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Effect of lipoarabinomannan and mycobacteria on tumour necrosis factor production by different populations of murine macrophages

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SUMMARY

Tumour necrosis factor (TNF) production is an important pathological mediator in mycobacterial infections, and yet little is known of the factors which influence its production. We have studied the influence of murine macrophage heterogeneity and activation state on TNF production following mycobacterial stimulation *in vitro*. Lipoarabinomannan (LAM) from strains of *Mycobacterium tuberculosis* and *Myco. avium* differentially stimulated TNF production in thioglycollate-elicited macrophages in a dose-dependent manner. In comparison, resident peritoneal macrophages produced much less TNF when stimulated with LAM, dead mycobacteria or lipopolysaccharide (LPS). In contrast, zymosan stimulated resident macrophages to a higher degree than thioglycollate-elicited cells. Another comparison between bone marrow and thioglycollate-elicited macrophages showed that both responded to LPS, but only the latter was stimulated significantly by H37Rv LAM. This may indicate that LAM stimulation of macrophages takes place through a different pathway than both zymosan- and LPS-stimulated TNF production. Also, *in vitro* activation of peritoneal macrophages with interferon-gamma (IFN-γ), increased TNF response to several stimuli. Our studies indicate that the pathology of mycobacterial infections through TNF production may be influenced by the type and activation state of the macrophage which responds to that infection.

Keywords lipoarabinomannan macrophage mycobacteria tumour necrosis factor

INTRODUCTION

Tumour necrosis factor-alpha (TNF-α) is a potent cytokine peptide produced mainly by activated macrophages in response to a wide range of stimuli, including bacterial, parasitic and viral organisms and their products [1]. Functionally, TNF is an endogenous pyrogen [2] involved in a wide range of biological activities including inflammation, cachexia, endotoxic shock and cytotoxicity [3,4], antiviral responses [5,6] and haemorrhagic necrosis of transplanted tumours [7].

Interestingly, during infection, TNF production can be associated with both a protective host response and the pathology of the disease. A good example of this is seen in mycobacterial infections, where the protective actions of TNF include TNF-stimulated killing of mycobacteria and inhibition of bacterial growth through TNF alone, or in synergy with other cytokines [8–10]. Furthermore, TNF production *in vivo* may lead to granuloma formation, which limits bacterial dissemination within the host [11].

TNF is also involved in the pathology of mycobacterial infections [12], such as in causing tissue necrosis [13,14], and clinical manifestations of tuberculous pleuritis such as fever and exudative pleural effusion [15].

Mycobacteria stimulate macrophages to produce TNF primarily by lipoarabinomannan (LAM) [16–18], although proteins [19–21] and protein–peptidoglycan complexes [15] may also be involved. Recent evidence suggests that the structure of LAM is important in determining TNF-inducing capacity. It has been shown that LAM from different strains of mycobacteria varies in structure [22,23] and also TNF-inducing capacity. For example, LAM from *Mycobacterium tuberculosis* avirulent strain H37Ra is 100-fold more potent at inducing TNF secretion than LAM from the virulent Erdman strain [18]. Furthermore, most of the biological activity of the molecule appears to be associated with the phosphatidylinositol end of the molecule [16].

Another potential variable in TNF production is the macrophage population which is stimulated. Macrophages are very heterogeneous, and stimulation to produce TNF can vary with the population of cells chosen, e.g. resident *versus* thioglycollate-elicited macrophages [24,25]. Thioglycollate-elicited macrophages differ from resident peritoneal cells in that they are monocytes recruited to the peritoneum following injection of an inflammatory mediator, in this case thioglycollate. Resident cells are not 'elicited' as such, but are recruited stochastically. Neither of these cell populations are immunologically activated,

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but it has been shown that activation of human monocytes with interferon-gamma (IFN-γ) before stimulation with mitogens, IL-2, *Myco. tuberculosis* or lipopolysaccharide (LPS), increases TNF production [13,26,27].

In these experiments we attempted to address some of these variables in relation to TNF production by mycobacteria-stimulated macrophages. Our results show that macrophage TNF production in mycobacteria-stimulated cells *in vitro* is greatly influenced by the type and activation state of the macrophage and the form of mycobacterial stimuli. It is proposed that these factors may be important in investigating *in vivo* TNF production, and hence the pathology of mycobacterial diseases.

