Suppression of *Plasmodium cynomolgi* in rhesus macaques by co-infection with *Babesia microti*.

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Footnotes:

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Abstract

Plasmodium and Babesia species are both intra-erythrocytic protozoa that infect a wide range of hosts, including humans, eliciting similar inflammatory responses and clinical manifestations showing markedly different degrees of severity. We recently reported that a rhesus macaque, chronically infected with Babesia microti, was better able to control infection with Plasmodium cynomolgi (a parasite of macaques with characteristics closely similar to P. vivax) than naïve monkeys.

To confirm this and investigate the underlying immunopathology, six naïve rhesus monkeys were infected with B. microti. After 24 days, 4 of these monkeys and 4 naïve rhesus monkeys were challenged with P. cynomolgi blood stage parasites. B. microti persisted at low levels in all monkeys, and clinical parameters were comparable to non-infected controls. There was a significant decrease in P. cynomolgi parasitaemia in animals co-infected with B. microti compared to those infected with P. cynomolgi alone. This decrease in P. cynomolgi parasitaemia correlated with an increase in pro-inflammatory monocytes at the time of P. cynomolgi infection and more elevated CRP serum levels one week after malaria infection. Therefore, we conclude that ongoing infection with B. microti parasites leads to suppression of the malaria infection.

Keywords: co-infection, heterologous immunity, Plasmodium, Babesia, rhesus macaques.
Introduction

Pathogens rarely infect immunologically naïve hosts. In fact, maturation of the immune system requires antigenic stimulation that begins in the neonatal period, and perhaps even during gestation (34). In addition to a history of prior infections, individuals from areas endemic for multiple pathogens are often co-infected with unrelated organisms. Concrete examples of exacerbated pathology related to co-infection in humans, such as the deleterious effect of schistosomiasis on Hepatitis C progression (2,22), have led to an increased interest in studying heterologous immunity (7). Co-infections in rodents with the apicomplexan, intra-erythrocytic parasites Babesia and Plasmodium, respectively the causative agents of babesiosis and malaria in humans, have been reported to induce cross-protection. Protection against some Plasmodium spp. after a natural cured or drug cured infection with B. microti (9,10) was thought to be due to common antigenic determinants between Babesia and Plasmodium spp. (10) although cross-reacting antibody titers were low (11) or could not be detected (9). This suggests that non-specific factors may also be involved in cross-protection.

Human malaria, an infectious disease vectored by anopheline mosquitoes, is caused by four different species of Plasmodium: P. falciparum, P. vivax, P. malariae and P. ovale. In addition P. knowlesi, a simian parasite, is also able to naturally infect humans (12,21,43). Of these, P. falciparum and P. vivax are the most clinically important species. P. falciparum causes more than 1 million deaths annually in sub-Saharan Africa; victims are mainly children under the age of 5 (16,44). P. vivax is prevalent in eastern/central Africa and in more temperate climates outside Africa, and has enormous
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socio-economic impact, particularly in South America and Asia (31). *P. cynomolgi*, a simian malaria parasite that has been shown to experimentally infect humans, is phylogenetically and phenotypically closely related to *P. vivax*, including the development of hypnozoites, (13) and provides a close and relevant biological model of *P. vivax* (24,36,46).

Babesial parasites are vectored by ticks and infect a wide variety of mammals. Awareness is growing of their role as a zoonotic agent of human disease in which pyrexia, hemolytic anemia, and hemoglobinuria may be induced (19). A cardinal sign of babesiosis is erythrocyte destruction (48,49). Recently, human babesiosis caused by *B. microti* has emerged as a worldwide health threat (15,19,29) that can be severe and life-threatening (18). Although around 25% of adults and 50% of children infected with *B. microti* are asymptomatic (26) severe babesiosis can occur in patients after splenectomy (38) and other co-morbid conditions, like Lyme disease, probably contribute to increased severity of illness (27).

