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Malaria primes the innate immune response due to interferon-γ induced enhancement of toll-like receptor expression and function

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Malaria-induced sepsis is associated with an intense proinflammatory cytokine production for which the underlying mechanisms are poorly understood. It has been demonstrated that experimental infection of humans with Plasmodium falciparum primes Toll-like receptor (TLR)-mediated proinflammatory responses. Nevertheless, the relevance of this phenomenon during natural infection and, more importantly, the mechanisms by which malaria mediates TLR hyperresponsiveness are unclear. Here we show that TLR responses are boosted in febrile patients during natural infection with P. falciparum. Microarray analyses demonstrated that an extraordinary percentage of the up-regulated genes, including genes involving TLR signaling, had sites for IFN-inducible transcription factors. To further define the mechanism involved in malaria-mediated “priming,” we infected mice with Plasmodium chabaudi. The human data were remarkably predictive of what we observed in the rodent malaria model. Malaria-induced priming of TLR responses correlated with increased expression of TLR mRNA in a TLR9−/−, MyD88−/−, and IFNγ−/− dependent manner. Acutely infected WT mice were highly susceptible to LPS-induced lethality while TLR9−/−, IL12−/− and to a greater extent, IFNγ−/− mice were protected. Our data provide unprecedented evidence that TLR9 and MyD88 are essential to initiate IL12 and IFNγ responses and favor host hyperresponsiveness to TLR agonists resulting in overproduction of proinflammatory cytokines and the sepsis-like symptoms of acute malaria.

Despite concerted effort, a consensus on which malarial molecule is responsible for activating cytokine production in immune cells has not been achieved. Two candidates have been proposed: malarial glycosylphosphatidylinositol (GPI) anchors and parasite-derived DNA bound to hemozoin. These putative “malarial toxins” have been shown to activate TLR2 and TLR9, respectively (8, 10). As human polymorphisms that affect the outcome of malaria have been described for TLR2, TLR9 (15, 16), and MAL/TIRAP (17), it seems likely that TLRs are involved in innate immune responses to malaria and possible that TLRs 2 and 9 are the main receptors involved.

One approach to define a role for a specific TLR in disease is to examine affected patients for the development of tolerance through a defined signal transduction pathway. LPS-challenged humans, for example, are hyporesponsive to LPS-mediated responses when subsequently re-challenged (18). Cells from patients with Gram-negative bacterial septicemia are similarly hyporesponsive to LPS (19), indicating that the TLR4 pathway has entered into a state of innate immune “tolerance” (20). Tolerance almost certainly exists in malaria, as patients from areas where re-infection occurs almost daily often do not exhibit the signs and symptoms of disease despite the presence of blood parasites (21, 22).

McCall et al. studied TLR function in peripheral blood mononuclear cells (PBMC) from individuals experimentally infected with malaria (23). Their results suggested that as a result of malaria infection, the innate immune response was enhanced to TLR1/TLR2 and TLR4 agonists. This finding was most surprising because it is markedly dissimilar to numerous reports of immune hyporesponsiveness (often referred to as “immune paralysis”) during bacterial sepsis even though this syndrome, in many respects,
mimics malaria. However, due to the nature of human experimental infection, subjects in this study were minimally symptomatic with very low parasitemia. This suggested to us that the effects they described were confined to the very earliest stages of malaria infection and that tolerance had not yet developed at the time that patient blood was collected for study. Furthermore, no obvious mechanism of the priming observed was delineated.

It was for this reason that we attempted to identify TLR usage during acute natural malaria with the idea that naturally infected patients would, in time, develop specific tolerance to the appropriate TLR ligand after experiencing high levels of parasitemia for a period of several days. In fact, we found that acute infection with Plasmodium falciparum in humans resulted in enhanced activation of innate immune cells to TLR agonists. This phenomenon was reproduced in Plasmodium chabaudi AS infected mice and occurred in a TLR9/IL 12/IFN-γ-dependent manner. The mechanism by which malaria infection primes the innate immune responses of the host was found to be that TLR9 activation during Plasmodium infection initiates endogenous IL 12 and IFN-γ production, which in turn enhances TLR expression and “priming” associated signaling pathways. Our data indicate that this augmented TLR expression leads to a stage of hyperresponsiveness to TLR agonists, and, as confirmed in mice, dramatically enhances the deleterious effect of an endotoxin challenge, as might occur in humans during infection with Gram-negative pathogens such as Salmonella.

