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In Vivo Induction of Nitrite and Nitrate by Tumor Necrosis Factor, Lymphotoxin, and Interleukin-1: Possible Roles in Malaria

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Tumor necrosis factor and related cytokines are thought to be implicated in cell-mediated immunity and pathophysiology in malaria, but their mechanism of action has not been ascertained. Tumor necrosis factor has been reported to generate nitric oxide in vitro, so we have measured levels of this molecule and its products in the plasma of mice after they have received an injection of tumor necrosis factor, lymphotoxin, interleukin-1, gamma interferon, or interleukin-6, all of which have been reported to be increased in malaria. Total reactive nitrogen intermediate levels in plasma were assayed spectrophotometrically after exposing plasma to a copper-cadmium-zinc catalyst to convert nitrate to nitrite and then to Griess reagent. Tumor necrosis factor, lymphotoxin, and interleukin-1 all induced reactive nitrogen intermediates in vivo, with interleukin-1 showing the most activity. Tumor necrosis factor was then examined more closely. It induced more reactive nitrogen intermediates in malaria-infected mice than in normal mice, and appreciably more was in the form of nitrate than was in the form of nitrite. N^G-methyl-L-arginine inhibited the in vivo generation of reactive nitrogen intermediates by tumor necrosis factor in a dose-dependent manner, implying that these molecules were arginine derived. These results are consistent with the possibility that tumor necrosis factor, lymphotoxin, and interleukin-1 may contribute to host pathology and parasite suppression through generation of nitric oxide.

In keeping with our group's earlier proposals (7, 13) and subsequent mimicry of the pathology seen in *Plasmodium vinckei* malaria by injecting recombinant tumor necrosis factor (TNF) into mice (10), there is now considerable evidence associating this cytokine with the pathological changes seen in this disease. Malaria parasites can trigger the release of TNF (5), and high levels of this cytokine in serum are associated with parasite load and disease severity in rodent (8) and human (6, 27) malaria. Likewise, TNF has been reported to inhibit malaria parasites in vivo (11, 47).

The precise way in which TNF may inhibit parasites and cause illness and the pathology that parallels the changes seen in malaria have not been defined, but recent studies suggest that various TNF-induced mediators could be responsible. Among these mediators are the reactive nitrogen intermediates (RNI), which comprise NO· and its oxidation products, the nitrite and nitrate anions. These have well-recognized antiprotozoal activity (1, 33) and physiological (4, 48) effects. Cellular sources of arginine-derived nitric oxide include macrophages (44), endothelial cells (38), and neurons (18). Since nitric oxide or its metabolites have been shown (28) or implied (37) to be induced by TNF in vitro, and this process can be inferred to have occurred in vivo (29), we decided to see whether TNF, as well as interleukin-1 (IL-1) and lymphotoxin (LT), two other cytokines with raised levels in serum from malarial patients (7a, 31), would induce RNI in vivo.

MATERIALS AND METHODS

Mice and parasites. CBA/Ca mice, male or female and 6 to 8 weeks of age, were used in all experiments. They had been bred and maintained under specific pathogen-free conditions at The Australian National University.

Plasmodium vinckei vinckei from F. E. G. Cox (King's College, London, United Kingdom) was stored in liquid nitrogen and routinely passaged in mice, with recourse to frozen stocks when required. All infections were initiated with 10^6 parasitized erythrocytes injected intraperitoneally (i.p.). Parasitemias were assessed by Giemsa staining of thin blood smears. Cytokines or saline was injected intravenously (i.v.) 6 days later when the mice had attained parasitemias of 20 to 30% and were apparently quite healthy. Depending on the periods of observation, parasitemias ranged up to 40% during the experiments. Illness is not observed for this infection until approximately 80% of the erythrocytes are infected.

Plasma collection. Mice were killed with ether and bled from their axillary vessels. The blood was collected in heparinized tubes, which were centrifuged at 15,000 rpm for 5 min in a microcentrifuge (Beckman Instruments, Gladesville, New South Wales, Australia). The plasma was decanted into clean tubes and stored at −20°C.