MATERIALS AND METHODS

Mice and media

Adult, female BALB/c mice aged 6–16 weeks were obtained from Biological Services Unit (Hammersmith Hospital, London, UK) and Olac Harlem Ltd (Shaws Farm, Bicester, UK).

RPMI (Sigma Chemical Co., St Louis, MO) was supplemented with 5% fetal calf serum (FCS) (GIBCO, Life Technologies Ltd, Paisley, UK), 2 mM L-glutamine (Flow Labs, Irvine, Scotland), 100 U/ml penicillin and 100 μg/ml streptomycin (ICN Biomedicals Ltd, Irvine, UK).

Mycobacteria, LAM, LPS and zymosan

LAM from *Myco. avium* (SmD serovar 2), *Myco. tuberculosis* Erdman and H37Ra were prepared and kindly provided by Dr P. Brennan (Fort Collins, CO) [18]. *Mycobacterium tuberculosis* H37Rv LAM was prepared from irradiated bacteria [28]. H37Rv was killed by irradiation and resuspended in sterile RPMI media by brief sonication. *Mycobacterium avium* complex serovar 9 (kindly provided by Dr J. K. McClatchy, National Jewish Hospital and Research Centre, Denver, CO) was heat killed at 60°C for 1 h before use.

LPS (*Escherichia coli* 0127:B8; Sigma) was prepared at the appropriate concentrations in RPMI and sterilized by filtration. Zymosan A (Sigma), a carbohydrate-rich cell wall preparation derived from *Saccharomyces cerevisiae*, and consisting of α D-mannan and β D-glucan residues, was prepared as a 5 mg/ml solution in distilled water and autoclaved. The preparation was then diluted to appropriate concentrations in media.

Peritoneal macrophages

Isolation of macrophages and stimulation *in vitro* to produce TNF has been described [17]. Thioglycollate-elicited macrophages were obtained by peritoneal lavage of mice injected intraperitoneally with 1 ml of 4% thioglycollate (Brewer thioglycollate medium; Difco, East Molesey, UK) 3–5 days previously.

Resident peritoneal or thioglycollate-elicited cells were taken up in 5 ml Hanks (Flow) containing 1 U/ml heparin (Leo Laboratories Ltd, Aylesbury, UK) and 5 μg/ml of polymyxin B (Calmic Medical Division, The Wellcome Foundation Ltd, London, UK), to neutralize endotoxin. Washed cells were suspended in RPMI/5% FCS containing 5 μg/ml polymyxin, and counted using trypan blue exclusion. Cells were also checked for phagocytosis by the uptake of 1% neutral red solution. Cells were adjusted to a defined concentration and plated at 2×10^5 viable cells/well (Figs 1 and 2) in 24-well flat-bottomed microtitre plates (Nunc, Roskilde, Denmark). Alternatively, cells were plated in 96-well plates (Nunc) at 8×10^5 cells/well (Fig. 1) or 5×10^5 cells/well (in all other experiments) depending on the number of cells available. The cells were then incubated for 2–4 h at 37°C in an atmosphere of 5% CO_2 in air. Non-adherent cells were removed by washing with warm RPMI, and the medium was replaced with RPMI 5% FCS/5 μg/ml polymyxin containing the stimulants to be tested and incubated overnight (except for LPS, where polymyxin was omitted). Next day supernatants were collected and stored at −20°C until they were assayed for TNF. Cultures incubated with LPS or medium alone were always included as positive and negative controls respectively, on the capacity of the macrophages to yield TNF. All wells were tested in duplicate.

In some experiments peritoneal macrophages were activated for 6 h by the addition of 50 U/well recombinant mouse INF-γ (Genzyme, Cambridge, MA) before washing in warm RPMI and the addition of stimulants.

