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Effects of IL-4 on macrophage functions: increased uptake and killing of a protozoan parasite (*Trypanosoma cruzi*)

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SUMMARY

We studied whether interleukin-4 (IL-4) could modulate two macrophage functions relevant to their microbicidal activity (uptake and killing), using non-invasive [amastigote (AMA)] forms of the protozoan parasite *Trypanosoma cruzi.* Treatment of cultures of mouse resident peritoneal macrophages (MPM) with the supernatant of cultures of cells transfected with IL-4 cDNA increased both the capacity of the MPM to take up the organisms and the rate of intracellular killing with respect to MPM mock-treated with medium alone. The presence in the medium of a monoclonal antibody specific for IL-4 during MPM treatment inhibited both effects, pointing to recombinant IL-4 (rIL-4) as the active principle in the supernatant. Kinetic studies revealed that at least a 24-hr pretreatment of the MPM with the rIL-4-containing supernatant was required for these effects to be produced. The rate of intracellular parasite killing was also significantly increased when the rIL-4 treatment was applied after AMA ingestion by MPM. This result confirmed that MPM could be activated by rIL-4 for greater intracellular killing and showed that this enhancement was not necessarily dependent on the initial rIL-4-mediated increase in parasite load. The use of scavengers of reactive oxygen reduction intermediates indicated that hydrogen peroxide, superoxide anion and singlet oxygen, but apparently not hydroxyl radicals, were involved in parasite killing modulated by rIL-4. These results document for the first time the capacity of IL-4 to enhance the microbicidal activity of macrophages and suggest that this lymphokine might play a role in host defence against *T. cruzi* infection.

INTRODUCTION

Interleukin-4 (IL-4 or B-cell stimulatory factor-1) has been reported to affect several cell types involved in immunological processes (Howard *et al.*, 1982; Roehm *et al.*, 1984; Vitetta *et al.*, 1985; Zlotnik *et al.*, 1987). Originally reported to regulate antigen expression on B cells (Roehm *et al.*, 1984; Vitetta *et al.*, 1985), IL-4 has recently been shown to modulate the activities of a variety of haemopoietic cells (Ohara & Paul, 1987). Furthermore, macrophages and certain macrophage-like cell lines express receptors for IL-4 (Crawford *et al.*, 1987; Ohara & Paul, 1987) and treatment of macrophages with IL-4 stimulates functions associated with the activated state. Thus, IL-4 increases the Ia-mediated antigen-presenting capacity of murine bone marrow-derived macrophages (Zlotnik *et al.*, 1987), and murine peritoneal macrophages treated with IL-4 display increased tumouricidal activity and Fc(II) receptor expression (Crawford *et al.*, 1987).

As one of the major cellular components of both inflammatory and immunological responses, macrophages play a key role in determining the course of numerous infections, including that caused by *Trypanosoma cruzi* (Chagas' disease) (Romaña, 1963; Kuhn, 1981), in which we have been particularly interested. The fate of *T. cruzi* within macrophages depends on the physiological status of these cells. Thus, this parasite can multiply within resident, unstimulated macrophages (Dias, 1932; Pizzi, 1957), whereas macrophages activated with various agents, including some T-cell-derived lymphokines (Nogueira & Cohn, 1976; Plata *et al.*, 1984; Wirth *et al.*, 1985) or other agents such as leukotrienes (Wirth & Kierszenbaum, 1985) and cord factor (trehalose 6, 6′-dimycolate) (Kierszenbaum, Zenian & Wirth, 1984), can kill *T. cruzi* more effectively than non-activated cells. The study of macrophage interaction with the non-invasive, amastigote (AMA) form of *T. cruzi* lent itself to the purpose of the present study: to establish whether IL-4 could modulate macrophage functions important for microbicidal activity.

Abbreviations: AMA, *T. cruzi* amastigotes; DMEM, Dulbecco's modified minimal essential medium; DMEM+FBS, DMEM supplemented with 5% heat-inactivated fetal bovine serum; IL-4, interleukin 4; MPM, mouse resident peritoneal macrophages; rIL-4, recombinant IL-4; SOD, superoxide dismutase.

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MATERIALS AND METHODS

Animals

The 6–9-week-old inbred CBA/J mice used to obtain resident peritoneal macrophages (MPM) were purchased from The Jackson Laboratory (Bar Harbor, ME).