Catalyst for RNI assay. By using a modification of the method of Davison and Woof (14), 1 g of powdered zinc (BDH) was first washed with 1 M HCl and three times with distilled water before being suspended in 100 ml of distilled water by stirring. A saturated solution of cadmium acetate (BDH) was added dropwise to the zinc until the suspension cleared. The precipitate was washed extensively in distilled water, added to 100 ml of 5% $CuSO_4$ for 30 s, and then again washed extensively in distilled water. Finally, the catalyst was stored in an NH_4Cl-borate buffer (5% NH_4Cl brought to pH 8.5 with $Na_2B_4O_7$, as previously described [20]) at 4°C. The catalyst was always used within 1 week of preparation.

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TABLE 1. Comparison of RNI concentrations at 10 h post-cytokine stimulation in *P. vinckei*-primed mice

Dose (μg)	RNI concn[a] (μM) with:				
	TNF (n^b)	LT (n)	IL-1 (n)	IFN-γ (n)	IL-6 (n)
0.0[c]	8.7 ± 2.7 (10)	8.7 ± 2.7 (10)	8.7 ± 2.7 (10)	8.7 ± 2.7 (10)	8.7 ± 2.7 (10)
0.1	ND	ND	ND	18.8 ± 11.6 (3)	ND
0.2	ND	ND	124.4 ± 19.4 (3)	ND	13.7 ± 2.0 (3)
1.0	65.0 ± 20.0 (3)	ND	540.0 ± 105.8 (3)	20.4 ± 2.2 (3)	33.1 ± 6.5 (3)
2.0	ND	89.8 ± 16.2 (12)	349.2 ± 28.1 (10)	ND	ND
5.0	669.7 ± 162.0 (6)	734.4 ± 43.0 (3)	498.7 ± 59.8 (6)	ND	10.4 ± 2.3 (3)
10.0	464.5 ± 109.0 (8)	648.9 ± 99.7 (9)	ND	ND	ND
15.0	ND	1264.0 ± 200.0 (3)	ND	ND	ND

[a] Data are means ± standard deviation of the means of the total plasma RNI (NO_2^- + NO_3^-).
[b] n, number of mice.
[c] Saline (200 μl) was injected i.v. All cytokine doses were diluted in saline and administered in a 200-μl volume.

Catalyst that was black rather than a metallic-copper color invariably gave the best results.

Assay for RNI. Thirty-microliter volumes of plasma were dispensed into 1.5-ml plastic tubes (Elkay Products Inc., Shrewsbury, Mass.) in duplicate, and sodium nitrite (Sigma Chemical Co., St. Louis, Mo.) and sodium nitrate (Sigma) standards (concentration range: 1 mM to 1 μM) were set up separately in 30-μl volumes of pooled (50 mice) normal mouse plasma. Control tubes contained 30 μl of normal mouse plasma or 30 μl of phosphate-buffered saline (pH 7.2). The latter control acted as a blank. Twenty microliters of catalyst buffer was added to one set of samples and the nitrite standard curve, while the other set and the nitrate standard curve received 20 μl of catalyst. All the tubes were incubated at room temperature for 5 min, after which the tubes containing the catalyst were centrifuged at 15,000 rpm for 1 min in the Beckman microcentrifuge and the supernatants were transferred to clean tubes. Griess reagent (100 μl) and 10% trichloroacetic acid (100 μl) were added to all tubes and mixed thoroughly before being centrifuged for 15 min at 15,000 rpm in the Beckman microcentrifuge. Two 100-μl samples of the supernatant from each tube were transferred to a 96-well flat-bottom plate (Nunc, Roskilde, Denmark), and the absorbance was read on a microplate reader (Dynatech MR 600; Dynatech, McLean, Va.) with a test wavelength of 540 nm and a reference wavelength of 630 nm.

Plasma nitrite was calculated by reading the absorbance directly from the nitrite standard curve, whereas reading plasma nitrate from the nitrate standard curve first required that the absorbance of the sample without catalyst be subtracted from the absorbance of the sample with catalyst. The results were expressed either as micromolar concentrations of nitrite or nitrate or as their sum.

L-NMMA. N^G-methyl-L-arginine (L-NMMA) was synthesized essentially as previously described (39). It was dissolved in saline immediately before use and injected i.p.

Recombinant cytokines. The cytokines were obtained as follows: recombinant human TNF (activity: 1 U ≅ 0.0455 ng) from Asahi Chemical Company, Tokyo, Japan; recombinant human IL-1β from J. Schmidt, Merck Institute, Rahway, N.J.; recombinant mouse gamma interferon (IFN-γ) (activity: 1 U ≅ 0.1 ng) from G. R. Adolf, Boehringer Ingleheim, Vienna, Austria; and recombinant human IL-6 (activity: 1 U ≅ 6.9 pg) from W. Fiers, University of Ghent, Ghent, Belgium. Highly purified recombinant human LT was isolated as described previously (3, 19). It had a specific activity of 1 U ≅ 0.01 ng as determined by cytolysis of actinomycin D-treated mouse L-929 cells (2).