Bone marrow macrophages

Bone marrow cells were flushed from the femur of female BALB/c mice and grown in Dulbecco's minimum essential medium (DMEM; GIBCO) supplemented with 10% FCS, 10% L-cell conditioned medium [29,30], 25 mM HEPES (Flow), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

The L-cell conditioned medium was prepared by seeding 5×10^5 L929 cells in 50 ml DMEM supplemented with 10% FCS and 0·15 mg/ml gentamycin (Sigma). After 1 week of incubation at 37°C, culture supernatants were harvested, cell debris was removed by centrifugation (3000 ***g***, 10 min), and the supernatant was filtered through a 0·45-μm filter and stored at −20°C until use. Cells were plated at 2×10^5 cells/well in 24-well flat-bottomed microtitre plates and grown for 5 days at 37°C in an atmosphere of 5% CO_2 in air. The cells were washed, and fresh medium was added on day 5 and day 7, with the addition of stimulants at the later time. The cells were stimulated overnight (as with the peritoneal cells) and the supernatants collected for TNF analysis.

ELISA assay for TNF

Microtitre plates (Nunc-Immuno Plate Maxisorp F96) were coated with 50 μl of TN3 19.12, MoAb to TNF-α (kindly provided by Celltech Ltd, Slough, UK) at a concentration of 5 μg/ml sodium carbonate buffer 0·05 M, pH 9·6 (overnight at room temperature). The plates were then blocked for 2 h at 37°C with PBS (GIBCO)/2% bovine serum albumin (BSA; Sigma). Plates were incubated at room temperature for at least 1 h with 50 μl samples (neat or diluted) or recombinant murine TNF-α (Genzyme) in duplicate wells, using two-fold dilutions from 20 ng/ml as a standard.

Following this, the plates were incubated for 1 h at room temperature with 50 μl of a second-step rabbit anti-mouse TNF-α polyclonal antibody (Genzyme; 1:1000 dilution) and a third-step anti-rabbit IgG (whole molecule) peroxidase conjugate (Sigma; 1:500 dilution). The plates were washed three times with PBS/0·2% BSA between each incubation step.

The colour was developed with 100 μl/well of *p*-phenylenediamine dihydrochloride (OPD; Sigma) at a concentration of 1 mg/ml in citrate buffer pH 5 containing 1 μl/ml 30% hydrogen peroxide (Sigma). The absorbance was read at 450 nm in a Titertek Multiskan MCC/340 spectrometer. The amount of

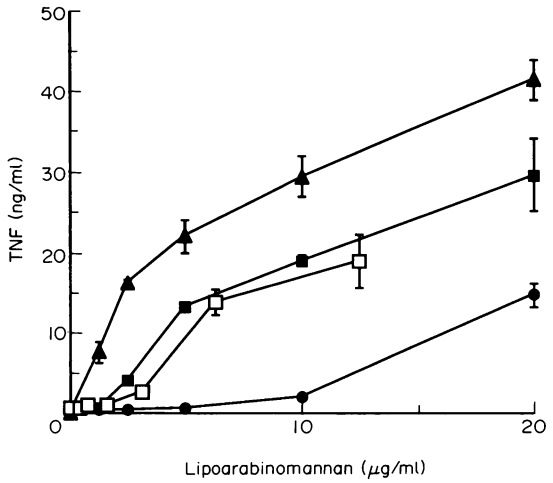

Fig. 1. Dose response of thioglycollate-elicited macrophages to lipoarabinomannan (LAM) from *Mycobacterium avium*, *Myco. tuberculosis* Erdman, H37Ra and H37Rv. Peritoneal macrophages were harvested and plated at 8×10^5 cells/well for stimulation with LAM from *Myco. avium*, Erdman and H37Ra. In a separate experiment, macrophages were plated at 2×10^5 cells/well for stimulation with LAM H37Rv. LAM stimuli were added for overnight incubation and the supernatants assayed for tumour necrosis factor (TNF) by ELISA. Data are expressed as the mean ± s.d. of duplicate wells. ■, *Myco. avium*; ●, Erdman; ▲, H37Ra; □, H37Rv.

