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Effect of cyclo-oxygenase inhibitors and modulators of cyclic AMP formation on lipopolysaccharide-induced neutrophil infiltration in mouse lung

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1 The adult respiratory distress syndrome (ARDS) is an acute lung inflammation developed after direct or indirect contact with pathogenic agents. In the present study, a mouse model was developed to mimic this condition using aerosolized bacterial lipopolysaccharide (LPS) and to investigate the mechanisms involved in the lung inflammatory response.

2 Inhalation of LPS led to a time and dose-dependent increase in tumour necrosis factor-α (TNF-α) production and neutrophil recruitment into the bronchoalveolar lavage fluid (BALF) of Balb/c mice. Under the same conditions, neutrophil infiltration was also found in the BALF of the LPS-sensitive mouse strain C3H/HeN, but was absent in the LPS-resistant strain C3H/HeJ. Intranasal administration of murine recombinant TNF-α also triggered neutrophil recruitment.

3 One hour after inhalation of LPS, half of the maximal level of TNF-α was measured in the BALF, but only a few neutrophils were detected at this time. The peak TNF-α concentration was reached at 3 h, when the neutrophil amount started to increase. At 24 h, maximal neutrophil number was found in the BALF and TNF-α was no longer present.

4 Pretreatment of mice under different experimental conditions demonstrated that: (a) cycloheximide almost completely blocks both neutrophil recruitment and TNF-α production; (b) anti TNF-α antibodies block neutrophil recruitment; (c) indomethacin or aspirin enhance by two fold neutrophil recruitment; (d) indomethacin significantly increases TNF-α production 1 h after inhalation of LPS; (e) dibutyryl cyclic AMP and prostaglandin E_2 (PGE_2) block both neutrophil recruitment and TNF-α production.

5 It is concluded that aerosolized LPS in mice triggers an acute lung inflammation which can be used as a potential model of inhalational ARDS and that, strategies leading to the elevation of cyclic AMP levels *in vivo* can be effective in modulating LPS-induced TNF-α synthesis and neutrophil recruitment.

Keywords: Aspirin; indomethacin; cyclic AMP; lipopolysaccharide; tumour necrosis factor-α; neutrophil; lung

Introduction

In vivo administration of bacterial lipopolysaccharide (LPS) triggers a network of inflammatory responses. One of the primary events is the activation of mononuclear phagocytes through a receptor-mediated process (Ulevith & Tobias, 1994; Watson *et al.*, 1994), leading to the release of different cytokines, including tumour necrosis factor-α (TNF-α), considered as one of the most important mediators of endotoxin-induced tissue injury (Beutler *et al.*, 1985; Tracey *et al.*, 1986; Watson *et al.*, 1994). Indeed, high levels of TNF-α have been correlated with the severity of pathological disturbances caused by endotoxin (Tracey *et al.*, 1986; Gorgen *et al.*, 1992). Once released, TNF-α favours the migration and sequestration of neutrophils which play a critical role in the pathogenesis of lung inflammation (Ulich *et al.*, 1991; 1993; Deni *et al.*, 1994; Steinberg *et al.*, 1994), including adult respiratory distress syndrome (ARDS). The increased adherence of neutrophils to endothelial cells induced by TNF-α leads to their massive infiltration in pulmonary spaces (Albelda *et al.*, 1994; Ulich *et al.*, 1995). This process has initially a defensive function, but once activated, neutrophils release proteolytic enzymes and free radicals that can cause tissue injury and in some cases organ failure. Data obtained from patients or from animal models of lung injury show a close correlation between neutrophil accumulation and tissue damage (Steinberg 1994; Kollef & Schuster, 1995). Moreover, lung injury following aspiration, trauma, shock or sepsis is characterized by a marked infiltration of neutrophils which is associated with the severity of ARDS (Steinberg *et al.*, 1994).

In the present study, a mouse model was developed to investigate the mechanisms involved in lung neutrophil recruitment induced by aerosolized LPS. This procedure mimics the inflammation caused by LPS present in the inhaled air and minimizes the toxic effects of LPS, particularly on endothelial cells (Meyrick, 1987). It may thus provide a model for ARDS caused by direct (inhalational) rather than indirect (hematogenous) pulmonary insults.