Macrophages

CBA/J mice were killed by excess ether inhalation and injected i.p. with 5 ml of sterile Dulbecco's modified minimal essential medium containing 100 U penicillin and 100 μg streptomycin per ml (DMEM). After gentle abdominal massage, the peritoneal cells were collected, washed by centrifugation (280 ***g***, 10 min, 4°) with DMEM and then resuspended at 3×10^6 cells/ml in the same medium. Fifteen microlitre aliquots of this suspension were placed on the sterile, 3-mm diameter glass wells of otherwise Teflon-coated microscope slides (Cel-Line, Newfield, NJ). The slides were placed inside sterile petri-dishes and incubated at 37° for 1 hr in a 5% CO_2-in-air atmosphere saturated with water vapour. After removing the non-adherent cells, 15 μl of DMEM supplemented with 5% heat-inactivated (56°, 30 min) fetal bovine serum (FBS; Sterile Systems, Logan, UT) (DMEM+FBS) were added, and the cultures were incubated further for 18 hr under the same conditions. The adherent cell monolayers consisted of >99% cells with typical macrophage morphology (Giemsa staining) and positive staining for non-specific esterase activity; cell viability was always >99% as determined by trypan blue dye exclusion. For the different experiments reported in this paper the average number of adherent cells per well was 11,000–12,000. Media and FBS used in this work had no detectable levels of bacterial endotoxin (<0·05 ng/ml).

Parasites

Amastigote forms of *T. cruzi* (AMA) (Tulahuen isolate) were grown in cell-free ML15-HA medium and prepared as described in detail elsewhere (Villalta & Kierszenbaum, 1982). The parasites were washed once in DMEM before use; their viability was 99–100% as determined by their typical vibratile motion. Parasite concentrations were determined microscopically using a Neubauer haemacytometer. Concentration adjustments were made with DMEM.

Reagents

Recombinant mouse interleukin-4 (rIL-4) was used as the supernatant from COS-7 monkey kidney cells transfected with rIL-4 cDNA (DNAX Research Institute, Palo Alto, CA). Mock-transfected COS-7 cell supernatant, used at dilutions equivalent to those of the rIL-4 supernatant, did not alter any of the parameters monitored in the present work. One unit of rIL-4 is defined as the reciprocal of the dilution producing a half maximal response in the HT-2 cell proliferation assay (Mosmann *et al.*, 1986). The monoclonal antibody specific for IL-4 (11.B.11), originally produced by Ohara & Paul (1985), was used as the culture supernatant of the corresponding hybridoma culture maintained at the DNAX Research Institute. A 1/2 dilution of this supernatant neutralized 200 U/ml of rIL-4 activity in the HT-2 cell proliferation assay. Bovine liver catalase, superoxide dismutase (SOD), D-histidine, D-mannitol and benzoic acid were purchased from Sigma Chemical Co. (St Louis, MO). When required, catalase and SOD were heat-inactivated at 100° for 10 min. None of the above reagents affected MPM viability at the concentration used in this work (see below).

Uptake of AMA by MPM

Monolayers of MPM were washed twice with DMEM and incubated in a 5% CO_2 incubator for 2 hr with 15 μl of parasite suspension containing 45,000 AMA. After removing the free organisms by three washes with DMEM, the cells were fixed with absolute methanol and stained with Giemsa. In some experiments the MPM monolayers were treated with reagents (see the Results) before exposure to the parasites. In these cases, the MPM were incubated with 15 μl of DMEM+FBS alone or containing the appropriate concentration of the reagent to be tested for the length of time described in the Results. After washing three times with DMEM, 15 μl of parasite suspension were added. The cultures were then treated and processed as described above. Not less than 200 cells were screened microscopically ($\times 1000$) in each culture, recording the percentage of MPM with one or more parasites and the average number of AMA per 100 MPM. The results were expressed as the mean of triplicate determinations $\pm$ SD.

Intracellular parasite killing by MPM

Cultures of MPM prepared and incubated with AMA as described above were either fixed and stained immediately after removal of the free parasites (i.e. at Time 0) or incubated further with 15 μl of fresh DMEM+FBS alone or containing the appropriate reagent, for varying periods of time (see the Results). In experiments designed to test the participation of reactive oxygen reduction intermediates in parasite killing by MPM, scavengers were present in the medium both during MPM-AMA co-culture and during MPM incubation after parasite removal. All conditions were tested in triplicate, monitoring the parameters and expressing the results as described in the preceding paragraph.