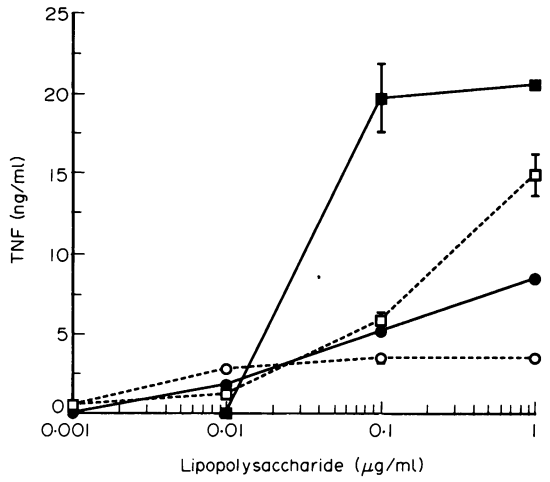

Fig. 2. Dose response of thioglycollate-elicited macrophages taken from individual mice to lipopolysaccharide (LPS). Peritoneal macrophages were harvested and plated at 2×10^5 cells/well as described. LPS stimuli were added for overnight incubation and the supernatant assayed for tumour necrosis factor (TNF) by ELISA. Data are expressed as the mean ± s.d. of duplicate wells.

TNF present in samples was calculated based on the standard curve obtained using recombinant murine TNF-α. It should be noted that macrophages produce a glycosylated form of TNF which may be free or bound to a receptor, whereas the recombinant TNF used as a standard is not glycosylated and not bound to a receptor. Therefore, the amount of TNF calculated based on the standard curve is used primarily in a comparative form between and within experiments.

RESULTS

Previously it has been shown that LAM stimulates TNF production by macrophages, with the avirulent strain of *Myco. tuberculosis* H37Ra being 100-fold more potent at inducing TNF production than the virulent Erdman strain [18]. To extend these findings, we tested LAM from two additional strains of mycobacteria, *Myco. avium* and H37Rv, for TNF-inducing capacity in thioglycollate-elicited macrophages. We confirm that H37Ra LAM is a more potent inducer of TNF production than Erdman, and also that *Myco. avium* and H37Rv LAM appear to be highly stimulatory, similar to H37Ra in TNF-inducing capacity (Fig. 1). This is particularly evident with H37Rv LAM, where the concentration of cells per well was four times lower than with the other LAMs tested, and yet the amount of TNF produced was comparable to that produced with *Myco. avium* LAM.

It became apparent when studying macrophage stimulation that many factors influence TNF production. In the following experiments we therefore attempted to investigate some of these variables. Initially, an experiment was performed to determine the individual variation between mice in TNF production. Thioglycollate-elicited macrophages were isolated from four BALB/c mice of the same age, strain and sex, and tested

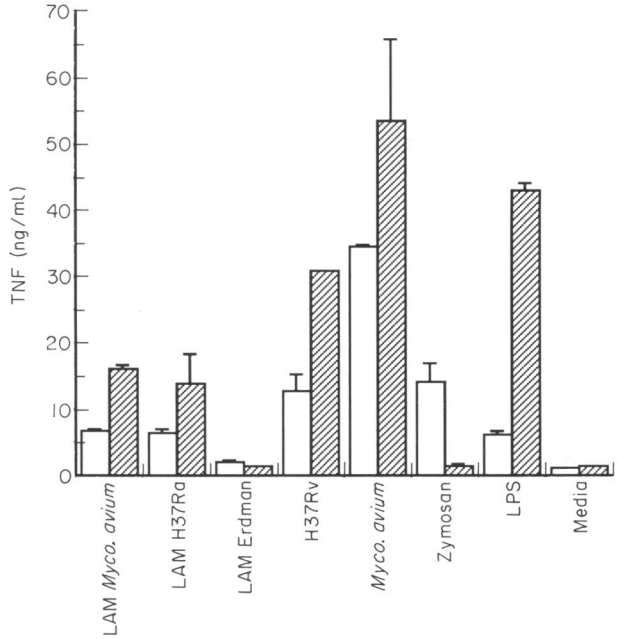

Fig. 3. Resident and thioglycollate-elicited macrophages were harvested and plated at 5×10^5 cells/well as described. Cells were stimulated overnight with 10 μg/ml lipoarabinomannan (LAM), 100 μg/ml *Mycobacterium tuberculosis* H37Rv, 5×10^6 bacteria/well *Myco. avium*, 32 μg/ml zymosan, 5 μg/ml lipopolysaccharide (LPS) or media. The supernatants were assayed for tumour necrosis factor (TNF) by ELISA. Data are from one representative experiment and are expressed as the mean ± s.d. of duplicate wells. □, Resident; ▨, thioglycollate.