Previously, we have shown in a single rhesus macaque, that long term (>5 years) chronic infection with the *Babesia microti* MM-1 isolate appeared to control a blood stage *Plasmodium cynomolgi* infection (45). With the study described here, we have investigated this observation more extensively and determined whether short term infection with *B. microti* has a similar effect.
Materials and Methods

Primates and study design. Ten female rhesus macaques were selected for this study; all monkeys were 6 years of age and weighted 5-7.5 kg (supplementary table 1). They were weight-matched and assigned to the experimental groups as shown in figure 1. Animal work was carried out under protocols approved by the Animal Ethics Committee (DEC), following Dutch laws.

The study design is shown in figure 1. Six monkeys were infected on day -24 with heparinized blood obtained from the monkey previously reported to be chronically infected with *B. microti* MM-1 isolate (45). Monkeys were each intravenously injected with 0.5 ml packed cell volume containing approximately $5 \times 10^5$ *B. microti* parasites. On day 0, 4 of these monkeys and 4 naïve controls were challenged with $1 \times 10^6$ *P. cynomolgi* M strain (8,39) blood stage parasites obtained from a parasite donor monkey. This parasite was originally a kind gift of W.E. Collins (CDC, Atlanta, Georgia).

Throughout the study finger prick blood was taken every other day (when the majority of the malaria parasites was in the ring stage) to monitor parasitaemia by Giemsa-stained thin film analysis and by PCR. On days -24, -10, 0, 7 and 24, blood was drawn. At the end of the study all animals were euthanized and full necropsy was performed.

PCR analysis for measuring *Babesia microti*. Erythrocytes were lysed from whole blood (Qiagen, cat. nr. 158902). PCR was performed as previously described (41) to specifically amplify the hypervariable region V4 of the SSU rRNA genes of piroplasms.
Lymphocyte isolation and FACS analysis. PBMCs and lymphocytes from spleen and lymph nodes were isolated according to standard procedures. Lymphocytes from liver were obtained as previously reported (17).

The following antibodies were purchased from BD pharminen: CD3-AlexaFluor700, CD4-PerCP-Cy5.5, CD16-PE, CD20-FITC, CD25-PE, and CD154-FITC. CD14-PE-TexasRed and CD20-PE-TexasRed were derived from Beckman Coulter. CD8-PacificBlue was purchased from DAKO. For the detection of selected surface markers, cells were incubated with the appropriate antibodies for 15-30 min at 4°C in the dark. After washing, cells were fixed in 1% paraformaldehyde. Data acquisition and analysis were performed on a FACS Aria using FACSDiva 5.0 software (BD Biosciences).

Pathology. Full necropsy was performed on all animals at the end of the study. Tissues were fixed in 4% buffered formalin and embedded in paraffin for routine histology. Four-micron-thick sections were stained with hematoxylin/eosin. Special staining (Perls’ method for iron) was used for detection of hemosiderin on selected tissue samples.

Statistics. The *P. cynomolgi* parasitaemia development through time was modeled using non-linear mixed effect models (NLME) (24). Differences in clinical parameters were analyzed with a one-way ANOVA, followed by a Bonferroni test. Differences between groups in the average cumulative *P. cynomolgi* parasitaemia were calculated with an unpaired Student’s t test and the standard error is depicted. CRP levels were log-transformed to obtain normality and subsequently analyzed by Student’s t-test. Values of *p* < 0.05 with a 95% confidence interval were considered significant.
Results

*Babesia microti* infection in rhesus macaques. To initiate *B. microti* infection, parasitized erythrocytes from a chronically infected monkey (45) were intravenously inoculated on day -24 into six naïve rhesus monkeys (Fig. 1). All monkeys were PCR-positive after 1 week (suppl. Fig. 1). Subsequently, *B. microti* parasites stayed present during the entire study in all infected monkeys, but at low levels (<0.1% parasitaemia) as analyzed by Giemsa-stained thin blood films (data not shown). These data indicate that rhesus macaques can be infected with *B. microti* parasites and these remained present during the 48-day follow-up period.

Infection with *B. microti* parasites suppresses blood stage *P. cynomolgi* parasitaemia.