Incorporation of [^{3}H]thymidine by T. cruzi

Amastigotes (5×10^5) were incubated in the wells of sterile 96-well plates in 0·2 ml of ML-15HA medium (Villalta & Kierszenbaum, 1982) containing 1 μCi [^{3}H]thymidine (specific activity 2 Ci/mmole; New England Nuclear, Boston, MA) at 37° in a 5% CO_2-in-air incubator for 24 hr. The cultures were then terminated with a multiple automated harvester. Incorporated radioactivity was determined by using a liquid scintillation spectrometer.

Presentation of results and statistics

Each set of results presented in this paper is typically representative of two to four separate repeat experiments. Differences were considered to be significant if $P<0·05$, as determined by the Mann–Whitney U-test.

RESULTS

Effects of rIL-4 on MPM interaction with AMA

Incubation for 24 hr with rIL-4-containing supernatant providing at least 400 U rIL-4/ml caused MPM to display a greater capacity to take up *T. cruzi* AMA than MPM mock-treated with medium alone (Table 1). This was readily demonstrable imme-

Table 1. Effects of rIL-4 on MPM interaction with *T. cruzi* AMA

Time after infection (hr)	MPM treatment*	% MPM with parasites	No. parasites per 100 MPM
0	Medium alone	47·6±1·2	92·5±4·5
0	rIL-4 200 U/ml	54·8±1·2 (15·1)†	98·3± 3·2 (6·3)
0	rIL-4 400 U/ml	60·3±2·0‡ (26·7)	128·4± 8·0‡ (38·8)
0	rIL-4 800 U/ml	63·1±3·5‡ (32·6)	126·3± 4·7‡ (36·5)
0	rIL-4 1600 U/ml	60·9±3·0‡ (27·9)	140·5±11·2‡ (51·9)
24	Medium alone	36·8±3·8§ [−10·8]†	74·6± 5·1§ [−17·1]
24	rIL-4 200 U/ml	39·5±4·0§ [−15·3]	73·8± 4·6§ [−24·5]¶
24	rIL-4 400 U/ml	32·8±4·3§ [−27·5]¶	64·1± 1·9§ [−64·3]¶
24	rIL-4 800 U/ml	44·1±2·9§ [−19·0]¶	78·9± 5·8§ [−47·4]¶
24	rIL-4 1600 U/ml	43·6±2·6§ [−17·3]¶	92·6±14·1§ [−47·9]¶

* Cultures of MPM were treated for 24 hr with medium alone or containing rIL-4, washed and then incubated with AMA for 2 hr. After removing the free parasites, some cultures were terminated (Time 0) whereas replicate cultures were incubated further for 24 hr with fresh medium or rIL-4 as indicated.

† In parenthesis is the percentage change with respect to the corresponding control value (medium alone). In brackets is the absolute decrease of the value in the 24-hr period.

‡ The difference between this value and the corresponding control value (medium alone) measured at Time 0 was statistically significant ($P<0·05$).

§ The difference between this value and that obtained at Time 0 with the same initial treatment was statistically significant ($P<0·05$).

¶ This absolute reduction differed from that of the corresponding control (medium alone, 24 hr) at a statistically significant level ($P<0·05$).

diately after removing the free parasites (defined as Time 0). Although significant reductions in parasite load over the 24-hr period that followed AMA removal were observed in cultures of both rIL-4- and mock-treated MPM, MPM treated with 400 U rIL-4/ml killed over three times as many AMA as did mock-treated MPM. Because doubling or quadrupling the concentration of rIL-4 did not augment the extent of the stimulatory effects in a significant or consistent manner, we used 400 U rIL-4/ml in all subsequent experiments. Treatment with rIL-4 had no significant effect on AMA viability and did not affect the capacity of the parasite to incorporate [^{3}H]thymidine over a 24-hr period (data not shown). It is noteworthy that the extents of the rIL-4-induced enhancements of phagocytosis and intracellular killing varied among repeat experiments. However, the effects themselves were consistently produced and were always statistically significant, as can be appreciated by comparing the data presented in the tables of this paper.