Table 1. Stimulation of resident and thioglycollate-elicited macrophages in the presence or absence of IFN-γ

	Resident macrophages		Thioglycollate-elicited macrophages	
	IFN-γ			
	−	+	−	+
LAM *Mycobacterium avium*	1·54±0·04	2·56±0·42	3·52±0·74	7·99±1·66
LAM H37Ra	1·56±0·39	1·89±0·06	3·35±0·71	8·27±0·23
LAM Erdman	0·59±0·08	0·62±0·09	0·60±0·80	0·86±0·03
Myco. tuberculosis	6·17±1·01	9·60±2·18	7·22±0·63	19·21±1·02
Myco. avium	15·42±3·73	14·78±2·33	16·20±1·83	24·53±1·04
LPS	3·66±0·29	8·10±1·30	6·31±1·52	25·65±5·15
Zymosan	3·84±0·17	5·92±0·71	0·80±0·07	4·81±3·47
Media	0·38±0·12	0·33±0·02	0·45±0·05	1·28±1·15

Resident and thioglycollate-elicited macrophages from nine and seven mice respectively were plated at 5×10^5 cells/well, six wells per treatment, and activated for 6 h with 50 U/mL of IFN-γ. Cells were then stimulated with 10 μg/ml lipoarabinomannan (LAM), 100 μg/ml *Myco. tuberculosis* H37Rv, 5×10^6 bacteria/well *Myco. avium*, 5 μg/ml lipopolysaccharide (LPS), 32 μg/ml zymosan or media. Duplicate wells were pooled and triplicates of supernatants were assayed for tumour necrosis factor (TNF) by ELISA. Data are from one representative experiment and are expressed as the mean±s.d. of triplicate wells.

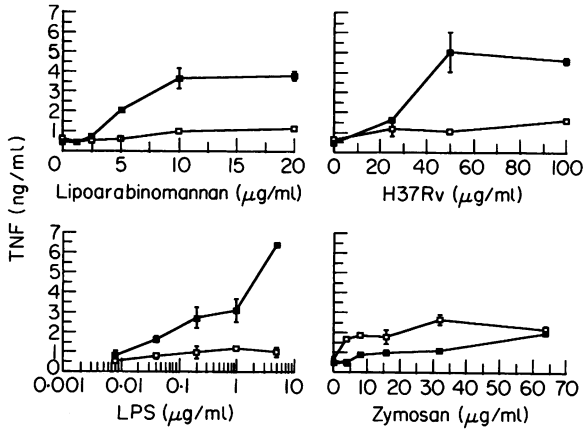

Fig. 4. Dose response of resident (□) and thioglycollate-elicited (■) macrophages to *Mycobacterium avium* lipoarabinomannan (LAM), *Myco. tuberculosis* H37Rv, lipopolysaccharide (LPS) and zymosan. Peritoneal macrophages were harvested and plated at 5×10^5 cells/well as described. Cells were stimulated overnight and supernatants assayed for tumour necrosis factor (TNF) by ELISA. Data are expressed as the mean±s.d. of duplicate wells.

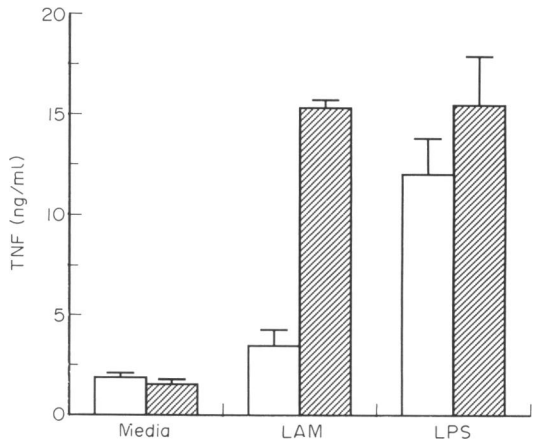

Fig. 5. Bone marrow cells (□) and thioglycollate-elicited macrophages (■) were harvested and plated at 2×10^5 cells/well as described. Cells were stimulated overnight with 1 μg/ml lipopolysaccharide (LPS) and 5 μg/ml H37Rv lipoarabinomannan (LAM) and supernatants assayed for tumour necrosis factor (TNF) by ELISA. Data are expressed as the mean±s.d. of duplicate wells.