Results
Increased TLR Responses During Acute P. falciparum Infection. PBMCs were isolated from P. falciparum naturally infected subjects to investigate TLR responses during acute malaria infection. PBMCs obtained from control subjects living in the same endemic area were analyzed in parallel (Fig. 1). With the sole exception of the TLR3 ligand, P1C, increases in all TLR responses were observed in PBMCs from individuals acutely infected compared to healthy subjects (Fig. 1). Together, these results suggested that P. falciparum infection primes cells to subsequent TLR stimulation.

Chemotherapy Reverses Hyperresponsiveness of TLR Responses. Plasma samples from subjects with acute P. falciparum infection collected before and 3–4 weeks after curative mefloquine chemotherapy were used for evaluation of cytokines. P. falciparum infection led to a significant enhancement of systemic levels of proinflammatory cytokines. All patients reported greatly improved sense of well being and the absence of symptoms after therapy. The clinical characteristics of these patients are listed in Table S1. Coincident with this clinical improvement, cytokine levels were significantly diminished after chemotherapy (Fig. 2A). After curative chemotherapy, cytokine levels produced in response to TLR stimulation were back to baseline levels (Fig. 2B).

TLR/IFN Pathways Are Boosted During Acute Malaria Infection. To define the mechanism by which malaria primes up-regulation of proinflammatory cytokines, we profiled mRNA obtained from infected individuals before and after curative chemotherapy. Fig. 2C and Table S2 show a panel of select genes up-regulated as a result of P. falciparum infection. Although neither TLR3 nor TLR9 expression was dramatically altered, TLR7 expression was enhanced approximately 2.6-fold (P < 0.01, data not shown). Statistically significant changes were also observed with TLRs 1, 2, 4, and 8 (P < 0.05). Genes involved in TLR signaling pathways consistently showed changes during P. falciparum infection (Fig. 2C). Expression of CD36, a coreceptor for TLR2 associated with the recognition of malaria parasites and subsequent induction of proinflammatory cytokines (24), was also up-regulated. In addition, we observed enhanced expression of various elements of IFN signaling pathways (Table S2). An analysis of the promoters of the up-regulated genes revealed that a large number of these genes had sites for IFN-inducible transcription factors (Table S3). The transcriptional changes of select genes in TLR or related signal transduction pathways were validated by qPCR (Fig. 2D). To determine if the up-regulation of TLR mRNA results in increased protein expression, we assessed the surface expression of TLR2 and TLR4 by flow cytometry. Expression of TLR2 and TLR4 were significantly augmented in CD14+/CD14− monocytes (Fig. 3 and S1).

Increased TLR Responses During Acute P. chabaudi Infection. Next, we evaluated the response of mouse spleen cells to TLR ligands during...
Fig. 2. Cytokine response in symptomatic malaria patients is reversed after curative chemotherapy. (A) Levels of 6 cytokines (TNFα, IL1β, IL6, IL8, IL10, and IL12p70) were determined in the plasma of P. falciparum infected subjects before treatment (closed triangles) or 30 days after treatment (open boxes) using CBA. (B) PBMC isolated from P. falciparum infected individuals before (closed triangles) or after treatment (open boxes) were cultured in the presence of the indicated stimuli for 20 h. Levels of TNFα, IL1β, and IL12p40 were measured in culture supernatants by ELISA. Significant differences are indicated with p-values using paired t test or Willcoxon matched-pairs signed-ranks test when a normality test failed. (C) Results of the clusterization. Each row represents a gene and each column the Log2 of the ratio of the quantity of cDNA “Before treatment”/”After treatment” standardized by the β2microglobulin cDNA.

Fig. 3. Increased expression of TLR2 and TLR4 in monocytic cells from symptomatic malaria patients. PBMC were isolated from acute infected individuals before and 30 days after treatment and analyzed ex vivo through flow cytometry. The expression of TLR2 and TLR4 was evaluated in CD14⁺ and CD11c⁺ cells. Representative histogram showing fluorescence intensity of TLR2 in CD14⁺ (Left) and CD11c⁺ (Right) cells.

