Cytokines Kill Malaria Parasites during Infection Crisis: Extracellular Complementary Factors Are Essential

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Summary

Malaria infection crisis, at which the parasitemia drops precipitously and the parasite loses infectivity to the mosquito vector, occurs in many natural malaria systems, and has not been explained. We demonstrate that in a simian malaria parasite (*Plasmodium cynomolgi* in its natural host, the toque monkey), the loss of infectivity during crisis is due to the death of circulating intraerythrocytic gametocytes mediated by crisis serum. These parasite-killing effects in crisis serum are due to the presence in the serum of cytokines tumor necrosis factor and interferon γ, which are produced by the host as a result of the malaria infection. The killing activity of each cytokine is absolutely dependent upon the presence of additional, as yet unidentified factor(s) in the crisis serum.

Infectivity of malarial infections to mosquitoes is due to the presence of circulating gametocytes, the sexual stages that fertilize in the midgut of a blood-fed mosquito. In several malaria host-parasite systems, it has been noted that peaks of parasitemia are associated with "crisis" in the infection (1–3) and the simultaneous reduction or loss of infectivity of the parasite to mosquitoes (4, 5); neither of these phenomena has been explained. During a blood infection of *Plasmodium cynomolgi* in its natural host, the toque monkey, *Macaca sinica*, peak parasitemia is often accompanied by a crisis in the infection; this is most pronounced in splenectomized animals and is characterized by the appearance of morphologically abnormal intraerythrocytic parasites. At crisis, there is a sudden loss of infectivity of the parasites to mosquitoes, which persists for 5 to 7 days. We show here that the loss of infectivity at crisis is due to death of circulating intraerythrocytic gametocytes mediated by crisis serum. These killing effects in the crisis serum are due to the presence of the cytokines TNF and IFN-γ; the killing activity of each cytokine is absolutely dependent upon the presence of additional, as yet unidentified, factor(s) in the crisis serum.

Materials and Methods

Animals. The toque monkey, *Macaca sinica*, the natural host of *P. cynomolgi ceylonensis*, was used in this study. Wild-caught adult animals of either sex, weighing between 1 and 4 kg, which were free of malaria infections as determined by the indirect immunofluorescence test, were used for experiments. Splenectomies were performed using standard sterile techniques as previously described (6).

Parasites. An uncloned strain of *P. cynomolgi ceylonensis* that was kindly provided by Drs. R. W. Gwadz and L. H. Miller, Malaria Division, Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, MD, was used. The parasite was maintained either by cryopreservation or by blood passage in monkeys. Animals were infected 1–2 wk after splenectomy by intravenous inoculation of parasitized erythrocytes either from thawed stabilates or from an infected donor animal. The parasitemia and gametocytemia of infected animals were determined by microscopic examination of Giemsa-stained thin blood films.

Mosquito Vector. Anopheles tessellatus, a natural vector of human malaria in Sri Lanka, a colony of which is maintained in our laboratory, was used for infectivity studies.

Infectivity to Mosquitoes. Animals were anesthetized (Ketamine hydrochloride; Parke Davis & Co., Berlin, FRG) and mosquito feedings were performed each day between 9 and 10 a.m. as follows: (*a*) mosquitoes were directly fed on the animal; and (*b*) for membrane feeding, 2 ml of blood was drawn by venepuncture without anticoagulant into 10 volumes of a suspended activation (SA)[1] solution (7). The red cells were washed free of serum and resuspended to a hematocrit of 50% in normal (uninfected) monkey serum (NMS) and fed to mosquitoes through a water-jacketed membrane

[1] *Abbreviations used in this paper:* NMS, normal monkey serum; NRS, normal rabbit serum; SA, suspended activation.

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feeding apparatus circulating water at 40°C (7, 8). Blood-fed mosquitoes were maintained for 8 d at 26–27°C, dissected, and their midguts examined for oocysts (products of gamete fertilization). The arithmetic mean of the number of oocysts per mosquito midgut was taken as the infectivity of the parasite to mosquitoes in each direct or membrane feeding.

