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Inflammatory mediator release by *Brugia malayi* from macrophages of susceptible host *Mastomys coucha* and THP-1 and RAW 264.7 cell lines

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ABSTRACT

Objective: To investigate which life stage of the parasite has the ability to stimulate release of pro- or anti-inflammatory mediators from macrophages. **Methods:** The human macrophage/monocyte cell line THP-1, the mouse macrophage cell line RAW 264.7 and naive peritoneal macrophages (PM) from the rodent host *Mastomys coucha (M. coucha)* were incubated at 37 ℃ in 5% CO_2 atmosphere with extracts of microfilariae (Mf), third stage infective larvae (L_3) and adult worms (Ad) of *Brugia malayi*. After 48 hr post exposure, IL-1 β, IL-6, TNF- α, IL-10 and nitric oxide (NO) in cell-free supernatants were estimated. **Results:** Extracts of all the life stages of the parasite were capable of stimulating pro- (IL-1 β, IL-6 and TNF- α) and anti-inflammatory (IL-10) cytokines in both the cell lines and peritoneal macrophages of *M. coucha*. Mf was the strongest stimulator of pro-inflammatory cytokines followed by L_3 and Ad; however, Ad was a strong stimulator of IL-10 release. Mf was found to have potential to modulate LPS-induced NO release in RAW cells. Ad-induced NO release was concentration dependent with maximum at 20 μg/mL in both RAW and PMs. **Conclusions:** The results show that parasites at all life stages were capable of stimulating pro- (IL-1 β, IL-6 and TNF- α) and anti-inflammatory (IL-10) cytokines and NO release from macrophages of susceptible host *M. coucha*, human and mouse macrophage cell lines. Mf can suppress the LPS-induced NO release in RAW cells. The findings also show that the two cell lines may provide a convenient *in vitro* system for assaying parasite-induced inflammatory mediator release.

1. Introduction

Human lymphatic filariasis (LF), a mosquito-borne disease of the tropics, caused by the nematode parasites *Brugia malayi (B. malayi), Brugia timori (B. timori)* and *Wuchereria bancrofti* (*W. bancrofti*), affects 120 million people worldwide, of which 40 million people show chronic disease manifestations: elephantiasis and hydrocele[1] (www.globalnetwork.org; WHO, 2006).

Macrophages play an important role in the initiation of specific innate immunity, formulation of strategies for elimination of pathogens[2] including filarial parasites[3] (http://www.jimmunol.org/cgi/content/full/167/6/3207) and in inflammatory reactions. Porthouse *et al*[4] reported that cytokines and other inflammatory mediators elaborated by macrophages have been implicated in the inflammatory reactions in filarial infections. Proinflammatory cytokines such as IL-1 β, IL-6, IL-12 and TNF- α generally have potent effector functions that overlap extensively with each other to bring about multiple components of inflammation, e.g. tissue necrosis, chemotaxis of cellular infiltrates, induction of collagenase and prostaglandin secretion. In filariasis, the diverse pathological manifestations are thought to be mediated by the life stages of the filarial parasite[5]. A variety of inflammatory cytokine[6] and nitric oxide (NO) responses have been reported in filarial patients and the disease process was thought to be the net effect of inflammatory mediator responses to different co-existing life stages of the filarial parasite[6, 7]. The present study was aimed at understanding which life stage of the parasite has the ability to stimulate release of pro- or anti-inflammatory

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mediators from macrophages. For this purpose we have used the human monocyte/ macrophage cell line THP-1, the mouse macrophage cell line RAW 264.7 and naïve peritoneal macrophages (PMs) of the rodent host *Mastomys coucha (M. coucha)*.

2. Materials and methods

2.1. Animals

The animals were obtained from Animal House of the Institute. These were handled and used in the study in compliance with the Institutional Animal Ethics Committee guidelines. Throughout the study, they were housed in climatically controlled animal quarters [Temp.: (23±2) ℃; RH: 60% and photoperiod: 12 hr light-dark cycles] and fed standard rodent chow supplemented with dried shrimps (*M. coucha*) and water *ad libitum*.

Healthy 6-8 week-old male *M. coucha* and *Meriones unguiculatus* were infected with infective larvae (L_3) of *B. malayi* which were obtained from laboratory bred female *Aedes aegypti (A. aegypti)* mosquitoes, fed on *M. coucha* experimentally infected with *B. malayi*[8].

2.2. Isolation of parasite stages

L_3 of *B. malayi* obtained from *A. aegypti* mosquitoes as above, were washed thoroughly with sterile phosphate-buffered saline (PBS, 0.01M, pH 7.2)[9] and used for the present study.

