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Although primary infection of mice with Babesia microti has been shown to protect mice against subsequent lethal infection by Babesia rodhaini, the mechanism behind the cross-protection is unknown. To unravel this mechanism, we investigated the influence of primary infection of mice with nonlethal B. microti using different time courses on the outcome of subsequent lethal B. rodhaini infection. Simultaneous infections of mice with these parasites resulted in rapid increases in parasitemia, with 100% mortality in BALB/c mice, as observed with control mice infected with B. rodhaini alone. In contrast, mice with acute, resolving, and chronic-phase B. microti infections were completely protected against B. rodhaini, resulting in low parasitemia and no mortalities. Mice immunized with dead B. microti were not protected from B. rodhaini infection, although high antibody responses were induced. Interestingly, the protected mice had significantly decreased levels of antibody response, cytokines (including gamma interferon [IFN-γ], interleukin-2 [IL-2], IL-8, IL-10, and IL-12), and nitric oxide levels after infection with B. rodhaini. SCID mice and IFN-γ-deficient mice with chronic B. microti infections demonstrated protective responses comparable to those of immunocompetent mice. Likewise, in vivo NK cell depletion did not significantly impair the protective responses. Conversely, macrophage depletion resulted in increased susceptibility to B. rodhaini infection associated with changes in their antibody and cytokines profiles, indicating that macrophages contribute to the protection against this challenge infection. We conclude that future development of vaccines against Babesia should include a strategy that enhances the appropriate activation of macrophages.

Babesiosis is caused by intraerythrocytic parasites of the genus Babesia. The infection is one of the most important tick-borne diseases parasitizing a wide range of mammalian hosts, including humans, worldwide. Babesiosis causes huge economic losses in the livestock industry. Recently, the disease has become an important emerging zoonosis, with Babesia microti being the most important cause of human babesiosis in America, Europe, and Asia (23, 26, 27, 35, 55). Most of the human cases are either from tainted blood transfusions or from bites of infected Ixodes scapularis nymphs, which inject sporozoites into the bloodstream of the host during their feeding. The infection is often asymptomatic in healthy humans but can occasionally be fatal in immunocompromised individuals (27, 36, 37).

A better understanding of the immune response to infection by Babesia parasites is important for designing a safe and efficacious vaccine (9, 28). Over the past decade, several studies have demonstrated the importance of T helper (Th) cells in regulating the immune response to Babesia infection (2, 12). These cells produce the cytokines needed for the both maturation of high-affinity immunoglobulin isotype production and the activation of macrophages for phagocytosis and parasiticidal activity (10, 11). However, the timing and magnitude of these cytokines can determine the outcome of the infection. The early response of the inflammatory cytokines gamma interferon (IFN-γ) and interleukin-12 (IL-12) is critical for controlling the initial burst of intraerythrocytic parasite multiplication. Moreover, the failure to maintain Th1-predominant response during the acute stage is correlated with a rapid increase in parasite load. On the other hand, the switch to the predominance of the Th2 response (IL-4 and IL-10) at the resolution stage accompanied by elevated antigen-specific immunoglobulin G (IgG) appears to be crucial for the control of parasite replication (2, 12). Phagocytosis of parasitized erythrocytes by activated macrophages occurs in the spleen and is believed to be essential for the removal of the parasites (48).

The use of mice, rather than large mammals, infected with rodent Babesia provides an economic model for investigating the host immune response to babesiosis (28, 50). Mice infected with B. microti reveal transient high parasitemia, followed by complete recovery from the acute infection, and the cured mice are usually resistant to the reinfection by the same parasite. This protection is mainly due to T-cell-mediated immunity in the spleen (28, 44). In contrast, B. rodhaini causes a more severe disease, resulting in 100% mortality (34). Interestingly, mice that had a prior infection with B. microti are known to be protected against challenge infection by B. rodhaini Antwerp strain, with a survival rate of up to 83% (58). However, the mechanism behind this cross-protection is unknown. We believe that understanding the mechanism behind this protection could provide important clues for the future design of vaccines against babesiosis.