Fig. 4. Hyperresponsiveness of spleen cells from mice undergoing acute malaria. C57BL/6 mice were challenged with 10⁶ iRBCs and followed every 3 days for (A) parasitemia and (B) levels of IFN measured in culture supernatants from mouse spleen cells harvested at various weeks post infection and stimulated with LPS (1 g/ml), Pam3cysk4ser (1 g/ml), Cpg DNA (1 g/ml), or malaria extract (100 g/ml) for 48 h. As a control for cell viability, cells were stimulated with the mitogen Concanavalin A (5 μg/ml). The levels of IFNγ were measured in culture supernatants 48 h post stimulation. The results are averages of 5 animals from a representative out of 2 experiments that yield similar results.
MyD88 and IFN-γ-Dependent Up-Regulation of TLR mRNA Correlates with P. chabaudi Mediated-Priming of TLR Responses. One explanation for the enhanced response to TLR ligands during infection would be that levels of receptor expression are increased. We therefore evaluated the levels of TLR mRNA in spleens of WT C57BL/6, TLR9−/−, MyD88−/−, and IFNγ−/− mice during P. chabaudi infection. Significant up-regulation of all of the TLRs tested was observed in WT mice at 6 days post infection and was clearly trending back to baseline by day 9. Up-regulation of TLR expression was significantly diminished in TLR9−/−, MyD88−/−, and IFNγ−/− mice (Fig. S4). As expected, 7 days post infection, splenocytes from WT mice produced large amounts of IFNγ while cells from infected TLR9−/− mice produced significantly lower levels of this (Fig. 5B) and other cytokines (Fig. S3) in response to TLR ligands.

TLR9, IL12, IFNγ, and T Cells Mediate Hyperresponsiveness and Susceptibility to Endotoxin Shock During Malaria. We have gained further insights into the role of TLR9 in priming innate immune responses during malaria. We show that in vitro stimulation of bone marrow derived dendritic cells (BMDCs) with P. chabaudi iRBCs induced IL12 production in a manner that depends on TLR9 (Fig. S4A). To assess the mechanism and relevance of TLR hyperresponsiveness during acute malaria, we infected WT, TLR9−/−, IL12−/−, IFNγ−/− and RAG−/− mice with P. chabaudi and challenged them with LPS 7 days postinfection. WT mice produce high levels of proinflammatory cytokines (Fig. S4A) and became very susceptible to i.v. injection of a sub-lethal LPS challenge (i.e., 10 μg) (Table 1). TLR2−/− mice were as susceptible as WT (data not shown) while TLR9−/− mice produce significant lower levels of proinflammatory cytokines (Fig. S4B) and become more resistant to LPS challenge (Table 1). IL12−/− mice showed impaired production of proinflammatory cytokines (Fig. S4B) and full resistance to low doses of LPS, while IFNγ−/− mice were completely resistant to even higher LPS dose (i.e., 100 μg) despite the fact that these mice faced higher parasitemia (Table 1). RAG−/− mice showed intermediate resistance to low and high doses of LPS. Together, these results show that the IL12/IFNγ axis is crucial for malaria induced-priming of TLR responses and that TLR9 have an impact on this phenomenon.

Discussion

The major findings of this study can be summarized as follows: augmented TLR responses in patients naturally infected with P. falciparum were associated both with cytokinemia and clinical symptoms of malaria. The vast majority of the highly inducible genes involved in the innate immune response, including the TLRs, appear to be IFN inducible. We hypothesize that augmented expression of genes in the TLR pathway favor the recognition of Plasmodium by phagocytes. Finally, the state of being acutely infected with malaria heightens the innate immune response to challenge with unrelated microbial products in an MyD88−/−, TLR9−/−, IL12−/−, and IFN-γ-dependent manner.

