acute parasitemia in poly(I)-treated mice may be due to significant inhibition of phagocytosis of free merozoites. Partial blocking of phagocytosis of pRBCs by poly(I) may also contribute, albeit to a lesser extent, to the increased parasitemia in these mice.

Mannose receptors are expressed on macrophages, endothelial cells, and dendritic cells [27, 50, 51]. This receptor recognizes carbohydrates on the surface of infectious agents and has been shown to play a role in mediating opsonin-independent phagocytosis of microorganisms and in antigen capture by dendritic cells [38, 51, 52]. The results of the present study show that blocking mannose receptors on peritoneal macrophages from infected B6 mice with mannan inhibited the phagocytosis of pRBCs and free merozoites. This suggests that the mannose receptor expressed on macrophages recognizes some carbohydrate ligands on the surface of malaria-infected RBCs and free merozoites and mediates opsonin-independent phagocytosis via these target molecules. However, treatment of P. chabaudi–infected B6 mice with mannan to block mannose receptors in vivo did not result in increased acute parasitemia. It is possible that mannan is quickly metabolized in vivo before it binds to mannose receptors. A mannose receptor ligand that is not degradable and suitable for use in vivo will be required.
for further study of the in vivo role of mannose receptors in the phagocytosis of pRBCs and free merozoites.

In conclusion, we have demonstrated that the levels of opsonin-independent phagocytosis of pRBCs and free merozoites by peritoneal macrophages from resistant and susceptible mice closely correlated with the ability to produce IFN-γ and control acute blood-stage *P. chabaudi* AS infection. Treatment with IFN-γ in vitro markedly enhanced opsonin-independent phagocytosis by macrophages harvested from uninfected mice and from mice in the early stage of infection, whereas IL-10 inhibited this function. Although SR-A/II receptors are not critically required for the phagocytosis of pRBCs and merozoites and for the control of blood-stage malaria, other members of the scavenger receptor family may be involved in this early defense mechanism, as shown by in vitro and in vivo blockade of scavenger receptors with poly(I). Blocking macrophage mannose receptors in vitro with mannan partially inhibited the phagocytosis of pRBCs and merozoites, which suggests that this receptor may also contribute to the clearance of malaria parasites during the early stages of infection.

Acknowledgments

We gratefully acknowledge the technical assistance of Mi Fong Tam, for breeding the gene knockout mice, maintaining the parasites, and performing the infection studies, and Patrick Fortin, for genotyping the class A type I and II macrophage scavenger receptor knockout mice.

References

32. Sam H, Stevenson MM. In vivo IL-12 production and IL-12 receptors β1 and β2 mRNA expression in the spleen are differentially upregulated in...