In the present study, the cross-protection conferred by primary infection of mice with B. microti against challenge with lethal B. rodhaini was examined either in the absence or presence of immune effector cells. We show that innate immunity based on the
macrophage response but not adaptive immunity is crucial to the cross-protection offered by *B. microti* against lethal *B. rodhaini* infection. However, antibody, B and T lymphocytes, IFN-γ, and NK cells did not play a major role in this cross-protection.

**MATERIALS AND METHODS**

Mice. Six-week-old female BALB/c and C.B-17/Scid-scid (SCID) mice were purchased from CLEA Japan. IFN-γ-deficient (IFN-γ−/−) mice derived from BALB/c background were bred and maintained as previously described (28, 49). All animal experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine of Japan. All experiments were repeated at least twice to obtain reproducible data.

Maintenance of the parasites and mouse infections. The *B. microti* Munich strain and *B. rodhaini* Australian strain were maintained in mice by intraperitoneal (i.p.) passage as previously described (28, 50). Initial infection of mice with *B. microti* was done by i.p. inoculations of mice with 107 of parasitized erythrocytes (pRBCs). Test mice were infected with *B. microti* prior to challenge infection with *B. rodhaini*, while control mice were either inoculated with nonparasitized murine erythrocytes (npRBCs) or not inoculated (mock mice) before the challenge infection.

**Preparation of dead *B. microti* for mice immunization.** The inocula of *B. microti* pRBCs and npRBCs, both fixed with glutaraldehyde, were prepared as previously described (7). Briefly, *B. microti*-infected murine blood was harvested when the parasitemias reached 50%, treated with Histopaque-1077 (Sigma), and then washed with sterile phosphate-buffered saline (PBS; pH 7.2) three times. After the last wash, the RBCs were fixed with 0.25% glutaraldehyde for 15 min at room temperature and then washed three times with sterile PBS. The fixed RBCs were stored at 4°C in sterile PBS supplemented with penicillin and streptomycin. Before inoculation, the cells were washed twice with sterile PBS, counted, and reconstituted at the desired concentrations. Mice were immunized i.p. three times at 2-week intervals with either 2 × 109 glutaraldehyde-fixed *B. microti* pRBCs in 0.5 ml of PBS (the test group) or an equivalent amount of glutaraldehyde-fixed npRBCs in 0.5 ml of PBS (the control group). Blood samples were collected from the tail vein 2 weeks after the last inoculation and before the challenge with *B. rodhaini* pRBCs. Thereafter, the specific antibody response to *B. microti* antigens was determined by indirect immunofluorescence test (IFAT) (50).

**Determination of parasitemia and survival rates.** Thin blood smears were made by using blood obtained from tail veins of mice. The smears were fixed in methanol and stained for 45 min with 10% Giemsa diluted in Sörensen buffer (pH 6.8). Thereafter, parasitemia was determined by examining 103 erythrocytes, under an oil immersion microscope, for the presence of intraerythrocytic Babesia. The examination was performed at 2-day intervals after the initial infection. In addition, the infected mice were observed daily for any mortality until the experiment was terminated at day 20 postchallenge infection. For hematological evaluation, 10 μl of blood collected from each mouse at 2-day intervals was transferred into plastic tubes containing 2 ml of premixed solution. A full blood cell count was made using an automatic cell counter (Nihon Kohden, Japan).

**Detection of specific antibody to *B. rodhaini** P26. Recombinant *B. rodhaini* P26 (rBtP26) was expressed as a glutathione S-transferase fusion protein with a molecular mass of 57.7 kDa. The expressed fusion protein was purified by glutathione-Sepharose 4B columns (Amersham Biosciences), and the resulting antigen was used in an enzyme-linked immunosorbent assay (ELISA) to detect specific antibodies (50). Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 μl of rBtP26 at a concentration of 0.2 μg/well in a coating buffer (50 mM carbonate-bicarbonate buffer [pH 9.6]). The plates were washed once with 0.05% Tween-20 (PBST) and then incubated with 100 μl of a blocking solution (3% skim milk in PBS) for 1 h at 37°C. After one wash with PBST, the antigen-coated wells were incubated with 50 μl of sera diluted 1:100 with the blocking solution for 1 h at 37°C. The plates were washed six times with PBST and then incubated with 50 μl of the horseradish peroxidase-conjugated goat anti-mouse immunoglobulin IgG1 and IgG2 (Bethyl Laboratories) diluted to 1:4,000 with the blocking solution for 1 h at 37°C as a secondary antibody. The plates were washed six times as described above, and then 100 μl of a substrate solution (0.1 M citric acid, 0.2 M sodium phosphate, 0.3 mg of ABTS [2,2’-azino-bis(3-ethylbenzthiazolinesulfonic acid)]/ml [Sigma, St. Louis, MO], 0.01% of 30% H2O2) per well was added. After incubation for 1 h at room temperature, the optical density was measured by an MTP-500 microplate reader (Corona Electric, Tokyo, Japan) at a wavelength of 415 nm.