To study whether short term persistent *B. microti* infection suppresses *P. cynomolgi* parasitaemia, 4 *B. microti*-infected monkeys were co-infected with *P. cynomolgi*, together with 4 naïve monkeys. Blood stage *P. cynomolgi* parasitaemia in rhesus macaques normally follows a characteristic pattern of a first self-curing peak, followed by a recrudescence about a week later (24). *P. cynomolgi* parasitaemia in all infected monkeys displayed this characteristic pattern, however monkeys also infected with *B. microti* had statistically significant lower first peak parasitaemia (2.34% and a 95% CI of 0.84-3.83%) compared to monkeys only infected with *P. cynomolgi* (p=0.0025) (Fig. 2A-H).

This anti-malarial effect is clearly noticed in the average cumulative *P. cynomolgi* parasitaemia (p=0.0143) (Fig. 2I). Thus persistent infection with *B. microti* resulted in decreased *P. cynomolgi* parasitaemia. The *P. cynomolgi* infection did not markedly
Co-infection of *B. microti* and *P. cynomolgi* influence the *B. microti* parasitemia: *B. microti* parasitemia remained low (<0.1% parasitaemia) throughout the study in all infected animals.

*P. cynomolgi*–induced anemia is not prevented in double infected monkeys. *Babesia* and *Plasmodium* are protozoan parasites that infect erythrocytes and establish comparable clinical features that include the induction of anemia (25,37). The hematocrit, hemoglobin, erythrocyte counts and percentage of reticulocytes (Figures 3A-D respectively) were measured for all groups (*B. microti* only, *B. microti* and *P. cynomolgi*, and *P. cynomolgi* only). No changes for these parameters were observed in monkeys infected only with *B. microti*, nor in the chronically infected monkey ((45); additional data in supplementary table 2) indicating that this parasite does not induce anemia in otherwise healthy monkeys. However, monkeys infected with *P. cynomolgi* displayed a statistically significant decrease in hematocrit (p=0.0029), hemoglobin (p=0.0054), and erythrocyte counts (p=0.0025) and a trend towards an increase in the percentage of reticulocytes (p=0.1536) three weeks after *P. cynomolgi* infection. Strikingly, there were no differences observed between the group of monkeys infected with *P. cynomolgi* only and the group which was infected with both parasites. All blood values and cell counts measured during the study are depicted in supplementary tables 3 and 4, showing that the values are overall within the normal range for rhesus macaques (30) and no differences between the groups were observed.

Together, these data indicate that although infection with *B. microti* in rhesus macaques induces suppression of *P. cynomolgi* parasitaemia it does not prevent malaria-induced anemia.
**Histopathological findings after P. cynomolgi infection.** A typical pathological feature of both *Plasmodium* and *Babesia* infections is the presence of marked erythro- and hemosidero phagocytosis and excessive deposition of pigment, elaborated by the extreme lysis of erythrocytes and enzymatic transformation of released hemoglobin (1,25). At the end of the study organs and tissues were collected from all monkeys and examined for pathology.

At necropsy all animals, including the monkeys infected with only *B. microti*, showed mild to moderate enlargement of the spleen (2 fold; data not shown). After microscopic examination of all parenchymal organs, extensive increase of phagocytized pigmented granular material was noted in several viscera in monkeys infected with *P. cynomolgi* alone or in combination with *B. microti*. Spleen, liver, lung and lymph nodes were the major locations with prominent erythro- and hemosidero-phagocytosis and numerous pigmented macrophages. The organs from gastrointestinal tract (stomach, small and large intestine) showed mild inflammation (gastritis, enteritis, colitis) with mild edema and congestion. In all malaria infected monkeys the liver exhibited yellow-brown granular pigmented material in Kupffer cells (data not shown). The same pathology was not observed in rhesus macaques infected with *B. microti* only. Figure 4 displays representative H&E staining of spleen and bone marrow (lymph node- and lung-sections are not shown).

**Induction of inflammatory responses by B. microti infection.** Infection with *B. microti* parasites suppressed *P. cynomolgi* parasitaemia without altering the induction of anemia. To explore immune responses underlying the suppressive effect on malaria parasitaemia, PBMCs were isolated on days -24, 0, and 24 and during necropsy lymphocytes were
isolated from spleen, inguinal lymph nodes and liver. The percentage of several cell populations present in the different compartments of all monkeys was analyzed.