Pre-incubation of Gametocytes. Gametocyte-containing blood of an infected monkey was washed free of serum in SA solution and pre-incubated in culture medium (RPMI 1640, 5% $NaHCO_3$, and 10% heat-inactivated FCS) containing 20% of either crisis serum obtained from a splenectomized infected animal on the day of crisis, or NMS as controls. 2.2 ml of each cell suspension at a hematocrit of 10% was incubated at 37°C in an atmosphere of 5% CO_2 and 95% air at 37°C in 24-well Linbro plates (Flow Laboratories, Herts, UK).

To test the effect of anticytokine antibodies on the gametocytocidal effects of crisis serum, 440 μl of crisis serum was mixed with either 100 μl of rabbit anti-TNF antibodies (1.94 mg/ml) (IRE Medgenix, Fleurus, Belgium), 466 μl of rabbit anti-IFN-γ antibodies (2.86 mg/ml) (IRE Medgenix), or equivalent volumes of normal rabbit serum (NRS), and incubated for 30 min at 37°C. The same amounts of each antiserum as above were added to the sample in which both anti-TNF and anti-IFN-γ were used; for controls, an equivalent volume of NRS was used. The treated crisis serum was then used to make up 20% of culture medium, as described above for pre-incubation of gametocyte-infected blood. After 3 h of incubation, cells were washed in RPMI 1640 with 5% $NaHCO_3$, resuspended to a hematocrit of 50% in NMS, and fed to mosquitoes in a membrane feeder. Infectivity of the preparations to mosquitoes was assessed as described above.

Induction of Parasite-killing Factors In Vitro. PBMC were prepared from venous blood from an uninfected spleen-intact monkey by sodium metrizoate density gradient centrifugation (Lymphoprep, Nygaard, Norway) according to standard procedure. PBMC were reconstituted in RPMI 1640 containing 0.2% $NaHCO_3$ and 10% NMS at a concentration of 2×10^6/ml. 2.5 ml of the cell suspension was seeded each into a 4-cm petri dish and LPS (Difco Laboratories Inc., Detroit, MI) was added to a final concentration of 2 μg/ml; an equivalent volume of PBS was added to control cultures. Culture medium containing the same concentration of LPS without cells was also used as a control. The test and control plates were maintained in 5% CO_2 and 95% air in an incubator at 37°C, and 48 h later, supernatants were collected and stored at −20°C until used. LPS-stimulated PBMC culture supernatants or controls were used at a 50% concentration with culture medium for pre-incubating gametocytes.

To assess the effect of anticytokine antibodies on the gametocytocidal activity of LPS-stimulated culture supernatants, either 100 μl of rabbit anti-TNF antibodies, 466 μl of rabbit anti-IFN-γ antibodies, or the equivalent volumes of NRS as controls were added before incubation to 1.6 ml of a gametocyte cell suspension described above.

To evaluate the gametocytocidal effects of TNF: (*a*) recombinant human TNF (rTNF) (IRE Medgenix, Fleurus, Belgium) in PBS was added to the LPS-stimulated culture supernatants (at 40,000 IU/ml), which were depleted of TNF by the addition of anti-TNF antibodies; an equivalent volume of PBS was used as a control. These were then used to make up 50% of the gametocyte-incubating medium with culture medium. Gametocyte-containing blood was pre-incubated for 3 h in culture medium containing 50% of the above described reagents. (*b*) rTNF alone was added to culture medium at 10-fold serial dilutions from 10,000 to 0.1 IU/ml of culture medium, and gametocyte-containing blood was pre-incubated in it for 3 h. The infectivity of pre-incubated gametocytes was assessed as described above.

Effects of LPS Injection In Vivo on Infectivity of Gametocytes. LPS was injected intravenously into a monkey at a dose of 5 ng/kg body weight.