**Detection of serum cytokines.** To obtain serum for cytokine detection, blood was collected from each mouse by cardiac puncture and then processed to get serum. Test and control mice (20 mice for each group) were sacrificed at days 0, 2, 4, or 6 postchallenge infection with *B. rodhaini* (five mice for each day). The concentrations of the individual sample cytokines were determined by ELISA kits using standard curves prepared with known concentrations of mouse recombinant IFN-γ, tumor necrosis factor alpha (TNF-α), IL-2, IL-4, IL-10, and IL-12 (Endogen) and IL-8 (Cusabio Biotech Co., Germany) according to the manufacturer’s instructions.

**Measurement of nitric oxide (NO).** The levels of nitrate and nitrite production in the mice sera were measured using a nitrate/nitrite assay kit (Cayman Chemical Co.) according to the manufacturer’s instructions. The NO levels were calculated with a standard absorbance curve derived from tests run on the same plate.

**Flow cytometry assays.** Splenocytes and peritoneal cells from three mice were obtained and resuspended in cold PBS containing 0.5% bovine serum albumin. Thereafter, the cells were incubated with the respective fluorescein isothiocyanate-labeled anti-mouse monoclonal antibodies (MAb) CD49b/Pan-NK cell (DX5), CD11b, or F4/80 (BD Biosciences, La Jolla, CA) at 4°C for 30 min (41). After three washes with cold PBS, the cells were analyzed using an EPICS XL flow cytometer (Beckman Coulter).

**IFAT.** To differentiate the two parasitic infections, standard IFAT was performed with specific antibodies. Briefly, IFAT slides were coated with pRBCs collected from mice at day 6 postchallenge infection with *B. rodhaini* (50). The slides were dried and fixed in absolute acetone for 10 min. The fixed slides were incubated with either anti-rBmP94/CT or anti-rBtP26 rabbit antibody (29, 43) diluted at 1:100 in PBS in a moist chamber at 37°C for 1 h. After the slides were washed four times with PBS containing 0.05% Tween 20 (PBST), Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes) was applied as a secondary antibody (1:250) and washed three times with PBST and examined using a fluorescence microscope (E400 Eclipse; Nikon, Japan).

**In vivo depletion of NK cells and macrophages.** To examine the effect of NK cell depletion on the outcome of the *Babesia* infections, 50 μl of anti-asialo-GM1 antibody diluted in 200 μl of PBS (Wako, Japan) was administered to mice i.p. on days −2, +3, and +6 relative to the infection with *B. rodhaini* (20). This protocol resulted in effective depletion of NK cells in the spleen when examined by flow cytometry using anti-mouse DX5 (BD Biosciences, La Jolla, CA). In separate experiments, macrophages were depleted by i.p. administration of 300 μl of clodronate liposomes 2 days before and 3 days after challenge infection with *B. rodhaini* (20). Clodronate encapsulated in liposomes (54) was a gift from Roche Diagnostics, GmbH, Netherlands. Seven days after the last injection, macrophage depletion was determined by using flow cytometry by staining cells derived from peritoneal fluid and splenocytes with CD11b and F4/80 MAbs, respectively.

**Statistical analysis.** Statistical analysis of any significant differences between the means of all variables was done by one-way analysis of variance (GraphPad Prism 5; GraphPad Software, Inc.). Tukey’s multiple-comparison test was used for pairwise comparison of data from the multiple groups. Survival analysis was done by using log-rank and Wilcoxon
tests incorporating the Kaplan-Meier nonparametric model for establishing any statistically significant differences. All results were considered statistically significant when $P$ was $<0.05$.

