Optimal Tumor Necrosis Factor Induction by *Plasmodium falciparum* Requires the Highly Localized Release of Parasite Products

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Overproduction of tumor necrosis factor (TNF) has been linked with the pathogenesis of *Plasmodium falciparum* malaria. Here, we examined why the high levels of TNF-inducing activity associated with *P. falciparum*-parasitized erythrocytes (PE) appear to be lost after cell lysis. Static coculture of PE and peripheral blood mononuclear cells (PBMC), with or without separation by porous membranes, demonstrated that rupture of live PE in the presence of responder cells was required for optimal TNF induction. Although the insoluble fraction of lysed PE was found to partially inhibit TNF responses, supernatants prepared from large numbers of lysed PE still contained only low levels of TNF-inducing activity, which showed no evidence of instability. A dramatic reduction in TNF levels resulted when noncytoadherent PE lines were maintained under low-cell-proximity conditions by suspension coculture. This reduction was much less marked with PE capable of adhering to PBMC, despite the fact that cytoadherent and noncytoadherent parasite lines induced comparable levels of TNF in high-cell-proximity, static coculture. These results suggest that rupture of PE in a highly localized setting, facilitated by either static coculture or the more biologically relevant phenomenon of cytoadherence to PBMC, can result in considerable enhancement of the *P. falciparum*-induced TNF response.

Malaria due to *Plasmodium falciparum* is responsible for high morbidity and mortality, especially in children living in areas of endemicity. The pathogenesis of the disease is complex, but high levels of the circulating cytokine tumor necrosis factor alpha (TNF- α) are notably associated with severe complications, such as coma and death (9, 16). Further supportive evidence that TNF has a role in severe pathology comes from genetic studies in which certain single-nucleotide substitutions in the upstream promoter regions of the TNF gene were found to modify the risk of cerebral malaria several fold (14, 19). However, a causal relationship between TNF and severe pathology is open to debate, because high levels are observed in *Plasmodium vivax* infection (12), in which severe manifestations are rarely observed. Asexual blood stages of *P. falciparum* are unique among human malarias in that they cytoadhere to endothelial cells, which leads to their sequestration in deep tissue capillaries during the second half of their replication cycle. It is feasible that production of TNF in a highly localized setting, not quantifiable by measurement of plasma TNF levels obtained from peripheral blood, results from parasite sequestration and gives rise to the organ-specific pathology associated with severe falciparum malaria (5).

Rupture of schizont-stage-parasitized erythrocytes (PE) and the release of their contents is thought to provide the major stimulus for TNF production (12, 15). However, until recently, contamination of certain in vitro-maintained *P. falciparum* lines with *Mycoplasma* organisms, which are potent inducers of TNF (2), has obscured the fact that lysed PE, compared to live intact PE, induce only very modest levels of TNF (29, 30).

Therefore, a paradox is evident in that lysis of PE not only appears to provide the major stimulus for TNF production but also represents the point at which most of this stimulus is lost. As yet, there is no direct evidence that contact between intact PE and responder cells plays a role in TNF induction.

Differences between the immunostimulatory properties of live and dead pathogens have been found, which in some cases can be ascribed to the differential induction of regulatory cytokines (21, 32). Reevaluation of the proinflammatory cytokine-inducing properties of *P. falciparum* parasites by coculture of schizont-stage PE, free of *Mycoplasma* contamination, with naïve peripheral blood mononuclear cells (PBMC) has revealed that synergistic interactions between different PBMC subpopulations are necessary for optimal induction of early TNF responses. Thus, monocytes (CD14⁺ CD3⁻) alone respond weakly to live PE, but responses are significantly enhanced by the presence of both monocytes and CD3⁺ CD14⁻ cells in stimulation assays (30). In addition to this basic finding, it has been found that optimal stimulation of gamma interferon (IFN- γ), interleukin-2 (IL-2), and IL-12 also requires live, intact PE as opposed to either lysates or culture supernatants from parasite cultures in which schizont rupture had taken place (10, 36). It now appears that the early TNF response induced by *P. falciparum* may be a composite of different proinflammatory and cellular responses, although why some of these responses are induced optimally by live PE is not known.

