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Differential cytokine gene expression and secretion after phagocytosis by a human monocytic cell line of *Toxoplasma gondii* compared with *Mycobacterium tuberculosis*

J. S. FRIEDLAND, R. J. SHATTOCK, J. D. JOHNSON*, D. G. REMICK†, R. E. HOLLIMAN* & G. E. GRIFFIN *Division of Communicable Diseases and *Department of Medical Microbiology & Public Health Laboratory, St. George's Hospital Medical School, London, UK and †Department of Pathology, University of Michigan Medical School, Ann Arbor, MI, USA*

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SUMMARY

Toxoplasma gondii infection may be clinically silent in immunocompetent individuals but may cause fatal disease in immunocompromised patients such as those with HIV infection. Proinflammatory cytokines are known to be important in murine resistance to *T. gondii* but there are no data from human models of infection. We have investigated whether phagocytosis of *T. gondii*, of *Mycobacterium tuberculosis* (a pathogen which elicits a granulomatous host immune response) and of inert latex particles by THP-1 cells, a human monocytic line, caused gene expression and secretion of tumour necrosis factor (TNF), IL-6 and IL-8. These cytokines are important in recruitment and activation of T lymphocytes, and both TNF and IL-6 may have direct antitoxoplasmacidal and antimycobacterial activity. Phagocytosis of *T. gondii* by THP-1 cells resulted in minimal gene expression and secretion of TNF, IL-6 and IL-8 similar to that following phagocytosis of inert latex particles. In contrast, phagocytosis of *M. tuberculosis* resulted in increased gene expression of TNF and IL-8 as well as increased secretion of all three cytokines, particularly IL-8. These observations may partially explain the frequency of non-inflammatory host responses to *T. gondii* in immunocompetent individuals.

Keywords cytokine *Toxoplasma* *Mycobacterium tuberculosis* monocyte

INTRODUCTION

Human infection with *Toxoplasma gondii* is usually either asymptomatic or a benign self-limiting illness in immunocompetent individuals, although cysts may persist in tissues [1]. Infection is more serious in immunodeficient patients with AIDS who have reduced T lymphocyte function and number. *Toxoplasma* are able to survive within macrophages, and trophozoites can block lysosome fusion and potentially escape intracellular killing mechanisms [2]. In rodent models, antigen-specific $CD8^+$ T cells reacting with the major parasite surface antigen p30 are thought to be central to effective host defence [3]. Such $CD8^+$ cells appear to act in synergy with $CD4^+$ lymphocytes [4]. In part, the effector action of T cells may be mediated via secretion of interferon-gamma (IFN-γ) [5] and this cytokine has been used successfully to treat toxoplasmic encephalitis in the mouse model [6]. However, toxoplasmastatic activity of murine peritoneal macrophages induced by IFN-γ is mediated via endogenous tumour necrosis factor-alpha (TNF-α) [7]. Resistance to murine toxoplasma encephalitis has been associated with expression of particular polymorphisms of the TNF gene [8]. Studies *in vivo* using anti-TNF confirmed that endogenous production of TNF is important in mediating such murine resistance to *T. gondii* infection [9]. IFN stimulates many macrophage functions, including release of proinflammatory cytokines including TNF, IL-6 and IL-8.

Data on human *Toxoplasma* infection are scarce, although human cytokine responses are frequently different from those in animals [10]. Such data are necessary before it is possible to investigate interactions between *T. gondii* and an immunocompromised host. We were interested to determine whether phagocytosis of *T. gondii* by human monocytic cells resulted in the gene expression and secretion of proinflammatory cytokines, TNF, IL-6 and IL-8. TNF has potential anti-toxoplasma actions as described in rodents and the other two cytokines have important effects on T lymphocytes, central to host defence to *T. gondii* in man. There are high affinity receptors for IL-6 on T cells [11] and IL-6 is involved in T lymphocyte activation [12]. IL-8 is a T lymphocyte chemoattractant *in vitro* and *in vivo* [13].