**RESULTS**

Primary infection with *B. microti* offers complete protection against lethal *B. rodhaini* challenge infection. Infection with *B. rodhaini* caused severe parasitemia resulting in mortalities of all mice within the first week of infection. In contrast, infection with *B. microti* caused transient parasitemia for about 3 weeks and, thereafter, the infection became persistent in mice with low level of parasitemia (data not shown). To determine whether primary infection of mice with *B. microti* at the acute, resolving, and chronic stages could provide protection to lethal *B. rodhaini* infection, BALB/c mice were initially inoculated with *B. microti* and then challenged with *B. rodhaini* at days 0, 7, 14, 28, and 56 after primary infection. Mock mice received *B. rodhaini* alone. Arrows indicate the time of challenge infection with *B. rodhaini* (A, C, E, G, and I). The parasitemia course (K) and survival rate (L) of mice immunized with either dead *B. microti* (pRBCs) or nonparasitized murine RBCs (npRBCs) and then challenged with *B. rodhaini*. The results are expressed as a mean percent parasitemias ± the standard deviations (SD) of five mice.

![FIG 1 Parasitemia and survival rates after *B. microti* inoculation and *B. rodhaini* challenge infection of BALB/c mice. Parasitemia course (A, C, E, G, and I) and survival rates (B, D, F, H, and J) of mock and test mice are presented. Test mice were initially infected with *B. microti* then challenged with *B. rodhaini* on days 0, 7, 14, 28 and 56 after primary infection. Mock mice received *B. rodhaini* alone. Arrows indicate the time of challenge infection with *B. rodhaini* (A, C, E, G, and I). The parasitemia course (K) and survival rate (L) of mice immunized with either dead *B. microti* (pRBCs) or nonparasitized murine RBCs (npRBCs) and then challenged with *B. rodhaini*. The results are expressed as a mean percent parasitemias ± the standard deviations (SD) of five mice.](http://iai.asm.org/)

Immunization with dead *B. microti* fails to protect the mice from lethal *B. rodhaini* challenge infection. To examine whether the dead parasites could offer protective immunity against *B. rodhaini* infection, *B. microti*-infected RBCs were administered i.p. into BALB/c mice, followed by two consecutive boosters at 14-day intervals. The immunized mice developed high titers of specific antibody against *B. microti* (1:3,200 to 1:6,400). Control mice, which were inoculated with murine noninfected RBCs did not show antibody response to *B. microti* (data not shown). Strikingly, after challenge infection with *B. rodhaini*, all of the mice showed rapid increases in parasitemia and consequently succumbed to the infection within a week (Fig. 1K and L). These results indicated that immunization of
mice with dead *B. microti* did not confer protective immunity against challenge infection with *B. rodhaini*.

Protected mice induce low levels of antibody and cytokine production. Serum antibody and cytokines were measured in mice acutely (7 days) and chronically (28 days) infected with *B. microti* at days 2, 4, and 6 after challenge infections with *B. rodhaini*. Specific antibody to *B. rodhaini* (rBrP26) was detected at day 6, whereas the IgG1 and IgG2 levels were significantly lower in mice initially infected with *B. microti* and then challenged with *B. rodhaini* than those detected in either mock or control mice (Fig. 2A to C). Likewise, detectable IL-8, IL-12, IL-2, and IFN-γ levels were significantly lower in *B. microti*-infected mice (*P* < 0.05) compared to mock and control mice. The results are expressed as mean values ± the SD for five mice.