Three weeks after *B. microti* infection a marked increase in the percentage of activated monocytes (CD3-CD14+CD16+) in the peripheral blood compartment was evident (Fig. 5A). In monkeys then co-infected with *P. cynomolgi* parasites the percentage of activated monocytes dropped, whereas in monkeys only infected with *B. microti* a higher percentage of activated monocytes persisted both in peripheral blood (p=0.019) and spleen (p=0.0156) (Fig. 5B).

A peak in C-reactive protein (CRP), an acute phase protein produced rapidly in response to pro-inflammatory stimuli with binding and functional characteristics suggestive of a role in host defense against infection (28), was observed one week after malaria infection (Fig. 5C). CRP levels in the group with the double infected monkeys were 0.49 fold more of those in the group with *P. cynomolgi* only infected monkeys (p=0.15; 95% CI 0.17 – 1.41). Infection with *B. microti* alone did not elevate CRP-levels. Serum cytokines and chemokines which were also measured in this study are depicted in supplementary figure 2.

2. IFN-γ does follow a comparable pattern as CRP, but, unlike CRP, levels are similar in single and double-infected monkeys. Furthermore a clear drop in serum levels of MCP-1 (monocyte chemoattractant protein 1) and MIP-1β (macrophage inflammatory protein 1β) was observed at the time of necropsy in all animals infected with *P. cynomolgi*.

Together, these data indicate that *B. microti* activates the immune system at a low level compared to the malaria infection, suggested by the activation of monocytes in the periphery and increased CRP-levels following the malaria infection.
Co-infection with *B. microti* leads to an earlier induction of activated CD4+ T cells after *P. cynomolgi* infection. We studied the activation of other immune compartments by measuring levels of activated T- and NK cells in peripheral blood over time and in lymphoid organs at the time of necropsy. Figure 6A displays the percentage of activated CD4+ T cells, selected by CD25 and CD40L, and figure 6B shows the percentage of CD3-CD14-CD16+ NK cells in peripheral blood. A trend to more activated CD4+ T cells and CD3-CD14-CD16+ NK cells in the periphery of double infected monkeys is observed, whereas monkeys infected with only *P. cynomolgi* displayed a higher percentage of CD3-CD14-CD16+ NK cells in the periphery.
Discussion

Previously, we have shown that a rhesus monkey chronically infected with *B. microti* for 5 or more years was better able to control a blood stage infection with *P. cynomolgi* than naïve rhesus monkeys (45). Here we have confirmed the existence of a clear interplay between these two parasites and a primate host; even when the prior exposure of *B. microti* infection was reduced to a three-week period. In addition immunological and pathological parameters were investigated in the current study.

In general, three weeks of infection is sufficient for induction of a full-blown immune response with triggering adaptive immune responses (5,6,20). Following the early course of infection with sensitive PCR determinants we were able to demonstrate that the blood became positive for *B. microti* one week after inoculation (suppl. Fig. 1) after which *Babesia* parasitaemia remained consistently present at low levels. The persistent low-level *B. microti* infection in both the double infected monkeys and the two Babesia-only infected monkeys suggests that the malaria infection has no major effect on the *B. microti* infection. Development of a quantitative PCR might reveal more subtle parasitemia changes in subsequent studies.

Although both the peak parasitaemia and cumulative exposure to *P. cynomolgi* were significantly suppressed in animals previously and concurrently infected with *B. microti*, the pathology induced by the malaria parasites at the level of both anemia and hypoxia was not reduced. As the first peak of *P. cynomolgi* infection still reached significant levels (at least 1.67% infected erythrocytes in the double infected monkeys), it may not be surprising that pathology, like the drop in hematocrit and hemoglobin, still occurred.
In terms of protective effects of *Babesia* co-infection, it would be interesting to investigate whether pathology in the double infected monkeys is shorter of duration than in malaria-only infected monkeys and whether other parameters of pathology are equally unaffected.

