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Role of tumour necrosis factor and reactive oxygen intermediates in lipopolysaccharide-induced pulmonary oedema and lethality

S. GATTI, R. FAGGIONI, B. ECHTENACHER* & P. GHEZZI *'Mario Negri' Institute for Pharmacological Research, Milan, Italy, and *Deutsches Krebsforschungszentrum, Heidelberg, Germany*

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SUMMARY

The purpose of this study was to characterize the role of tumour necrosis factor (TNF) and neutrophils (PMN) in the pathogenesis of pulmonary oedema induced by endotoxin (lipopolysaccharide (LPS)). Intraperitoneal administration to BALB/c mice of 0·6–1 mg of LPS caused pulmonary oedema and lethality. This was associated with production of TNF in serum and bronchoalveolar lavage fluid and with accumulation of PMN in the lung. In this experimental model, we could block TNF production by different means: pretreatment 30 min before LPS with 4 mg/kg of i.p. chlorpromazine (CPZ), 3 mg/kg of i.p. dexamethasone (DEX), 1 g/kg p.o. of N-acetylcysteine (NAC, an antioxidant precursor of glutathione), or an anti-TNF MoAb. CPZ, DEX and anti-TNF completely prevented LPS lethality but not pulmonary oedema or pulmonary PMN infiltration, indicating that: (i) lung oedema is not the main cause of death after LPS; and (ii) lung oedema induced by LPS is not mediated by TNF. Pretreatment with NAC not only inhibited TNF production but also protected against LPS-induced pulmonary oedema, indicating that reactive oxygen intermediates are implicated. NAC also blocked TNF production in blood and in bronchoalveolar lavage. We also tested the effect of PMN depletion induced with cyclophosphamide (CP) or 5-fluorouracil (5-FU). While no pulmonary PMN infiltrate was observed in PMN-depleted mice, neutropenia did not prevent LPS lethality or oedema, indicating PMN do not play an important role in the toxic effects of LPS in this experimental model.

Keywords lipopolysaccharide adult respiratory distress syndrome tumour necrosis factor N-acetylcysteine chlorpromazine

INTRODUCTION

Endotoxin (lipopolysaccharide, LPS) causes several pathological changes characteristic of septic shock, including hypotension, fever, disseminated intravascular coagulation, multiple organ failure, pulmonary oedema and death [1,2].

Several lines of evidence indicate that tumour necrosis factor (TNF) is a key mediator in the pathogenesis of septic shock. In particular, TNF administration reproduces many of the pathological consequences of endotoxic shock and anti-TNF antibodies protect against septic shock in various animal models [3,4]. Furthermore, TNF has been measured in patients with septic shock or sepsis-associated adult respiratory distress syndrome (ARDS) [5], and TNF was found to induce pulmonary damage *in vivo* [6,7]. It was also shown that TNF is induced during hepatic or intestinal ischaemia/reperfusion, and that in these experimental models lung injury develops that is protected by anti-TNF antibodies [8,9].

Although most of the attention has focused on TNF, it should be noted that other cytokines might have a role in mediating LPS-induced pulmonary damage, including IL-1 [10], and the chemotactic cytokine IL-8 [11].

Most of these studies were focused on LPS lethality or its haemodynamic effects, while there is not clear cut evidence for a role of TNF in the development of LPS-induced pulmonary damage. In fact, LPS increases lung microvascular permeability with an early deterioration of respiratory function and induction of severe pulmonary oedema associated with neutrophil (PMN) infiltration and lung endothelial injury [12].

Different inflammatory mediators have been implicated in LPS-induced pulmonary damage or in ARDS. These include eicosanoids [13], platelet-activating factor (PAF) [14], complement components [15] and reactive oxygen intermediates (ROI) [16].

In this respect, a central role for PMN in the pathogenesis of ARDS has often been pointed out [17] and in isolated perfused lung, activated PMN can induce pulmonary oedema [18]. The studies on the protective effect of PMN depletion on LPS-induced pulmonary oedema are controversial. In particular, PMN depletion with hydroxyurea was protective in sheep infused with LPS [19]. On the other hand, other papers have shown that neutropenia induced with nitrogen mustards in

Correspondence: Pietro Ghezzi, 'Mario Negri' Institute for Pharmacological Research, via Eritrea 62, 20157 Milan, Italy.

rabbits or goats does not prevent LPS-induced pulmonary damage [20,21]. It was also pointed out that the protective effect in hydroxyurea-treated animals might not be related to neutropenia but to some non-specific effects of this agent [21]. Likewise, the induction of pulmonary oedema by direct administration of TNF has also been shown to be independent of neutrophils [7].