RESULTS

Cytokine-induced RNI production in *P. vinckei*-infected mice. *P. vinckei*-primed mice were injected i.v. with saline, TNF, LT, IL-1, IFN-γ, or IL-6 and bled 10 h later to enable sufficient time for the induction of the NO-synthase enzyme and for measurable levels of RNI to be synthesized.

Table 1 shows that TNF, LT, and IL-1 can all induce the release of >50 μM RNI into the plasma, whereas saline, IFN-γ and IL-6 did not increase RNI above background levels detectable in normal mice (6 to 12 μM), even though these last two cytokines were used at doses comparable to the others. Weight-for-weight, IL-1 required less material than did either TNF or LT to induce an increase in plasma RNI, although TNF and LT induced higher levels overall than IL-1 and LT required less material than TNF. Limitations in availability of some of these cytokines precluded analysis of the kinetics of RNI generation over the 0 to 10 h range being examined.

Inhibition of cytokine-induced increase in plasma RNI levels by L-NMMA. In order to determine whether the RNI detected in the plasma of the mice was of arginine origin, the animals received two i.p. doses of L-NMMA, one at the same time as the TNF injection (5 μg i.v.) and the other 6 h later. The mice were then bled after a further 6 h. Figure 1 shows that L-NMMA blocked an increase in plasma RNI in a dose-dependent manner. The 2- and 4-mg injections reduced RNI output to below 100 μM 12 h after TNF injection, and the 8-mg injections totally blocked RNI synthesis.

Higher TNF-induced RNI production in malaria-primed mice than in normal mice. TNF (5 μg) was used to induce the release of RNI in both normal and *P. vinckei*-primed mice, and the concentration of RNI in plasma was monitored by bleeding groups of six mice at the times indicated in Fig. 2. The most striking feature was that nitrate was the dominant RNI species measured, with nitrite contributing no more than 20 μM to group means that were as high as 1,400 μM total plasma RNI. Saline injections did not cause any elevation in RNI levels over the same time course in either infected or normal mice.

As shown in Fig. 2, no increase in RNI was measurable in the first 2 h after injection of TNF, but by 3 h, a similar increase was detected in both the normal and *P. vinckei* groups. These values rose in parallel for another hour. From 4 to 12 h, the total plasma RNI concentration in the unparasitized mice increased at 40 μM/h to a maximum of 355 μM before it began to decrease, whereas the plasma RNI in the malarial group increased at an average of 156 μM/h from 4 to

