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Endotoxin Induces Late Increase in the Production of Pulmonary Proinflammatory Cytokines in Murine Lupus-Like Pristane-Primed Modelp

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Lupus-like syndrome is characterized by multiple organ injuries including lungs and kidneys. Endotoxin induces a transiently intent systemic inflammatory response and indirectly transient acute lung injury in normal condition. However, whether endotoxin may trigger the persistent development of lung injury in chronic, inflammatory lupus-like syndrome compared with normal condition remains unclear. We examined the pulmonary vascular permeability and production of proinflammatory cytokines, such as TNF-α, IL-6, IL-10 and IFN-γ, which play prominent roles in the pathogenesis of lupus-like tissue injury, 6 h and 72 h after *i.p.* lipopolysaccharide (LPS; endotoxin) injection in pristane-primed chronic inflammation ICR mice characterized by a lupus-like syndrome. These results demonstrated that levels of serum IL-6, IL-10 and IFN-γ and bronchoalveolar lavage (BAL) IL-6 and IFN-γ were remarkably increased 6 h in LPS-exposed pristane-primed mice compared with pristane-primed controls, while pulmonary vascular permeability and levels of serum and BAL TNF-α were not. And levels of BAL TNF-α, IL-6 and IL-10 were significantly enhanced 72 h in LPS-exposed pristane-primed mice compared with pristane-primed controls. Also, LPS significantly induced the increased *in vitro* production of TNF-α, IL-6 and IL-10 by lung cells obtained from LPS-exposed pristane-primed mice compared with LPS-exposed normal mice. Our findings indicate that LPS may trigger persistent progression of lung injury through late overproduction of BAL TNF-α, IL-6, and IL-10 in lupus-like chronic inflammation syndrome compared with normal condition.

Key words: TNF-α, IL-6, IL-10, IFN-γ, LPS, Lung vascular permeability, Pristane, Chronic inflammation

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic, inflammatory and systemic autoimmune disease that causes in the tissue of the brain, endothelial cells, joints, kidneys, lungs, muscles, and skin (Childs, 2006). Lung injury results from intense inflammatory responses in the lungs directly or indirectly *via* an extrapulmonary source of lupus-like autoimmune diseases or sepsis (Czermak *et al.*, 1999; Karim *et al.*, 2002).

Endotoxin, a component of the cell walls of gram-negative bacteria, is also known to induce a transiently intent systemic inflammatory response and indirectly transient acute lung injury (Neumann *et al.*, 1999; Rojas *et al.*, 2004). Overexpression of proinflammatory cytokines in plasma and BAL fluids contributes to pulmonary pathogenesis or lung injury. LPS markedly induces upregulated production of proinflammatory cytokines in plasma and BAL fluids at early phase, and then the peaks immediately return to normal levels (Martin, 1997; Bauer *et al.*, 2000). However, persistently elevated proinflammatory cytokines including BAL TNF-α, IL-1β, and IL-6 led to continuous lung injury process leading to high mortality in sepsis (Meduri *et al.*, 1995a; Meduri *et al.*, 1995b). Also, IL-10 is an important mediator of the enhanced susceptibility to pneumococcal pneumonia after influenza infection and associated with lung fibrosis and lung disease (Martinez *et al.*, 1997; van der Sluijs *et al.*, 2004; Barbarin *et al.*, 2005). However, whether endotoxin triggers persistent development of lung injury in lupus-like syndrome is

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unclear (Christen and von Herrath, 2005).

Proinflammatory cytokines such as TNF-α, IL-6, IL-10, and IFN-γ play prominent roles in the pathogenesis of lupus-like tissue injury (Malide *et al.*, 1995; Balomenos *et al.*, 1998; Mihara *et al.*, 1998; Park *et al.*, 1998). High levels of the proinflammatory cytokines, TNF-α, IL-1 and IL-6, were maintained throughout lupus-like disease course (Segal *et al.*, 1997; Dean *et al.*, 2000). Also, IL-10 is produced at a high level by B lymphocytes and monocytes of patients with SLE, which contributes to pathogenesis and high mortality (Ishida *et al.*, 1994; Llorente *et al.*, 1995). IFN-γ has been found to be required for lupus-like disease and lymphoaccumulation in MRL-*lpr* mice and to play prominent roles in the pathogenesis of murine lupus-like tissue injury (Balomenos *et al.*, 1998). Lupus is associated with collagen vascular disease and pulmonary dysfunction (Karim *et al.*, 2002).

Therefore, we examined the effect of LPS on the early and late production of proinflammatory cytokines and pulmonary vascular permeability in pristane-primed lupus-like syndrome. These results showed that LPS induced late overproduction of pulmonary TNF-α, IL-6, and IL-10 in pristane-primed mice.

