CYTOKINE RESPONSES DURING ACUTE SIMIAN PLASMODIUM CYNOMOLGI AND PLASMODIUM KNOWLESI INFECTIONS

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Abstract. Experimental infection of non-human primates with simian malaria parasites offers a controlled system to study malarial immunity. *Plasmodium cynomolgi* (*P. vivax*-like) and *P. knowlesi* (*P. falciparum*-like) infections in the rhesus monkey were used as a model to test the hypothesis that initial acute infection stimulates type 1/pro-inflammatory cytokine expression followed by a gradual type 2/anti-inflammatory response upon re-infection. This study analyzed cytokine gene expression (interleukin-12, interferon-γ, tumor necrosis factor-α = type 1; interleukin-4, interleukin-10 = type 2) using a semi-quantitative reverse transcriptase–polymerase chain reaction in monkeys infected with each of the parasites (three per group). Clinicoparasitologic and serologic parameters were also monitored. Monkeys were re-infected to assess whether enhanced immunity could increase parasite clearance. The immune response to *P. cynomolgi* infection in rhesus monkeys seemed to be mediated by anti-parasite, pro-inflammatory responses during primary infection with a transition to protective type 2 responses after repeat infection. The immune responses to *P. knowlesi* infection were more varied. Anti-inflammatory responses were more prevalent during primary infection. Repeat infection stimulated a wide variety of responses; most included expression of tumor necrosis factor-α, a cytokine that has been associated with inflammatory and host-destructive effects (weight loss, fever, anemia). These observations further confirmed that the simian malaria/rhesus monkey model is well suited for studies on the regulation of immunity to acute *Plasmodium* infection.

INTRODUCTION

Malaria is the most prevalent human infectious disease in the world, with estimates of 500 million persons infected each year. Lessons learned from previous eradication efforts and the recent re-establishment of malaria indicate that the world community needs to focus on limiting the severity and complications of malarial illness. Therefore, knowledge of basic immune mechanisms to malaria is an important component of prevention strategies designed to minimize the clinical burden of *Plasmodium* infections. A number of malaria infections occur naturally in non-human primates. *Plasmodium cynomolgi* is a natural parasite of macaques, and has proven to be an excellent model for the study of *P. vivax*-like malaria. Similatities include a 48-hour asexual cycle, parasitemias within the same range as those of *P. vivax*, and the capacity to produce relapses. *Macaca fascicularis* is the natural host for *P. knowlesi*; thus, the rhesus *Macaca mulatta* is considered an experimental host. It has a 24-hour cycle of schizogony, which replicates rapidly to produce extremely high parasitemias. *Plasmodium knowlesi* is often used as a model for falciparum malaria since it is known to sequester in blood capillaries. The purpose of the present study was to follow the development of acquired immunity to malaria during the course of primary and repeat *P. cynomolgi* and *P. knowlesi* infections in rhesus monkeys. We hypothesized that the pattern of cytokine expression (interleukin [IL]-4, IL-10, IL-12, interferon-γ [IFN-γ], and tumor necrosis factor-α [TNF-α]) in the infected host would be affected by prior malarial history, anti-malarial antibody levels, the course of parasitemia before and after drug treatment, and the overall cytokine milieu.

MATERIALS AND METHODS

Experimental animals. Six healthy, malaria-naive, juvenile rhesus macaque (*Macaca mulatta*) monkeys born in captivity at the Tulane Regional Primate Research Center were used in the study. Three additional monkeys were used to reactivate frozen parasite stablates. Monkeys were fed a balanced diet, water was supplied *ad libitum*, and their general health status was monitored throughout the experimental period. The maintenance and care of the experimental animals complied with the National Institutes of Health guidelines for the humane use of laboratory animals. In addition, the experimental protocol was approved by the Animal Use Committee of the Tulane Regional Primate Research Center.

Parasite isolates. The *P. cynomolgi* bastianelli and *P. knowlesi* hackerti isolates were stored frozen in liquid nitrogen and reactivated in splenectomized monkeys as needed. Fresh blood was then collected during ascending parasitemia and used to inoculate experimental monkeys.