That rIL-4 was the active component of the supernatant causing the noted effects was indicated by the inhibition afforded by monoclonal anti-IL-4. As can be seen in Table 2, no significant increases in parasite uptake or killing were observed when the MPM were treated with rIL-4 in the presence of anti-IL-4.

To find out if the increased trypanocidal activity of rIL-4-treated MPM was due to the fact that these cells had greater parasite loads at Time 0, we designed an experimental protocol in which untreated MPM were first allowed to take up the AMA and were then incubated with either medium alone or medium containing 400 U rIL-4/ml for various periods of time. We noted that mock-treated MPM reduced their parasite load over the following 24 hr, after which intracellularly established organisms started to replicate, significantly increasing the number of organisms per 100 MPM at the end of 72 hr compared to values obtained at 24 hr (Fig. 1). In contrast, MPM that had been treated with rIL-4 not only cleared more AMA after 24 hr but were able to further reduce the parasite load and contain AMA growth over the following 2-day period.

Table 2. Inhibition by a monoclonal anti-IL-4 antibody of the rIL-4-induced increased in parasite uptake and killing by MPM

Time after infection (hr)	MPM treatment*	No. parasites per 100 MPM
0	Medium alone	184·1±11·8
0	rIL-4	231·1± 7·6‡ (25·5)†
0	Anti-IL-4	191·7± 7·7 (4·1)
0	rIL-4+anti-IL-4	169·5± 5·8 (−7·9)
24	Medium alone	119·1±22·0§ [−65·0]†
24	rIL-4	72·1± 8·0§ [−159·0]¶
24	Anti-IL-4	107·9± 6·6§ [−83·8]
24	rIL-4+anti-IL-4	94·8± 5·4§ [−74·7]

* Cultures of MPM were treated for 24 hr with medium alone or containing rIL-4 (400 U/ml) with or without anti-rIL-4, washed, and incubated with AMA for 2 hr. After removing the free parasites, some cultures were terminated (Time 0) whereas replicate cultures were incubated further for 24 hr with fresh medium or rIL-4 (with or without anti-rIL-4 as appropriate) as indicated.

†, ‡, §, ¶ See footnotes to Table 1.

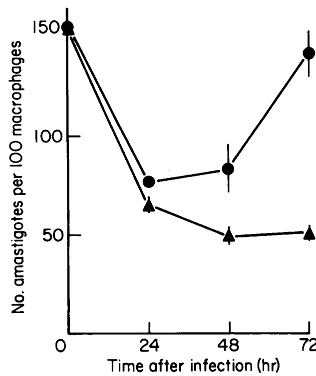

Figure 1. Effect of treatment of MPM with rIL-4 after parasite uptake on the rate of intracellular parasite killing. The MPM monolayers were co-cultured with AMA for 2 hr, washed to remove free organisms and then treated with medium alone (—●—) or containing 400 U rIL-4/ml (—▲—). Fresh medium without or with rIL-4 at the same concentration was added to the cultures every 24 hr until their termination.

Table 3. Kinetics of the induction of the rIL-4 effects of MPM

Time after infection (hr)	MPM treatment*	Length of treatment (hr)	No. parasites per 100 MPM
0	Medium alone	6	81·5±13·5
0	rIL-4	6	82·3±11·9 (1·0)†
0	Medium alone	24	76·7±7·9
0	rIL-4	24	135·5±5·6‡ (76·7)
0	Medium alone	48	121·2±2·5
0	rIL-4	48	166·1±5·4‡ (37·0)
0	Medium alone	72	122·1±1·5
0	rIL-4	72	178·0±11·8‡ (45·8)
24	Medium alone	6	62·6±6·1§ [−18·9]†
24	rIL-4	6	61·5±9·9§ [−20·3]
24	Medium alone	24	66·2±7·2§ [−10·5]
24	rIL-4	24	44·2±2·0§ [−91·3]¶
24	Medium alone	48	87·2±7·2§ [−34·0]
24	rIL-4	48	71·1±3·0§ [−95·0]¶
24	Medium alone	72	93·5±3·7§ [−28·6]
24	rIL-4	72	68·7±5·3§ [−109·3]¶

* Cultures of MPM were treated for the indicated times with medium alone or containing rIL-4, washed and then incubated with AMA for 2 hr. After removing the free parasites, some cultures were terminated immediately whereas replicate cultures were incubated further for 24 hr with fresh medium or rIL-4 as indicated.