Methods

Animals and experimental protocol

Male BALB/c mice weighing 25–30 g (Iffa-Credo, France) were employed in this study. In some experiments C3H/HeN and C3H/HeJ mice (Charles River, France) were also used. Drugs injected intraperitoneally (i.p.) or intra-nasally (i.n.) were prepared in saline. For oral administration (p.o.) they were dissolved in 0.5% carboxymethyl cellulose (CMC) prepared in saline. Protocols for administration and intervals before LPS inhalation were as follows: (a) indomethacin

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(3 mg kg^{-1}) - p.o. (1 h); (b) aspirin (50 mg kg^{-1}) - i.p. (30 min); (c) PGE_2 (70 μg) or db cyclic AMP (2.5 μg) - i.n. (30 min); (d) cycloheximide (1 mg) - i.p. (1 h); (e) sheep IgG or anti-TNF-α antibodies (150 and 250 μl) - intravenously (1 h).

Animals slightly anaesthetized with ether received directly into their muzzles 50 μl of a solution of db cyclic AMP or PGE_2 in saline; rTNF-α in 0.2% bovine serum albumin (BSA) or inactivated rTNF-α, obtained by boiling the solution for 1 h. Control groups received saline or 0.2% BSA, respectively.

The inhalation chamber was made with a buchner of 1 L adapted with conical tubes of glass coupled to a manometer. Groups of 5 to 6 mice were put in the tubes to inhale aerosols of LPS dissolved at different concentrations in 2 ml of saline for 10 min. After different time intervals, animals were anaesthetized with 12 mg kg^{-1} of sodium pentobarbitone i.p., tracheae were cannulated and lungs washed 8 times with 0.5 ml saline to provide 4 ml of bronchoalveolar lavage fluid (BALF). Aliquots of each BALF were used to evaluate the total and differential cell numbers and to assay TNF-α.

Leukocyte analysis

Total cells present in the BALF were counted with a Coulter counter ZM (Coultronics, Margency, France) and values expressed as number of cells ml^{-1}. Differential cell counts were performed after cytocentrifugation (Hettich-Universal) and staining with Diff-Quik stain (Baxter Dade AG, Dudingen, Germany). At least 250 cells were counted and results are expressed as number of each cell population ml^{-1}.

TNF-α assay

TNF-α levels in the BALF were determined by a highly specific ELISA with a detection limit of 50 pg ml^{-1}. The assay system was established by Mrs C. Dumarey in our laboratory by using a rat anti-murine TNF-α polyclonal antibody and a rabbit anti-murine TNF-α polyclonal antibody from Endogen Inc. (MA, U.S.A.) and a peroxidase-labelled goat anti-rabbit IgG from BioSys (Compiègne, France).

Materials

Escherichia coli lipopolysaccharide (lot 55:B5) was purchased from Difco Lab. (Detroit, MI, U.S.A.); indomethacin, aspirin, prostaglandin E_2 (PGE_2) and dibutyryl adenosine 3':5'-cyclic monophosphate (db cyclic AMP) from Sigma Chem. Co. (St. Louis, MO, U.S.A.); cycloheximide from Merck (Darmstadt, Germany); sodium pentobarbitone from Sanofi (Libourne, France). Murine recombinant TNF-α (*E. coli*-derived) was kindly provided by Dr G.R. Adolf from E. Boehringer Institute (Vienna, Austria). The rat anti-mouse TNF-α monoclonal antibody (V1q0494) was a gift from Dr B. Echtenacher (Universtat Rigensburg, Rigensburg, Germany). The sheep anti-mouse TNF-α serum, which was prepared as previously described (Mahadevan *et al.*, 1990), specifically neutralises mouse TNF-α and is inactive against other murine cytokines, including interleukin-1 (IL-1), IL-6 and TNF-β.

Statistical analysis

Data were analyzed by the Statsworks program. Differences between means were evaluated by use of Student's unpaired *t* test and considered to be statistically significant when $P<0.05$. Results are expressed as means ± s.e.mean. *Indicates $P<0.05$.