As far as the role of TNF in LPS-induced pulmonary oedema is concerned, it should be pointed out that LPS has a direct toxic effect on endothelial cells [22], thus making it difficult to determine the relative role of TNF in respect to other LPS-induced cytokines.

The aim of the present study was to investigate the possible role of TNF in a murine model of LPS-induced pulmonary damage using different approaches. In particular, we have characterized LPS-induced TNF production in serum and in bronchoalveolar lavage fluid (BALF), pulmonary oedema, PMN accumulation in the lung and survival. Using a phamacological approach, we studied the effect of pretreatment with drugs previously reported to inhibit TNF production and protect against LPS toxicity, like dexamethasone (DEX) and chlorpromazine (CPZ) [23]. The effect of an anti-TNF MoAb previously reported to protect against LPS toxicity (24) was also studied in order to have a TNF inhibitor to serve as reference.

To identify the biochemical mediators involved, with particular attention to ROI and arachidonic acid oxygenation products, we also studied the effect of an antioxidant, N-acetylcysteine (NAC), and of two inhibitors of prostaglandin synthesis, ibuprofen and indomethacin. We have tested NAC since previous works had shown that this antioxidant attenuated several pathophysiological changes in the LPS model of ARDS in sheep [25] and inhibits the production of TNF and LPS toxicity in mice [26]. The role of PMN in this experimental model has been studied by depleting PMN with cyclophosphamide (CP) and 5-fluorouracil (5-FU).

MATERIALS AND METHODS

Animals and treatments

Male BALB/c mice 20–22 g were obtained from Charles River Italy (Calco, Italy). LPS (Westphal preparation, from *Escherichia coli* O55: B5; Sigma, St Louis, MO), DEX (DEX phosphate, from Istituto Farmacologico Milanese, Milan, Italy) and CPZ (from Farmitalia Carlo Erba, Nerviano, Italy) were injected intraperitoneally in 0·2 ml of sterile, pyrogen-free saline. Appropriate controls received saline alone at the same times. NAC (Zambon, Bresso, Italy) was dissolved in water and administered by oral gavage at the dose of 1 g/kg in a final volume of 0·2 ml, as previously described [26]. When indicated, mice were rendered neutropenic by pretreatment with 5-FU (150 mg/kg intravenously 1 week before the experiment) or CP (150 and 100 mg/kg intraperitoneally on days -5 and -1, respectively) as previously described [27,28]. A rat MoAb (V1q) was prepared and administered to mice as previously described [24]. Briefly, supernatant from cultures of anti-TNF-producing hybridoma was concentrated by ammonium sulphate precipitation, dialysed against sterile PBS and administered intraperitoneally in 0·1 ml 2 h before LPS. Control mice received medium alone added with ammonium sulphate and dialysed as described above. The specificity and activity of this MoAb has been described before [24].

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, 12 Dec., 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

Pulmonary oedema and BALF preparation

At the times indicated mice were killed by ether anaesthesia, blood was collected by cardiac puncture, and lungs were excised and cleared of all extrapulmonary tissue. Total lung wet weight was determined and the specimens were allowed to dry overnight at 80°C and weighed again to determine the dry weight. Pulmonary oedema was expressed as the wet weight-to-dry weight ratio.

For the preparation of BALF, mice were anaesthetized with i.p. chloral hydrate (600 mg/kg), the trachea was cannulated and the lungs infused with 0·3 ml of PBS. BALF was recovered (average fluid recovery was 0·2 ml), clarified by centrifugation at 1000 ***g*** for 10 min and used for TNF assay.

TNF assay

TNF bioactivity was measured in serum or BALF by a standard cytotoxicity assay using L929 cells as target and recombinant TNF as a standard, as previously described [29]. Data are expressed as ng/ml. The detection limit of the TNF assay was 0·08 ng/ml. More than 95% of the cytotoxic activity of sera or BALF in this assay was neutralized by anti-mouse TNF antibody.

