

# Regulation of TNF- $\alpha$ , IL-1 $\beta$ and ICAM-1 Gene Expression in THP-1 Monocytes Stimulated with *Plasmodium falciparum*-Cultured Medium by Excretory/Secretory Products from *Spirometra erinaceieuropaei* Plerocercoids

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We have reported that excretory/secretory (ES) products from *Spirometra erinaceieuropaei* plerocercoids suppress production and gene expression of tumor necrosis factor (TNF)- $\alpha$  in murine macrophages stimulated with lipopolysaccharide. The present study demonstrates that ES products suppress TNF- $\alpha$ , interleukin (IL)-1 $\beta$  and intercellular adhesion molecule (ICAM)-1 gene expression in human monocytic leukemia cell line THP-1 stimulated with *Plasmodium falciparum*-cultured medium (*Pf*-CM). Inhibition of extracellular-signal regulated protein kinase 1/2 (ERK1/2) with PD98059 reduced TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 gene expression; on the contrary inhibition of p38 mitogen-activated protein kinase (MAPK) with SB203580 increased the expression of these genes. These findings indicated that ERK1/2, but not p38 MAPK pathway is important for induction of TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 gene expression in *Pf*-CM stimulated THP-1 monocytes. ES products additionally suppress IL-1 $\beta$ , but not TNF- $\alpha$  and ICAM-1 gene expression in *Pf*-CM stimulated THP-1 cells treated with PD98059. We hypothesize that ES products may be useful in reducing *falciparum* malaria-induced inflammatory response and sequestration in the late stage of malarial diseases such as cerebral malaria.

**Key words:** human monocytes; mitogen-activated protein kinase; *Plasmodium falciparum*; *Spirometra erinaceieuropaei*; tumor necrosis factor- $\alpha$

*Plasmodium falciparum* malaria is the most prominent parasitic infection in humans. It has been known that the pathogenesis of *Plasmodium falciparum* malaria depends on many factors, including the destruction of erythrocytes, drug resistance, cytoadherence and immunological responses (Clark et al., 1989; Aikawa et al., 1990). In malaria, although complement and antibody-mediated im-

mune responses are important (Rigione et al., 1996; Guevara et al., 1997; Kumaratilake et al., 1997), cell-mediated immunity is also involved in the protection and pathogenesis of this disease and some reports suggest that the host immunological responses, especially those mediated through the cytokine cascade, may determine the severity of malaria (Artavanis and Riley, 2002). Severe *falci-*

Abbreviations: dNTPs, deoxyribonucleotide triphosphate; ERK1/2, extracellular-signal regulated protein kinase 1/2; ES, excretory/secretory; ICAM, intercellular adhesion molecule; IL, interleukin; LPS, lipopolisaccharide; MAPK, mitogen-activated protein kinase; M-MLV RT, Moloney mouse leukemia virus reverse transcriptase; *Pf*-CM, *Plasmodium falciparum*-cultured medium; PMA, phorbol 12-myristate-13 acetate; RT, reverse transcription; TNF, tumor necrosis factor

*parum* malaria is usually accompanied by serious secondary complication, such as cerebral malaria, severe anemia, hypoglycemia and renal impairment (Aikawa et al., 1990; Kwiatkowski, 1990). Several lines of evidences implicate that tumor necrosis factor (TNF)- $\alpha$  is a major mediator for these complications. Indeed, it has been reported there is an elevation in serum levels of not only TNF- $\alpha$ , but also interleukin (IL)-1 $\beta$  and IL-6 in patients with *falciparum* malaria (Kwiatkowski et al., 1989, 1990; Kwiatkowski, 1990), which are correlated with severity of the disease and death (Grau et al., 1989; Clark et al., 1990; Kwiatkowski et al., 1990; Kurtzhals et al., 1998). A study by Udomsangpetch et al. (1997) showed that there were localized high levels of inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and interferon- $\gamma$  in the brain tissue of a patient who died from cerebral malaria, in which an up-regulation of adhesion molecules such as CD36 or intercellular adhesion molecule (ICAM)-1 and sequestration of parasitized erythrocytes, lymphocytes and monocytes to the endothelial cells were associated. Identification of ICAM-1 as a major sequestration receptor, especially in the brain, has led to the assumption that it contributes to the pathophysiology of severe malaria (Aikawa et al., 1990). In fact, in the early stages of malaria, the mild and moderate amount of inflammatory cytokines, such as TNF- $\alpha$  or IL-1 $\beta$ , are beneficial for the host by suppressing and killing the parasite (Grau et al., 1989; Clark et al., 1990; Kwiatkowski, 1990; Kwiatkowski, et al., 1990). However, in later stages, an excessive production of these cytokines are detrimental, since activation of phagocytes to kill intracellular or extracellular parasites requires a great amount of inflammatory cytokine production which can cause adverse systemic effects such as severe anemia and cerebral malaria (Kwiatkowski, 1990; McGuire et al., 1994; Luty et al., 2000).