The manner in which the innate immune system is activated in malaria is difficult to precisely define. We are still not certain what ligand or ligands are responsible for stimulating cytokine production, although it is intriguing that a large number of TLR components are regulated downstream from an IFN response. Overall, the innate immune response to acute malaria results in an immunological state that is notably enhanced. Such a “primed” immune system could be expected to prevent super infection with bacteria or viruses and might be crucial for host survival. On the other hand,

Table 1. TLR9−/−, IL12−/−, IFNγ−/− and RAG−/− mice undergoing acute malaria display increased in vivo resistance to low LPS doses

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Condition</th>
<th>LPS dose μg/mouse</th>
<th>Number of dead/tested mice</th>
<th>Mortality, %</th>
<th>Mean parasitemia, %</th>
<th>p value chi-square test</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>Control</td>
<td>110 100</td>
<td>0/3 1/15 0/9</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>110 100</td>
<td>0/6 3/31 4/22/23</td>
<td>99 9</td>
<td>99 9</td>
<td></td>
</tr>
<tr>
<td>TLR9−/−</td>
<td>Control</td>
<td>10 100</td>
<td>0/9 0/5</td>
<td>0 0</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>10 100</td>
<td>1/16 8/8</td>
<td>62 100</td>
<td>12 15</td>
<td>P = 0.00186</td>
</tr>
<tr>
<td>IL12−/−</td>
<td>Control</td>
<td>10 100</td>
<td>0/8 0/3</td>
<td>0 0</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>10 100</td>
<td>0/16 2/6</td>
<td>33.3</td>
<td>19 22</td>
<td>P &lt; 0.0001 P &lt; 0.0003</td>
</tr>
<tr>
<td>IFNγ−/−</td>
<td>Control</td>
<td>10 100</td>
<td>0/4 0/4</td>
<td>0 0</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>10 100</td>
<td>0/10 9/9</td>
<td>0 0</td>
<td>18 13</td>
<td>P &lt; 0.0001 P &lt; 0.0001</td>
</tr>
<tr>
<td>RAG−/−</td>
<td>Control</td>
<td>10 100</td>
<td>0/6 0/3</td>
<td>0 0</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>10 100</td>
<td>2/10 3/5</td>
<td>20 60</td>
<td>8 12</td>
<td>P &lt; 0.0001 P = 0.195</td>
</tr>
</tbody>
</table>
a highly deleterious hypercytokinemia would be expected to ensue should a robust bacterial or viral invasion occur.

McCall and colleagues recently reported that adult individuals experimentally infected with *P. falciparum* were primed for responses to some, but not all, TLR ligands. These authors found an enhanced response to LPS (TLR4) and Pam3CysK4 (TLR2/TLR1) (23). Our results are notably different because the enhanced innate immune response was far more pronounced, and we found an enhanced response to all TLR ligands except pI:C. Although McCall et al. made an effort to correlate TLR2/4 expression with the priming they observed; they did not see enhanced expression in TLR2/4 in monocytes. The most likely reason for the limited degree of priming and the lack of enhanced TLR expression was due to the differences in the total body burden of parasites and the duration of parasitemia. It is useful to recall that the experimental volunteers were treated as soon as they become symptomatic and/or parasitemic, while our patients often did not present at our malaria clinic until they had had fevers and rigor for more than a week. As the average experimentally infected subject was aparasitemic for approximately 9 days (23), we infer that our patients were infected for a period of no more than 2 weeks before study.

While virtually every cytokine response we tested was enhanced in malaria-infected individuals, this was not the case for the anti-inflammatory cytokine, IL10. IL10 levels were greatly increased in the plasma of malaria patients. However, when PBMCs from infected patients were stimulated with TLR ligands, we often observed either no change in the IL-10 response or a suppression of IL-10 release by PBMCs (data not shown). Therefore, it can be concluded that the priming of cells for enhanced TLR responses is not coordinated throughout the entire transcriptome, but specifically applies to a defined group of important proinflammatory genes.

The in vivo consequences of malaria-induced priming appear to be important and applicable to infection with other *Plasmodium* species. Thus, *P. chabaudi*-infected mice had evidence of priming and developed a lethal hypersensitivity to very small amounts of LPS in a TLR9-, IL12-, and IFNγ-dependent fashion. Indeed, these data are some of the best to date that malarial DNA recognition might be pathophysiologically significant by engaging TLR9, as we previously proposed (10). As IFNγ is not produced in abundance by phagocytes, and is expressed in mouse lymphocytes (26), the mechanism appears to involve cross talk between the innate and the acquired arms of the immune system.