**FIG 2** Kinetics of serum IgGs and cytokines of protected and susceptible mice after *B. rodhaini* challenge infection. The production of IgG (A), IgG1 (B), IgG2a (C), IL-8 (D), IL-12 (E), IL-2 (F), IFN-γ (G), IL-10 (H), TNF-α (I), and NO (J) in mice after challenge infection with *B. rodhaini* was determined. Test mice with acute and chronic *B. microti* infection, control mice (which received npRBC), or mock mice (which received no injection) were infected with *B. rodhaini*. Detection of IgGs, cytokines, and NO was performed in the mice at days 2, 4, and 6 after challenge infection. Asterisks indicate statistically significant differences (*, *P* < 0.05; **, *P* < 0.005; ***, *P* < 0.0001 [compared to mock and control mice]).
than those detected in either mock or control mice at days 4 and 6 postchallenge infection (Fig. 2D to G). IL-10 cytokine was only detected in the sera of either mock or control mice 6 days after challenge infection with *B. rodhaini*. However, IL-10 level was below the detection limit in *B. microti*-infected mice (Fig. 2H). TNF-α production was detected at day 6 after the challenge infection, and these levels were not significantly different between test and control mice (Fig. 2I). In addition, the detectable nitric oxide (NO) was significantly lower (*P < 0.05*) in *B. microti*-infected mice than those detected in either mock or control mice (Fig. 2J). Moreover, the levels of IL-4 in all mice were below the detection limit of the ELISA kit (data not shown). These results indicated that the protective status when the blood smears of protected mice were examined by IFAT was maintained in *B. microti*-infected mice during either the acute or the chronic stage had diminished their antibody and cytokine production in response to lethal *B. rodhaini* infection.

The absence of IFN-γ and B and T lymphocytes does not impair the complete protection conferred by *B. microti* infection. To determine the role of IFN-γ and B and T lymphocytes in the protection against lethal infection with *B. rodhaini*, IFN-γ−/− mice and SCID mice were initially infected with *B. rodhaini*, followed by challenge infection with *B. rodhaini* after 28 days. The kinetics of parasitemia in IFN-γ−/− mice and SCID mice was significantly different from those of immunosufficient BALB/c mice. Indeed, IFN-γ−/− and SCID mice demonstrated higher parasitemia levels persisting over 28 days after the primary infection (Fig. 3A and C). After challenge infection with *B. rodhaini*, control IFN-γ−/− and SCID mice developed rapid increases in parasitemia approaching >80%, and all mice eventually succumbed to the infection within the second week of challenge infection (Fig. 3B and D). In contrast, IFN-γ−/− mice and SCID mice with chronic *B. microti* infection survived over a period of 3 weeks (Fig. 3B and D), although they persistently maintained high parasitemia ranging between 20 and 30% (Fig. 3A and C). Notably, the majority of erythrocytes were found to be infected with *B. rodhaini* when the blood smears of protected mice were examined by IFAT (data not shown). These results indicated that the protective status induced by *B. microti* infection against the lethality of *B. rodhaini* challenge infection was not impaired in the absence of IFN-γ and B and T lymphocytes.

The absence of macrophages/monocytes but not natural killer cells impairs the protection conferred by *B. microti* infection in BALB/c mice. To examine the possible contribution of NK cells and macrophages/monocytes in protection against lethal infection with *B. rodhaini*, anti-asialo-GM1 antibody and clodronate liposome were administered i.p. to BALB/c mice with chronic *B. microti* infections, respectively. The depletion experiment was performed prior to challenge and optimized for long-lasting efficiency (data not shown). The parasitemia levels in mice with depleted NK cells were slightly elevated and consequently resolved within the second week of challenge with no significant difference compared to mice that received control antibody (*P > 0.05*). Consistently, there was no significant difference in mortalities and survivals between intact and NK cell-depleted mice (Fig. 4A and B). In sharp contrast, clodronate liposome-treated mice had more significant rapid parasite growth than did PBS-liposome-treated mice. Consequently, 80% of these mice died within the second week of challenge infection (Fig. 4C and D). These results indicated the importance of macrophages in offering immune protection against challenge infection with *B. rodhaini*. Furthermore, to examine whether the absence of macrophages/monocytes impairs...
the immune response to the infection, serum IgG antibody and cytokine productions were measured at day 8 after challenge infection with *B. rodhaini*. Notably, there was no significant difference in the antibody response between clodronate liposome-treated mice and PBS-liposome-treated mice (Fig. 5A), although the parasitemia profile after *B. rodhaini* challenge infection was different. On the other hand, cytokine production, including the production of IL-12 and IL-2, was significantly higher in clodronate liposome-treated mice than in control mice (Fig. 5B and C). In sharp contrast, macrophage/monocyte-depleted mice did not produce TNF-α (below the detection limit), whereas high levels were detected in PBS-liposome-treated mice (Fig. 5B). The production of IL-8, IL-10, IFN-γ, and NO was generally higher in clodronate liposome-treated mice than in untreated mice (Fig. 5B to D). These results showed that the absence of macrophages/monocytes not only resulted in higher parasitemia and mortality but also caused changes in inflammatory cytokine production.