Without the use of strong adjuvants, live parasites are far more effective stimulators of protective immune responses in vivo than parasite extracts (7, 11). In light of recent in vitro findings, the in vivo stimulatory properties of live PE could relate directly to their capacity to optimally stimulate certain early cytokine responses. The growing number of live PE-dependent cellular and cytokine responses that are likely to influence TNF production also raises the possibility that more

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than one parasite-derived factor contributes to the early induction of TNF in malaria. Further progress in unraveling cellular responses as well as the nature and source of the parasite stimuli responsible for TNF induction will require some basic clarification of why the activity associated with viable PE cannot be reproduced with nonviable parasite material. In this study, we have carried out a series of experiments that directly addressed the question of why live PE are much more potent stimulators of TNF than lysed PE.

MATERIALS AND METHODS

Parasite culture. In vitro-adapted lines of *P. falciparum* were maintained under standard culture conditions (34). RPMI 1640 medium was supplemented with 0.25 g of gentamicin per liter, 0.5 g of hypoxanthine per liter, 0.2% glucose, and either 0.5% Albumax II (Gibco BRL, Paisley, United Kingdom), or 10% normal human serum (RPMI-HS). Parasite cultures were tested for *Mycoplasma* contamination by PCR (Stratagene, Cambridge, United Kingdom). *Mycoplasma*-positive parasite lines were treated with *Mycoplasma* removal reagent (MRA; ICN, Thame Oxon, United Kingdom) according to the manufacturer's instructions and then retested by PCR. *Mycoplasma*-negative stocks were cryopreserved and reestablished routinely to avoid the risk of contamination during long-term culture. Parasite growth was synchronized by sorbitol lysis (17). For retardation of parasite maturation, ring-stage cultures were retained at 4°C for defined intervals of time before returning to 37°C. This manipulation did not affect the parasite count or degree of synchrony (data not shown).

TNF induction assays. PBMC were isolated from the heparinized blood of malaria-naïve individuals by using low-endotoxin Isopaque-Ficoll (Pharmacia, Amersham, Little Chalfont, United Kingdom) (3). After being washed in RPMI 1640, PBMC were diluted in RPMI-HS and cultured in 96-well plates at 2×10^5 cells per well. Stimulation assays, as opposed to parasite culture, were carried out in RPMI-HS, because this medium was found to be optimal for TNF induction (unpublished data). Immediately after PBMC preparation, enrichment of mature-stage PE (~60 to 80% of cells infected) was carried out by Percoll (Sigma, Poole, United Kingdom) density gradient centrifugation (28). Washing of PE was performed in RPMI 1640, and after dilution, live parasites were promptly returned to culture conditions to reduce any loss of viability. For preparation of PE lysates, Percoll-enriched schizont-stage PE, suspended in RPMI-HS, were frozen at -70°C and thawed rapidly at 37°C. To ensure viable parasites were not present in whole lysates, a further freeze-thaw cycle was performed. To prepare lysate supernatants, whole lysates were centrifuged in microcentrifuge tubes at $50,000 \times g$ for 5 min at room temperature. Supernatants prepared in this manner contained no particulate matter, as determined by phase-contrast microscopy.

Membrane-separated cocultures employed the Falcon Cell Culture Insert system (Becton Dickinson, Oxford, United Kingdom). Membranes are composed of high-porosity polyethylene terephthalate, and a distance of 0.8 mm separates the upper and lower well facing surfaces. PBMC (2×10^6) were added to the lower well (with standard 24-well plate dimensions) in a volume of 600 μ l and allowed to equilibrate at 37°C and 5% CO₂. Percoll-enriched PE were added the upper well (with standard 96-well plate dimensions) at a parasitemia level of 40%, which was adjusted by adding washed uninfected erythrocytes.

Continuous suspension of cells during culture was achieved by continuous vertical rotation at 33 rpm (device radius = 10 cm) in ventilated microcentrifuge tubes in 100- or 200- μ l volumes. Only highly synchronous, fully segmented schizont-stage parasites were used for suspension cocultures in order to ensure cell rupture occurred before significant migration of cells away from the center of the rotation device had taken place due to the small centrifugal force generated.