Lung myeloperoxidase assay

Lungs were rinsed with saline and immediately homogenized in five volumes of ice-cold 50 mM phosphate buffer, pH 6·0, containing 0·5% hexadecyltrimethylammonium bromide using an Ultra Turrax rotating blade homogenizer. The homogenate was centrifuged at 40 000 ***g*** for 10 min at 4°C and myeloperoxidase (MPO) activity was determined in the supernatants by a spectrophotometric technique [30]. Briefly, the test sample (0·1 ml) was mixed with 2·9 ml of 50 mM phosphate buffer, pH 6·0, containing 0·167 mg/ml *o*-dianisidine dihydrochloride, and 0·005% hydrogen peroxide in a final volume of 3 ml. The optical density change (Δ OD) at 460 nm was measured over 2 min in a spectrophotometer and data expressed as Δ OD/g lung tissue.

Statistical analysis

The data shown are the mean $\pm$ s.e.m. (5–8 mice per group). TNF levels, MPO and lung wet/dry weight ratio were compared using Fisher's test, Student's *t*-test or Dunnett's test. Mortality data were obtained with groups of 10–15 mice. All experiments had been repeated three times, and a typical experiment reported.

RESULTS

Effect of LPS on survival, TNF levels, lung PMN accumulation and pulmonary oedema

We studied the effect of 1 mg/mouse of LPS on survival. It can be seen that at this dose of LPS 100% of mice died within 48 h (Fig. 1a). Since with this dose of LPS 50% of the mice died within 24 h, we have used a sublethal dose (0·6 mg/mouse) to allow determination of the other parameters to be investigated

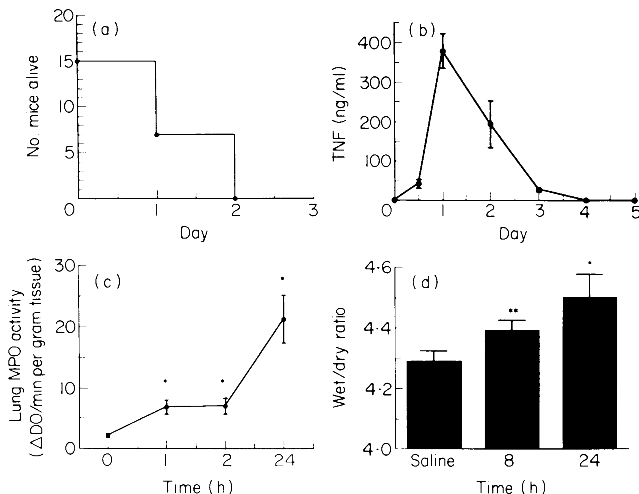

Fig. 1. Effect of lipopolysaccharide (LPS) on survival (a), serum TNF levels (b), lung neutrophil (PMN) accumulation (c), and pulmonary oedema (d). Survival was followed after treatment with 1·0 mg/mouse of LPS (15 mice/group). Serum TNF levels were measured at various times after treatment with 0·6 mg/mouse of LPS and expressed as ng/ml (mean ± s.e.m.; 5 mice/group). Lung PMN accumulation was measured as myeloperoxidase (MPO) activity of lung homogenates in mice treated with 0·6 mg/mouse and expressed as ΔOD/min per gram tissue (mean ± s.e.m.; 5 mice/group). Pulmonary oedema was measured as wet/dry lung weight ratio in mice treated with 0·6 mg/mouse of LPS (mean ± s.e.m.; 8 mice/group; $^*P < 0·01$; $^{**}P < 0·05$ *versus* saline control by Student's *t*-test).

at this time point (pulmonary PMN and oedema, that had to be measured up to 24 h after LPS, see below). Kinetics of serum TNF levels following this dose of LPS displays a clear peak at 1 h, with levels returning to basal by 3 h (Fig. 1b). Lung MPO began to increase at 1 h following LPS and was maximal 24 h later (Fig. 1c). At this dose of LPS a significant pulmonary oedema was induced (Fig. 1d). On the basis of these experiments, we decided to use the following times for the determination of the different parameters: TNF, 1 h after LPS; lung MPO and oedema 24 h after LPS; survival was followed up to 7 days.