Experimental infections of rhesus monkeys. Experimental monkeys were inoculated intravenously with approximately 10^6 asexual parasites on day 0 and again on day 100. Parasitemia levels, body weight, temperature, and hematocrits were monitored throughout the experimental period. The *P. cynomolgi* group consisted of monkeys P961, R225, and R323, whereas the *P. knowlesi* group consisted of monkeys R042, R562, and R737. Treatment with chloroquine phosphate intramuscularly (7 mg/kg of body weight per day for seven days) was initiated when parasitemias reached 3% or higher.

Sample collection. Peripheral blood was collected for cytokine analysis and serology on day 0 and at four-day intervals after primary and repeat infections. Ten samples were collected from each monkey for analysis.

 Peripheral blood mononuclear cell cultures for cytokine analysis. Peripheral blood mononuclear cells were isolated with Lymphocyte Separation Medium (Organon Teknika, Durham, NC) according to the manufacturer’s instructions. Cell suspensions were adjusted to a concentration of 3 × 10^6 cells (1.5-mL cell suspension) in RPMI 1640 medium containing 10% fetal calf serum and antibiotics (penicillin = 200 units/mL and streptomycin = 100 µg/mL), placed in 12-well sterile culture plates (Costar Corporation, Cambridge, MA),
and incubated for 24 hours at 37°C in 5% CO₂ in humidified air. Cultures were also prepared in the presence of phytohemagglutinin (PHA) mitogen at a concentration of 10 μg/ml (for use as positive controls in cytokine studies).

**Isolation of RNA and cDNA synthesis.** Total RNA was isolated from peripheral blood mononuclear cell cultures using RNA STAT-60™ (Tel Test, Inc., Friendswood, TX) according to the manufacturer’s instructions. Isolated RNA samples were adjusted to equal concentrations of 2 μg/ml and stored at −70°C in 75% ethanol until cDNA synthesis. Reverse transcription for cDNA synthesis was carried out as described by Samudio and others.⁶

**Normalization of cDNA content by a competitive polymerase chain reaction (PCR).** To compare cytokine gene expression between samples, it was necessary to use equivalent amounts of cDNA. Therefore, all samples were normalized for cDNA content by constructing a cDNA competitor for β-actin.⁷ Beta-actin primers (Table 1) were used to amplify both the competitor and target (sample) cDNA. Since the competitor generated a shorter PCR product (262 base pairs) than the target cDNA (502 base pairs), both PCR products were easily identified. The PCR product that was dominantly amplified (target or competitor) depended upon the initial ratio of target to competitor. A suitable concentration of β-actin competitor (that resulted in a band of equal intensity as the target band) was selected to normalize all samples to a constant amount of cDNA. When representative normalized cDNA samples were serially diluted, the relative mRNA levels in these samples, as determined by band intensity of PCR products, was dose-dependent when the number of cycles was kept in the exponential range of amplification for β-actin (30 cycles). We concluded that reverse transcriptase-polymerase chain reaction (RT-PCR) analysis carried out under the conditions described would permit accurate determination of relative differences in cytokine mRNA concentrations of experimental samples.

**Nested PCR for cytokine analysis.** We selected an RT-PCR method that combines competitive PCR methodology with a nested PCR to improve the determination of relative amounts of multiple cytokine mRNA levels in experimental samples.⁷ Using this nested RT-PCR, we determined the relative amounts of five mRNA cytokine transcripts from the normalized sample of cDNA. With this method, two sets of primers (outer and inner) were used to increase the sensitivity and specificity for detection of cytokines IL-4, IL-10, TNF-α, IFN-γ, and IL-12 (Table 2). To avoid the variability of analyzing samples in separate PCRs, we analyzed samples simultaneously for each cytokine. Pure, non-transcribed RNA and RT mixture without RNA were used as negative controls.

The amount of PCR product was determined by comparing the cytokine signal intensity to the intensity of the β-actin internal control signal generated in dose-titration experiments (Figure 1A). Differences in the levels of mRNA transcripts in different samples were determined visually by comparing the ratio of cytokine product intensity to β-actin reaction intensity. Signals were given a visual score representing degrees of intensity ranging from undetectable (0.0) to extremely bright (5.0) (Figure 1A). Cytokine PCR product signals were similarly scored 0.0 to 5.0. A ratio of cytokine: β-actin signal was then determined for each sample (ratios of 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0). This ratio represented inducible cytokine gene expression as a fraction of constitutive gene (β-actin) expression for normalized cDNA samples (Figure 1B). Finally, descriptors of qualitative cytokine expression, ranging from undetectable to high, were assigned to increasing increments in ratio values (Figure 1B).