Correspondence: Dr Jon S. Friedland, Division of Communicable Diseases, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK.

As contrast, monocyte cytokine release following phagocytosis of *M. tuberculosis* and inert latex particles was examined. Unlike *T. gondii*, which is not always associated with an active immune response in man, *M. tuberculosis* causes granulomatous inflammation *in vivo*. Granulomas are complex histological structures comprised of monocyte and T cells, and proinflammatory cytokines are important in their development. Granuloma formation in a mouse model of *M. bovis* mycobacterial infection was prevented by pretreatment of animals with anti-TNF [14].

MATERIALS AND METHODS

Cell culture

THP-1 cells are a phagocytic human myelomonocytic cell line [15] and were cultured in RPMI 1640 (Sigma, Poole, UK) with 10% endotoxin-free fetal calf serum (FCS; Serolab), 2 mM glutamine and 100 μg/ml ampicillin. Gentamicin was not used in cultures in view of its antimycobacterial activity. Immediately before experiments, cells were suspended in fresh medium in PTFE vials (Pierce, UK) (10^7 cells/vial; one vial/time point). The use of such vials prevents cellular adherence to tissue culture plastic following stimulation and hence the cytokine secretion that is known to follow adherence [16,17].

Mycobacterium tuberculosis *and* Toxoplasma gondii

Mycobacterium tuberculosis, strain H37-Rv, was initially obtained from the National Collection of Type Cultures (Colindale, UK) and then cultured in Dubos medium enriched with albumin Cohn fraction V and dextrose and containing 200 U/ml polymixin B, 100 μg/ml carbenicillin, 10 μg/ml trimethoprin and 10 μg/ml amphotericin B (all Sigma). Virulent *T. gondii* strain RH were passaged in 6–8-week-old inbred MF1 mice. *Toxoplasma* used for experiments were obtained by open peritoneal lavage of mice killed by CO_2 asphyxiation having been inoculated 3 days previously, by intraperitoneal injection, with $2{\cdot}5 \times 10^5$ trophozoites. Organisms were then washed three times in PBS. The ratio of *toxoplasma* to contaminating peritoneal macrophages was approximately 250:1. Before experiments both *T. gondii* and *M. tuberculosis* were suspended in fresh RPMI 1640.

Experimental protocol

Cells were stimulated with either *T. gondii* or *M. tuberculosis* which had been sonicated in order to separate clumps of mycobacteria that form in culture medium or 3 μm inert latex beads, a negative particulate control (Sigma). *Toxoplasma gondii* or *M. tuberculosis* (10^8) were added to 10^7 THP-1 cells. Preliminary experiments had demonstrated that phagocytosis by THP-1 cells was 90% complete within 30 min. Microscopy revealed that approximately 65% of THP-1 cells contained *T. gondii* or *M. tuberculosis* after exposure to either pathogen. There were approximately 1–10 organisms per THP-1 cell. Higher concentrations of either pathogen resulted in cell death. Thus all experiments involved conditions of maximal stimulation. At time = 0, 1, 2, 4, 8, 24 h the contents of a single PTFE vial were centrifuged at 1000 ***g*** for 5 min at 4°C. Supernatants were assayed for secreted cytokine and cellular RNA was extracted (see below). All experiments were repeated at least three times.