All TNF induction assays were carried out in triplicate wells at 37°C in a 5% CO₂ atmosphere with RPMI-HS medium. Allowing parasite culture to occur in a 5% CO₂-air mixture instead of the standard mixture of 7% CO₂, 5% O₂, and 88% N₂ during assays did not appear to have an adverse effect on invasion rates or subsequent growth (data not shown). Unless stated otherwise, incubations were stopped when TNF responses were maximal (16 to 20 h). Culture supernatants were removed and diluted in Tris-buffered saline-0.05% Tween 20 by a factor of 2 or more, before the TNF concentration was determined with the matched reagent, Duoset, capture enzyme-linked immunosorbent assay (ELISA) system (Genzyme). All data shown are representative of at least two similarly conducted experiments, and statistical significance was obtained by Student's *t* test.

RESULTS

TNF induction by PE lysates. TNF induction by parasite lysates was investigated, considering the possibility that the loss of TNF-inducing activity previously reported (29) may vary according to the parasite isolate or different methods used for achieving cell disruption. We found comparably low levels of TNF were induced by freeze-thawed lysates prepared from the mature schizont-stage PE of *Mycoplasma*-free FCB-1, T9/96, FCR3S, R29, and a patient-derived wild isolate recently adapted to in vitro culture (not shown). Uninfected lysed erythrocytes did not induce a detectable TNF response, indicating that at least some activity attributable to erythrocyte parasitization was retained after lysis. Commonly used cell-disruption methods, such as sonication, saponin treatment, osmotic shock, or sodium carbonate treatment, appeared to be no more effective at either releasing or preserving the activity associated with live intact PE (not shown).

The TNF-inducing capacities of live and freeze-thawed FCB-1 PE were compared over a range of different PE doses (Fig. 1). The responses shown demonstrated the potent TNF-inducing activity derived from live FCB-1 PE and the pronounced reduction of this activity that resulted from freeze-thawing the same numbers of PE. A maximal response of 478 ± 54 pg/ml was induced by supernatants of lysed PE at a 16:1 PE/PBMC ratio, whereas whole lysates and washed lysate pellets induced maximal responses of 172 ± 8 and 148 ± 22 pg/ml, respectively. The rise in TNF induction seen with increasing concentrations of lysate supernatants appeared to peak at a 16:1 PE/PBMC ratio. However, compared to supernatants, this plateauing of responses occurred at lower doses with whole lysates, lysate pellets, and live PE. This finding suggested that an inhibitory activity might be present in the insoluble fraction of lysed PE.

TNF induction by live PE is partially inhibited by the insoluble fraction of lysed PE. The possibility that an inhibitory activity is present in the insoluble fraction of lysed PE, which could modulate TNF induction by live PE, was examined. Lysate fractions were added at the start of PE-PBMC coculture, and the effect on TNF induction was assessed. Clear inhibition of the live PE-induced TNF response was evident when cocultures were supplemented with whole lysates or pellets from either 2×10^6 or 2×10^5 PE (Fig. 2). In contrast, addition of lysate supernatants, even at the lower dose, enhanced TNF induction by live PE, suggesting that the inhibitory activity in lysates was effectively removed by centrifugation. Addition of whole lysates or pellets to parasite cultures did not adversely affect PE rupture or erythrocyte reinvasion rates (not shown), indicating that inhibition of TNF responses was due to direct effects of the insoluble parasite material on PBMC. However, the maximal level of inhibition, 5.5-fold, seen with whole lysate from 2×10^6 PE was considerably less than the 35-fold reduction (not shown) in TNF induction that resulted from lysis of the same number of live PE by freeze-thawing, indicating that this inhibition could not fully account for the low levels of activity displayed by whole PE lysates. Equivalent numbers of freeze-thawed uninfected erythrocytes had no effect on TNF induction by live PE (not shown), while incubation of PE lysate material with recombinant human TNF did not affect detection of the latter by ELISA (not shown).

FIG. 1. Dose-response comparison of TNF induction by live and lysed PE. Serial twofold dilutions of Percoll-enriched schizont-stage FCB-1 PE were made in RPMI-HS and then added directly to PBMC (■). Alternatively, the stock solution of PE was frozen and thawed twice before whole lysate (▲) or washed insoluble (○) and soluble fractions (●) were diluted and added to PBMC. Incubation at 37°C was carried out for 16 h. Mean TNF values were derived from one representative experiment of three performed and are shown on a log scale. Incubation of PBMC with medium alone resulted in a TNF response of 26 ± 5 pg/ml.