Results

When mosquitoes were fed directly on an infected splenectomized (S-) animal, *P. cynomolgi* parasites were found to be infectious to mosquitoes from the first day of patency of a blood-induced infection (Fig. 1). However, at the peak of parasitemia and gametocytemia, between days 5 and 10 of patency, which corresponds to the period of crisis during which intraerythrocytic parasites appeared morphologically abnormal (Fig. 2), the infectivity of the parasite dropped to zero (Fig. 1). This total loss of infectivity lasted for 5–7 d in all of seven infected animals studied. Gametocyte-infected blood taken during crisis was noninfectious to mosquitoes, even when washed and resuspended in NMS and fed to mosquitoes through an artificial membrane (Fig. 1). Therefore, the circulating intraerythrocytic gametocytes had already been killed before they entered the mosquito.

To investigate the role of host serum during crisis on the infectivity of gametocytes, healthy infectious gametocytes of *P. cynomolgi* were incubated for 3 h in vitro in culture medium containing 20% crisis serum. Compared with controls incubated in nonimmune NMS, infectivity of the gametocytes incubated in crisis serum was consistently abolished (Table 1, rows *1* and *2*).

The addition of antibodies against either TNF or IFN-γ alone to crisis serum did not affect its gametocyte killing activity (Table 1, rows *3–5*), however, simultaneous addition of antibodies against both TNF and IFN-γ completely reversed

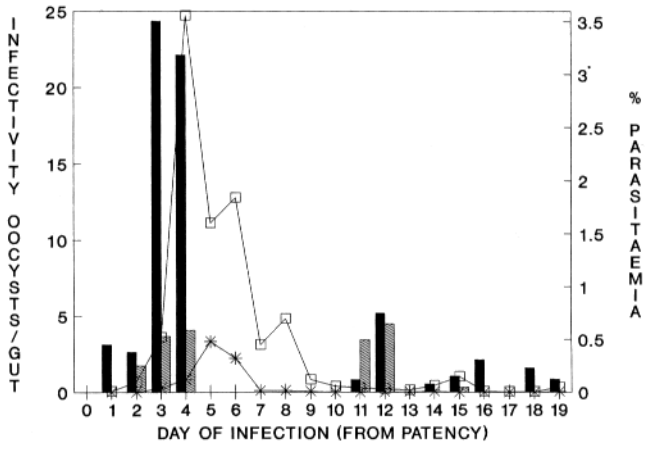

Figure 1. Loss of infectivity to mosquitoes during crisis (day 5–10) in a *P. cynomolgi* infection in a toque monkey as shown by: the direct infectivity of the infected toque monkey to mosquitoes (■); and the intrinsic infectivity of the gametocyte-infected blood fed to mosquitoes through a membrane in the presence of nonimmune NMS (▨) in relation to the total parasitemia (□) and gametocytemia (*) on each day of the infection. Comparable results were obtained in all of the seven animals studied. In each of these monkeys, peak of gametocytemia and fall post-peak coincide, respectively, with the peak of the asexual parasitemia and crisis in the infection.

Table 1. *Effect of Pre-incubation in Crisis Serum on Infectivity of Gametocytes to Mosquitoe and the Reversal of this Effect by Anti-TNF and Anti-IFN-γ Antibodies*