Pathological processes that occur following *Babesia* and *Plasmodium* infection are complex and incompletely understood. Some evidence indicates that the most important mechanism is excessive production of pro-inflammatory cytokines. Markedly elevated serum concentrations of TNF, IFN-γ, IL-2, IL-6, E-selectin (expressed in the endothelium), vascular cell adhesion molecule-1 (VCAM-1), and intracellular cell adhesion molecule-1 (ICAM-1) occur during an acute phase of human *B. microti* infection and return to baseline one month after resolution of infection (40). Here, we observed a marked increase in peripheral blood CD3-CD14+CD16+ monocytes three weeks after *B. microti* infection. CD3-CD14+CD16+ monocytes are a unique, distinct population of monocytes (50) with a pattern of surface antigen expression similar to that of tissue macrophages (33,35). The cytokine expression pattern of this distinct subset of monocytes is production of high levels of TNF and low levels of IL-10, indicating that these monocytes are so-called pro-inflammatory monocytes (4,14). It seems likely that the higher levels of this monocyte subset at the time of *P. cynomolgi* inoculation of *Babesia* exposed animals is partly responsible for the malaria suppressive effect observed in these monkeys. Due to moderate inflammation induced by *B. microti* infection, as evidenced by the increase of CD3-CD14+CD16+ monocytes, CRP serum levels were elevated after *P. cynomolgi* infection in the double infected monkeys compared to monkeys that were only infected with the malaria parasite. CRP is a prototypical acute
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Phase protein of the innate immune system in humans and non-human primates. CRP is able to recognize damaged cells of the host to help with their elimination. It can activate the complement pathway as an opsonic protein or by binding C1q, and by binding Fcγ receptors it can also lead to complement-independent phagocytosis (32,47). Recently, it has also been shown that CRP can bind differentially with malaria parasite infected erythrocytes and assist in clearance from circulation, implying a potentially important protective role in malaria infection (3). We wish to point out that as this study was focused on measuring effects of an existing Babesia infection on P. cynomolgi blood stage infection, key cytokine measurements were performed close to the P. cynomolgi peak parasitemia (day 7 post P. cynomolgi infection). Retrospectively, noting that at day 14 post B. microti infection (day -10 in Fig. 1 and suppl. Fig. 2) some effects on cytokine levels are also observed, it would be interesting in a future study to compare cytokine levels more closely between days 7 and 14 post infection with B. microti as well as P. cynomolgi (i.e. between days -17 and -10 and days 7 and 14 in Fig. 1).

P. cynomolgi is a parasite closely allied to P. vivax, and macaque immunology is closely related to human immunology, suggesting that such interplay between babesiosis and malaria may also occur in the human situation. There is a paucity of information regarding prevalence of babesial infection worldwide, and particularly in countries with under-developed health systems where malaria is prevalent. The diagnosis of babesiosis has increased markedly in the past few years, and it is seen as an emerging disease (23). Given this clear interaction between these related species we would suggest that some effort is warranted to better understand the extent of babesial infection in malaria endemic regions. Further research is necessary to enable a better understanding of the
malaria-suppressive effects demonstrated here, with a view to the development of novel tools to control malaria. It is not inconceivable that the shared characteristics between Babesia and Plasmodium may make genetically modified Babesia an attractive agent for delivery of live anti-malaria vaccines. Such thoughts are encouraged by the fact that Babesia has been successfully used as an attenuated vaccine for veterinary application (42).

With this study, we have shown that B. microti remains present at low levels in rhesus monkeys, inducing a moderate immune response after a few weeks as evidenced by the increase in peripheral blood CD3-CD14+CD16+ monocytes and a trend to increased CRP serum levels following subsequent malaria co-infection. Moreover, we observed a clear increase at the end of the study in activated CD3+CD4+ T cells, as measured both by CD25 and CD40L in these double infected monkeys, whereas this was not surveyed in the single infected monkeys. Monkeys infected with P. cynomolgi alone displayed elevated levels of NK cells in the peripheral blood compartment, which was not the case in double infected monkeys. These, and undoubtedly other, factors may have contributed to suppression of P. cynomolgi blood stage infection.
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Co-infection of B. microti and P. cynomolgi

References


Co-infection of *B. microti* and *P. cynomolgi*


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Figure legends

Figure 1. Study design. At day -24, 6 rhesus monkeys were infected with erythrocytes containing B. microti parasites (parasitaemia ≈ 0.02%; white arrow). On day 0, 4 of these monkeys were co-infected with P. cynomolgi together with 4 naïve monkeys (grey arrow). 24 days later all monkeys were sacrificed and full necropsy was performed. At several time points (black arrows) blood was drawn.