Rupture of live PE in the presence of PBMC is required for high levels of TNF induction. The previously reported association between PE rupture and TNF production (12, 15) seems to be inconsistent with the fact that PE lysates contain relatively little TNF-inducing activity (29). In order to understand why viable intact PE induce much higher levels of TNF than lysates, we thought it necessary to reexamine the link between PE rupture and TNF induction. First, the relationship between the time of live PE rupture and TNF induction was investigated by performing parallel incubations with PBMC that differed only with respect to the amount of schizont rupture taking place in each coculture (Fig. 3A). To stagger parasite growth, a single sorbitol-synchronized parasite culture containing ring-stage FCB-1 parasites was divided into four subcultures, and parasite maturation was retarded for intervals of either 2, 4, or 6 h by incubation at 4°C or continued by returning one subculture directly to culture at 37°C (0 h). At the end of the 4°C incubation periods, subcultures (2, 4, and 6 h) were also returned to culture at 37°C. Parasite maturation was then monitored in the subculture in which growth had not been delayed (0 h). After 46 h, when a high proportion of mature schizont-stage parasites became evident, aliquots from each subculture were washed, and cells were added to PBMC. After 5 h of coculture, the number of PE that had ruptured per well was determined microscopically, and supernatants were taken for TNF analysis. As shown in Fig. 3A, the amount of schizont rupture that occurred during the coculture period was highest with the 0 h parasite subculture and lowest with the subculture in which growth had been retarded for 6 h. Levels of TNF induction were found to mirror the amount of schizont rupture

that had taken place in each coculture, indicating an association between these two processes.

In the next experiment (Fig. 3B), the total amounts of activity present in parasite cultures containing different amounts of PE were compared. Media and cells from a set of age-staggered parasite cultures in which schizont rupture was ongoing were incubated in parallel with PBMC for 18 h. Levels of TNF production were found to be proportional to the amount of schizont rupture taking place during the coculture period. Conversely, the amount of rupture that had taken place in parasite cultures prior to the start of coculture appeared to have little bearing on TNF levels. Thus, parasite cultures in which the majority of schizonts had already ruptured retained relatively little TNF-inducing activity. Supernatants prepared from parasite cultures in which schizont rupture had taken place also induced very low levels of TNF (not shown).

The stability of the activity present in supernatants from either PE lysates or parasite cultures was assessed by preincubating supernatants at 37°C prior to their incubation with PBMC. However, preincubation of supernatants for periods of up to 3 h appeared to have little or no effect on TNF induction (not shown), suggesting the activity present was relatively stable. Attempts were made to optimize the TNF-inducing capacity of parasite culture supernatants, either by culturing parasites at high densities or by concentrating supernatants by ultrafiltration or dialysis against polyethylene glycol. Although levels of TNF induction were enhanced by such methods, the responses induced remained low relative to those induced by PE-PBMC coculture (not shown).

The time course experiments described suggested that either

FIG. 2. Partial inhibition of live PE-induced TNF responses by insoluble material from freeze-thawed PE. Live PE alone and or together with either whole freeze-thawed PE, freeze-thawed PE supernatant, or washed pellet fractions were added to the PBMC cultures in the quantities shown. Incubations were carried out for 18 h. Incubation of PBMC with medium alone resulted in a TNF response of <25 pg/ml. Shown are mean TNF values $\pm$ standard deviation derived from one representative experiment of four performed.