**Cytokine profiles.** By defining patterns of cytokines, we were able to differentiate cytokine profiles among subjects. The presence of the prototypical type 1 cytokine IFN-γ distinguished a type 1 profile, defined here as dominant expression of IFN-γ with moderate IL-12 or TNF-α expression and no detectable IL-4 expression. A pro-inflammatory profile had predominant TNF-α or IL-12 expression, or high IFN-γ expression in combination with either of the former cytokines. In the anti-inflammatory profile, strong or dominant IL-10 expression was noted above any detectable IL-12, IFN-γ, or TNF-α. A type 2 response referred to a profile in which IL-4 expression was present without IFN-γ. If co-expression of IL-4 and IFN-γ was detected, the profile was considered as type 0. Finally, a null profile referred to the inability to detect any of the cytokines being measured.

**Indirect fluorescent antibody (IFA) test.** Serial two-fold serum dilutions were used for the IFA test using antigen slides containing *P. cynomolgi* and *P. knowlesi*-infected erythrocytes. Sera containing peak antibody titers were also analyzed using an isotype-specific IFA. Reaction development was carried out using either fluorescein isothiocyanate-conjugated goat anti-human IgG or mouse anti-human IgG1, 2, 3, and 4 ( Sigma Chemical Co., St. Louis, MO) with 0.01% Evans Blue in phosphate-buffered saline as a counterstain.

**RESULTS**

**Plasmodium cynomolgi infections.** Primary and repeat *P. cynomolgi* infections were characterized by similar respective prepatent periods and times to peak parasitemia. Parasitemias increased to an average of 19.7% during primary infection and to approximately 9.6% during re-infection. By the time of drug treatment, parasitemias had begun to decrease and then dropped dramatically after one day of treatment. Monkeys with the highest parasitemias experienced some weight loss and anemia (Table 3A).

**Anti-malarial antibody responses.** Anti-malarial antibody responses were of the IgG1 isotype. Titers started to increase by day 6, peaking at a titer of 2,560 by 18 days post-infection (Table 3A). Antibody titers decreased thereafter. Following

**Table 1**

<table>
<thead>
<tr>
<th>Target</th>
<th>Sense primers</th>
<th>Antisense primers</th>
<th>Product size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin (target)</td>
<td>S GGG TCA GAA GGA TTC CTA TG</td>
<td>AS1 CTC CTT AAT GTC ACG CAC GAT TTC</td>
<td>502 (T)</td>
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<tr>
<td>β-actin (competitor)</td>
<td>S GGG TCA GAA GGA TTC CTA TG</td>
<td>AS2 GGT CTC AAA CAT GAT CTG GG</td>
<td>262 (C)</td>
</tr>
</tbody>
</table>

* S = sense primer; AS = antisense primer; (T) = target; (C) = competitor.
repeat infections, a rapid increase in antibody titers was observed, reaching maximal levels as parasitemias were peaking (Table 3A). Cytokine responses. Comparisons of individual cytokines expressed by all three *P. cynomolgi*-infected monkeys are shown for IL-4 in Figure 2A, IL-10 in Figure 3A, IL-12 in Figure 4A, IFN-γ in Figure 5A, and TNF-α in Figure 6A. Cytokine transcripts generated with mitogen (PHA)-stimulated cultures showed maximal intensity of expression. Negligible cytokine transcripts were observed on day 0 of primary infection (null response). The pro-inflammatory cytokine response, characterized by dominant expression of TNF-α or IL-12 with or without IFN-γ or IL-10, was the predominant response during primary infection of all three monkeys. During re-infection, pro-inflammatory responses occurred less frequently. Anti-inflammatory responses characterized by strong or dominant expression of IL-10 occurred prior to repeat infection and early after re-infection.

![Image](63x105 to 302x348)

**Figure 1.** Analysis of relative levels of cytokine gene expression using a competitive reverse transcriptase–polymerase chain reaction with a β-actin target and competitor. A, varying band intensities with corresponding visual scores. B, expression ratios (cytokine/β-actin) of representative samples, together with qualitative descriptors.