Effect of different drugs on LPS-induced pulmonary damage

As shown in Fig. 2a, b, pretreatment (30 min before, i.p.) with CPZ or DEX and to a lesser extent NAC (45 min before LPS, p.o.) protected against LPS-induced lethality and inhibited circulating TNF production (at 1 h) as well as BALF TNF production (at 24 h, Fig. 2e). LPS-induced MPO was markedly inhibited by CPZ and, to a lesser extent, by NAC and DEX, as shown in Fig. 2c. However, only NAC effectively protected against LPS-induced pulmonary oedema (Fig. 2d). Using the same experimental model, we also tested the effect of pretreatment with inhibitors of arachidonic acid metabolism, ibuprofen (15 mg/kg intraperitoneally, 30 min before LPS) and indomethacin (5 mg/kg intraperitoneally, 30 min before LPS). None of these drugs had a protective effect on LPS-induced pulmonary oedema or lethality (data not shown). It should be noted that CPZ and DEX almost completely blocked TNF not only with an LPS dose of 0·6 mg (as in Fig. 2b) but also with the lethal dose of 1 mg (data not shown; [23]). It should also be noted that peak TNF levels varied from 100 to 1500 ng/ml, depending on the experiment. For this reason, all experiments were repeated several times. The inhibitory action of DEX, CPZ and anti-TNF

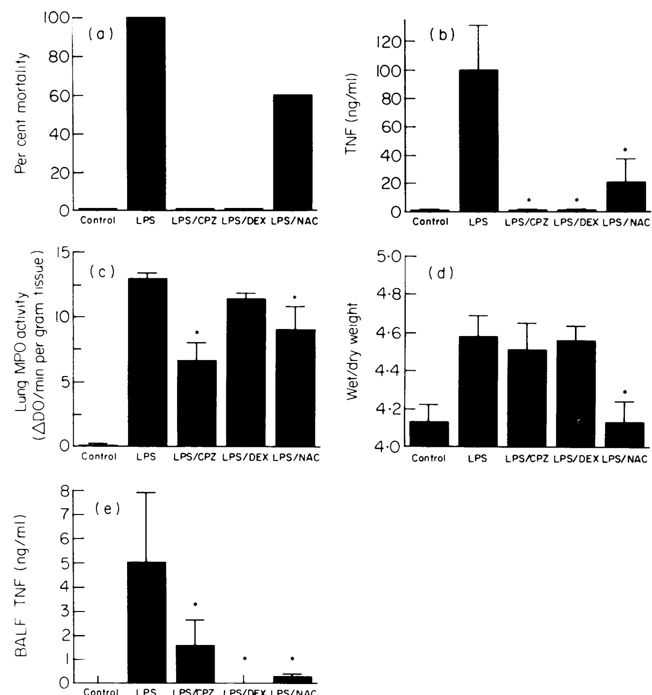

Fig. 2. Effect of different drugs on lipopolysaccharide (LPS)-induced mortality (a), serum TNF levels (b), lung neutrophil (PMN) accumulation (c), pulmonary oedema (d) and bronchoalveolar lavage fluid (BALF) TNF levels (e). Mice were pretreated with chlorpromazine (CPZ) (4 mg/kg intraperitoneally, 30 min before LPS), dexamethazone (DEX) (3 mg/kg intraperitoneally, 30 min before LPS) or N-acetylcysteine (NAC) (1 g/kg, p.o., 45 min before LPS). Times and doses of LPS were as follows: mortality, 7 days after 1 mg/mouse; serum TNF, 1 h after 0·6 mg/mouse; lung myeloperoxidase (MPO), 24 h after 0·6 mg/mouse; lung wet/dry weight ratio, 24 h after 0·6 mg/mouse; BALF TNF, 24 h after 0·6 mg/mouse. $^*P < 0·01$ *versus* LPS alone by Dunnett's test (all data are mean ± s.e.m., 5–8 mice/group).

reported here were reproduced independently of the amount of TNF induced.

Effect of an anti-TNF MoAb

To study the role of TNF induction in the observed effects of LPS, we used as a more specific tool a MoAb against mouse TNF. The results of these experiments are reported in Table 1. It can be seen that administration of this MoAb effectively neutralized circulating TNF levels in LPS-treated mice and protected against LPS lethality as previously reported [24]. However, despite the complete protection observed against LPS toxicity, the anti-TNF antibody was completely ineffective against LPS-induced lung MPO and oedema.