a potent but short-lived activity is released at the time of schizont rupture, or intact PE provide a cell contact-dependent stimulus that is destroyed by cell lysis. The second possibility was investigated by using permeable cell culture inserts to prevent PE-PBMC contact. It was predicted that if signaling by intact PE does play a role in TNF induction, then the levels of TNF induced by live PE compared to those induced by lysates should be similar if PE-PBMC contact is prevented. Responses to lipopolysaccharide (LPS) or PE lysate supernatants were not reduced by membrane separation, indicating that the membranes used did not significantly impede migration of soluble factors from upper to lower wells (Table 1). Contrasting with lysates and LPS, live PE-induced TNF responses were markedly reduced (4.3-fold with $0.4\text{-}\mu\text{m}$ -pore-size inserts in the experiment shown) by separation from PBMC. The lower responses seen with the $1.0\text{-}\mu\text{m}$ -pore-size membranes compared to the 3.0- and $0.4\text{-}\mu\text{m}$ -pore membranes may reflect the lower porosity of these membranes. Despite the reductions observed, membrane-separated live PE induced considerably higher levels of TNF than PE supernatants (4.5-fold at the $0.4\text{-}\mu\text{m}$ -pore size). Moreover, in separate experiments, using only $0.4\text{-}\mu\text{m}$ -pore membrane inserts, responses even closer to those elicited by direct cell coculture were sometimes observed (not shown). Therefore, despite the absence of direct PE-PBMC contact, the levels of TNF induced by live PE were still several fold higher than those elicited by lysed PE supernatants.

Close proximity between cells during coculture enhances TNF induction. To this point, it had been established that parasite products released by PE rupture do indeed play a major role in TNF induction, but there was no clear explanation

as to why lysed or ruptured PE are unable reproduce the activity elicited by PE-PBMC coculture. We considered whether the manner in which parasite factors are presented to responder cells during PE-PBMC coculture was important. Coculture of PE and PBMC under static conditions ensures that rupture of most PE takes place in close proximity to responder cells. To reduce PE-PBMC proximity, but not necessarily contact, FCB-1 PE and PBMC were cocultured in suspension by rotation. It was clear that a significant reduction in TNF production resulted from suspension culture, approaching or equivalent to that incurred by natural or artificially induced PE lysis (Fig. 4A). This effect was specific for live PE, because suspension culture did not alter LPS induction of TNF (Fig. 4A). Moreover, TNF induction by supernatants of freeze-thawed PE was actually increased by suspension culture (Fig. 4B), perhaps because of the benefits of mixing. Increasing PBMC density in suspension culture (Fig. 4B) resulted in increases in TNF induction by lysate supernatants that were proportional to those seen in static cultures in which cell proximity was already high. Therefore, proximity between responder cells (i.e., PBMC) does not appear to be important for TNF induction by soluble PE factors.

TNF-induction by cytoadherent and noncytoadherent PE in suspension coculture. The possibility that stable associations between PE and PBMC (i.e., cytoadherence), as opposed to contact only, might enhance TNF induction under suspension conditions by ensuring inducer-responder cell proximity was considered. Up until this point, the FCB-1 strain of *P. falciparum* had been used to examine TNF induction by viable PE. However, the FCB-1 strain did not appear to be capable of

A

B

FIG. 3. Optimal expression of the FCB-1 TNF-inducing activity requires rupture of live PE to occur in the presence of PBMC. Synchronous ring-stage cultures with PE constituting between 5 and 10% of the cell total were delayed for intervals of 0, 2, 4, and 6 h by incubation at 4°C and then returned to 37°C culture. (A) After 46 h, PE and uninfected erythrocytes were washed before being added (5×10^6 cells per well) to PBMC and then cultured for 5 h. (B) Culture medium was renewed after approximately 38 h of culture, and after 50 h, aliquots containing medium and cells from each subculture were added directly to PBMC, without washing, at 5×10^6 cells per well. Coculture was carried out for 18 h. For both experiments, blood smears were prepared from parallel cocultures at the beginning and end of the incubation period in order to assess schizont rupture. Incubation of PBMC with medium alone resulted in a TNF response of <16 pg/ml in both experiments. Shown are mean TNF values $\pm$ standard deviation derived from one representative experiment of three performed.

TABLE 1. TNF induction in membrane-separated cultures

Pore size (μm) ^a	Pore density (no. of pores/cm ²)	TNF induction (pg/ml) with stimulus ^b :		
		Live PE ^c	PE lysate ^d	LPS (5 ng/ml)
No insert		7,710 $\pm$ 479	287 $\pm$ 20	26,740 $\pm$ 1,235
8.0	1 $\times$ 10 ⁵	5,578 $\pm$ 249	605 $\pm$ 42	27,800 $\pm$ 1,039
3.0	2 $\times$ 10 ⁶	1,760 $\pm$ 54	592 $\pm$ 34	25,040 $\pm$ 1,180
1.0	1 $\times$ 10 ⁶	1,020 $\pm$ 127	283 $\pm$ 45	20,140 $\pm$ 872
0.4	1 $\times$ 10 ⁸	1,790 $\pm$ 341	396 $\pm$ 58	20,060 $\pm$ 288

^a Different stimuli were added to duplicate cell culture inserts of the defined pore size.