The plerocercoids of *Spirometra erinaceieuropaei*, one species of parasitic tapeworm, has been known to produce anti-inflammatory like substance(s) which suppressed the nitrite production and gene expression of inducible nitric oxide synthase (Fukumoto et al., 1997) and the TNF- $\alpha$  in

murine macrophages stimulated with lipopolysaccharide (LPS) (Miura et al., 2001; Dirgahayu et al., 2002). Based on these findings and on what we now understand about malaria pathogenesis, the present study was conducted to investigate whether excretory/secretory (ES) products from these plerocercoids might suppress TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 mRNA expression of the human monocytic leukemia cell line THP-1 induced by *Plasmodium falciparum*-cultured medium (*Pf*-CM). A possible potential role of ES products in malaria pathogenesis will be discussed.

## Materials and Methods

### ***Plerocercoids of Spirometra erinaceieuropaei and their ES products***

Plerocercoids of *Spirometra erinaceieuropaei* were collected from 2 species of snakes (*Elaphe quadrivirgata* and *Rhabdophis tigrinus*) in the southern part of Ehime Prefecture, Japan and stored for over 6 months in the subcutaneous tissues of golden hamsters, which were housed and maintained according to the guidelines for proper treatment of animals at the Division of Laboratory Animal Science, Research Center for Bioscience and Technology, Tottori University, Japan. ES products were obtained as described previously (Miura et al., 2001). The protein concentration was assayed using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) and adjusted to 50  $\mu$ g/mL. We made separate lots for ES products from each golden hamster in order to perform several experiments independently. One representative experiment is shown here.

### ***Preparation of Pf-CM***

The *Pf*-CM was obtained from the continuous culture of *Plasmodium falciparum* FCR-3 strain which was maintained according to the method of Trager and Jensen (1976), kindly provided by the Department of Molecular Parasitology, School of Medicine, Ehime University, Japan. The *Pf*-CM was obtained

from the culture when the parasite reached an initial hematocrit of 5% in RPMI 1640 medium (Sigma Chemical, St. Louis, MO) supplemented with HEPES buffer (25 mM) and 0.2% NaHCO<sub>3</sub> with 10% heat-inactivated human serum (albumax) (Gibco BRL, Paisley, United Kingdom). The cultured-medium were then treated as previously described (Ballet et al., 1981; Abdalla and Wickramasinghe, 1985). Briefly, 40 mL of *Pf*-CM (20% parasitemia) was centrifuged for 3000 rpm for 20 min at 4°C prior to dialyzation against cold distilled water for 48 h. After being lyophilized, the sample was then re-suspended in 4 mL RPMI 1640. The *Pf*-CM was then passed through a 0.45- $\mu$ m-pore-size membrane filter and stored at -30°C until use. Thawed media were filtered again through a 0.45- $\mu$ m-pore-size membrane filter before being added to culture.

### Cell culture

The human monocytic leukemia cell line THP-1 (TIB-202) was purchased from RIKEN cell bank (Tokyo, Japan). THP-1 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD) containing 100 U/mL penicillin G (Banyu Pharmaceutical, Tokyo) and 100  $\mu$ g/mL streptomycin (Meiji Seika., Tokyo), plated in tissue culture dishes (Greiner, Frickenhausen, Germany), and incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> in a humidified incubator. We differentiated the THP-1 cells by re-suspending them for 24 h in medium containing 200 nM of phorbol 12-myristate-13-acetate (PMA) (Wako, Tokyo), as previously described by Reyes et al. (1999). Such treatment with PMA induces the conversion of THP-1 into mature cells as macrophages (Tsuchiya et al., 1980), designated “THP-1-derived macrophages”. The macrophages were washed with phosphate buffered saline and were left overnight before treatment. We used polymyxin B (10  $\mu$ g/mL) (Sigma Chemical) for treatment of ES products for all subsequent experiments prior to stimulation with *Pf*-CM.