Figure 2. P. cynomolgi parasitaemia. A-D) P. cynomolgi parasitaemia of individual monkeys in the group infected with P. cynomolgi only. E-H) P. cynomolgi parasitaemia of monkeys infected with both B. microti and P. cynomolgi. The first peak was significantly lower in the double infected monkeys compared to the P. cynomolgi single infected monkeys (P=0.0025). Dots represent the counted parasitaemia, and solid lines depict the NLME-modeled P. cynomolgi parasitaemia (24). I) Average cumulative P. cynomolgi parasitaemia. The average cumulative parasitaemia was significantly lower in double infected monkeys compared to monkeys infected with P. cynomolgi only (p=0.0143). Error bars display the standard error.

Figure 3. B. microti infection does not lead to anemia in rhesus macaques in contrast to P. cynomolgi infection. A) Hematocrit levels, B) hemoglobin levels, C) erythrocyte counts and D) the % of reticulocytes are depicted for all groups over time. On day 24 hematocrit levels, hemoglobin levels and erythrocyte counts from double infected and P. cynomolgi single infected monkeys was significantly decreased compared to B. microti
infected monkeys (p=0.0029, p=0.0054 and p=0.0025 respectively). There was no statistically significant difference between the double infected and *P. cynomolgi* single infected monkeys.

**Figure 4.** Histopathology of spleen and bone marrow. H&E staining of representative spleen (A-C) and bone marrow (D-F) sections at day 24 from a monkey infected with *B. microti* only (A, D; monkey #Ri201046), both parasites (B, E; monkey #Ri205138) or *P. cynomolgi* only (C, F; monkey #Ri201112). WP = white pulp and RP = red pulp from the spleen. The yellow arrow heads depict bone marrow macrophages containing pigment. A 40x magnification was used, bar = 50 µm.

**Figure 5.** *B. microti* infection triggers the immune system of rhesus macaques. A) % CD3-CD14+CD16+ monocytes in PBMCs over time. The percentage of CD3-CD14+CD16+ monocytes was significantly increased in all *B. microti* infected monkeys compared to non-infected monkeys (day 0, p=0.019) B) % CD3-CD14+CD16+ monocytes in organs at the end of the study (%CD3-CD14+CD16+ cells in spleen of *B. microti* infected monkeys versus double and single *P. cynomolgi* infected monkeys: p=0.0156). C) Serum CRP levels in mg/l over time.

**Figure 6.** Double infected rhesus monkeys have more activated CD3+CD4+ T cells, as depicted by CD25+CD40L+. A) % CD25+CD40L+ of CD4+ T cells over time. B) % CD16+ of NK cells over time.
Figure 1
Bleeding
Group 2: B. microti and P. cynomolgi infection (n=4)
Group 3: P. cynomolgi infection (n=4)
Group 1: B. microti infection (n=2)

Study days
-24 -10 0 7 24

Necropsy on October 24, 2017 by guest
http://iai.asm.org/ Downloaded from
Figure 2
Figure 3
Figure 4

B. microti only

B. microti and P. cynomolgi

P. cynomolgi only

Spleen

Bone marrow

A

B

C

WP

RP

WP

RP

WP

RP
Figure 5
Figure 6

A

\[ \% \text{CD25-CD4+IL-6+} \]

\[ \text{Study days} \]

B

\[ \% \text{CD25-CD14-CD16+ cells} \]

\[ \text{Study days} \]

- O: B. microti only
- □: B. microti + P. cynomolgi
- ▲: P. cynomolgi only