Monkey R225 demonstrated a dominant type 1 cytokine profile on day 6 of primary infection. The response was characterized by maximal expression of IFN-γ, moderate to high expression of pro-inflammatory cytokines IL-12 and TNF-α, and undetectable expression of IL-4. Type 2 responses, characterized by dominant expression of IL-4 without IFN-γ, occurred more frequently after re-infection. In two consecutive samples collected early after re-infection, monkey P961 showed a type 2 response, as shown by high expression of IL-4 in combination with IL-10 and IL-12 without IFN-γ. This pattern coincided with peak parasitemia and an increase in antibody titer. Both monkeys R225 and R323 demonstrated type 2 responses late after re-infection (day 14), with dominant IL-4 expression. A type 0 profile, described as co-expression of IL-4 and IFN-γ, was seen together with broad expression of all cytokines measured in the study. Monkey R323 demonstrated a type 0 profile late after primary infection (day 18). Monkey R225 had a type 0 response 7 days after repeat infection. Strong expression of IL-4 and IFN-γ, along with IL-10, IL-12 and TNF-α, was detected. A summary graph depicting parasitemia curves with annotations of cytokine profiles and significant clinical events is shown in Figure 7.

*Plasmodium knowlesi* infections. Primary and repeat *P. knowlesi* infections were characterized by shorter prepatent periods (average = 2 and 5 days), shorter time to peak parasitemias, lower overall parasitemias, and lower hematocrits (Table 3B). Seven days after treatment (day 12) parasites were undetectable. However on day 23, all monkeys had experienced a recrudescence in parasitemias that resulted in the death of monkey R737.

**Table 2**

<table>
<thead>
<tr>
<th>Target</th>
<th>Sense primer</th>
<th>Anti-sense primer</th>
<th>Product size (base pairs)</th>
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<tr>
<td>IL-4</td>
<td>o-S CCT CTG TTC TTC CTG CTA GCA TGT GCC</td>
<td>o-AS CCA ACG TAC TCT GGT TGG CTT CCT TCA</td>
<td>373</td>
</tr>
<tr>
<td>IL-10</td>
<td>o-S ATG CCC CAA GCT GAG AAC CAA GAC CCA</td>
<td>o-AS TCT CAA GGG GCT GGG TCA GCT ATC CCA</td>
<td>352</td>
</tr>
<tr>
<td>IL-12</td>
<td>o-S ACA TCA AGG CGC ATG TGA ACT CCC</td>
<td>i-AS CCA GAT CCG ATT TGG GAG ACC TC</td>
<td>292</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>o-S AGT GAT CCG AGA AGT AGT GGC AAC TTT GTC CAC GGA CAC AAG</td>
<td>i-AS GAG GTT CCT GTC TGT TGG TCT CC</td>
<td>272</td>
</tr>
<tr>
<td>TNF-α</td>
<td>o-S CGG GAC GTG GAG GTG CTC GCC GAG GAG</td>
<td>i-AS TCT TCT CTC TCC TGA TCG TGG CAG</td>
<td>354</td>
</tr>
<tr>
<td></td>
<td>i-S TCT TCT CCT TCC TGA TCG TGG CAG</td>
<td>i-AS TTA TCT CTC AGC TCC ACG CCA TTG GC</td>
<td>262</td>
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</table>

* * IL = interleukin; o = outer primer; S = sense primer; AS = antisense primer; i = inner primer; IFN-γ = interferon-γ; TNF-α = tumor necrosis factor-α.
TABLE 3
Clinicopathologic features of *Plasmodium cynomolgi* and *P. knowlesi* infections

<table>
<thead>
<tr>
<th></th>
<th>Day of patency (days)</th>
<th>P961</th>
<th>R255</th>
<th>R323</th>
<th>Average</th>
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<tr>
<td></td>
<td>Primary infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Day of patency</td>
<td></td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Repeat infection</td>
<td></td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Duration of parasitemia (%)</td>
<td></td>
<td>9.9</td>
<td>14.85</td>
<td>34.25</td>
<td>19.7</td>
</tr>
<tr>
<td>Maximum % weight loss</td>
<td>Primary infection</td>
<td>-3.3</td>
<td>18.8</td>
<td>0.0</td>
<td>-7.4</td>
</tr>
<tr>
<td></td>
<td>Repeat infection</td>
<td>-6.7</td>
<td>-6.7</td>
<td>0.0</td>
<td>-6.8</td>
</tr>
<tr>
<td>Maximum % change in hematocrit (%)</td>
<td>Primary infection</td>
<td>-32.1</td>
<td>-34.9</td>
<td>-44.2</td>
<td>-37.0</td>
</tr>
<tr>
<td></td>
<td>Repeat infection</td>
<td>-33.2</td>
<td>-12.5</td>
<td>-21.6</td>
<td>-22.4</td>
</tr>
<tr>
<td>Maximum antibody titer (%)</td>
<td>Primary infection</td>
<td>1,280</td>
<td>1,280</td>
<td>2,560</td>
<td>1,613</td>
</tr>
<tr>
<td></td>
<td>Repeat infection</td>
<td>5,120</td>
<td>5,120</td>
<td>2,560</td>
<td>3,225</td>
</tr>
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</table>