**DISCUSSION**

The main goal of immunological research on babesiosis has been the development of a safe and effective vaccine to minimize the morbidity and mortality of the infected hosts. Better knowledge of the host immune response to *Babesia* infection is undoubtedly needed to achieve this (11, 27). Rodent babesiosis has been widely utilized as an experimental model to investigate the host immune response after clodronate liposome-treated mice and PBS-liposome-treated mice (Fig. 5A), although the parasitemia profile after *B. rodhaini* challenge infection was different. On the other hand, cytokine production, including the production of IL-12 and IL-2, was significantly higher in clodronate liposome-treated mice than in control mice (Fig. 5B and C). In sharp contrast, macrophage/monocyte-depleted mice did not produce TNF-α (below the detection limit), whereas high levels were detected in PBS-liposome-treated mice (Fig. 5B). The production of IL-8, IL-10, IFN-γ, and NO was generally higher in clodronate liposome-treated mice than in untreated mice (Fig. 5B to D). These results showed that the absence of macrophages/monocytes not only resulted in higher parasitemia and mortality but also caused changes in inflammatory cytokine production.

BALB/c mice with acute, resolving, and chronic stages of *B. microti* infection were completely protected against lethal infection with *B. rodhaini*. In contrast, mice simultaneously infected with the two parasites displayed high parasitemia levels and died within a week. These findings match those of a previous study in which monkeys chronically infected with *B. microti* were protected against *Plasmodium cynomolgi* infection (53). In addition, latent infection with nonlethal murine malaria parasites suppresses pathogenesis caused by lethal *P. berghei* NK65 challenge infection and prolongs the survival of mice (40). In sharp contrast to our observations, simultaneous infections by nonlethal malaria have shown suppressive effects against lethal malaria infection, and the resistance developed in this model is impaired in the absence of IL-10 (40). The failure in the protection after the simultaneous...
coinfection of mice with the rodent Babesia might be due to the initiation of rapid and progressive parasitemia by B. rodhaini in the bloodstream before B. microti parasites could initiate appropriate immune responses that suppress the pathogenesis of the lethal infection. Moreover, mice immunized with dead B. microti were not protected against the lethal infection, although highly specific antibody was induced. These observations suggest that the host antibodies elicited after infection with live organisms are more functional and are probably directed against critical neutralization epitopes exposed during RBC entry. Generally, the importance of antibody responses in control and protection has been documented in many infectious diseases. In Babesia infection, the possible function of specific antibodies is to neutralize free parasites, preventing their entry into the host erythrocytes and resulting in the lysis of the parasites by either complement or phagocytosis. On the other hand, the effects of antibodies may be restricted after the parasites enter the erythrocytes, which are devoid of major histocompatibility complex molecules. However, certain merozoite antigens can be expressed on the surfaces of pRBCs, making them targets for antibody and complements. Thereafter, the opsonizing antibodies make the pRBCs recognizable and vulnerable to phagocytosis (4, 10, 13, 27, 32).