†, ‡, §, ¶ See footnotes to Table 1.

Kinetics of induction of rIL-4-mediated enhancement of MPM functions

When MPM were incubated with medium alone or containing 400 U rIL-4/ml for various periods of time before adding the AMA, maximal stimulation of both uptake and intracellular killing was demonstrable after a 24-hr pretreatment, although significant increases in uptake and comparable levels of killing were seen with a 48- or 72-hr pretreatment (Table 3). The shorter pretreatment, 24 hr, was used in subsequent experiments.

Table 4. Effect of scavengers of reactive oxygen reduction intermediates on AMA killing by MPM treated with rIL-4

Time after infection (hr)	MPM treatment*	No. parasites per 100 MPM
0	Medium alone	53·8±2·7
0	rIL-4	115·5±3·0‡ (114·7)†
24	Medium alone	38·5±4·7§ [−15·3]†
24	rIL-4	44·7±8·0§ [−70·8]¶
24	rIL-4+catalase	68·4±8·6§ [−47·1]¶
24	rIL-4+SOD	67·1±6·5§ [−48·4]¶
24	rIL-4+histidine	62·9±6·5§ [−52·6]¶

* Cultures of MPM were treated for 24 hr with medium alone or containing 400 U rIL-4/ml and washed. MPM were then incubated with AMA alone or together with 22,500 U catalase/ml, 3,200 U SOD/ml or 10 mM histidine for 2 hr. After removing the free parasites, some cultures were terminated (Time 0), whereas replicate cultures were incubated further for 24 hr with fresh medium or rIL-4 without or with the appropriate scavenger, as indicated.

†, ‡, §, ¶ See footnotes to Table 1. The inhibitory effects of catalase, SOD and histidine were statistically significant ($P<0{\cdot}05$).

Role of reactive oxygen reduction intermediates in intracellular AMA killing by rIL-4-treated MPM

T. cruzi AMA are sensitive to the toxic effects of hydrogen peroxide (Villalta & Kierszenbaum, 1984), one of several reactive oxygen reduction metabolites usually produced during the respiratory burst that accompanies macrophage activation. For this reason, we looked into whether some oxygen reduction intermediates played a role in the increased macrophage killing induced by rIL-4. To this end, scavengers of hydrogen peroxide (catalase), superoxide anion (SOD) or singlet oxygen (histidine) were incorporated into the media used to both incubate the MPM with the AMA and culture the MPM after removal of the free parasites. The results presented in Table 4 show that all three scavengers were able to reduce the rate intracellular parasite killing by rIL-4-treated MPM to significant extents. Thus, for example, in the experiment presented in Table 4, MPM treated with rIL-4 in the absence of scavengers contained an average of 115·5 organisms per 100 MPM at Time 0 but only 44·7 AMA 24 hr later (representing the clearance of almost 71 parasites per 100 MPM in the intervening period). The presence of catalase, SOD and histidine reduced parasite clearance during the same period to 47, 48 and 53 parasites per 100 MPM, respectively. Differences of this nature were consistently observed in all repeat experiments and were always statistically significant. It is noteworthy that the same concentrations of catalase, SOD and histidine also inhibited the background levels of parasite killing by control macrophages (data not shown). In separate experiments we determined that heat-inactivated catalase and SOD did not alter the rate of AMA killing by rIL-4-treated MPM (data not shown). It should be noted that, at the concentrations shown in the table, catalase, SOD or histidine did not affect the level of AMA uptake by MPM (data not shown). Higher concentrations of these scavengers were not tested because preliminary tests showed that they exerted a certain degree of toxicity for the MPM in our assay system, and

results would have been misleading. Sodium benzoate (60 mM) and mannitol (50 mM), scavengers of the hydroxyl radical, had no detectable effect on parasite killing by either mock-treated or rIL-4-treated MPM (data not shown).