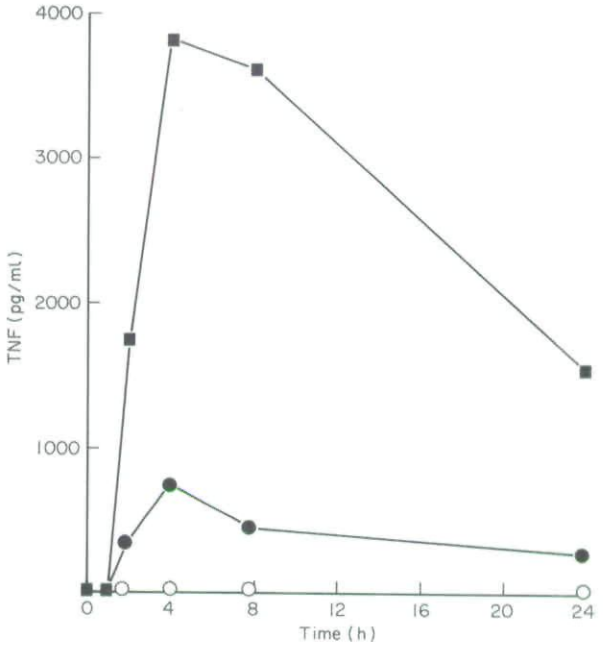

Fig. 1. Tumour necrosis factor (TNF) secretion from THP-1 cells during 24 h following phagocytosis of *Toxoplasma gondii* (●), *Mycobacterium tuberculosis* (■), and latex particles (○). Cells were exposed to the stimulus at time = 0 and TNF released into culture medium measured by bioassay using WEHI 169 cells (see text) [18]. Results are representative of at least three independent experiments.

Cytokine assays

The TNF concentration in supernatants was assayed using the WEHI 164 cell line, subclone 13 (the generous gift of Dr A. Waage, University of Trondheim, Norway) [18]. The IL-6 measurement used the B-9 cell line proliferation assay (the kind gift of Dr J. Gauldie, McMaster University, Canada) [19] which we have shown to be specific for this cytokine [20]. The lower limits of sensitivity of these bioassays were 22 pg/ml and 1 pg/ml respectively. IL-8 concentrations were assayed with a previously described ELISA with a lower limit of sensitivity of 95 pg/ml [21].

RNA extraction and Northern blotting

Cellular mRNA was extracted in 4 M guanidine thiocyanate, 25 mM Tris pH 7·0%, 0·5% N-lauroylsarcosine and 0·1 M 2-mercaptoethanol, and underwent double phenol-chloroform-isoamyl alcohol extraction before precipitation in isopropanol and washing in ethanol. RNA was resuspended in DEPC (diethyl pyrocarbonate) water and quantified on an optical densitometer (Pye/Unicam SP6-450). Equal amounts of RNA (10–15 μg) were run on denaturing formaldehyde 1% agarose gels, transferred by capillary blotting to Hybond-N (Amersham) and fixed by exposure to ultraviolet light.

Oligonucleotide probing

Northern blots were prehybridized with 6× SSC, 1× Denhardt's, 0·5% SDS, 0·05% sodium pyrophosphate, 50 μg/ml polyadenylic acid and 100 μg/ml transfer RNA and then hybridized with γ-^{32}P end-labelled oligonucleotide probes: TNF a 25 mer [22], IL-6 a 30 mer [23], IL-8 a 30 mer and β-actin a 42 mer [24]. Blots were washed and then autoradiographed with intensifying screens at −70°C for 24–48 h. β-actin probing as

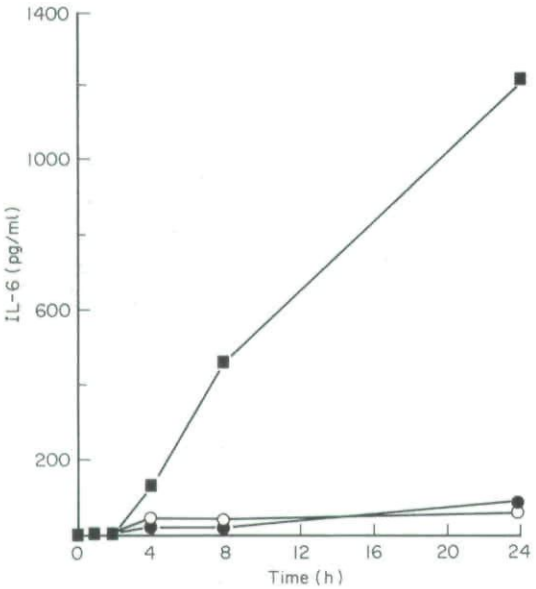

Fig. 2. IL-6 secretion from THP-1 cells during 24 h following phagocytosis of *Toxoplasma gondii* (●), *Mycobacterium tuberculosis* (■), and latex particles (○). Cells were exposed to the stimulus at time = 0 and IL-6 released into culture medium measured using the B9 cell proliferation assay (see text) [19]. Results are representative of at least three independent experiments.