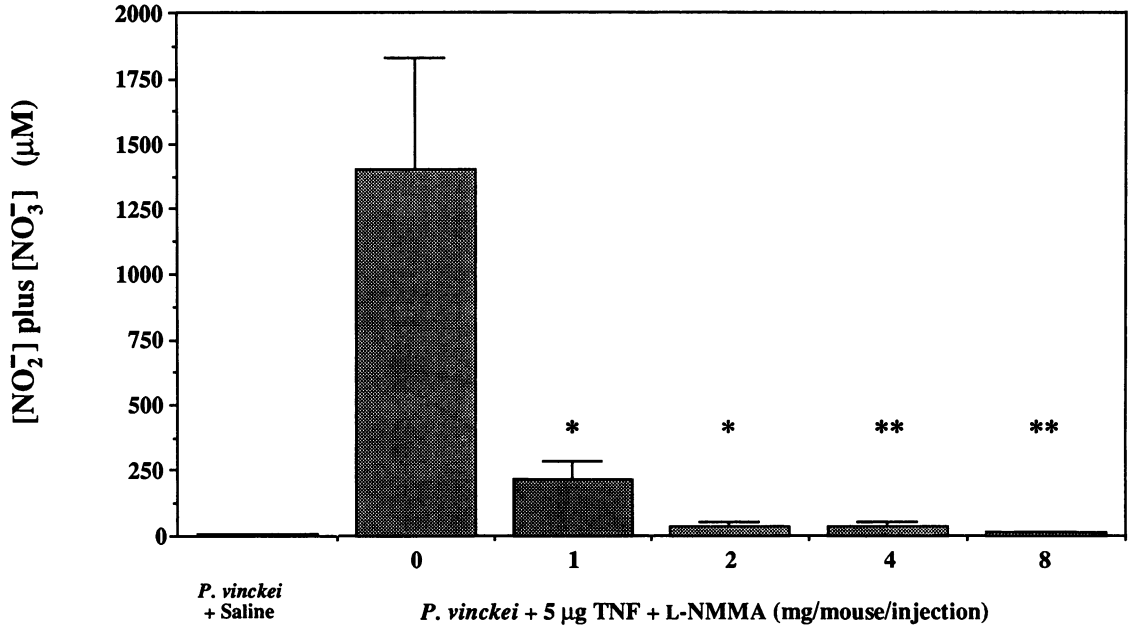

FIG. 1. Comparison of plasma RNI concentrations (NO_2^- + NO_3^-) from *P. vinckei*-primed mice alone and at 12 h after being injected i.v. with 5 μg of recombinant human TNF in 200 μl of saline. Each dose of L-NMMA was administered i.p. in 200 μl of saline at 0 and 6 h after TNF injection. Data are means ± standard errors of the means for each point, and six mice were tested for each point. *, $P < 0.02$; **, $P < 0.007$ versus that for the group with no L-NMMA.

12 h (four times that of normal mice injected with TNF). By 18 h, the plasma RNI concentration in the normal mice had returned to pre-TNF treatment levels, whereas in the parasitized mice, RNI remained high (~500 μM [$P < 0.001$ versus that for the normal group]) although lower than that at the 12-h post-TNF time point.

We then treated a group of *P. vinckei*-infected mice with a regimen of L-NMMA injections slightly different from those used previously. These mice received an i.p. injection of 2 mg of L-NMMA 1 h before TNF was given and another 2-mg dose at the same time as TNF administration. This resulted in no detectable increase in plasma RNI for up to 6 h posttreatment with TNF (Fig. 2). At 6 h, an increase was detectable, but this was still less than that seen in the unparasitized TNF-treated animals not given L-NMMA ($P < 0.025$). The difference was greater ($P < 0.01$) compared with the RNI generated at 6 h in the non-L-NMMA, TNF-treated malarial group (Fig. 2). From 6 to 12 h, the RNI levels in the L-NMMA group increased at 220 μM/h (1.5 times that of the non-L-NMMA-treated *P. vinckei*-infected group) over the same period, implying that the L-NMMA had been fully metabolized or excreted by 6 h and that the amounts of nitric oxide synthase induced in both *P. vinckei* groups were comparable. The highest RNI concentration attained in these mice was similar to that attained in the non-L-NMMA-treated *P. vinckei* group. This had also decreased by the 18-h time point but not to pre-TNF levels, even though it was not significantly different from that of the normal mice ($P = 0.09$). The rate of decrease was greater in the L-NMMA-treated group than in the non-L-NMMA-treated group.

DISCUSSION

We report here that exogenous TNF, LT, and IL-1, when injected into mice, will induce the generation of NO·, measured as total RNI, free in plasma (Table 1). Mice treated with these cytokines early in the course of a malaria infection generated considerably more RNI than did normal mice (Fig. 2). This is in keeping with the increased sensitivity, in terms of illness and hypoglycemia, that such parasitized mice exhibit to all three of these cytokines (7a, 10).

As well as their well-documented toxicity to several intramacrophage protozoan parasites (1, 22), these nitrogen-based molecules have been implied to retard malarial sporozoite development (21). We have recently demonstrated that RNI, particularly when they are in the S-nitrosothiol form, are directly toxic to *Plasmodium falciparum* blood stages in vitro (41). The dose required to inhibit parasite growth in these studies was of the same order as the plasma concentrations achieved in our cytokine-treated mice (Table 1; Fig. 2). The presence of RNI in plasma after TNF has been injected may, therefore, help explain how prolonged exposure to this cytokine inhibits nonlethal malaria parasites in vivo (11, 47). Our study used a single injection of cytokine and a lethal parasite, so it is not surprising that little effect on parasitemia (data not shown) was observed over the short periods of the experiment.