Results

Effects of LPS inhalation on neutrophil recruitment and TNF-α production

Inhalation of aerosolized LPS (0.1, 0.3 and 1.0 mg ml^{-1}) induced a time and dose-dependent neutrophil recruitment into the BALB/c mice BALF (Figures 1a and b). One hour after inhalation, few neutrophils were detected. Three and 6 hours later this number increased, reaching the maximum level 24 h after stimulation. Neurotrophil recruitment spontaneously decreased and disappeared after 96 h. The effect of 0.3 mg ml^{-1} LPS was also investigated on C3H mice. Figure 1c shows that 3 h after inhalation of LPS, neutrophils were recruited into the BALF of the C3H/HeN mice, a strain sensitive to LPS, whereas no neutrophils were detected in the BALF of the C3H/HeJ mice, a resistant strain (Sultzer *et al.*, 1993).

Inhalation of LPS was also followed by a dose-dependent release of TNF-α into the BALF (Figure 2a). One hour after inhalation of LPS, the TNF-α concentration reached 50% of its maximal level, at a time when only few neutrophils were found (Figure 1a), indicating that synthesis of TNF-α precedes neutrophil recruitment. Maximal levels of TNF-α were measured in the BALF 3 h after inhalation of 0.3 mg ml^{-1} LPS (Figure 2b). Pretreatment of mice with 1 mg of the protein synthesis inhibitor cycloheximide i.p., 1 h before inhalation, strongly reduced both TNF-α and neutrophil concentrations in the BALF (Figure 3). To establish a relationship between TNF-α production and lung neutrophil recruitment, mice received 150 or 250 μl of sheep anti-mouse TNF-α antiserum i.v. 1 h before stimulation with LPS. As shown in Figure 4a, this treatment significantly reduced

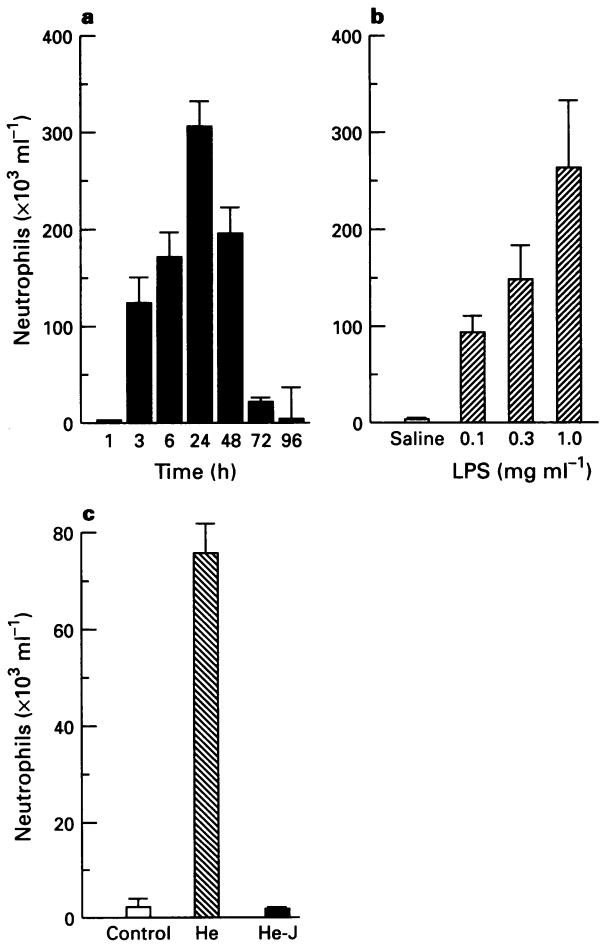

Figure 1 Effect of inhalation of LPS on neutrophil recruitment. Neutrophil number in BALF of BALB/c or C3H mice was determined as described under methods. BALF were collected: in (a) at different time intervals after inhalation of 0.3 mg ml^{-1} LPS; in (b) 3 h after inhalation of saline (open column); 0.1, 0.3 or 1.0 mg ml^{-1} LPS (hatched columns); in (c) BALF of C3H mice were collected 3 h after inhalation of saline by HeN or HeJ (open columns) or 0.3 mg ml^{-1} LPS by C3H/H3N (hatched column) or C3H/HeJ (solid column). Results are expressed as mean ± s.e.mean of 5–6 animals.

the number of neutrophils in the BALF. The same result was obtained with rat anti-mouse TNF-α antibody V1q0494 (data not shown). The involvement of TNF-α in neutrophil recruitment was confirmed by treating animals with rTNF-α intranasally. As shown in Figure 4b, direct treatment with rTNF-α also induced neutrophil recruitment. To eliminate a possible contamination of rTNF-α with LPS, a control group was treated with heat-inactivated rTNF-α. Results showed that under these conditions neutrophil recruitment was almost completely abolished (data not shown).