^b Mean TNF values $\pm$ standard deviation are derived from one representative experiment from three performed.

^c A total of 4 $\times$ 10⁶ intact schizont-stage FCB-1 PE.

^d Supernatant fraction from 4 $\times$ 10⁶ freeze-thawed schizont-stage PE.

either adherence to PBMC or rosette formation, even when cultured in medium supplemented with HS, which can be essential for expression of the rosetting phenotype *in vitro* (6). Alternative parasite lines were employed with known cell-adhesive properties when grown and assayed in normal HS. The TNF-inducing properties of FCR3S, which is capable of adherence to endothelial cells and rosette formation (4, 8), and T9/96, which displays no cell-adhesive properties (35), were compared. Both parasite lines were grown in RPMI-HS, and adherence of FCR3S, but not T9/96, PE to both PBMC and uninfected erythrocytes was observed by light microscopy (not shown). In static culture, both FCR3S and T9/96 parasites induced high levels of TNF (Fig. 5). Although FCR3S levels were 1.4-fold greater than those induced by T9/96, this difference was not significant ($P > 0.05$). However, in suspension cocultures, clear differences emerged. FCR3S consistently induced higher levels of TNF than T9/96 in suspension coculture with a mean from four experiments of 2,609 $\pm$ 772 pg/ml, compared to 236 $\pm$ 130 pg/ml ($P < 0.01$), representing a mean 11-fold difference. Another cytoadherent parasite line, R29, was also found capable of adhering to PBMC and inducing high levels of TNF under suspension coculture conditions (not shown). Levels of TNF induced by T9/96, FCR3S, and R29 in membrane insert cocultures were comparable (not shown), providing further evidence that the differences seen in suspension cocultures were due to the cytoadhesive properties of FCR3S PE.

Inspection of cells during suspension culture by light microscopy indicated that FCR3S formed rapid and stable associations with individual PBMC. Larger cell aggregates consisting of PE, uninfected erythrocytes, platelets, and mononuclear leukocytes or clusters containing mainly autoagglutinated PE were occasionally seen. However, a direct role for rosette formation in enhancement of TNF production during suspension coculture was largely excluded by the fact that erythrocyte availability was limited by using Percoll-enriched PE and the finding that addition of a 10-fold excess of uninfected erythrocytes to enhance rosette formation did not significantly alter levels of TNF-induction (not shown).

DISCUSSION

The outcome of acute malaria infection is dependent on the interplay of a number of host, parasite, and environmental

factors. For example, in the host, there is an association between certain mutations in the upstream TNF gene promoter region and an individual's risk of developing cerebral malaria (14, 19). In contrast, the possibility that genotypic or phenotypic properties of the parasite influence TNF induction has been more difficult to prove, although clearly evasion of host immunity leading to increased parasite loads should elevate cytokine production. The recent observation that lysis of PE *in vitro* results in a dramatic loss of TNF-inducing activity indicates that TNF induction *in vivo* by *P. falciparum* may be a delicately balanced and subtle process. There are two conclusions that can be drawn from this study that suggest that the seemingly transient nature of the parasite-derived TNF-inducing activity has *in vivo* significance. First, the levels of TNF induced by live *P. falciparum* parasites appear to vary dramatically according to the degree of proximity between rupturing PE and responder cells. Second, PE cytoadherence to PBMC, by ensuring close proximity to responder cells, may significantly enhance TNF production. Together, these factors predict that high levels of parasitemia may not necessarily be sufficient to induce the locally or even systemically induced high levels of TNF that are thought to trigger the organ-specific pathology associated with severe falciparum malaria.