Control mice infected with B. rodhaini developed rapid proliferation of parasites associated with increases in the levels of serum antibody, cytokines, including IFN-γ, IL-2, IL-8, IL-10, and IL-12, and NO; these levels were gradually elevated and peaked when the mortality started. Conversely, immune mice displayed very low levels of IFN-γ, 8-fold less than those of the controls, and the IL-10 levels were nearly not detectable after challenge infection with B. rodhaini. In a related study, the expression of IL-10, IFN-γ, and TNF-α was significantly elevated in mice infected with lethal Babesia strain WA1 but not in nonlethal B. microti-infected mice (24, 25). These results support the concept that the severe pathogenicity of babesiosis depends on the timing and magnitude of particular cytokines (3, 11, 12). In general, successful resolution of rodent Babesia is dependent on the ability of mice to mount an early proinflammatory cytokine response (IL-12 and IFN-γ) and the appropriate maintenance of their kinetics during acute stage of infection, thereby preventing the parasitemia from escalating to overwhelming levels. During the resolution stage, the predominance of these cytokines shifts to the Th2-based cytokines IL-4 and IL-10 accompanied by IgG responses (2, 27). Therefore, the rapid proliferation of B. rodhaini in mice might be due to the early elevation of an anti-inflammatory cytokine (IL-10) that inhibits the activity of Th1 cells, NK cells, and macrophages, thereby preventing the resolution of the infection (21). The lethality of rodent malaria parasite is thought to be due to the overproduction of IL-10 in an early stage of infection associated with the impairment of parasitemia clearance (38, 42). Further study on the differences in cytokine production in mice infected with lethal and nonlethal rodent Babesia is needed to understand the mechanism of host-parasite interactions, which is an important prerequisite for vaccine design (11).

The important roles of IFN-γ and B and T lymphocytes as the key inducers of the immune effector mechanisms needed for initial control of the rodent Babesia infection are supported by the finding that IFN-γ−/− and SCID mice are unable to resolve the

FIG 5 Kinetics of serum IgGs and cytokines of macrophage/monocyte-depleted and control mice after B. rodhaini challenge infection. (A) Production of IgGs. (B to D) Production of IL-8, IL-12, TNF-α, IL-2, IFN-γ, and IL-10 (B and C) and NO (D) at day 8 postchallenge infection with B. rodhaini. Asterisks indicate statistically significant differences (★, P < 0.05; ★★, P < 0.005; ★★★, P < 0.0001 [compared to the control]). The results are expressed as mean values ± the SD of five mice.
primary infection (2, 19, 28, 57). Here, IFN-γ−/− and SCID mice were completely protected against the lethality of *B. rodhaini* infection, resulting in the survival of all mice. Although the primary infection was not completely resolved, the findings indicated that the mechanism of resistance to *B. rodhaini* infection is IFN-γ, T cell, and antibody independent. Our findings suggest that both IFN-γ and antibody seem to have specific effects on the control and resolution of *B. microti* infection. An appropriate production of IFN-γ is needed to initiate an effective killing mechanism, as is the production of specific IgGs that neutralize the parasites and opsonize the pRBCs; in this way the parasite burden of *B. microti* can be reduced (12). The lack of any major role of lymphocytes or IFN-γ in controlling the virulent wave of *B. rodhaini* raised a question as to whether the innate immune responses play any role in limiting the resistance. Our data indicate that host resistance to *B. rodhaini* is impaired after macrophage/monocyte depletion in vivo, as evidenced by the rapid increase in parasitemia and high mortality rates in macrophage/monocyte-depleted mice. In contrast, after depletion of NK cells, the mice displayed a slight increase in parasitemia, with subsequent resolution of the infection resulting in a survival rate of 80%. These results provide strong evidence for the critical role of the innate response, particularly by macrophages and not NK cells in the protective mechanism conferred by *B. microti* infection against challenge infection with lethal *B. rodhaini*.