Effects of non-steroidal anti-inflammatory drugs (NSAIDS)

Mice were pretreated with two inhibitors of cyclo-oxygenase, indomethacin and aspirin. As shown in Figure 5a, oral administration of 3 mg kg^{-1} indomethacin or intraperitoneal injection of 50 mg kg^{-1} aspirin increased the neutrophil numbers in the BALF of animals after inhalation of LPS.

The enhancement of the neutrophil number in the BALF by NSAID might be due to an increase of TNF-α levels. In order to evaluate this possibility, TNF-α was measured in the BALF of indomethacin-treated mice, 1 h after inhalation of 0.3 mg ml^{-1} LPS, i.e., before the maximal level had been reached. As seen in Figure 5b, administration of indomethacin increased TNF-α concentrations.

TNF-α production and neutrophil recruitment under conditions that increase cyclic AMP

The paradoxical effect of indomethacin might be explained by the decreased production of cyclic AMP due to the blockade of PGE_2 synthesis in a target cell. In order to test this possibility, mice were pretreated with 70 μg PGE_2 or 2.5 μg db cyclic

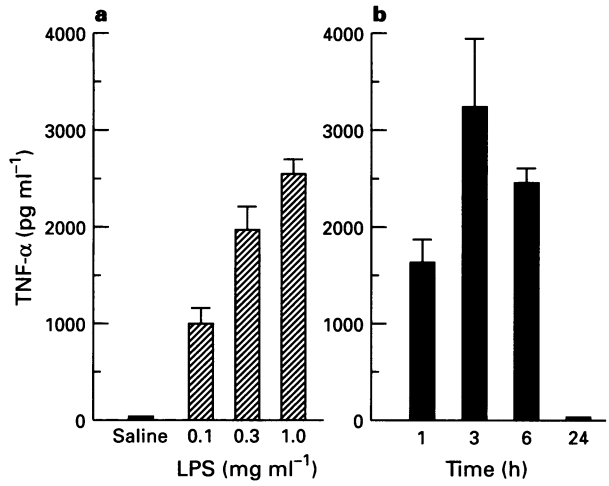

Figure 2 Effect of inhalation of LPS on TNF-α concentration. TNF-α was detected in BALF of BALB/c mice by ELISA method in (a) after inhalation of saline; 0.1, 0.3 or 1.0 mg ml^{-1} LPS, and in (b) at different time intervals after inhalation of 0.3 mg ml^{-1} LPS. Results are expressed as means ± s.e.mean of 5–6 animals.

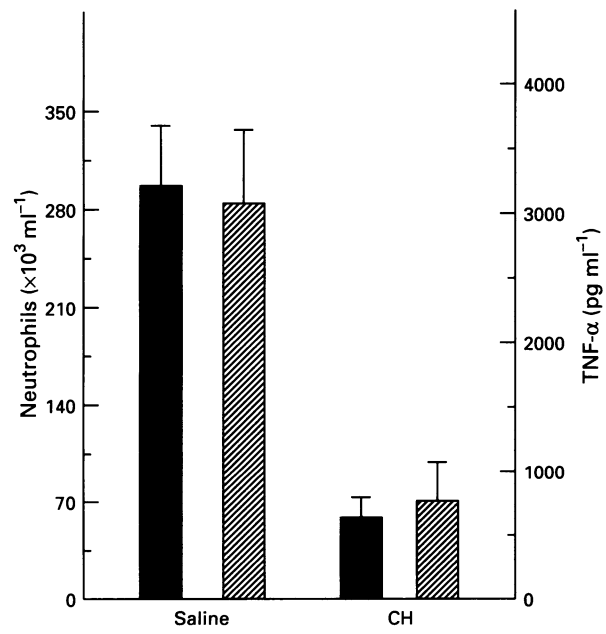

Figure 3 Effect of pretreatment with cycloheximide (CH) on neutrophil recruitment and TNF-α production. Each mouse received saline or 1 mg cycloheximide i.p. 1 h before inhalation of 0.3 mg ml^{-1} LPS. BALF were collected 3 h after inhalation and TNF-α concentration (hatched columns) and neutrophil number (solid columns) were determined. Results are expressed as mean ± s.e.mean of 5–6 animals.