There are a number of explanations that could account for the need for live parasites for optimal cytokine induction by *P. falciparum* PE. These would include (i) a potent stimulus delivered by intact PE, which is destroyed by cell lysis; (ii) release of inhibitory factors by PE lysis; or (iii), due to low abundance or instability of the active parasite factors, the need for rupture of PE to occur in close proximity or even juxtaposition with PBMC for optimal TNF induction. These possibilities were initially examined under normal, static *in vitro* culture conditions. The results confirmed that there is a close temporal relationship between schizont rupture and TNF induction, implying that any signal made by contact between intact PE and PBMC alone is small. The presence of a TNF response-inhibiting activity in the insoluble fraction of lysed PE was clearly demonstrated. The inhibition of LPS-induced TNF responses by hemozoin (33) makes this parasite product a prime candidate despite the fact that both synthetic and native forms of hemozoin have also been shown to stimulate TNF production (25, 31). However, quite large amounts of pelleted material were required for only moderate inhibition of the response to live PE, and the increase in activity that resulted from removal of the fraction from whole lysates could only in part account for the loss of activity after PE lysis. It was also found that the activity present in lysate supernatants was relatively stable. The possibility that additional, more labile factors are released during PE lysis and contribute to the TNF response cannot be ruled out at this stage.

By comparing TNF responses under standard static and suspension culture conditions, a number of differences pertinent to the dynamics of TNF induction by *P. falciparum* parasites were revealed. The dramatic reduction in levels of TNF induced by live PE in suspension coculture indicated that parasite viability *per se* is not the principal factor in determining levels of cytokine induction. Moreover, this result shows that the context in which schizont rupture takes place *in vivo* could result in a considerable disparity between local and systemic levels of TNF. The fact that even at very high concentrations,

FIG. 4. TNF induction is reduced by coculture of cells in suspension. (A) Cocultures of FCB-1 PE and PBMC were carried out under conditions promoting cell suspension (rotation) or sedimentation (static) as described in Materials and Methods. LPS (5 ng/ml) was included for comparison. Incubations were performed for 18 h. Shown are mean TNF values $\pm$ standard deviation derived from one representative experiment of five performed. (B) Supernatants from PE subjected to one freeze-thaw cycle at a final concentration of 10^7 PE equivalents per ml were incubated with different amounts of PBMC as shown. Incubations were performed for 24 h. PBMC (4×10^6 /ml) cultured with medium alone gave a TNF response of <16 pg/ml. Shown are mean TNF values $\pm$ standard deviation derived from one representative experiment of three performed. *, $P < 0.05$ (Student's t test).

lysate supernatants did not elicit high levels of TNF is further evidence that responses induced in naïve individuals by systemic release of parasite products cannot approach levels induced locally. Proximity between cells could enhance TNF induction by minimizing the time interval between the release

of labile parasite factors and their contact with PBMC or by ensuring that responder cells are exposed to parasite factors in a highly concentrated form. Two other possible explanations for higher responses in static culture are that adherence of PBMC subpopulations to culture plates or higher proximity

FIG. 5. Cytoadherent-rosetting FCR3S parasites induce significantly higher levels of TNF than the noncytoadherent-nonrosetting T9/96 parasites under suspension, but not static, coculture conditions. Schizont-stage PE cultured in RPMI-HS were pelleted, resuspended in RPMI, and incubated with heparin at 10 U/ml for 10 min at 37°C. After Percoll enrichment, 50 μl of PE ($2 \times 10^7/\text{ml}$) was mixed with 50 μl of PBMC ($4 \times 10^6/\text{ml}$) and cocultured under static or suspension conditions for 16 h. The mean TNF values and standard errors shown were calculated from four identical experiments in which PBMC were obtained from the same individual. *, $P < 0.01$ (Student's t test).

between individual PBMC in static culture is beneficial. However, neither interpretation is consistent with the fact that, in static, as compared to suspension culture, TNF responses to lysed PE material were, if anything, lower (Fig. 4B).