### Reverse transcription (RT)-PCR analysis

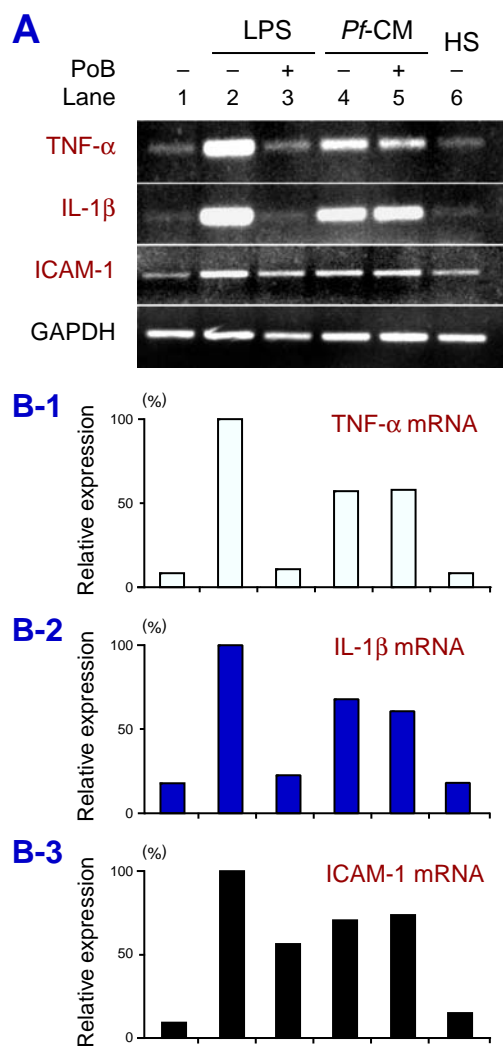
Total RNA was prepared from *Pf*-CM-stimulated or control macrophages using an ISOGEN Kit (Nippon Gene, Tokyo) according to the manufacturer’s protocol. Approximately 1  $\mu$ g of total RNA was reverse-transcribed in a 20  $\mu$ L reaction volume according to the manufacturer’s protocol. Approximately 1  $\mu$ g of total RNA was reverse-transcribed in a 20  $\mu$ L reaction volume consisting of 2.5  $\mu$ L random hexamers (50 pM), 4  $\mu$ L 5 $\times$  Moloney mouse leukemia virus reverse transcriptase (M-MLV RT)-reaction buffer, 2.5  $\mu$ L deoxyribonucleotide triphosphate (dNTPs) (2.5 mM each) (Nippon Gene, Tokyo), 1  $\mu$ L of RNase-inhibitor and 1  $\mu$ L of M-MLV RT enzyme (all reagents were purchased from Promega, Tokyo). PCR reactions were run on a Perkin Elmer DNA Thermal Cycler (Takara, Kyoto, Japan), in 20  $\mu$ L total volume per reaction mixture containing 2  $\mu$ L of cDNA RT products, 1.6  $\mu$ L dNTPs mix (2.5 mM each), 2  $\mu$ L 10 $\times$  Gene Taq Universal buffer (Nippon Gene, Tokyo), 1  $\mu$ L of each primer (diluted to 20  $\mu$ M) and 0.1  $\mu$ L Taq Gold. Primer sequences used were as follows: TNF- $\alpha$ : (sense: 5'-AAG CCT GTA GCC CAT GTT GT-3' and antisense: 5'-CAG ATA GAT GGG CTC ATA CC-3'), ICAM-1: (sense: 5'-AAT GCC CAG ACA TCT GTG TC-3' and antisense: 5'-CAG TTC AGT GCG GCA CGA GAA AT-3'), IL-1 $\beta$ : (sense: 5'-GCT CCT TCC AGG ACC TGG AC-3' and antisense: 5'-CGT GCA CAT AAG CCT CGT TA-3') and  $\beta$ -actin: (sense: 5'-CCA GAG CAA GAG AGG CAT CC-3' and antisense: 5'-GTG GTG GTG AAG CTG TAG CC-3'). The PCR cycles consisted of denaturation at 94°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 1 min for 27 cycles (TNF- $\alpha$ ), 25 cycles (ICAM-1), 23 cycles (IL-1 $\beta$ ) and 21 cycles ( $\beta$ -actin) for semi-quantitative analysis. A 15  $\mu$ L aliquot of each of the PCR products was separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and photographed. For quantification, photographs showing PCR products were scanned and analyzed using Densitograph version 4.0 software (ATTO, Tokyo). For each sample, the PCR product values were normalized to the  $\beta$ -actin PCR product values.