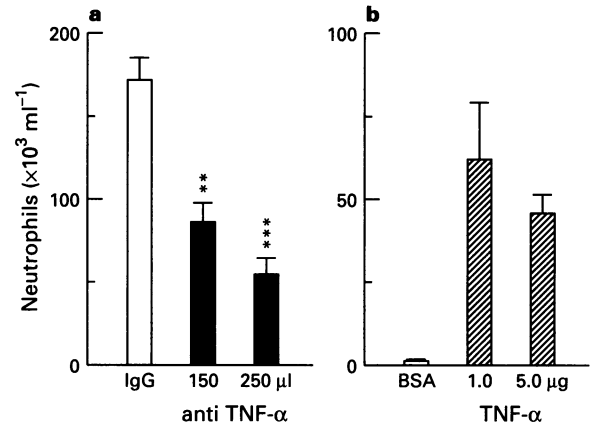

Figure 4 Effect of anti-TNF-α antibody and rTNF-α on neutrophil recruitment. In (a) each mouse received 250 μl of sheep IgG (open column), 150 μl or 250 μl of Sheep anti-mouse TNF-α (solid columns), i.v., 1 h before inhalation of 0.3 mg ml^{-1} LPS. In (b) mice were treated with 0.2% BSA (open column), 1.0 or 5.0 μg murine rTNF-α (hatched columns) intranasally. BALF were collected 3 h after inhalation or instillation and neutrophil number was determined. Results are expressed as mean ± s.e.mean of 5–6 animals.

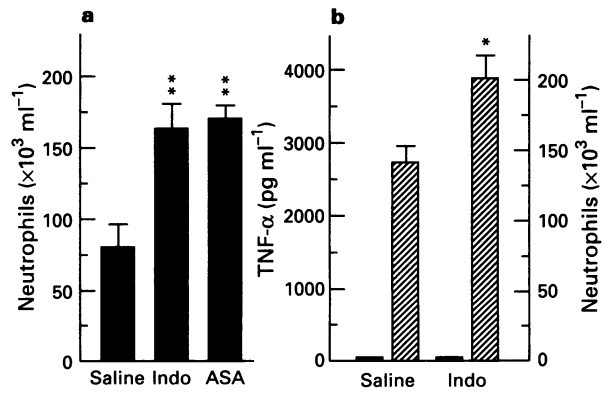

Figure 5 Effect of NSAIDs on neutrophil recruitment and TNF-α production. In (a) mice were pretreated with saline p.o., 3 mg kg^{-1} indomethacin p.o. (Indo) or 50 mg kg^{-1} aspirin, i.p., (ASA) as described under methods. Neutrophil number were determined 3 h after inhalation of 0.3 mg ml^{-1} LPS. In (b) mice were pretreated with saline or 3 mg kg^{-1} indomethacin, p.o., as described. Neutrophil number (solid columns) and TNF-α concentration (hatched columns) were determined in BALF collected 1 h after inhalation of 0.3 mg ml^{-1} LPS. Results are expressed as mean ± s.e.mean of 5–6 animals.

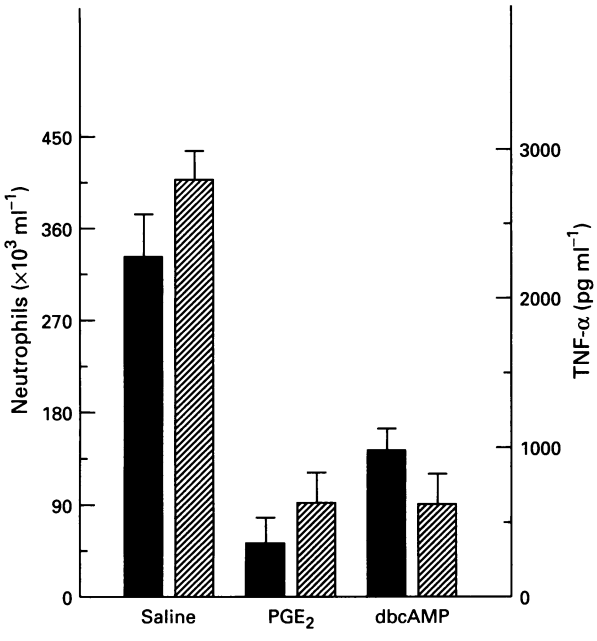

Figure 6 Effect of dibutyryl cyclic AMP and PGE_2 on neutrophil recruitment and TNF-α production. Mice were pretreated with saline or 70 μg PGE_2, i.n., or 2.5 μg db cyclic AMP, i.n., 20 min before inhalation of 0.3 mg ml^{-1} LPS. BALF were collected 3 h after inhalation and TNF-α concentration (hatched columns) or neutrophil number (solid columns) were determined. Results are expressed as mean ± s.e.mean of 5–6 animals.