MATERIALS AND METHOD

Animals

Adult female ICR mice were used at 8-12 weeks of age, purchased from the Dae-Han Experimental Animal Center (Taejeon, Korea), and maintained on a regular 12 h light-dark cycle under a temperature of 22 ± 2 °C and relative humidity of 55 ± 5% with water and food available *ad libitum*. Mice were received *i.p.* a single injection of 1.0 mL of pristane (Sigma Chemical Co., St., Louse, MO) or PBS, and then were used as a pristane-primed chronic inflammation model within 2-3 months after pristane injection for experiment. To induce acute lung injury, pristane-primed or normal mice were injected *i.p.* in a dose of 2.5 mg/kg of LPS (*Escherichia coli* Serotype 0127:B8, Sigma Chemical Co., St., Louse, MO) diluted in PBS once and controls received PBS in same regimen.

Quantification of lung vascular permeability

Pulmonary vascular leak was studied by measuring the extravasation of Evans blue, which, when given intravenously, binds to plasma proteins, particularly albumin (Green *et al.*, 1988). To elicit endotoxin-induced vascular leak, pristane-primed or normal mice were injected *i.p.* with LPS at a dose of 2.5 mg/kg body weight or PBS as a control once. The mice were injected by tail vein with 160 mg/kg of Evans blue (Sigma) in PBS 2 h prior to termination of the experiment. The lungs were removed 6 h or 72 h after LPS injection. Evans blue was extracted from lungs by incubating samples in formamide at 37°C for 14-18 h. The supernatant was separated by centrifugation at 5000×g for 30 min. The pulmonary vascular permeability was quantified at 650 nm.

Preparation of serum

Bloods were harvested from hearts under anesthetics 6 h and 72 h after *i.p.* injection with LPS of 2.5 mg/kg body weight or PBS in pristane-primed or normal mice. The bloods were allowed to clot for 30 min, and then centrifuged for 10 min at 1000 × *g*. The sera were collected and stored at -20°C for cytokine assays.

Preparation of bronchoalveolar lavage fluids

The lungs were removed under anesthetics 6 h or 72 h after *i.p.* LPS or PBS injection in pristane-primed or normal mice. BAL fluids were performed twice in a total volume of 1 mL of PBS through an intratracheal polyethylene tube attached to a 1 mL-syringe and centrifuged. The BAL fluids were collected and stored at -20°C for cytokine assays.

Isolation of lung cells

Lungs were removed under anesthetics from LPS-exposed pristane-primed or LPS-exposed normal mice 24 h after *i.p.* LPS injection. The lungs were perfused with 10 mL of PBS, minced, and incubated in RPMI 1640 medium containing 670 U/mL collagenase IV for 45 min at 37°C, 5% CO_2 incubation. The lung cell suspensions were obtained by filtration through a nylon mesh of 140 μm diameter. Treatment with collagenase IV resulted in complete digestion of lung tissue without any visible cell clumps left after filtration. Erythrocytes were lyzed for 5 min in 5 mL of lysis buffer (pH 7.3) containing 155 mM ammonium chloride, 15 mM sodium bicarbonate and 1 mM EDTA. The lung cells (1×10^6 cells/well) were transferred to 24-well plates containing complete RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin (10 U/mL)-streptomycin (10 μg/mL) for lung cell culture.

Cell culture for cytokine assay

Lung cells (1×10^6 cells/mL) from LPS-exposed pristane-primed or LPS-exposed normal mice were cultured in complement-RPMI supplemented with 10% FBS for 48 h or 60 h at 37°C, 5% CO_2 incubation in the presence of 10 μg/mL of LPS (Sigma Chemical Co., St., Louse, MO) or vehicle. The cell supernatants were then harvested and stored at -70°C for cytokine assay.

Assay of cytokines

The concentrations of TNF-α, IL-6, IL-10, and IFN-γ in plasma, BAL fluids and supernatants of cultured lung cells

were determined using ELISA (enzyme-linked immunosorbent assay) with cytokine monoclonal antibodies (BD Biosciences Pharmingen, U.S.A.). All measurements were carried out in duplicate. The results were measured in picograms per milliliter at 450 nm using an ELISA microplate reader (Molecular Devices Co., Ltd., U.S.A.). The lower limit of sensitivity for each of the ELISA was equal to or smaller than 5 pg/mL.

Statistical analysis

All data are expressed as means ± standard error (S.E.). Experiments were always run in duplicate and repeated at least twice. Analysis of variation and Student's *t*-test were used to determine statistical significance, and $p < 0.05$ was considered to be statistically significant.

RESULTS

Effect of LPS on the up-regulated pulmonary vascular permeability in pristane-primed mice

Alterations of pulmonary vascular permeability were investigated by tissue accumulation of Evans blue as a marker for the transcapillary flux of macromolecules in capillary leak syndromes. These results demonstrated that pulmonary vascular permeability was strongly upregulated 6 h after *i.p.* LPS injection in LPS-exposed normal mice compared to normal controls, whereas were not altered 72 h. However, upregulated pulmonary vascular permeability was maintained 6 h and 72 h in LPS-exposed pristane-primed mice and pristane-primed controls without influence of LPS compared to normal mice (Fig. 1).