A type 2 cytokine profile characterized by moderate IL-4 and low IL-10 transcripts, respectively, was detected late after primary infection prior to re-infection (monkey R042). After re-infection, type 2 responses with dominant IL-4 expression were found in all monkeys. Maximum expression of IL-4 in the presence of IL-10, IL-12, and TNF-α was noted in R562 on day 6, one day before peak parasitemia. Late after repeat infection and coincident with peak antibody titer (days 17 and 24), high IL-4 expression was detected in R042, consistent with the return of a late type 2 response.

A type 0 response was absent during primary infection. On day 6 after re-infection, monkey R042 displayed a type 0 response with simultaneous expression of all five cytokines. The null profile was noted in monkey R737 (succumbed to infection and died) on day 4 and again on day 12 after an intervening anti-inflammatory response. Monkey R042 also demonstrated a null profile on day 12 after primary infection, a time when both parasites and cytokine mRNA transcripts were undetectable.

**DISCUSSION**

In the present study, three rhesus monkeys were experimentally infected with *P. cynomolgi* bastianelli blood-stage parasites and three were inoculated with *P. knowlesi* hackerii parasites to determine the kinetics of cytokine responses to asexual blood-stage infection. The purpose of the study was to relate systemic expression of cytokines (IL-4, IL-10, IL-12, IFN-γ, and TNF-α) to parasitologic, clinical, and serologic parameters during the course of *P. cynomolgi* and *P. knowlesi* primary and repeat infections in rhesus monkeys.

The course of disease in *P. cynomolgi* and *P. knowlesi* infections of rhesus monkeys was quite different. Average peak parasitemia (19.7%) for the *P. cynomolgi*-infected natural host was almost four times as high as the average peak parasitemia (5.4%) for the *P. knowlesi*-infected experimental host. Parasitemias in two of the three *P. cynomolgi*-infected monkeys began to decrease before initiation of treatment, suggesting a naturally acquired protective response. Apparently, immunity was boosted upon re-infection since subsequent peak parasitemias after repeat infections were lower than that of primary infection in two of the three monkeys. Parasitemia levels in untreated, recrudescent infections have been reported as being lower than initial parasitemias. For the experimental host, both primary and repeat *P. knowlesi* infections were characterized by lower parasitemias. Parasite levels also spontaneously decreased before treatment after repeat infection, an indication that these monkeys developed some form of immunity. Immunity against clinical effects of superinfection with the same strain has been reported to occur in monkeys with chronic or latent *P. knowlesi* infections. Parasite recrudescence regardless of drug treatment and the *P. knowlesi*-related death attributed to the more severe nature of this parasite in the experimental host. In addition, monkeys infected with *P. knowlesi* malaria had a longer course of disease than did monkeys with *P. cynomolgi* malaria.

The difference in clinical outcome paralleled one of the differences between *P. vivax* and *P. falciparum* infections in humans, the latter (like *P. knowlesi*) being associated with increased morbidity and mortality. During both primary and repeat infections, anemia was more severe in the
P. knowlesi-infected monkeys, albeit, of greater severity after repeat infection. In the P. cynomolgi group, decreases in hematocrit were, on average, greater during primary infection than after repeat infection. Anemia as a consequence of parasite levels has been associated with lysis of red blood cells by schizonts, immune-mediated lysis, or erythrophagocytosis in the spleen. The fact that anemia occurred as monkeys were controlling their parasitemias, and that anemia was worse in monkeys where parasitemias decreased spontaneously, supports a host component to red blood cell lysis/clearance.