DISCUSSION

To the best of our knowledge, these results show for the first time that IL-4 can activate macrophages for increased phagocytosis and intracellular killing of a micro-organism. Such activities were exemplified in our work with *T. cruzi* AMA which, being non-invasive, could only have been internalized by the MPM via phagocytosis. In view of these observations, IL-4 could be grouped with other macrophage-activating factors of lymphocyte origin, for example interferon-gamma (Steeg, Johnson & Oppenheim, 1982; Wirth *et al.*, 1985; Vilcek *et al.*, 1987), that have multiple effects on macrophages. In this context, increased phagocytosis and intracellular killing add to the known capacities of IL-4 to enhance tumouricidal activity (Crawford *et al.*, 1987) and antigen presentation (Zlotnik *et al.*, 1987), and to stimulate Ia and Fc(II) receptor expression (Crawford *et al.*, 1987).

In our assay system, maximal stimulation of parasite uptake and killing was attained with 400 U rIL-4/ml (Table 1) and no significant improvement was seen with greater concentrations of the lymphokine, which in some experiments reached as high as $3{\cdot}2 \times 10^4$ U/ml (data not shown). With any of the effective doses not only was the number of phagocytosed AMA increased significantly over control values but the percentage of MPM engaging parasites was augmented as well, indicating that additional macrophages were stimulated by rIL-4 to interact with the parasite. Prevention of both effects by the presence of monoclonal anti-IL-4 confirmed that the noted increases were indeed mediated by IL-4 (Table 2).

In the experiments discussed above (Tables 1 and 2) MPM treated with rIL-4 contained greater numbers of parasites at Time 0 (i.e. immediately after removal of the free AMA) than control MPM, which could possibly account for the killing of greater numbers of organisms. However, when MPM were first allowed to internalize AMA and were treated with rIL-4 after removal of the free AMA (Fig. 1), increased rates of killing were also observed. Clearly, the greater killing activity of rIL-4-treated MPM was not necessarily dependent on larger initial parasite loads and could be induced after phagocytosis. Additionally, the observations that rIL-4 did not affect the viability of *T. cruzi* AMA or alter the capacity of the parasite to incorporate [^{3}H]thymidine rendered it highly unlikely that parasite clearance might have actually resulted from a toxic effect of rIL-4 on the AMA rather than from an IL-4-induced enhancement of the MPM killing capacity. Furthermore, it seems unlikely that the various scavengers of oxygen reduction metabolites that inhibited enhanced parasite killing, including catalase and SOD—whose inhibitory activities were abrogated by heat inactivation—could interfere with a putative toxic effect of IL-4 on the AMA.

At least a 24-hr pretreatment of MPM with rIL-4 was needed for these cells to display significant increases in parasite uptake and killing (Table 3). This length of time is not unlike those reported for other lymphokine-mediated effects on monocyte or macrophage functions (Perlmutter *et al.*, 1986; Steeg *et al.*, 1982). For example, MPM require a 24-hr period of incubation with interferon-gamma to manifest enhanced uptake and killing of the invasive, trypomastigote form of *T. cruzi* (Wirth *et al.*, 1985).

While our results do not define, at this point, the mechanisms underlying the induction of the noted IL-4 effects, they do provide a mechanistic basis for the enhanced intracellular killing: reliance, at least in part, on toxic oxygen reduction metabolites. This conclusion was supported by the inhibitory effects on killing of catalase, SOD and histidine (Table 4), which implicated at least hydrogen peroxide, superoxide anion and singlet oxygen, respectively, in the cytotoxic process. In examining the levels of inhibition achieved with the three effective scavengers, it was noticed that they were comparable, presumably reflecting the chemical inter-relation in the formation of these metabolites. Also, scavenger-mediated inhibition of rIL-4-induced AMA killing by MPM was not complete, suggesting that other lytic processes may have also been simultaneously operative such as, for example, those mediated by hydrolytic lysosomal enzymes. Other investigators have shown that both oxygen-dependent (Nathan *et al.*, 1983) and oxygen-independent (Rothermel *et al.*, 1986) mechanisms are involved in macrophages stimulated with interferon-gamma. However, we can not rule out that, in our case, scavenging of toxic oxygen reduction intermediates may not have been complete.

Although parasite killing by control macrophages was not as pronounced as that induced by IL-4, it was nevertheless significant and reproducible. Our results do not provide an explanation for this finding and it would be premature to speculate on the existence of one or more parasite components capable of stimulating macrophages.

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