Effect of PMN depletion on LPS-induced pulmonary damage

The role of PMN in the toxicity of LPS was investigated using mice that were rendered neutropenic by treatment with cytotoxic drugs including CP or 5-FU. The results of these experiments are shown in Tables 2 and 3. As expected, the increase of lung MPO was completely blocked in neutropenic mice, thus confirming the specificity of the MPO assay for the quantification of PMN infiltration. However, the toxic effects of LPS in terms of both mortality and pulmonary oedema were unchanged, suggesting that PMN are not required for these

Table 1. Effect of anti-TNF antibody on lipopolysaccharide (LPS)-induced mortality, pulmonary myeloperoxidase (MPO) and oedema

Treatment	Mortality*	TNF†	MPO‡	Lung wet/dry ratio§
Control	0	<0·08	3·3±0·3	4·28±0·04
LPS	60	120±14	6·9±1·0¶	4·53±0·05¶
Anti-TNF/LPS	0**	1±1††	9·1±0·8	4·58±0·02

Mice were pretreated with anti-TNF (0·1 ml; this preparation diluted 1:12800 neutralized 256 U of mouse TNF), 2 h before LPS.

* Per cent mortality. Ten mice per group. Mortality was evaluated 7 days after LPS (1 mg/mouse).

† TNF was measured 1 h after LPS (0·6 mg/mouse) and expressed as ng/ml (mean±s.e.m., 5 mice/group).

‡ Lung MPO activity was measured 24 h after LPS (0·6 mg/mouse) and expressed as ΔOD/min per gram tissue (mean±s.e.m., 7 mice/group).

§ Wet/dry lung weight ratio was measured 24 h after LPS (0·6 mg/mouse) (mean±s.e.m., 8 mice/group).

¶ $P<0{\cdot}01$ *versus* control by Student's *t*-test.

** $P<0{\cdot}01$ *versus* LPS alone by Student's *t*-test.

†† $P<0{\cdot}01$ *versus* LPS alone by Student's *t*-test.

Table 2. Effect of cyclophosphamide (CP)-induced neutropenia on lipopolysaccharide (LPS)-induced mortality, serum TNF, pulmonary myeloperoxidase (MPO) and oedema

Treatment	Mortality*	TNF†	MPO‡	Lung wet/dry ratio§
Control	0	<0·08	1·9±0·1	4·28±0·02
CP	0	<0·08	<0·05	4·61±0·05¶
LPS	100	47·1±17·9	6·2±0·4¶	4·47±0·02¶
CP/LPS	100	29·8±22·4	0·8±0·9**	4·59±0·04¶

Mice were rendered neutropenic by pretreatment with CP (150 and 100 mg/kg intraperitoneally on days −5 and −1, respectively).

* Per cent mortality. Ten mice per group. Mortality was evaluated 7 days after LPS (1 mg/mouse).

† TNF was measured 1 h after LPS (0·6 mg/mouse) and expressed as ng/ml (mean±s.e.m., 5 mice/group).

‡ Lung MPO activity was measured 24 h after LPS (0·6 mg/mouse) and expressed as ΔOD/min per gram tissue (mean±s.e.m., 7 mice/group).

§ Wet/dry lung weight ratio was measured 24 h after LPS (0·6 mg/mouse) (mean±s.e.m., 8 mice/group).

¶ $P<0{\cdot}01$ *versus* control by Student's *t*-test.

** $P<0{\cdot}01$ *versus* LPS alone by Student's *t*-test.

effects of LPS. It should be noted that, unlike 5-FU, CP *per se* induced some pulmonary oedema, as previously described [31].

DISCUSSION

In the present study we investigated the role of TNF in the toxic effects of LPS (lethality and pulmonary oedema) using different experimental approaches: inhibition of TNF production by CPZ, NAC or DEX and neutralization of TNF with an anti-TNF MoAb. Inhibition of TNF by either drugs or antibodies was always associated with a protective effect against LPS toxicity, thus confirming the key role for TNF as a mediator of endotoxic shock.

On the other hand it is clear, particularly from the data with DEX and the anti-TNF antibody, that protection against LPS toxicity can be achieved without pulmonary oedema, and therefore it is likely that pulmonary oedema is not the main cause of mortality in LPS-treated mice. Furthermore, these data indicate that TNF is not an essential mediator for LPS-induced pulmonary oedema. Likewise, since the anti-TNF antibody did not inhibit LPS-induced lung MPO, it could also be concluded that TNF does not mediate the accumulation of PMN in the lung after i.p. injection of LPS. The fact that some of the toxic effects of LPS are still observed when TNF is blocked is not surprising, since previous studies have shown that LPS can be directly toxic to endothelial cells *in vitro*, indicating that not all of the effects of LPS are necessarily mediated via cytokines. Furthermore, it seems obvious that other cytokines different from TNF might be involved in LPS-induced pulmonary damage, particularly IL-1 and IL-8.