## Results

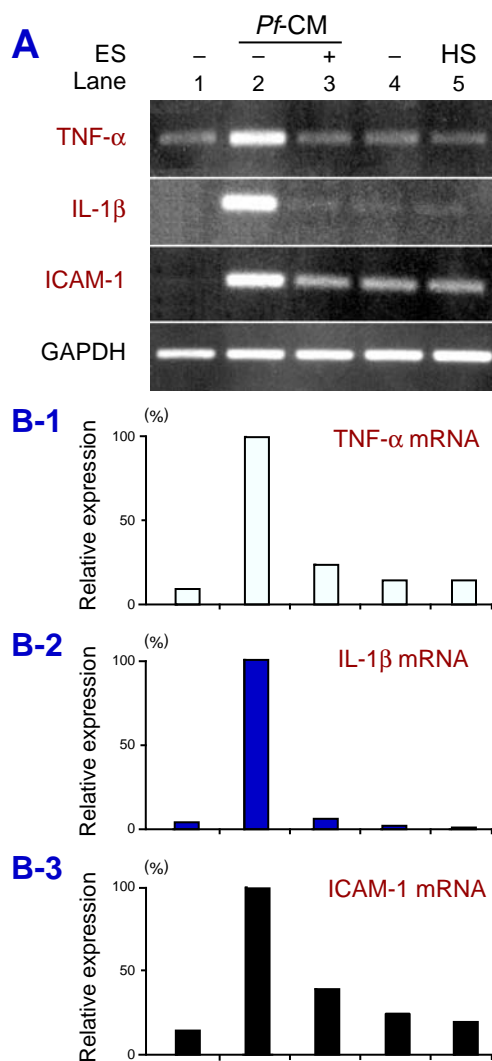
### **Induction of TNF- $\alpha$ , IL-1 $\beta$ and ICAM-1 mRNA expressions of THP-1 cells in response to *Pf*-CM**

We first examined the effect of *Pf*-CM on the induction of TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 mRNA expres-

sions. As seen in Fig. 1, *Pf*-CM induced TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 mRNA accumulation in THP-1 cells (lane 4). Although LPS-induced gene expression of TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 mRNA was reduced in the presence of polymyxin B (lane 3), *Pf*-CM-induced expression of these genes was not prevented in the presence of polymyxin B (lane 5). No appreciable band was observed in controls and hu-



**Fig. 1.** TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 gene expressions in human monocytes stimulated with LPS or *Pf*-CM. THP-1 monocytes were left unstimulated or stimulated with LPS (100 ng/mL), *Pf*-CM or heat-inactivated human serum (HS) for 4 h in the absence or presence of polymyxin B (10  $\mu$ g/mL). Total RNA (1  $\mu$ g/lane) was obtained and the levels of TNF- $\alpha$ , ICAM-1, IL-1 $\beta$  and  $\beta$ -actin mRNA expression were assessed by semiquantitative RT-PCR. One representative of 3 experiments is shown. PoB, polymyxin B.