	Infectivity as oocysts/gut (no. positive/no. dissected) in Exps:									Average ± SD as percent of controls‡
	1	2	3	4	5	6	7	8	9	
NMS	4.77 (6/22)	5.89 (5/19)	6.94 (6/18)	7.53 (5/15)	10.22 (5/18)	11.56 (6/16)	7.57 (3/14)	13.8 (6/15)	11.27 (6/15)	100 (100)
Crisis serum	0 (0/16)	0 (0/16)	0 (0/15)	0 (0/14)	0.17 (1/18)	0.45 (2/20)	ND	0 (0/15)	0 (0/18)	0.69 (5.83) ±1.42 (±10.95)
Crisis serum + anti-TNF antibodies	0 (0/21)	0 (0/18)	0.25 (2/16)	ND	ND	ND	ND	0.58 (3/19)	0 (0/14)	1.56 (15.39) ±2.15 (±21.09)
Crisis serum + anti-IFN-γ antibodies	ND	ND	0 (0/15)	0.28 (3/18)	0 (0/15)	0.44 (4/18)	ND	0.45 (2/20)	0 (0/14)	1.80 (22.38) ±1.98 (±26.95)
Crisis serum + NRS*	ND	0 (0/21)	0.33 (1/15)	0.28 (2/18)	0.10 (2/20)	0 (0/16)	ND	0.19 (1/16)	0.44 (2/16)	2.11 (19.46) ±1.98 (±15.15)
Crisis serum + anti-TNF abs + anti-IFN-γ antibodies	ND	ND	ND	ND	ND	ND	5.67 (5/15)	7.13 (5/16) 6.00 (6/16)	9.71 (5/14) 8.12 (5/17)	65.65 (98.05) ±17.57 (±33.17)
Crisis serum + NRS*	ND	ND	ND	ND	ND	ND	0 (0/12)	0 (0/13)	0 (0/12)	0 (0)
NMS + anti-TNF antibodies + anti-IFN-γ antibodies*	ND	ND	ND	ND	ND	ND	ND	8.41 (6/17)	8.56 (6/16)	68.45 (91.0) ±10.62 (±3.90)

* Experiments in row 5 were the controls for experiments in rows 3 and 4. Experiments in rows 7 and 8 were the controls for experiments in row 6. Experiments in rows 5 and 7 differed with respect to the volume of NRS used.

‡ Mean ± 1 SD of the mean oocysts/gut and (proportion of mosquitoes infected) of all experiments, each taken as a percentage of the corresponding NMS control (row 1). Experiments described in row 6 were performed in duplicate in each of Exps. 8 and 9.

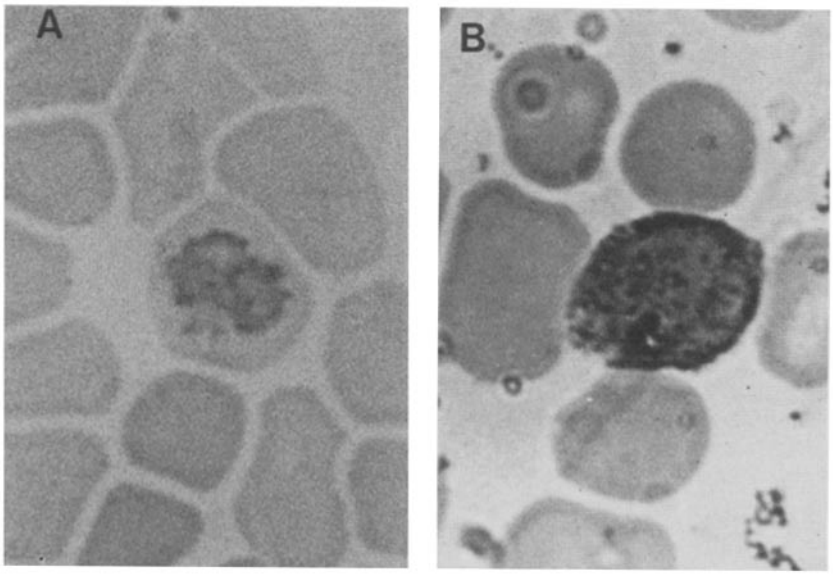

Figure 2. The morphological appearance in Giemsa-stained blood films of intraerythrocytic *P. cynomolgi* parasites taken during crisis of a blood infection showing a degenerate faintly stained parasite (*A*), and a normal growing blood stage parasite taken before crisis (*B*).

the killing activity of the crisis serum (Table 1, rows *6-8*). As parasites survived only when both cytokines were removed by antibody, these results suggest that the levels of TNF and IFN-γ in the crisis serum were each sufficient to kill gametocytes without the other.