individually by stimulating with varying concentrations of LPS, a known inducer of TNF production [31]. A dose response curve to LPS was determined for each of the four mice (Fig. 2). It was found that individual mice varied greatly in the amount of TNF produced, with up to a six-fold difference in the capacity of macrophages from individual mice to be stimulated by LPS. This occurred even though the tests were performed simultaneously using the same reagents and mice matched for strain, age and sex. A similar individual variation between human monocytes stimulated with LAM has been observed previously [17].

It was thought that these differences may be due in part to the type of macrophage present in the peritoneum, and its state of activation. Evidence that suggested the former was the observed difference in TNF produced by resident peritoneal and thioglycollate-elicited macrophages that were stimulated by zymosan and LPS [24]. We therefore decided to compare these two different macrophage populations in their capacity to be stimulated by LAM and whole mycobacteria. To simplify this study we only tested BALB/c mice, although this difference was originally noted in C57Bl/6 mice [24].

Macrophages were isolated from seven thioglycollate-elicited and nine control mice. Due to the small number of cells available from individual mice it was not possible to test mice individually with all the stimuli. Macrophages were therefore pooled before stimulation. Both resident and thioglycollate-elicited macrophages were stimulated with LAM from H37Ra,

Erdman and *Myco. avium*, killed forms of *Myco. tuberculosis* (H37Rv) and *Myco. avium*, zymosan and LPS.

Figure 3 shows that there were differences in TNF produced by different macrophage populations. Resident macrophages produced less TNF than thioglycollate-elicited macrophages with the stimulants *Myco. avium* LAM, H37Ra LAM, H37Rv, *Myco. avium* and LPS. However, with zymosan the resident population produced more TNF than the thioglycollate-elicited population (Figs 3 and 4 and Table 1), a finding which has been reported previously [24]. Stimulation with LAM Erdman produced such low levels of TNF that there was no apparent difference between macrophage populations.

Dose response curves of TNF produced by LPS, LAM and H37Rv stimulation show an increase in production of TNF with an increase in the concentration of stimulants tested (Fig. 4). Furthermore, the stimulation curves produced by LAM and mycobacteria are similar, but as with LPS they vary from the dose response curve observed with zymosan. Also, elicited macrophages from thioglycollate-treated mice appear to be sensitive to even low concentrations of LPS (<100 ng/ml), whereas resident macrophages show little TNF production at any concentration of LPS tested. Therefore the type of macrophage present in the peritoneum certainly influenced TNF production.

To extend these findings we stimulated another population of macrophages that were derived from bone marrow cells. These cells were grown for 7 days in culture and matured into macrophages in the presence of colony-stimulating factor from L929 cells [30]. Bone marrow and thioglycollate-elicited macrophages were stimulated overnight with LPS and H37Rv LAM. Figure 5 shows that bone marrow and thioglycollate-elicited macrophages differ in their response to LAM, although they show a similar response to LPS. In fact, bone marrow macrophages were not stimulated significantly by LAM H37Rv compared with control values.

A further explanation for the variation seen between individual mice and macrophage populations in TNF production was that their macrophage activation state varied. We attempted to investigate this by activating macrophages with IFN-γ for 6 h before stimulation. The results shown in Table 1 indicate that activation with IFN-γ enhanced TNF production by resident and particularly thioglycollate-elicited macrophages. Only in the case of *Myco. avium* stimulation of resident macrophages did IFN-γ have no effect on TNF production. Activated resident macrophages still produced less TNF than activated thioglycollate-elicited macrophages, indicating that the difference seen between resident and thioglycollate-elicited macrophages was not due to IFN-γ activation.