**Fig. 2.** Effect of ES products on the *Pf*-CM-induced TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 gene expression in human monocytes. THP-1 monocytes were left untreated or pretreated with ES products (5  $\mu$ g/mL) and polymyxin B (10  $\mu$ g/mL) for 6 h, washed, and then the medium was replaced before the stimulation with 250  $\mu$ L of *Pf*-CM or human serum (HS) for 4 h. Total RNA (1  $\mu$ g/lane) was obtained and the levels of TNF- $\alpha$ , ICAM-1, IL-1 $\beta$  and  $\beta$ -actin mRNA was analyzed by semiquantitative RT-PCR. One representative of 3 experiments is shown.

man serum-treated monocytes in the absence of LPS or *Pf*-CM (lanes 1 and 6). These results indicate that *Pf*-CM has the ability of some stimulatory activity to induce the gene expression of pro-inflammatory cytokines and ICAM-1, and this activity exhibited in *Pf*-CM-cultured THP-1-cells is a *Plasmodium falciparum* origin, without a trace of endotoxin contamination.

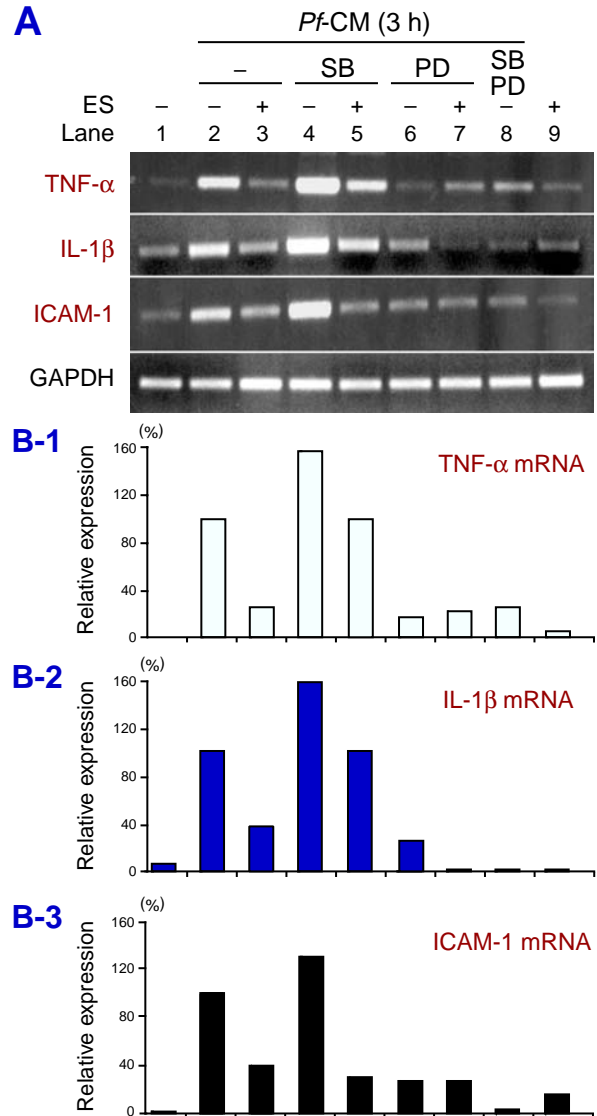
### Treatment of THP-1 cells with ES products prior to stimulation with *Pf*-CM prevent induction of TNF- $\alpha$ , IL-1 $\beta$ and ICAM-1 gene expression

In order to evaluate the impact of ES products on TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 gene expressions of THP-1 cells, these monocytes were left untreated or treated with 5  $\mu$ g/mL ES products and polymyxin B prior to stimulation with 250  $\mu$ L *Pf*-CM in the final medium volume of 3 mL. As shown in Fig. 2, ES products potently reduced TNF- $\alpha$  mRNA (by 76%), IL-1 $\beta$  (by 85%) and ICAM-1 mRNA expression (by 60%) in response to *Pf*-CM (lane 3). THP-1 cells treated with human serum showed a band pattern similar to the controls (lanes 1 and 5). The levels of constitutively expressed  $\beta$ -actin mRNA, however, did not change with the treatment of ES products. This result shows that ES products are a potent inhibitor for TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 gene expression in human monocytes in response to *Pf*-CM.