Similarly, there is now ample evidence that NO·, through activation of guanylate cyclase, is implicated in both the normal physiological control of vascular smooth muscle tone (24) and, in larger amounts, the hypotension associated with TNF injection in dogs (29). Hypotension is one of the dose-limiting side effects of TNF therapy in human tumor patients and is also common in patients with severe falciparum malaria (25). As noted, these individuals have high serum TNF levels, and hypotension was one of the complications Kern and coworkers (27) found to correlate with this cytokine. Hence, as we have proposed earlier (9), TNF-induced NO· is likely to play an important role in human malarial hypotension. L-NMMA has been shown to reverse hypotension after its onset in animals treated with endotoxin

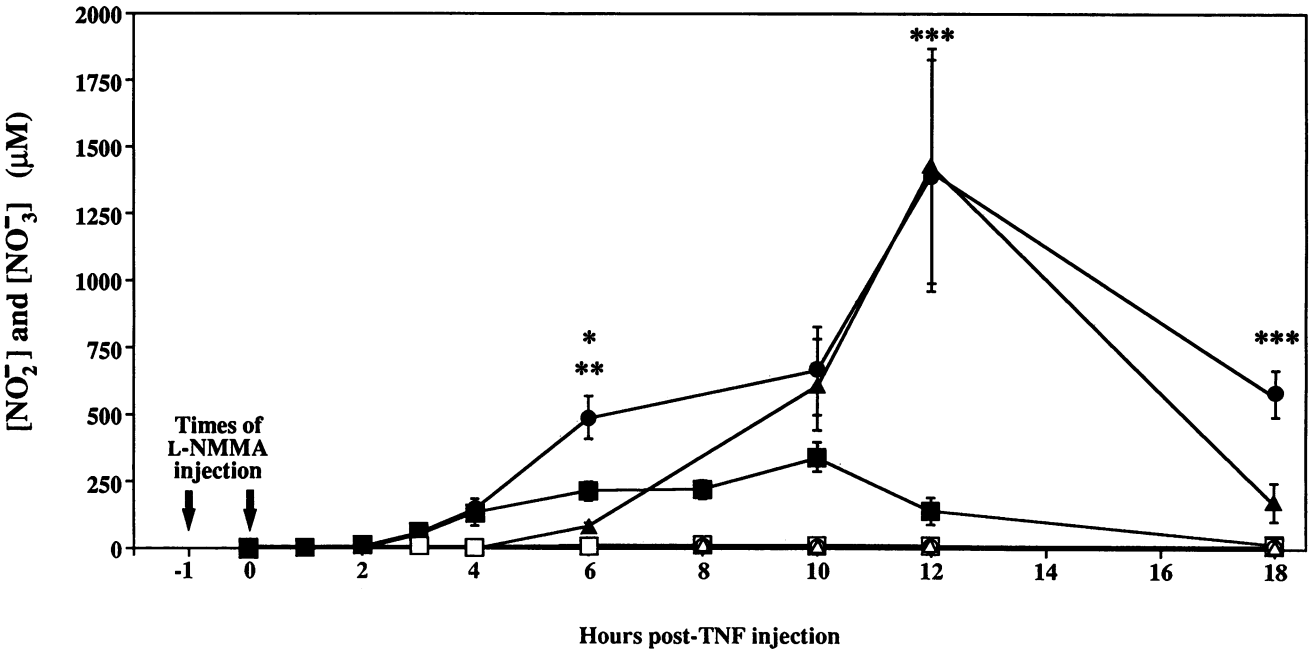

FIG. 2. Comparison of post-TNF plasma RNI concentrations from normal (□, NO_2^-; ■, NO_3^-), *P. vinckei*-primed (○, NO_2^-; ●, NO_3^-), and L-NMMA-treated *P. vinckei*-primed (△, NO_2^-; ▲, NO_3^-) mice. All mice received 5 μg of recombinant human TNF i.v. in 200 μl of saline and were bled at the times shown. Neither *P. vinckei*-primed nor normal mice produced measurable RNI levels following injections of 200 μl of saline i.v. at any time point. L-NMMA-treated mice received 2 mg i.p. in 200 μl of saline at 1 h pre-TNF and 2 mg i.p. in 200 μl of saline at the time of TNF injection. Data are means ± standard errors of the means for six mice at each point. *, $P < 0.01$ for *P. vinckei* group versus *P. vinckei* plus L-NMMA group; **, $P < 0.025$ for *P. vinckei* plus L-NMMA group versus the normal group; ***, $P < 0.025$ for *P. vinckei* group versus the normal group.