We next tested the effects of human rTNF and rIFN-γ on the viability of healthy infectious gametocytes of *P. cynomolgi* incubated with these cytokines in vitro as before (Table 2, rows *2–6*). Neither cytokine when tested individually, nor a combination of both cytokines, affected the infectivity of

Table 2. *Restoration of Gametocyte-killing Effects of TNF and IFN-γ Depleted Crisis Serum by Addition of rTNF and rIFN-γ*

	Infectivity as oocysts/gut (no. positive/no. dissected) in Exps:		Average as percent of controls‡
	1	2	
NMS	8 (12/15)	13.4 (13/18)	100 (100)
NMS + rTNF	10.7 (10/14)	10.7 (10/17)	100 (85.6)
NMS + rIFN-γ	10.5 (10/17)	12 (12/15)	105.1 (91.2)
NMS + rTNF + rIFN-γ	11.2 (11/17)	9 (11/16)	94.4 (87.7)
NMS + PBS (control for rows 2 and 3)	10.2 (10/16)	10.1 (8/13) 9 (11/16)	91.3 (84.4)
NMS + PBS (control for row 4)	10.4 (9/15)	11.0 (10/15)	100 (83.2)
MCrS*	0.3 (2/15)	0.16 (2/18)	2.2 (16.1)
MCrS + anti TNF + anti-IFN-γ	9.4 (10/15)	12.6 (12/16)	102.8 (93.1)
MCrS + anti TNF + anti-IFN-γ + rTNF	0.6 (4/18)	0.06 (1/16)	3.1 (18.7)
MCrS + anti TNF + anti-IFN-γ + rIFN-γ	0.7 (4/15)	0.35 (2/17)	4.9 (25.3)
MCrS + anti TNF + anti-IFN-γ + rTNF + rIFN-γ	0.8 (3/15)	0 (0/15)	3.7 (13.1)
MCrS + NRS (control for row 8)	0.4 (3/14)	0.1 (1/10)	2.3 (20.7)
MCrS + anti TNF + anti-IFN-γ + PBS (control for row 9)	8.9 (12/18)	11.6 (10/15)	95.8 (87.6)
MCrS + anti TNF + anti-IFN-γ + PBS (control for row 10)	8.0 (9/14)	11.1 (11/16)	89.3 (87.4)
MCrS + anti TNF + anti-IFN-γ + PBS (control for row 11)	10.5 (8/15)	8.1 (7/12)	86.9 (73.4)

rTNF and rIFN-γ in PBS were added to the culture medium at concentrations of 60,000 and 90 U/ml, respectively. An equivalent volume of PBS was used as controls.

* Monkey crisis serum.

‡ Arithmetic mean of the mean oocysts/gut and (proportion of mosquitoes infected) of Exp. 1 and 2, each taken as a percentage of the mean of the NMS control experiment in row 1.

the gametocytes to mosquitoes. However, when either cytokine was added to crisis serum that had been depleted of both, the killing effect of the crisis serum was totally restored (Table 2, rows *8–15*). These results confirm that TNF and IFN-γ each mediate gametocyte killing independently of the other, but that each cytokine does so only in the presence of another factor(s) provided by crisis serum. Neither cytokine can represent the complementary factor for the other, as removal of either did not affect the killing activity of the other, and as the addition of both recombinant cytokines together to normal serum did not induce killing. The complementary factor(s) were present only in the crisis serum and not in normal monkey serum (Table 2, rows *2–4*). Therefore, these factors must have been produced as a result of the malarial infection itself.