### Role of ERK1/2 and p38 MAPK pathways in the induction of TNF- $\alpha$ , IL-1 $\beta$ and ICAM-1 gene expression in *Pf*-CM-stimulated THP-1 cells

Using well-known pharmacological inhibitors of the ERK1/2 and p38 MAPK pathways, PD98059 and SB203580, respectively (Dudley et al., 1995), we investigated the suppressive mechanisms of ES products on TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 gene expressions in human monocytes stimulated with *Pf*-CM. THP-1 cells were pretreated with 5  $\mu$ g/mL ES products and polymyxin B for 6 h, washed and then the medium was replaced. The monocytes were then treated with SB203580 and/or PD98059 for 1 h

before the addition of 250  $\mu$ L *Pf*-CM in the final volume of 3 mL for further 3 h incubation. As shown in Fig. 3, PD98059 treatment suppressed TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 mRNA expression by 83%, 75%



**Fig. 3.** MAPK inhibitors differentially modulate the *Pf*-CM-induced TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 mRNA expressions in THP-1 monocytes. THP-1 monocytes were pretreated with 5  $\mu$ g/mL ES products and 10  $\mu$ g/mL of polymyxin B for 6 h. The cells were then washed and the medium was replaced. The cells were then either treated with PD98059 (50  $\mu$ M), SB203580, (10  $\mu$ M) or a combination of these inhibitors for 1 h prior to the stimulation with 250  $\mu$ L *Pf*-CM for an additional 3 h incubation. Total RNA (1  $\mu$ g/lane) was obtained and TNF- $\alpha$ , ICAM-1 and  $\beta$ -actin mRNA was assessed by semiquantitative RT-PCR. One representative of 3 experiments is shown.

and 73% (lane 6), respectively; however, SB203580 treatment induced approximately 1.5-fold levels of the TNF- $\alpha$ , IL-1 $\beta$  mRNA and ICAM-1 mRNA expression (lane 4), compared to the positive controls (lane 2). Combination of PD98059 and SB203580 profoundly reduced the 3 genes to near control levels (lane 8). These data indicate that the ERK1/2 MAPK pathway, but not p38 MAPK is important for induction of TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 mRNA expression in THP-1 cells stimulated with *Pf*-CM. We observed an additional suppressive effect by ES products on IL-1 $\beta$ , but not TNF- $\alpha$ , and ICAM-1 mRNA expression in the cells treated with PD98059 (lane 7).

## Discussion

A complex parasite such as human *Plasmodium* is likely to generate a variety of substances that injure the hosts directly or cause immunopathology. It has been widely known that severe malaria is associated with a failure of the host defense system to control parasite replication, excessive secretion of pro-inflammatory cytokines and sequestration of parasitized-erythrocytes in vital organs, such as the kidney, the lungs, the brain, etc. The excessive secretion of pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6 (Aikawa et al., 1990) has been assumed because of the stimulation of effector cells by certain parasite-derived toxins [substance(s)], released by ruptured schizonts that cause paroxysms of fever. This substance(s) has been shown to contain glycosylphosphatidylinositol (Schofield and Hackett, 1993; Schofield et al., 1996; Tachado et al., 1996) and malarial pigments (Pichyangkul et al., 1994). Several studies have shown that glycosylphosphatidylinositol anchor, a membrane attachment mechanism for cell surface proteins widely used in eukaryotes, plays a crucial role in the pathology of malaria by inducing pro-inflammatory cytokines, including iNOS (Schofield et al., 1996; Tachado et al., 1996), TNF- $\alpha$  (Schofield et al., 1993) and ICAM-1 (Schofield et al., 1996). Pichyangkul et al. (1994) demonstrated that sequestered malaria pigment in the microvasculature also induced production

and induction of TNF- $\alpha$  and IL-1 $\beta$  gene expression of murine macrophages. In agreement with several previous studies (Abdalla and Wickramasinghe, 1985; Taverner et al., 1990a, 1990b), we demonstrated that *Pf*-CM induced TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 mRNA expression in THP-1 cells, which was not abolished by the treatment of 10  $\mu$ g/mL polymyxin B (Fig. 1), suggesting that the stimulatory activity of *Pf*-CM was of parasite origin. We found that pretreatment of THP-1 monocytes with ES products prior to stimulation by *Pf*-CM reduced TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 gene expression (Fig. 2). We are confident that the results are not a consequence of toxicity since treatment of macrophages by ES products for over 24 h neither inhibited the growth of cell numbers (unpublished data) nor affected the expression level of the house-keeping gene  $\beta$ -actin.