(30) and TNF (29). We therefore suggest that L-NMMA (or a suitable analog) has a reasonable prospect of being therapeutically useful in malarial hypotension as it is now proving to be in patients with sepsis (40). Interestingly, if malarial hypotension proves to have these origins, it should be exacerbated in malarial anemia, in which lower levels of hemoglobin, an NO· scavenger (42) would result in more NO· being available to relax vascular smooth muscle (42).

As recently reviewed (17), NO· is now recognized to be an essential link in the processes initiated by the activation of excitatory neurotransmitter receptors in the brain. Neurotoxicity is a side effect of TNF administration to tumor patients (43), and TNF levels have been shown to correlate with the severity of cerebral malaria (31). We have therefore recently reasoned that cytokine-induced generation of NO· in the walls of cerebral blood vessels could be an important mediator in this condition by providing nonsense signals to neurons (12). It is also reported that NO· can inhibit the activity of glutamate-controlled calcium channels (35), thereby interfering with synaptic activity in a way that would result in coma.

When nitric oxide synthase reacts with arginine, the short-lived NO· radical is produced (36). This molecule can be directly measured chemically by using the Griess reagent (23), as can nitrite, an oxidation product of NO·. If only these two compounds were measured, no differences in plasma levels would be detected in any of the groups of mice we tested, with concentrations remaining between 4 and 20 μM (Fig. 2). However, the high oxidative capacity of hemoglobin implies that nitrate, as well as nitrite, would be formed from plasma NO· (49). For this reason, we exposed plasma to a copper-cadmium-zinc catalyst to reduce any nitrate to nitrite, in which form it could be easily measured by the Griess reagent. As shown in Fig. 2, nitrate made up nearly all of the plasma RNI; indeed, the total RNI consisted of some 20 μM nitrite, with the balance (up to 1,400 μM) being nitrate. Similarly, we have found that a high proportion of the RNI released in vitro culture supernatants was nitrate (data not shown). The use of the nitric oxide synthase inhibitor, L-NMMA, confirmed that the RNI were derived from arginine. Appropriate doses of L-NMMA largely prevented the increase in plasma RNI concentrations (Fig. 1) caused by injecting TNF, and a total dose of 4 mg of L-NMMA, administered during the hour preceding TNF injection, prevented the increase in RNI levels for up to 6 h (Fig. 2). These findings therefore suggest that measurements of NO·, nitrite, and nitrate should always be performed when assaying for nitric oxide synthesis. This concept may explain the negative results reported by others (26) who measured nitrite only.

The rate of increase in plasma RNI was greater in the *P. vinckei*-infected mice than in the normal group (155 μM/h versus 40 μM/h between 4 and 10 h after TNF injection) (Fig. 2). The reason for this is unknown, but possibilities include increased macrophage numbers in malaria-infected mice or their enhanced state of activation (16). Subclinical malarial infections are known to sensitize mice to other actions of TNF (10). It has been demonstrated that malaria parasites can induce TNF release (5, 46), and we have shown that TNF as well LT and IL-1 can induce RNI release (Table 1). On a weight-for-weight basis, these cytokines can be ranked according to the levels of RNI induced after 10 h: IL-1 is greater than LT is greater than or equal to TNF is greater than IL-6 is equal to INF-γ, but this ranking may be different when it becomes possible to compare these mediators in

units. The last two cytokines, at the doses tested, did not induce high plasma RNI production compared with the others at similar doses. This is in contrast to in vitro data in which, of various cytokines used alone, only IFN-γ induced macrophages to release RNI (15, 45), and IFN-γ induced NO·-mediated inhibition of intracellular protozoan parasites in vitro (22). While IFN-γ may be the cytokine of choice to induce RNI release in vitro, our results suggest that TNF, LT, or IL-1 would be a more effective inducer of RNI-mediated effects in vivo. This is consistent with the ability of anti-TNF antibody to reverse IFN-γ-induced activation of macrophage-leishmanicidal activity and RNI production in vitro (21). A similar antibody has also been used to demonstrate that TNF is involved in controlling cutaneous leishmaniasis (34), which is mediated via NO· (32).

In summary, our results predict that the generation of NO· would be increased in severe malaria as a direct result of the rise in circulating levels of TNF (6, 27), LT (7a), and IL-1 (31) found in this disease. This molecule may be a major mediator of the host pathology seen during malaria infections.

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