Microfilariae and adult worms of *B. malayi* were recovered from peritoneal cavity of *B. malayi* infected jirds (*M. unguiculatus*) as described by Dixit *et al*[9]. Briefly, microfilariae were passed through 5 μm membrane filter to remove peritoneal cells and washed with sterile PBS. Adult parasites were plated on sterile plastic petri dishes containing PBS and incubated at 37 ℃ for one hour to remove peritoneal exudate cells of jirds. Adults and microfilariae thus isolated were collected and used for extract preparation.

2.3. Preparation of parasite extracts

Soluble somatic extracts of microfilariae (Mf), L_3 and adults (Ad) were prepared as per method described by Dixit *et al*[9]. The homogenate was centrifuged at 3 000 *g* for 30 min. at 4 ℃. The protein content of the extracts was measured according to Lowry *et al*[10], aliquoted and kept at -20 ℃ until used.

2.4. Cell lines

Both THP-1 and RAW 264.7 cells were obtained from National Centre for Cell Science, Pune, India and maintained as per the instructions received. RAW 264.7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. The THP-1 cell line was maintained in RPMI 1640 medium containing above mentioned supplements. Both the media and supplements were purchased from the Sigma chemicals.

2.5. Isolation of PM from M. coucha

PMs of *M. coucha* were isolated as per method described by Mukhopadhyay *et al*[6].

2.6. Stimulation of cell lines and PMs with extracts and LPS

The cells were pretreated with 1∝, 25 dihydroxyvitamin D3 (Calbiochem-Nova Biochem International, La Jolla, California) at 0.05 μM, for 48 hr before being stimulated with the extracts or LPS[11]. The experiment was carried out in sterile 24-well plate (Nunc, Denmark). The RAW 264.7 cells and PMs were dispensed with 0.5×10^6/mL/well and were allowed to adhere for 16 hr at 37 ℃ in 5% CO_2 atmosphere before exposure to stimulants. THP-1 cell suspension was used at 2×10^6 cells/mL. Three such independent experiments were performed.

2.7. Cytokine release by cells

For detectable amount of cytokine release, the dose of each stimulant was optimized using THP-1 cells. The cells were incubated with parasite extracts (1, 5, 10 μg/mL) and LPS (Sigma Chemical Company, St. Louis, USA) at 1, 2, 5 μg/mL in culture plates for 48 hr as above. Optimum concentration of the stimulants (10 μg/mL for Mf and L_3, 5 μg/mL for Ad and 1 μg/mL for LPS) was then used for experiments with RAW or PM cells. Cell-free supernatants were collected at 48 hr post-exposure (PE) with stimulants following centrifugation (400 g) at 4 ℃ for 10 min. and stored at -20 ℃ until used.

2.8. Cytokine assays

IL-6, IL-10, TNF-α (Pharmingen) and IL-1β (Biosource International, Camarillo, CA) were estimated in culture supernatants by sandwich ELISA using paired cytokine-specific monoclonal antibodies (human or mouse) broadly following the manufacturer's instructions with some modifications according to our conditions[12]. Duplicates of each sample were run separately. Cytokine concentration in the samples was calculated using OD readings of measured amount of standards suitable for the above mentioned paired antibodies obtained from the respective sources.

2.9. NO release by RAW 264.7 cells and PMs of M. coucha

NO release was determined indirectly using a quantitative and colorimetric assay based on the Griess reaction[13]. Briefly, the cells (0.5×10^6 in 1 mL volume/well) were incubated with various concentrations of Ad (cell lines: 1,

2, 5 and 10 μg/mL; PMs: 5, 10 and 20 μg/mL) and Mf (both cell lines and PMs: 5, 10, 20, 50 and 100 μg/mL) extracts and LPS at 1 μg/mL as above for 48 hr in 24-well plate. The cell free culture supernatant was collected and the reaction was carried out in triplicate by mixing 100 μL of each sample with 100 μL Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamide dihydrochloride, 2.5% ortho-phosphoric acid). After incubation for 10 min at room temperature, the OD was taken at 550 nm. Nitrite level was calculated using OD readings of measured amount of standard.

In certain experiments aminoguanidine (AMG; Sigma), a NO inhibitor, was added at 500 μM to the cultures of Ad extract (10 μg/mL). Similarly, to validate whether Mf extract has any modulatory effect on LPS-induced NO release in cell line, LPS (1 μg/mL) was co-cultured with Mf extract (100 μg/mL).

2.10. Statistics

Results were presented as mean±SD of three independent experiments and the data were analyzed statistically using Tukey's multiple comparison test. The differences were considered statistically significant at $P< 0.05$.

3. Results

3.1. TNF-α, IL-1β, IL-6, and IL-10 production in THP-1, RAW 264.7 cells and PM

Cells with medium (control) did not induce significant amount of cytokine-release.