We then tested whether a known inducer of TNF, LPS injected into toque monkeys, would produce a killing effect similar to that produced by crisis serum. From an uninfected monkey, serum was obtained immediately before (0 h) and 1.5 and 3 h after injection of LPS. Healthy infectious gametocytes lost almost all infectivity after incubation in the 1.5-h serum (Table 3, Exp. 1); this effect was greatly reduced in the serum taken 3 h after LPS injection. Similar effects were observed directly in vivo in a *P. cynomolgi*–infected toque monkey. Immediately before injection of LPS into the infected monkey, the parasites were infectious to mosquitoes; 1.5 h later all infectivity was lost, and there was little if any recovery of infectivity after 3 h (Table 3, Exp. 2). These results are consistent with the induction of both TNF and complementary factor(s) by injection of toque monkeys with LPS.

We further examined the induction of the complementary factor(s) by treating toque monkey PBMC in vitro with LPS.

Table 3. *In Vivo Effect of LPS on Infectivity of P. cynomolgi in Toque Monkeys*

Exp.		Infectivity as oocysts/gut (no. positive/no. dissected) 0 h	1.5 h	3 h
1	Effect of serum on infectivity of preincubated gametocytes	7.57 (3/14)	0.41 (1/17)	2.36 (3/14)
2	Direct infectivity	13.28 (8/18)	0.19 (3/21)	1.81 (7/26)
	Intrinsic infectivity	3.15 (5/20)	0 (0/25)	0 (0/28)

In Exp. 1, LPS was injected intravenously to an uninfected spleen-intact monkey. Gametocytes from an infected monkey were pre-incubated for 3 h in culture medium containing 50% serum from the LPS-injected animal obtained pre-injection (0 h), and 1.5 and 3 h after LPS injection. In Exp. 2, a *P. cynomolgi*–infected spleen-intact toque monkey was injected intravenously with LPS as above. Direct feeding of mosquitoes on the animal, and membrane feeding of gametocytes in NMS, were performed as described above just before LPS injection (0 h), and at 1.5 and 3 h after injection.

P. cynomolgi gametocyte-infected blood pre-treated with supernatants of PBMC cultures stimulated with LPS lost their infectivity to mosquitoes (Table 4, rows *1–3*). Removal of TNF from the LPS-stimulated culture supernatants by addition of anti-TNF antiserum abolished the gametocytocidal effects (Table 4, rows *4* and *6*). As expected, however, TNF alone had no gametocytocidal effects (Table 4, rows *8* and *9*), but did so when added to the TNF-depleted LPS-stimulated PBMC culture supernatants (Table 4, rows *6* and *7*). These experiments demonstrate that complementary factor(s), which are necessary for TNF-mediated killing of gametocytes, are present in the supernatants of monkey PBMC after LPS stimulation. Depletion of IFN-γ from these supernatants with anti-IFN-γ antibody did not affect the killing activity of the supernatants (Table 4, row *5*). Thus, as was found for monkey crisis serum, IFN-γ cannot represent the complementary factor(s) for TNF in these supernatants.

Discussion

Our results demonstrate that the cytokines TNF and IFN-γ mediate the gametocyte killing effects of serum taken during crisis of a malarial blood infection, and probably account for the total loss of infectivity of the parasites to mosquitoes during crisis. Gametocytes during crisis or normal gametocytes preincubated with crisis serum in vitro were already dead before entering the mosquito. This is shown by the fact that gametocytes from crisis or cultured in the presence of crisis serum failed to infect mosquitoes even when washed and resuspended in normal serum. Our results also implicate the existence of additional humoral or extra-cellular factor(s) in crisis serum that are necessary for the parasiticidal effects of TNF or IFN-γ. Neither cytokine, however, can by itself represent the complementary factor for the other as: (*a*) the removal of either did not diminish the effect of the other; and (*b*) a simple combination of both cytokines was without effect on gametocyte viability. We have also demonstrated that the extracellular complementary factor(s) for TNF-mediated killing can be produced in vitro in the supernatants of toque monkey PBMC after stimulation with LPS.