IFNγ has been shown not only to increase TLR expression but also to prime cells to LPS responses in a number of experimental infections (27–30) and has also been pointed as crucial to prime macrophages to release high amounts of TNF upon LPS administration and to contribute to LPS induced septic shock (31). We (12) and others (14) have shown that TLR9 accounts for a considerable amount of IFNγ produced during malaria. Furthermore, production of IL12, a potent IFNγ inducer, is diminished in BMDCs from TLR9+/− mice upon in vitro stimulation with *P. chabaudi* iRBCs. Thus, it is reasonabe to assume that the mechanism by which TLR9 mediates priming of host innate responses during malaria is by mediating parasite recognition and initiating IFNγ production. This assumption is strongly supported by findings in the *Propionibacterium* model in which TLR9-mediated production of IFNγ and IL12 is required to induce priming (32). Thus, we believe that the initial activation of innate immune responses is initiated by activation of TLR9 and induction of IL12 by innate immune cells (e.g., DCs and macrophages) resulting in production of IFNγ by NK and T cells (33, 34). In turn, the produced IFNγ will prime host cells to overexpress TLRs and become hyperresponsive to TLR agonists (Fig. 4C).

One can speculate on the consequences for the human host caused by malaria priming. The enhanced ability to respond to microbial ligands during innate surveillance probably protects the host from bacterial invasion. The areas of the world with the highest incidence and prevalence of malaria also have a high incidence of invasive bacterial infections, including *Salmonella*, *Pneumococcus*, and *Meningococcus* (35). In general, the innate immune response represents the classic “two-edged sword.” Priming means that the innate immune system has an enhanced capacity to be over activated during secondary infection and initiate the septic shock syndrome. Coinfection with bacteria is not only common, but, as we might have predicted from these results, patients with malaria and bacteremia die at 3 times the frequency of individuals with malaria alone (36). In addition to the ability of bacterial products to activate a hyper immune response during coinfection, the likelihood that parasite and/or endogenous TLR agonists act as second stimuli for cells primed during malaria should not be disregarded. To begin, different *P. falciparum* derived TLR agonists, such as GPI anchors (8) and DNA bound to hemoligin (10), might activate an enhanced innate immune response and result in unchecked inflammation. Moreover, host endogenous ligands that are weak TLR agonists have been described (37). Such endogenous molecules might, under the right circumstances (e.g., parasite-induced apoptosis), exacerbate the proinflammatory state of patients with malaria, even though under ordinary circumstances, they lack potency. Thus, it is our hypothesis that proinflammatory priming during malaria favors activation of TLRs by components from host and/or microbial origins and has the capability to induce injurious inflammatory states, including cerebral malaria.

The observation that an innate immune system is primed during febrile malaria could not have been predicted intuitively. In bacterial sepsis, most immune responses appear to be strongly suppressed, thus leading to an immune state that is often described as “immunoparalysis” (38). Although immunosuppressive therapy was once thought to be highly protective during septic shock (39) and the use of high dose steroids was commonplace, it is now recognized that this therapy increases mortality (40). By the time a patient with bacterial sepsis is diagnosed, the innate immune system is globally and profoundly immunosuppressed. Hence, additional anti-inflammatory therapy is unlikely to alter the course of events. Just the opposite appears to occur in acute malaria. Although anti-inflammatory approaches to life-threatening malaria have never been proven to be beneficial, it seems that the potential for targeted immunosuppressive therapy should not be ignored. By defining the innate immune response during the severest forms of malaria, as we have done here in patients with moderate illness, such strategies could be rationally designed and tested. Clearly, a better understanding of the innate immune response is critical if novel therapies are to be rationally designed.

**Materials and Methods**

**Reagents.** Unless stated elsewhere, all reagents were from Sigma-Aldrich. CL075 was obtained from Invivogen; ODN 7909 was synthesized by ALPHA DNA as phosphorothioate-linked ODNs; Pam2cysK4 was from EMCO Microcollection; CpG ODN 2007 was from the Coley Pharmaceutical Group. LPS was repurified by phenol chloroform extraction as described (41). MAbs to CD11c and TNFα were from BD PharMingen. MAbs to TLR2 and TLR4 were from eBiosciences. RPMI and DMEM were from Gibco. Cytokine ELISA kits were from R&D Systems.