Further investigation through the MAPK pathway showed that pretreatment of THP-1 cells with PD98059 partially inhibited TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 mRNA expression, whereas SB203580, instead of inhibition, induced all gene expressions approximately 1.5 times compared to the positive controls (Fig. 3) and a combination of PD98059 and SB203580 profoundly reduced TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 mRNA expression to near control levels, indicating that the ERK1/2 pathway is important for optimal induction of TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 mRNA expression. We observed that ES products additionally suppress IL-1 $\beta$  mRNA expression in LPS-stimulated THP-1 cells treated with PD98059, suggesting that ES products may suppress IL-1 $\beta$  mRNA by inhibiting phosphorylation of the ERK1/2 pathway. The reason for this additional suppressive effect only observed for IL-1 $\beta$  mRNA expression is still unclear.

Paroxysmal fever is the hallmark of malaria. Several observations indicate that TNF- $\alpha$  is a critical mediator of malarial fever: i) it is a potent endogenous pyrogen (Dinarello et al., 1986); ii) when *P. falciparum* is cultured with human monocytes, the rate of TNF production rises sharply at the time of schizont rupture (Kwiatkowski, 1990; Kwiatkowski et al., 1990); iii) in *P. vivax* infection, paroxysmal fever is associated with a sharp rise in circulating TNF levels (Karunaweera et al., 1992) and iv) anti-TNF

therapy inhibits fever in children with cerebral malaria (Kwiatkowski et al., 1993). However, TNF- $\alpha$  is not the only mediator of malaria fever. Other cytokines also have pyrogenic properties, including IL-1 $\beta$  (Rocket et al., 1994), IL-1 $\alpha$  (Kwiatkowski et al., 1990) and IL-6 (Kern et al., 1989), which are produced mainly by the monocytes/macrophages series. In addition to the pyrogenic effect, TNF- $\alpha$ , has been shown to induce ICAM-1 gene expression of the endothelial cell, the major adherence receptor in the brain (Aikawa et al., 1990; Udomsangpetch et al., 1997). A study by Udomsangpetch et al. (2002) demonstrated that febrile temperatures in the range usually encountered during malaria induced cytoadherence of the ring-stage of *Plasmodium falciparum*-infected erythrocytes in vitro. This fever-induced cytoadherence was shown to be associated with increased expression of the *Plasmodium falciparum*-erythrocyte membrane protein-1, a protein which is exported from the parasite and linked to the erythrocyte cytoskeleton during the 2nd half of the life cycle, on the surface of the infected red blood cell (Sharma, 1991). Up-regulating ICAM-1 expression of the endothelial cells allows parasites to bind and sequester in cerebral vessels, disrupting the function of the central nervous system (Adams et al., 2000), and in turn, leading to cerebral malaria. Based on the understanding of the pathogenesis of malaria, we suppose that suppression of TNF- $\alpha$  and IL-1 $\beta$  mRNA expression in THP-1 cells by ES products would, in turn, reduce the expression of ICAM-1 of endothelial cells by decreasing fever indirectly. In addition, ES products might also directly suppress ICAM-1 expression in monocytes.

In epidemic areas, helminthic infections commonly coexist with malaria in an individual. The significance of such concurrent infection, however, is not yet clearly understood. However, results have revealed that worm infection is associated with protection from cerebral malaria (Nacher et al., 2000), acute renal failure and jaundice (Nacher et al., 2001). Thus, worms might be central to natural anti-malarial immunity. Worms, which can induce a bias towards Th-2 cytokines and depress other cytokines could render the host more susceptible to liver-stage parasites and favor *P. falciparum*

reproduction but protect the host from severe complication of blood stage malaria. The beneficial or detrimental role of TNF- $\alpha$ , IL-1 $\beta$  and ICAM-1 during malaria attack could have important consequences, and modulation of these mediators during acute disease could lead to a novel approach in the treatment of malaria. Although co-infection of malaria and *Spirometra erinaceieuropaei* is unusual, the evidence presented here points to the possibility that ES products might be useful in reducing inflammatory responses and the phenomenon of sequestration in severe malaria, especially in cerebral malaria.

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