Previous studies have demonstrated malaria parasite killing effects of cytokines introduced in vitro or in vivo (9–13) or after immunization against the stages of the malaria parasite that enter and grow in liver cells (14). However, endogenous host cytokines have not been shown to be naturally elaborated during a malarial blood infection and affect parasite viability during the infection. Serum from humans infected with malaria has, in some circumstances, been found to induce crisis forms in malaria parasites grown in red blood cells in vitro (15). Similar forms were induced in mice with Bacille, Calmette, Guérin (BCG) and LPS, and this ability was reversed by addition of anti-TNF anti-serum (16).

A common feature of all these studies is that the cytokines were either induced in vivo or mediated their parasite killing effects in the presence of living nucleated host cells in tissue culture. In none of these instances did added TNF affect the development of malaria parasites grown in red blood cells

Table 4. *Effect of LPS-stimulated PBMC Culture Supernatants and Role of TNF on Infectivity of Gametocytes to Mosquitoes*

	Infectivity as oocysts/gut (no. positive/no. dissected) in Exps:							Averge ± SD as percent of controls*
	1	2	3	4	5	6	7	
Unstimulated PBMC sup.	3.35 (4/17)	10.05 (9/21)	9.73 (7/15)	10.13 (6/16)	4.64 (4/14)	ND	ND	100 (100)
LPS only	12.0 (7/16)	ND	7.13 (6/15)	13.07 (7/15)	4.07 (4/15)	ND	ND	
LPS-stimulated PBMC sup.	0.61 (3/18)	1.13 (2/15)	0 (0/18)	0 (0/15)	0 (0/15)	ND	ND	3.84 (16.13) ± 5.38 (± 23.03)
LPS-stimulated PBMC sup. + NRS	0.25 (1/20)	ND	0 (0/15)	0 (0/15)	ND	ND	ND	1.09 (4.95) ± 1.88 (± 8.58)
LPS-stimulated PBMC sup. + anti-IFN-γ antibodies	1.5 (3/18)	ND	0 (0/14)	ND	ND	ND	ND	9.77 (24.77) ± 13.81 (± 35.03)
LPS-stimulated PBMC sup. + anti-TNF antibodies	7.44 (5/16)	7.94 (6/18)	3.93 (4/15)	4.73 (4/15)	2.06 (2/16)	ND	ND	62.11 (68.17) ± 24.55 (± 18.00)
LPS-stimulated PBMC sup. + anti-TNF antibodies + rTNF	ND	0.87 (3/15)	ND	ND	0 (0/15)	ND	ND	4.33 (23.33) ± 6.12 (± 33.00)
PBS (control for rTNF)	ND	ND	ND	19.18 (8/17)	5.69 (4/16)	7.53 (5/15)	11.56 (6/16)	100 (100)
rTNF‡	ND	ND	ND	13.11 (9/18)	4.36 (3/14)	6.64 (4/14)	11.17 (6/18)	82.49 (91.64) ± 12.4 (± 9.85)

sup., supernatants.

* Mean ± 1 SD of the mean oocysts/gut and (proportion of mosquitoes infected) of all experiments, each taken as a percentage of the mean of the two control experiments in rows 1 and 2.

‡ rTNF was used in concentrations of 10,000, 40,000, 40,000, and 1,000 IU/ml of cell suspension, respectively, in experiments 4, 5, 6, and 7 given here. 10-fold serial dilutions of rTNF from 10,000 to 0.1 IU/ml cell suspension were tested in most experiments, and none had any gametocytocidal effects.

in the absence of nucleated host cells in vitro (17). The role of host cells in cytokine-mediated killing of parasites is commonly assumed. We have now shown that additional extracellular factor(s) are essential components of the cytokine-mediated killing; these factor(s) cannot be nucleated host cells because they are carried in cell-free serum. However, it is not yet clear whether the continued presence of nucleated host cells (naturally present in low numbers in the peripheral blood of *P. cynomolgi*–infected monkeys, as used in the present study) is necessary, thereafter, to achieve parasite killing.

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