Macrophage/monocyte-depleted mice revealed levels of IL-8, IL-12, and IL-2 cytokines produced after infection with *B. rodhaini* comparable to those of control mice. The IL-8 and IL-12 cytokines detected in macrophage/monocyte-depleted mice may be derived from other type of cells, including epithelial and dendritic cells, respectively (52). The elevation of the IL-2 level in macrophage/monocyte-depleted mice is most probably due to the increase in *B. rodhaini* burden and the absence of macrophages that selectively inhibit IL-2 production, as supported by a previous study on murine malaria (39). Moreover, the failure to detect TNF-α in clodronate-treated mice might be due to the absence of macrophages/monocytes, which are known to produce this cytokine in response to IFN-γ activation (17). TNF-α is an inflammatory cytokine that has been implicated in the regulation of Th1 responses, stimulation of NO production, enhancement of the production of IL-6 cytokine, and inhibition of erythropoiesis (8). Furthermore, in murine malaria models, TNF-α has shown dual roles in mediating the protection and in contributing to high mortality and cerebral malaria (17, 31). On the other hand, our study found that macrophage/monocyte-depleted mice had levels of NO comparable to those of controls, although the parasitemia reached an overwhelming level. We hypothesize that NO may have been derived from active granulocytes in a TNF-α-independent pathway. Indeed, granulocytes such as neutrophils are considered to be the first line of defense against infections that rapidly accumulate at the site of infection to ingest and kill the invaders, including blood parasites, as a response to several cytokines such as IFN-γ and TNF-α, and lymphotixin (5). Collectively, our observations suggest that macrophages are critical for the suppression of *B. rodhaini* parasitemia and that this suppression is independent of the action of NO. On the contrary, Aguilar-Delfin et al. (2) have documented that resistance to primary infection of the rodent *Babesia* WA1 is correlated with an increase in NO production. Macrophage-mediated control of blood-stage malaria infection has been well described in rodents and humans (20, 47, 56). The mechanisms by which macrophages kill or cripple malaria blood-stage parasites are proposed to be through reactive oxygen and nitrogen intermediates. These intermediates are utilized in the body as oxidative and cytotoxic agents that are produced by phagocytic cells during the oxidative burst induced by the infection. Their effects on malaria can be both beneficial and pathological, depending on the amount and place of production (15, 16). Their importance as babesiacidal agents has been demonstrated in vitro, in which Babesia replication was inhibited, inducing degeneration of the parasites that display crisis forms inside the erythrocytes (15, 23, 33).

Classical cross-protection occurs when effector lymphocytes respond to the initial infection and secrete IFN-γ, thereby activating bystander macrophages and generating a heightened state of innate immunity to the secondary infection. In this regard, immunization with either bacillus Calmette-Guerin (BCG) or killed *Propionibacterium acnes* protects mice against *Babesia* and *Plasmodium* infections (14, 30). On the other hand, mice that recovered from infection with *Corynebacterium parvum* are protected against lethal *P. vinckei* (22). Barton et al. (6) reported that herpesvirus latent infection conferred symbiotic protection against *Listeria monocytogenes* and *Yersinia pestis* infections. In a related study, mice with chronic-phase *B. microti* infections were resistant to challenge infection with the virulent malaria parasite *P. vinckei* (22). The common mechanism of protection reported in these studies is most likely not antigen-specific immunity but rather relies on systemic activation of macrophages and chronic secretion of IFN-γ (6). Conversely, in heterologous immunity, cross-reactive antigenic epitopes between the primary and secondary pathogens result in antigen-specific memory lymphocyte that can be activated after secondary infection based on adaptive immunity (6, 45). Macrophages produce cytokines, including IL-12 and TNF-α, that are critical for generating and regulating innate and acquired immune responses against many pathogens. IL-12 activates NK cells to produce IFN-γ and contributes to the development of acquired immunity by promoting the differentiation of Th cells to enhance IFN-γ production by effector CD4+ T cells. The sufficient production of IFN-γ and TNF-α facilitates the functions of phagocytosis by macrophages and neutrophils (46, 51). In our data, therefore, the prolonged activation of macrophages caused by the primary infection can not only mediate the removal of pRBCs but also regulate the consequent response of effector cells to *B. rodhaini* challenge.

A greater understanding of the immunological mechanisms evoked or inhibited during infection may provide important clues regarding the type of response that needs to be induced by vaccination. We clearly showed here that innate immunity based on macrophages, but not adaptive immunity based on antibody and B and T lymphocytes, contributes to the resistance induced by *B. microti* infection to lethal *B. rodhaini* infection in mice. The establishment of strategies for activating macrophage-specific responses to the parasites may be essential for developing effective vaccines against *Babesia* infection.

ACKNOWLEDGMENTS

This study was supported by a grant from the Global COE Program and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by a grant for Research on Regulatory Science of Pharmaceuticals and Medical Devices of Ministry of Health, Labor, and Welfare of Japan (H23-iyaku-ippan-003). M.A.T.,
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