Data from patient studies in which TNF levels were measured in peripheral blood have shown that both *P. vivax* and *P. falciparum* can be potent inducers of TNF in vivo, but only with *P. falciparum* is there an association between TNF levels and disease severity. Therefore, there has been considerable interest in the relationship between *P. falciparum* sequestration and TNF induction. TNF-induced upregulation of endothelial adhesion molecules responsible for PE cytoadherence (22) is a clear example of a link between these two pathogenic factors. The reciprocal relationship between cytokine induction and cytoadherence (i.e., the cytoadherence phenotype has a direct impact on levels of TNF induction) has not been shown. In a previous study, levels of TNF induction by noncytoadherent T9/96 and cytoadherent ITO4 parasites were not found to differ significantly under static coculture conditions (26). We obtained the same result when comparing T9/96 and cytoadherent FCR3S parasites under similar assay conditions. However, in suspension coculture, we found that although FCR3S PE induced high levels of TNF, responses to T9/96 PE were as low as those obtained with lysed PE. The involvement of rosette formation was largely excluded because of the minimal numbers of erythrocytes that were available after Percoll enrichment of PE and the fact that addition of erythrocytes to cocultures did not enhance TNF induction. A previous study suggested a link between rosette formation and TNF induction (1), but *Mycoplasma* contamination of the parasite cultures used was a possibility (29).

Binding of PE surface ligands involved in cytoadhesion to the appropriate monocyte receptors can result in cell activation (18, 23). Cytoadherence has also been found to inhibit dendritic cell maturation, an effect that was not evident with PE lysates or intact, noncytoadherent T9/96 PE (35). However, TNF induction has been found not to result from binding of trophozoite-stage parasites (CD36 dependent) to enriched monocyte preparations (18). It is possible that signals induced in PBMC preparations by PE cytoadherence indirectly enhance the TNF response to PE rupture-derived stimuli. However, once cell proximity was maximized in this study by static coculture, such cytoadherence-dependent "priming" of responder cells was not obvious, because cytoadherent and noncytoadherent PE induced similar levels of TNF.

It may be argued that there is role for cell contact-dependent signaling by PE in TNF induction, irrespective of the capacity of PE to cytoadhere. However, the opportunity for multiple PE-PBMC contact events to occur, because of mixing, may in fact be higher in suspension than in static coculture. Indeed, the binding of FCR3S PE to PBMC appeared to occur rapidly in suspension coculture, indicating that there is ample opportunity for random cell contact events under these conditions. If sustained and more stable cell contact is required, then TNF induction by cytoadherent parasites would be expected to be significantly higher under both the suspension and static coculture conditions used, but in static coculture, this was not the case. Therefore, it appears more likely that sustained close proximity, rather than sustained contact, between PE and responder cells results in high levels of TNF induction. Maintaining close proximity between cells either by static coculture or as a result of parasite cytoadherence would ensure that

parasite factors are released from rupturing schizonts directly onto the surface of responder cells.

Because of the marked loss of TNF-inducing activity that occurs under conditions of low inducer-responder cell proximity, a large portion of the early TNF response *in vivo* might be expected to emanate from sites in the body at which leukocyte densities are high. If cell proximity is important for TNF induction by species of *Plasmodium* other than *P. falciparum*, then in the case of nonsequestering parasites, rupture of PE during transit through the spleen is likely to result in optimal TNF induction. The spleen may also be relevant in terms of both the fate and action of the TNF response-inhibiting insoluble parasite factors demonstrated here. The fact that sequestration of *P. falciparum* PE results from their adherence to the capillary or postcapillary endothelia of various tissues appears to diminish the opportunity for PE adherence to and potent activation of circulating leukocytes. However, in the normal state, approximately half (13), and considerably more during systemic inflammation (20), of the blood mononuclear cells are present in a marginal pool, which is reversibly bound to capillary or postcapillary endothelia. The presence of marginated monocytes on capillary endothelia has been described in brain specimens from patients who died from cerebral malaria (24). Such cells may be ideally placed to respond locally to parasite products with concomitant local activation of endothelial cells. Thus, high levels of TNF observed in the plasma of cerebral malaria patients (9, 16) could reflect a systemic response to soluble parasite factors augmented by primed CD4⁺ T-cell-derived IFN- γ (27) or, in the light of our findings, local, but highly potent induction of TNF. Apart from its implications for pathogenesis, the occurrence of locally high, organ-specific, concentrations of proinflammatory cytokines, modulated by insoluble parasite factors, could also have a significant regulatory effect on both innate and acquired immune responses.

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