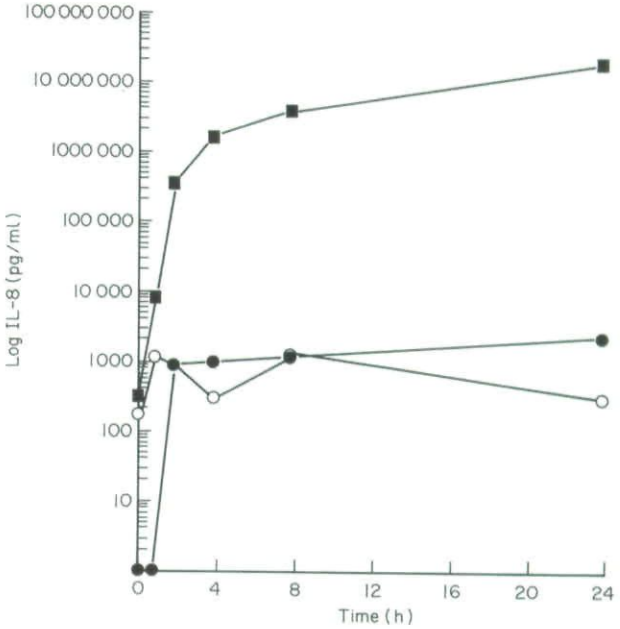

Fig. 3. IL-8 secretion (note log scale) from THP-1 cells during 24 h following phagocytosis of *Toxoplasma gondii* (●), *Mycobacterium tuberculosis* (■), and latex particles (●). Cells were exposed to the stimulus at time = 0 and IL-8 released into culture medium measured by ELISA (see text) [21]. Unstimulated cell culture supernatants contain < 500 pg/ml IL-8. IL-8 secretion following phagocytosis of *M. tuberculosis* is much greater than in other phagocytosis experiments. Results are representative of at least three independent experiments.

well as assessment of 18S and 28S ribosomal RNA bands were used to ensure uniform RNA loading on gels. Between probings, blots were stripped by heating for 1 h at 65°C in a solution of 0·005 M Tris-HCl pH 8·0, 0·002 M Na_2 EDTA and 0·1 × Denhardt's

RESULTS

Secretion of pro-inflammatory cytokines

Entry of *T. gondii* into THP-1 cells resulted in secretion of TNF into tissue culture fluid within 2 h, which was maximal at 4 h. The kinetics of TNF secretion following phagocytosis of *M. tuberculosis* was very similar. However, the magnitude of TNF release was much greater (Fig. 1). In contrast, phagocytosis of latex beads did not result in TNF secretion into culture medium. The uptake of *T. gondii* by THP-1 cells resulted in minimal secretion of IL-6 into tissue culture medium. The magnitude of such IL-6 release was similar to that observed following phagocytosis of latex (Fig. 2). In contrast, following phagocytosis of *M. tuberculosis* 15–20-fold higher levels of IL-6 were found in tissue culture supernatants than after entry of *T. gondii* into THP-1 cells. IL-6 was first detected at 4 h after phagocytosis and increased throughout the 24-h period. Entry of *T. gondii* into THP-1 cells did not cause similar IL-8 release but rather this organism, like latex, did not stimulate IL-8 secretion (Fig. 3, note log scale). Supernatant IL-8 concentrations remained similar to unstimulated levels (< 500 pg/ml and usually undetectable). We did confirm our previous finding that phagocytosis of *M. tuberculosis* is a very potent stimulus for IL-8 secretion [25]. Lavage fluid from MF 1 mice without trophozoites also did not stimulate any cytokine secretion (or gene expression).