**Subjects.** The study was approved by the Ethic and Research Council of the Rene Rachou Institute and the Brazilian Council of Ethics and Research (approval number 10567). Patients with acute febrile *P. falciparum* malaria (*n* = 57) were seen in the outpatient malaria clinic in the Tropical Medicine Research Center in Porto Velho, Brazil, an endemic malaria region in the Amazon basin. Informed consent was obtained before enrollment. All patients gave a history of recent fever and constitutional signs; none presented with severe anemia or cerebral malaria (see Table S1). Average ages of patients and a group of controls were 29.4 ± 12.8 and 31.2 ± 7.36 respectively. Up to 100 cc of blood was obtained immediately after confirmation of *P. falciparum* infection by a standard peripheral smear and 3–4 weeks after mefloquine therapy. PCR was used to confirm infection, and blood smears were taken at the first examination with *P. vivax* (42). Each patient served as his or her own control; additional control individuals (*n* = 16) included noninfected subjects living in Porto Velho and malaria naive individuals living in Belo Horizonte, Brazil, where malaria is not endemic.
PBMC Stimulation Assays. PBMCs were isolated from undiluted whole blood on Ficoll-paque Plus (GE Healthcare) per the manufacturer’s recommendations. Cells were plated into 96-well cell culture plates at a final density of 2 × 10^5 in DMEM containing 10% FCS (Gibco) and 10 μg ciprofloxacin (Cellofarm) per ml and stimulated for 20 h as indicated.

Microarray Experiments and Data Analysis. Information on microarray experiments and data analysis can be found in SI Text.

Analysis of Cell Surface Expression of TLRS. PBMCs from acutely infected patients (n = 11) were stained with combinations of the following mAbs: CD11c-FITC (Serotec), CD14-FITC, BD Biosciences), TRL2-PE, TRL4-PE (eBiosciences). Cells were gated by forward or side scatter to separate lymphocytes from monocytes and assessed for fluorescence using CellQuest software (BD Biosciences). Data were analyzed using Flowjo software (TreeStar).

Rodent Model of Malaria and Knockout Mice. The Plasmodium chabaudi chabaudi AS strain was used for experimental infections. Mice were infected by i.p. (i.p.) injection of 10^5 infected erythrocytes (IRBCs) and checked daily; moribund animals were scored as dead and euthanized. Laboratory values that reflect malaria-associated pathology were determined every 2–3 days throughout the infection (25). C57BL/6 as well as RAG^−/− and IFNγ^−/− mice were originally purchased from Jackson Laboratories. MyD88^−/−, TLR2^−/−, TLR4^−/− were a gift of S. Akira (Osaka University, Japan). Knockout mice were 8–12 weeks old and were all backcrossed for at least 8 generations onto a C57BL/6 background. Mice were bred under pathogen-free conditions in the animal house of René Rachou Institute-Fundação Oswaldo Cruz.

Mouse Splenocyte Stimulation Assays. Cytokine measurements from stimulated mouse splenocytes during P. chabaudi infection were performed with groups of 4 to 8 C57BL/6 mice and the corresponding number of knockout animals, as indicated in the figure legends. Each experiment was repeated 2–3 times. Each animal was analyzed individually. Mice were infected i.p. with 10^5 IRBCs. Splenocytes from infected-patients were quantified with the CBA inflammation kit (Becton-Dickinson).

Real Time PCR Analysis (qPCR). Total RNA was isolated from mouse spleens over the course of infection, as described. Sequences of primers are listed in Table S4. Relative level of gene expression was determined by the comparative threshold (Ct) method using the formula 2^−ΔΔCt whereby data for each sample were normalized to β-actin mRNA levels and expressed as a fold change compared with uninfected controls.

Statistical Analysis. All data were analyzed using Graphpad InStat 4.0 Software. Cytokine measurements were analyzed using two-tailed student’s t-test. Mann-Whitney testing was used for non-parametric analysis when data did not fit a Gaussian distribution. A P value of ≤0.05 was considered to be statistically significant.

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