During the anemia episodes, the predominant profile during primary P. cynomolgi infection was the pro-inflammatory type associated with strong expression of TNF-α. The greatest decrease in hematocrit in the P. cynomolgi group occurred at the time of maximum parasitemia and strong simultaneous expression of TNF-α. The monkey with the smallest decrease in hematocrit demonstrated an overall anti-inflammatory response accompanied by maximal expression of IL-10. Repeat P. cynomolgi infections were associated with more prevalent anti-inflammatory profiles (and more prevalent expression of IL-10) and less severe anemia. Low IL-10 to TNF-α ratios have been associated with anemia.¹¹⁻¹³

During primary P. knowlesi infection, the dominant cytokine milieu was of the anti-inflammatory type. Since IL-10 down-regulates inflammatory responses, it has the potential to control the anemia associated with these responses. This finding may explain why anemia during primary infection was not as severe as observed during repeat infection.

**FIGURE 2.** Expression of interleukin-4 (IL-4) during A. Plasmodium cynomolgi and B. P. knowlesi infections. Cytokine expression is depicted as the IL-4/β-actin ratio.
Severe anemias occurred late following repeat *P. knowlesi* infection, while monkeys were still parasitemic. The largest decreases in hematocrit occurred on days when anti-inflammatory profiles dominated. Malarial anemia as a consequence of inadequate amounts of TNF-α has also been theorized. ¹⁴ Perhaps in the *P. knowlesi* infections, the anti-inflammatory milieu down-regulated the mechanisms that control parasitemia and in doing so allowed the rapid growth of parasites and subsequent anemia due to parasite-mediated erythrocyte rupture. A delicate balance between inflammatory and anti-inflammatory immune responses appears to be critical to avoid pathology.

Specific associations between a particular cytokine (e.g., IL-4 or IFN-γ) and antibody production were not apparent during primary infection. Following repeat *P. cynomolgi* and *P. knowlesi* infections, IgG1 antibody levels were higher and type 2 responses with IL-4 expression were more prevalent. Activation of IL-4-producing T cell subsets and production of malaria-specific antibodies have been reported previously.¹⁵ Interleukin-4 is also involved in the induction of isotype switching from IgM to IgG1, IgG4, and IgE.¹⁶ Since type 2 responses are associated with the development of humoral immunity, specifically with antibody isotype switching to IgG1, our observation of greater expression of IL-4

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**Figure 3.** Expression of interleukin-10 (IL-10) during A, *Plasmodium cynomolgi* and B, *P. knowlesi* infections. Cytokine expression is depicted as the IL-10:β-actin ratio.
during repeat infection seemed consistent with previous studies.

The dominant cytokine profile observed during primary *P. cynomolgi* infection was of the pro-inflammatory type. All monkeys expressed varying levels of IFN-γ, TNF-α, and occasionally IL-12, early during primary infection. This inflammatory response was associated with suppression of parasitemias. Production of pro-inflammatory cytokines has also been reported in rhesus monkeys infected with *P. coatneyi*.\textsuperscript{17}

This finding is consistent with the *P. c. chabaudi* and *P. berghei* murine model systems in which type 1 or pro-inflammatory cytokine production is associated with innate immune mechanisms that help control parasitemia.\textsuperscript{18,19} However, potentially protective cytokines, when overproduced, may play a role in host-mediated pathology (weight loss, fever, and anemia).\textsuperscript{20}

Conversely, anti-inflammatory profiles were more common during primary *P. knowlesi* infection. Early anti-inflammatory cytokine profiles characterized by dominant IL-10 might have prolonged *P. knowlesi* parasitemia eventually leading to parasite-induced pathology. In the monkey that succumbed to an overwhelming infection (R737), only IL-10 transcripts were detected, albeit, at low levels. Severe fulminating infections may indirectly stimulate IL-10-mediated anti-inflammatory responses in an attempt to minimize host pathology induced by type 1/pro-inflammatory cytokines.\textsuperscript{21}

The pro-inflammatory and type 1 responses evident during primary *P. knowlesi* infection involved relatively greater ex-

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**FIGURE 4.** Expression of interleukin-12 (IL-12) during **A.** *Plasmodium cynomolgi* and **B.** *P. knowlesi* infections. Cytokine expression is depicted as the IL-12:β-actin ratio.
pression of IL-12 and IFN-γ than TNF-α. Monkey R042, with the lowest parasitemia, demonstrated a pro-inflammatory response characterized by strong expression of IL-12 at the time of peak parasitemia during both primary and repeat infections. The other two monkeys (with parasitemias at least twice as high), displayed anti-inflammatory or null profiles when parasitemias peaked. In murine models, early endogenous IL-12 responses have been associated with protection from lethal Plasmodium infections,13 and exogenous IL-12 administration has been shown to reduce the susceptibility of mice to lethal malaria.22,23 An earlier pro-inflammatory response was triggered in the P. cynomolgi group probably because of the higher parasite density characteristic of P. cynomolgi infections. The spontaneous decrease in parasitemia coincided with expression of IFN-γ except in monkey P961, which showed maximal expression of IL-10.