Gene expression of pro-inflammatory cytokines

The TNF gene is not constitutively expressed in THP-1 cells. Phagocytosis of *T. gondii* resulted in a transient expression of the TNF gene between 1 and 4 h, maximal at 1–2 h. The kinetics of TNF gene expression following phagocytosis of *M. tuberculosis* were similar, although the maximal accumulation of TNF mRNA tended to occur after approximately 1 h. In both instances, TNF gene expression preceded secretion of this cytokine and maximal gene expression occurred 1–2 h before the highest TNF supernatant concentrations were recorded. However, the magnitude of TNF mRNA accumulation following phagocytosis of *M. tuberculosis* was greater than that following entry of *Toxoplasma* into THP-1 cells (Fig. 4). IL-6 mRNA was not detected in Northern blots from these experiments. This mRNA species was only found in experiments involving stimulation of THP-1 cells by lipopolysaccharide (LPS) when higher IL-6 concentrations (> 2000 pg/ml) were detected in culture medium. THP-1 cells constitutively express the IL-8 gene but phagocytosis of either *Toxoplasma* or latex did not cause accumulation of IL-8 mRNA in these cells. This is perhaps not surprising in view of the minimal effects their phagocytosis had on IL-8 secretion. In contrast, there was an approximate three-fold increase in IL-8 gene expression following phagocytosis of *M. tuberculosis* (Fig. 4).

DISCUSSION

The data presented demonstrate that entry of *T. gondii* into the THP-1 phagocytic human monocyte cell line may be associated with minimal secretion of TNF. In addition, release of IL-6 and