A second aspect of this study is the role of PMN in LPS toxicity and lung oedema. The experiments in which neutropenia was induced using two different drugs (CP and 5-FU) clearly indicate that PMN depletion, while completely blocking LPS-induced lung PMN infiltration, does not ameliorate LPS lethality or pulmonary oedema. These results are in agreement with other studies indicating that neutrophil depletion is not protective against LPS-induced pulmonary damage [20,21], and that ARDS can occur in humans in the setting of severe neutropenia [32]. This lack of a role of PMN in LPS-induced

Table 3. Effect of 5-fluorouracil (5-FU)-induced neutropenia on lipopolysaccharide (LPS)-induced mortality, serum TNF, pulmonary myeloperoxidase (MPO) and oedema

Treatment	Mortality*	TNF†	MPO‡	Lung wet/dry ratio§
Control	0	<0·08	<0·05	4·45±0·01
5-FU	0	<0·08	<0·05	4·49±0·01
LPS	100	6·8±0·9	11·6±1·4	4·65±0·01¶
5-FU/LPS	100	5·8±1·3	<0·05	4·71±0·07¶

Mice were rendered neutropenic by pretreatment with 5-FU (150 mg/kg intravenously 1 week before the experiment).

* Per cent mortality. Ten mice per group. Mortality was evaluated 7 days after LPS (1 mg/mouse).

† TNF was measured 1 h after LPS (0·6 mg/mouse) and expressed as ng/ml (mean±s.e.m., 5 mice/group).

‡ Lung MPO activity was measured 24 h after LPS (0·6 mg/mouse) and expressed as ΔOD/min per gram tissue (mean±s.e.m., 7 mice/group).

§ Wet/dry lung weight ratio was measured 24 h after LPS (0·6 mg/mouse) (mean±s.e.m., 8 mice/group).

¶ $P<0{\cdot}01$ *versus* control by Student's *t*-test.

pulmonary oedema leaves open the possibility that other effector cells (e.g. monocytes/macrophages) are involved.

A third aspect that was taken into consideration in the present study is the role of ROI and eicosanoids in the lethality of LPS and pulmonary oedema. Our results indicate that, while NAC was less potent than DEX or CPZ in inhibiting TNF production and protecting against LPS toxicity, it was the only drug that protected against LPS-induced pulmonary oedema. One likely interpretation for these results is that NAC not only acts by inhibiting TNF production, but also inhibits LPS actions at different sites, possibly involving an oxidative damage. In fact, amelioration of LPS toxicity by antioxidants was reported, indicating that ROI are important second messengers for LPS toxicity [33].

On the other hand, the results obtained with indomethacin and ibuprofen suggest that, at least in our experimental model, eicosanoids do not play a major role in either LPS toxicity or pulmonary oedema. It may be important to note that non-steroidal anti-inflammatory drugs potentiate cytokine induction by LPS *in vivo* [34], indicating that eicosanoids, and particularly prostaglandins, rather represent feedback regulators of cytokine synthesis, in addition to protecting against their toxicity [35], thus complicating the interpretation of their use as tools in studies on cytokine-mediated pathologies.

This study suggests that PMN and TNF do not have an essential role in LPS-induced pulmonary oedema. Further studies will be necessary to clarify whether a direct effect of LPS is involved or if it is mediated by other LPS-induced cytokines. For instance, IL-1 and interferon-gamma (IFN-γ) were both shown to increase endothelial cell permeability *in vitro* [36,37], and their role *in vivo* is clearly worth being considered.

The data with NAC strongly support the hypothesis that ROI might play a key role in LPS-induced pulmonary oedema. It will be important to define whether LPS induces an oxidative stress directly or through the action of cytokines. While TNF itself has been shown to be toxic by mechanisms involving ROI, as demonstrated by the protective effect of antioxidants on TNF toxicity *in vivo* and *in vitro* [38], other cytokines might induce an oxidative stress, particularly IFN, which was shown to induce superoxide-generating xanthine oxidase [39,40]. From this and other studies it seems likely that LPS-induced pulmonary oedema does not involve a single mediator but is a rather complex phenomenon, and its understanding is obviously necessary for a rational therapeutic approach to ARDS.

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