DISCUSSION

Macrophages are a very heterogeneous population of cells [32] which originate from bone marrow precursor cells and are directed via the bloodstream into tissues, where they mature into macrophages. Their heterogeneity is thought to be brought about by differentiation-related mechanisms which generate distinct monocyte phenotypes in the bone marrow [33], and by environmental stimuli which generate further heterogeneity between macrophage populations [34,35]. In these studies, by measuring TNF production, we have shown that bone marrow, resident and thioglycollate-elicited macrophage populations exhibit heterogeneity in their response to mycobacteria and mycobacterial LAM. In fact, in the resident, unelicited state, macrophages show very little TNF production in response to LAM or LPS. It is only when macrophages are elicited by an inflammatory mediator such as thioglycollate, or activated does their TNF production increase. However, resident macrophages do show some response to whole mycobacteria, and in particular *Myco. avium*, where the unelicited population produced TNF to a similar extent to that found in the thioglycollate-elicited population. A possible explanation for the difference seen between TNF produced from resident macrophages stimulated with *Myco. avium* or *Myco. avium* LAM is that in the former case, other antigens aside from LAM may be involved in stimulating TNF production.

In these experiments we measured by ELISA both free bioactive TNF and non-active TNF bound to shed TNF receptors. This method is different from the commonly used L929 bioassay, which measures only the bioactive TNF. Therefore, the amount of bioactive TNF may be completely different from the amount we determined, and the differences seen between resident and thioglycollate-elicited macrophages may be due to differences in the receptor-bound TNF alone. It must be stressed that TNF receptor release was not part of the work presented here, and we were concerned only with the total amount of TNF released under different conditions of stimulation.

It is interesting to speculate on the pathway of LAM stimulation of macrophages. In our experiments resident macrophages were stimulated by zymosan but not by LPS and LAM, suggesting that their pathways of TNF production differ. Zymosan-triggered TNF production by human monocytes may involve complement receptor 3 (CR3; Mac-1; CD11b, CD18) [36]. Therefore, it would seem improbable that CR3 is involved in LAM stimulation of macrophages. However, CR3 may be involved in mycobacteria-stimulated TNF production, where LAM appears not to be involved, such as *Myco. avium* stimulation of resident macrophages. Another receptor involved in zymosan-binding to macrophages is the macrophage mannose/fucose receptor [37,38]. This receptor has been structurally characterized [39,40] and mediates the binding of macrophages to organisms with surface mannose residues or soluble mannose-containing glycoproteins. It has been suggested that this receptor may be involved in LAM binding [18]. However, we consider it unlikely that it would play a direct role in LAM-stimulated TNF production, because, first, the receptor is present on both resident and thioglycollate-elicited macrophages [41], but only the latter is stimulated by LAM to produce TNF. Second, expression of the mannose receptor is inhibited by IFN-γ [42], and yet in our experiments treatment of thioglycollate-elicited macrophages with IFN-γ increased TNF production.

In contrast to zymosan, LPS triggering of human macrophages is mediated by the interaction between CD14 and LPS-binding protein (LBP)–LPS complexes [43,44]. It seems unlikely that LAM-triggered TNF production in murine macrophages involves CD14 binding, because LAM and LPS stimulation differ in that only the latter stimulated bone marrow macrophages.

Activation of macrophages occurs mainly through T cell production of IFN-γ following interaction with antigen [45]. It has been shown that IFN-γ increases TNF production by

human monocytes stimulated with *Myco. tuberculosis* [13]. Similarly, we found that activation of thioglycollate-elicited macrophages before stimulation with LAM also increased TNF production. It seems likely therefore that LAM stimulation of macrophages is also regulated by IFN-γ, which is important in determining the amount of TNF produced and hence the pathology of the disease.

These findings are important in that they indicate that *in vivo* the course of pathology of mycobacterial infections through TNF production may be influenced by the type of macrophage which responds to the infection and its activation state. Furthermore, in relation to the tissue infected, the species of *Mycobacterium* (or strain) and the presence of additional signals such as T cell-derived cytokines and/or concomitant infections represented in this model by zymosan and LPS stimuli, all influence TNF production and the final outcome of the disease.

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