In the P. cynomolgi group, anti-inflammatory profiles were prominent between the primary and repeat parasitemic peaks. Anti-inflammatory responses in monkey P961 first appeared after resolution of parasitemia, perhaps stimulated by the previous pro-inflammatory response. Anti-inflammatory responses dominated the early period after repeat infection in monkeys R225 and R323; these monkeys demonstrated higher parasitemias than monkey P961 during repeat infec-

**Figure 5.** Expression of interferon-γ (IFN-γ) during A. Plasmodium cynomolgi and B. P. knowlesi infections. Cytokine expression is depicted as the IFN-γ/β-actin ratio.
The early anti-inflammatory cytokine profiles found in these monkeys demonstrate the potential harm such responses may cause by inhibiting anti-parasite pro-inflammatory/type 1 responses, thereby hindering cell-mediated parasiticidal activity.

Type 2 responses only appeared after repeat infection in both parasites. Type 2 cytokine responses correlate with humoral immunity and are associated with protection against the recrudescence of *P. c. chabaudi* parasites during the chronic phase of infection. In our *P. cynomolgi* group, the one monkey that did not demonstrate a type 2 response during repeat infection displayed the highest parasitemia, suggesting that its lack of type 2-mediated immunity might be detrimental. The *P. knowlesi*-infected survivors developed type 2 cytokine profiles during re-infection that correlated with increasing antibody titers and a protective response to re-infection (demonstrated by lower levels of parasitemia and spontaneous reduction of parasitemia prior to drug treatment).

Experimental infection of non-human primates with natural simian malarial parasites under laboratory conditions offers a controlled setting in which the development of immunity may be studied in the absence of confounding variables, e.g., unknown malarial history, concurrent infections, auto-medication. As shown by this study, the course of parasitemia, clinical effects, and concurrent cytokine profiles are
FIGURE 7. Cytokine profiles during *Plasmodium cynomolgi* infection (parasitemia levels post-infection). Cytokine profiles = type 1, type 2, type 0 (non-polarized to either type 1 or type 2), pro-inflammatory (PI), anti-inflammatory (AI); Hct = hematocrit.

FIGURE 8. Cytokine profiles during *Plasmodium knowlesi* infection (parasitemia levels post-infection). Cytokine profiles = type 1, type 2, type 0 (non-polarized to either type 1 or type 2), null (no detectable cytokine expression), pro-inflammatory (PI), anti-inflammatory (AI); Hct = hematocrit; D = death of monkey R737 on day 24 post-infection.
different during *P. cynomolgi* and *P. knowlesi* infections. More pathologic effects occurred after primary *P. cynomolgi* infections, when pro-inflammatory cytokine profiles dominated. Repeat infections in these monkeys were characterized by lower parasitemias, fewer symptoms, limited production of pro-inflammatory cytokines, and prolonged expression of IL-10. In the *P. knowlesi* infections, overt disease (weight loss, febrile responses, severe anemia) was noted after repeat infection when expression of TNF-α was frequent.

In conclusion, the immune response to *P. cynomolgi* infection in rhesus monkeys seems to be mediated by anti-parasite, pro-inflammatory responses during primary infection with a transition to protective type 2 responses after repeat infection. The transition occurs via expression of anti-inflammatory cytokines such as IL-10. In contrast, the immune responses to *P. knowlesi* infection in the experimental host are more varied. Anti-inflammatory responses are more prevalent during primary *P. knowlesi* infections. Repeat infection stimulates a wide variety of responses, most including expression of TNF-α, that produce more inflammatory and host-destructive effects (weight loss, fever, anemia). After repeat infections with either *Plasmodium* parasite, type 2 cytokine responses (expression of IL-4) are prevalent when antibody responses are more pronounced.

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