AMP i.n. before the inhalation of LPS. These treatments led to suppression of both TNF-α production and neutrophil infiltration (Figure 6).

Discussion

In this study, inhalation of LPS was followed by the early synthesis of TNF-α and by a delayed massive neutrophil infiltration into mice lungs. Indeed, the formation of TNF-α reached its half-maximal level in the BALF 1 h after provocation, when neutrophils were not yet detected. This is consistent with data showing that the transcription of TNF-α starts after 15 min, with TNF mRNA peaking within 45 min after LPS addition to macrophage-like cell cultures (Taffet *et al.*, 1989). The massive infiltration of neutrophils that follows TNF-α release strongly suggests that this cytokine mediates their migration into the lungs after inhalation of LPS, as demonstrated in other inflammatory processes (Ulich 1991; 1993; Steinberg *et al.*, 1994). From the literature, it can be hypothesized that the relationship between neutrophil infiltration and TNF-α may be due to the chemotactic property of TNF-α (Ming *et al.*, 1987) associated with an induction of the synthesis of proteins such as adhesion molecules (Albelda *et al.*, 1994) and members of the interleukin 8 family (Smart & Casale, 1994; Ulich 1995). Indeed, the recruitment of neutrophils was strongly blocked when mice were pretreated with cycloheximide. Although this treatment is not specific, the simultaneous suppression of TNF-α formation suggests that its synthesis may be an essential step for neutrophil recruitment. In fact, pretreatment of mice with two different anti-TNF-α antibodies, significantly reduced neutrophil recruitment, while intranasal administration of rTNF-α triggered this recruitment. Although it cannot be ruled out that other mediators participate, our data clearly indicate that TNF-α is the most important mediator which accounts for the massive neutrophil influx in the airspaces after inhalation of bacterial endotoxin.

In order to investigate the role of cyclo-oxygenase metabolites in this model, aspirin and indomethacin were administered to mice before LPS. Unexpectedly, this treatment augmented neutrophil infiltration. This effect might be explained by the ability of these drugs to increase leukocyte-endothelium adherence, as observed in gastric mucosal injury (Wallace *et al.*, 1993), or to enhance TNF-α production (Pettipher & Wimberly, 1994). In the present case, indomethacin was indeed able to increase TNF-α production *in vivo*, while treatment with PGE_2 had a protective effect, impairing TNF-α production and neutrophil recruitment. It was inferred that this enhanced recruitment might be associated with the suppression of PGE_2 synthesis by the anti-inflammatory drugs. In fact, it is widely accepted that PGE_2 is an important mediator during the host response to infection and inflammation (Spengler *et al.*, 1989; Pettipher & Wimberly, 1994; Watson *et al.*, 1994). Furthermore, recent data provide evidence that PGE_2 can suppress the synthesis of TNF-α induced by LPS *in vitro* (Spengler *et al.*, 1989; Eisehut 1993).

In fact, the protective effect of PGE_2 is associated with increased intracellular cyclic AMP levels (Schultz *et al.*, 1978), a recognized mechanism for the down-regulation of inflammatory cell functions. Accordingly, a procedure was used to verify if this mechanism operates under our experimental conditions. Mice received directly PGE_2 or the analogue db cyclic AMP, before LPS inhalation. In both cases, TNF-α formation and neutrophil infiltration were blocked. This is in agreement with the finding that db cyclic AMP suppresses LPS-induced TNF mRNA accumulation by peritoneal macrophages *in vitro* (Spengler *et al.*, 1989).

In summary, this study allowed us to conclude that we have developed a potential model for understanding the mechanisms involved in inhalational ARDS. Additionally, we demonstrated that strategies leading to elevation of cyclic AMP levels *in vivo* can be effective in controlling TNF-α production and neutrophil infiltration.

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