Optimum concentration of stimulants (Mf and L_3: 10 μg/mL; Ad: 5 μg/mL; LPS: 1 μg/mL) obtained to induce detectable level of cytokines in THP-1 cells was subsequently used for RAW cells and PMs.

All the parasite extracts were able to simulate pro- (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-10) cytokine release in THP-1 cell line. The responses of cells to Mf were more or less as efficient as that observed with LPS. Analysis of cytokine stimulating potential of the extracts revealed that Mf extract ($P<0.001$, vs control) was the strongest cytokine stimulator followed by L_3 ($P<0.01$, vs control), and Ad was the weakest ($P<0.05$, vs control). Ad- induced IL-6 release in THP-1 cells was observed to be the lowest. Interestingly, Ad extract was comparatively better stimulator of IL-10 than other stages of the parasite (Ad vs Mf: $P<0.001$; Ad vs L_3: $P<0.01$) (Table 1).

The parasite extracts- or LPS-induced cytokine production pattern in mouse cell line was almost same as that observed in THP-1 cells except that Ad-induced IL-10 release was found lower compared to Mf ($P<0.001$) and L_3 ($P<0.01$) (Table 1).

Trend of all the cytokine production in PMs stimulated by all the life stages of the parasite was almost same as that observed in THP-1 and RAW (Table 1).

The magnitude of all the cytokine response to LPS in both the cell lines and PMs was significantly high.

3.2. Production of NO in RAW 264.7 cells and PMs

Mf even at 100 μg/mL was unresponsive to induce NO production in RAW cells, but at higher concentrations (50 and 100 μg/mL) Mf-induced NO production was significantly higher ($P<0.01$) in PMs than control. LPS-induced NO production in both the systems were significantly elevated (RAW cells: $P<0.05$; PMs: $P<0.01$). LPS-induced NO production was significantly down-regulated ($P<0.01$) when co-cultured with Mf at 100 μg/mL as compared to LPS-stimulation alone. It suggested that Mf extract can suppress NO production (Table 2).

Ad at 10 μg/mL was able to induce NO release significantly at 48 hr ($P<0.001$) in RAW cells compared to

Table 1
Cytokine production by THP-1, RAW 264.7 and peritoneal macrophage cells of *M. coucha* (Mean±SD) (pg/mL).

Stimulant		TNF-α	IL-1β	IL-6	IL-10
ThP-1 cells	Control	10.43±1.01	10.43±1.01	10.43±1.01	13.33±5.51
	Mf	1 885.71±124.36[c]	728.00±42.88[c]	1 738.32±237.85[c]	91.74±9.47[c]
	L_3	256.85±16.73[b]	239.09±6.27[c]	985.94±174.24[c]	103.39±9.19[c]
	Ad	216.12±14.35[a]	198.40±23.39[c]	70.73±16.58	127.03±8.04[c,d,e]
	LPS	2 321.88±194.72[c]	1 254.48±65.09[c]	2 213.11±315.77[c]	141.59±14.48c
RAW 264.7 cells	Control	12.57±4.90	36.45±41.89	40.53±14.32	12.55±5.48
	Mf	454.78±215.91[c]	1 446.80±131.47[c]	695.44±467.12[b]	104.99±35.33[c]
	L_3	385.14±143.36[c]	1 382.44±243.35[c]	697.86±98.57[b]	50.23±1.70[b]
	Ad	218.11±34.63[a]	322.84±77.62[a]	501.78±79.33[a]	42.54±4.08[a,d,e]
	LPS	643.61±49.05[c]	1 907.36±186.73[c]	1 731.72±330.58[c]	79.32±5.21[c]
Peritoneal macrophages of the host	Control	93.53±5.98	328.84±26.55	95.83±9.12	160.50±3.50
	Mf	145.00±11.30[c]	411.42±29.21	224.78±15.00[b]	180.20±3.40[c]
	L_3	152.00±6.60[c]	564±28.01	205.32±80.94[a]	185.50±10.00[c]
	Ad	172.00±9.90[c]	441.42±39.28[c]	200.78±18.00[a]	179.00±1.40[b]
	LPS	185.00±1.30[c]	435.64±38.03[c]	665.32±80.94[c]	178.50±12.00[b]

LPS: Lipopolysaccharide; a: $P<0.05$; b:$P<0.01$; c:$P<0.001$ (vs control); d:$P<0.001$ (vs Mf); e:$P<0.01$ (vs L_3).

Table 2
NO production in RAW 264.7 and *M. coucha* peritoneal macrophage cells (Mean±SD) (μM).