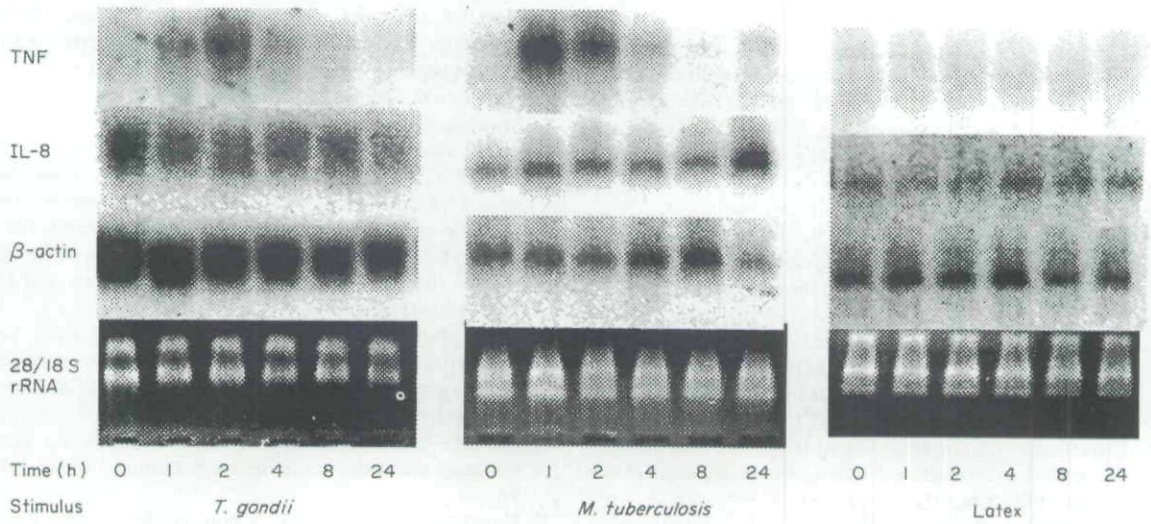

Fig. 4. Gene expression of tumour necrosis factor (TNF) and IL-8 in THP-1 cells during 24 h following phagocytosis of either *Toxoplasma gondii* or *Mycobacterium tuberculosis* or latex particles. Probing for β-actin, a reference gene, and analysis of ribosomal RNA bands indicate approximately equal loading of RNA on gels. Greatest total RNA on Northern blots (maximal β-actin expression) was detected in experiments involving *T. gondii* which emphasizes the qualitatively greater accumulation of TNF and IL-8 mRNA that followed phagocytosis of *M. tuberculosis* by THP-1 cells. Gels are all running up the page. Autoradiographs shown are representative of three independent experiments.

IL-8 into culture medium was similar to that observed following phagocytosis of inert latex beads. In contrast, phagocytosis of *M. tuberculosis* resulted in secretion of increased quantities of all three proinflammatory cytokines. In particular, we confirmed our previous finding that phagocytosis of *M. tuberculosis* causes secretion of very high levels of IL-8 into tissue culture medium by THP-1 cells [25]. Previously, we demonstrated that such IL-8 release from THP-1 cells was over 1000-fold greater than that which occurred following phagocytosis of zymosan or stimulation by bacterial LPS. The failure of *T. gondii* to stimulate release of TNF may in part relate to the minimal switching on of the TNF gene, although other post-transcriptional control mechanisms may also be involved. In addition, entry of *T. gondii* into THP-1 cells did not cause any increased levels of IL-8 gene expression above the constitutive levels found in these cells.

The reason why *T. gondii* stimulated only minimal release of the three proinflammatory cytokines investigated is uncertain, but may relate to the mode of entry of *T. gondii* into human monocytes. Following entry into macrophages, intracellular *T. gondii* is not killed even after stimulation of oxidative metabolism by granulocyte-macrophage colony stimulating factor [26]. *Toxoplasma gondii* is usually found within a vacuole which does not fuse with host endocytotic or biosynthetic organelles [27]. However, fusion occurs following Fc receptor-mediated phagocytosis of antibody-coated *T. gondii*, suggesting that the uncoated parasite may not interact with monocyte membrane proteins and therefore not initiate activation of intracellular events.

The failure of *T. gondii* to activate monocytes to secrete proinflammatory cytokines may provide an explanation as to why infection with this parasite may not be associated with histological inflammatory changes or symptoms in immunocompetent individuals [1]. It may also partly explain why the parasite is not eliminated by intracellular killing mechanisms normally activated by monocyte-derived cytokines [28]. In contrast, in infection with *M. tuberculosis* there is granuloma formation and killing of intracellular micro-organisms before infection is contained, if not eliminated. Both TNF and IL-6 have direct antimycobacterial activities [29,30]. In addition, TNF was central to granuloma formation and restriction of *M. bovis* multiplication in a mouse model of mycobacterial infection [14]. IL-8 may be important as a T lymphocyte chemoattractant, initiating recruitment of antigen-specific T cells to granulomas.

This study has examined gene expression and secretion of TNF, IL-6 and IL-8 in non-immunosuppressed human monocytic cells. However, *Toxoplasma* infection may be severe in the immunosuppressed and in pregnant women. HIV-infected individuals are an increasingly important group of immunosuppressed patients, have major T lymphocyte depletion, and toxoplasmosis is an AIDS-defining illness. In a patient with impaired independent T cell function, the fact that monocytes or macrophages may not be activated by *T. gondii* infection and there is minimal release of cytokines such as IL-6, involved in T cell activation [12] and proliferation [31], may be critical and may result in relatively unchecked replication of *T. gondii*. In preliminary studies we have found no evidence of altered gene expression of proinflammatory cytokines in THP-1 cells preinfected with HIV-1 before exposure to *T. gondii*, which might support the hypothesis that it is T cell depletion plus normally minimal monocyte cytokine release which is important in HIV-infected individuals.

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