Stimulant		NO concentration	
		RAW 264.7 cell line	Peritoneal macrophage s of the host
Mf	0 μg/mL (control)	41.47±2.79	26.21±4.50
	5 μg/mL	36.06±4.60	28.74±3.00
	10 μg/mL	40.06±1.24	23.59±2.00
	20 μg/mL	40.13±1.24	22.15±1.00
	50 μg/mL	35.69±9.29	33.70±1.87[c]
	100 μg/mL	38.15±3.09	37.02±3.00[c]
	LPS (1 μg/mL)	54.27±9.59[a]	40.33±2.00[c]
	Mf (100 μg/mL)+LPS (1 μg/mL)	39.47±9.29[d]	34.15±3.00[d]
Ad	0 μg/mL (control)	30.60±5.50	14.04±2.08
	1 μg/mL	32.19±2.40	12.24±4.18
	2 μg/mL	33.40±0.06	14.26±3.56
	5 μg/mL	31.00±3.09	16.64±2.29
	10 μg/mL	61.59±7.73[c]	22.20±3.74[a]
	20 μg/mL	79.23±5.73[c]	25.06±0.42[c]
	LPS (1 μg/mL)	50.75±3.09[c]	23.92±6.14[b]
	Ad (10 μg/mL)+AMG (500 μM)	31.00±3.10[f]	13.32±4.56[e]

a :$P<0.05$, b: $P<0.01$, c: $P<0.001$ (vs control); d: $P<0.01$ (Mf 100 μg/mL+LPS vs LPS); e: $P<0.01$ and f: $P<0.001$ (vs Ad 10 μg/mL).

unstimulated cells but lower doses failed to stimulate the cells to the release NO. Trend in Ad-induced NO release was almost same in PMs. AMG significantly suppressed the Ad (10 μg/mL)-induced NO production in the cells as compared to Ad (10 μg/mL) alone. LPS-induced NO release was significantly high in both RAW ($P<0.001$) and PMs ($P<0.01$) compared to control (Table 2). L_3 did not stimulate NO production up to 20 μg/mL.

4. Discussion

Filarial parasites are comprised of stimulatory and suppressive molecules[9]. The extract of Ad was found to be a weak stimulator of pro-inflammatory cytokines indicating that this stage contains predominantly inhibitory molecules. Investigators have demonstrated that Th2 (IL-10, IL-4) response is induced predominantly by female adult extract of *B. malayi*; however, Th1 response (IFN-γ and IL-2) is elicited by Mf. Nevertheless, prolonged exposure of the host to Mf, results in significantly high Th2 response[9, 14].

Both IL-1β and IL-6 have been reported to be implicated in the lymphatic damage and subsequent maintenance of inflammatory response in filarial subjects [15]. Raman *et al*[16] utilizing blood mononuclear cells (PBMCs) from Mf carriers reported high IL-1β release *in vitro*. In our present study we found that Mf and L_3 were capable of stimulating IL-1β and IL-6 release in both the cell lines and PMs. However, there was a low response of IL-6 in THP-1 cells stimulated with the Ad extract.

To a larger extent Mf and L_3 strongly stimulated TNF-α release indicating that parasites of these stages have specific pro-inflammatory molecules in abundance. A close relationship between TNF-α and severity of diseases has been reported earlier[17]. However, Das *et al*[18] reported low or no circulating TNF-α in Mf carrier subjects. In the present study this finding was substantiated by Mf or Ad-induced IL-10 release in the cell line and PMs of the host. Therefore, it seems that low TNF-α response in microfilaraemic subjects could be due to parasite life stages-induced IL-10 production. Harnett and Harnett[19-21] reported that persistent release of Mf is responsible for development of hypo-responsiveness.

NO has an important role in anti-filarial immunity[22]. It is reported that filarial parasites residing in close proximity to endothelial cells can induce NO production[23]. Therefore, it is thought that NO released by endothelial cells may directly affect parasites' survival in the lymphatics. In the present study it was observed that Ad could induce elevated level of IL-10 and NO production but weakly stimulated pro-inflammatory cytokine release in both RAW cells and host cells. Nevertheless, it is well reported that many antigens of Mf and L_3 are common to adult antigen. Interestingly, Mf extract at any dose tried in this study was found unresponsive to stimulate NO release in the RAW cells but could down-regulate the LPS-induced NO release from both RAW cells and PMs significantly indicating its potential role in immunosuppression. Thus, outcome of the filarial infection in the host might be the net effect of inflammatory mediator responses to filarial parasites at different co-existing life stages. In conclusion, the results show that all the life stages were capable of stimulating pro- (IL-1β,

IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines and NO release from macrophages of susceptible host *M. coucha* and human and mouse macrophage cell lines. Mf was found to suppress the LPS-induced NO release in RAW cells. The findings also show that the two cell lines may provide a convenient *in vitro* system for assaying parasite-induced inflammatory mediator release.

Conflict of interest statement

We declare that we have no conflict of interest.

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