Effect of LPS on the production of serum cytokines in pristane-primed mice

The levels of serum TNF-α, IL-6 and IFN-γ were greatly enhanced 6 h in LPS-exposed normal mice compared to normal controls, whereas were not altered 72 h (Fig. 2). Levels of serum IL-6, IL-10 and IFN-γ were remarkably increased 6 h in LPS-exposed pristane-primed mice compared to pristane-primed controls, whereas were not altered 72 h (Fig. 2). However, levels of serum TNF-α were not altered 6 h and 72 h in LPS-exposed pristane-primed mice compared to pristane-primed controls (Fig. 2).

Effect of LPS on the production of BAL cytokines in pristane-primed mice

Our results demonstrated that levels of BAL TNF-α, IL-6 and IFN-γ were strongly upregulated 6 h in LPS-exposed normal mice compared to normal controls, whereas were not altered 72 h. Also, levels of BAL IL-6 and IFN-γ but not BAL TNF-α and IL-10 were remarkably augmented 6 h in LPS-exposed pristane-primed mice compared to pristane-primed controls, whereas levels of BAL TNF-α, IL-6 and IL-10 were greatly up-regulated 72 h in LPS-exposed pristane-primed mice compared to pristane-primed controls (Fig. 3).

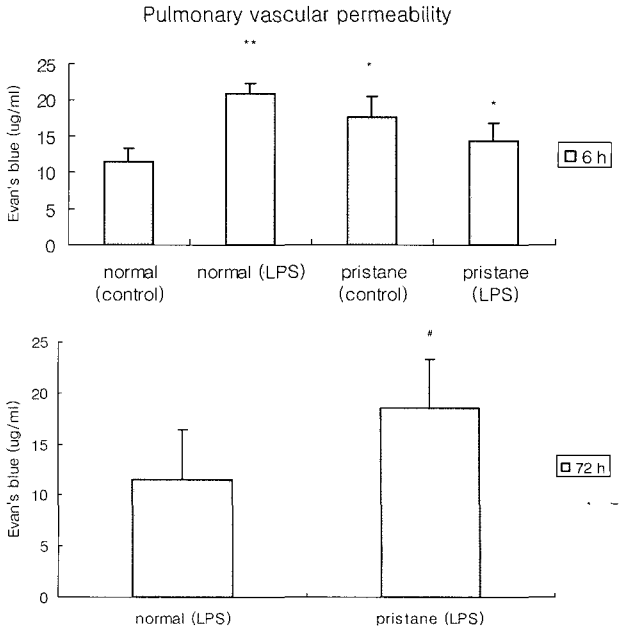

Fig. 1. Up-regulation of pulmonary vascular permeability in pristane-primed mice. (A): Pulmonary vascular permeability 6 h after *i.p.* LPS injection or PBS in pristane-primed or normal ICR mice. (B): Pulmonary vascular permeability 72 h after *i.p.* LPS injection or PBS in pristane-primed or normal ICR mice. ICR mice received a single *i.p.* injection of 1.0 mL of pristane were used as a pristane-primed chronic inflammation model within 2-3 months after pristane injection for experiment. Pristane-primed or normal mice were received *i.p.* with LPS at a dose of 2.5mg/kg body weight or PBS as a control once for induction of acute systemic inflammatory responses. The mice were injected by tail vein with 160 mg/kg of Evans blue in PBS 2 h prior to termination of the experiment. The lungs were removed 6 h or 72 h after LPS injection. Evans blue was extracted from lungs by incubating samples in formamide for 14-18 h at 37°C incubation. The concentration of Evans blue in lung supernatants was measured by a dual wavelength spectrophotometric method at absorptions of 650 nm for the vascular leak syndrome in LPS-exposed- pristine-primed or normal mice. Each mouse was individually analyzed for vascular leak, and data from five mice were expressed as mean ± S.E. * ($p<0.05$) and ** ($p<0.01$): Significantly different from the value 6 h after LPS injection in PBS-treated normal control. # ($p< 0.05$): Significantly different from the value 72 h after LPS injection in LPS-exposed normal group.

Comparison of the *in vivo* late production of cytokines in LPS-exposed pristane-primed mice and LPS-exposed normal mice

In the present study, levels of serum TNF-α, IL-10, and IL-6 but not IFN-γ were significantly upregulated 72 h in LPS-exposed pristane-primed mice compared to LPS-exposed normal mice (Fig. 4). Similarly, levels of BAL TNF-α, IL-10, and IL-6 but not IFN-γ were significantly

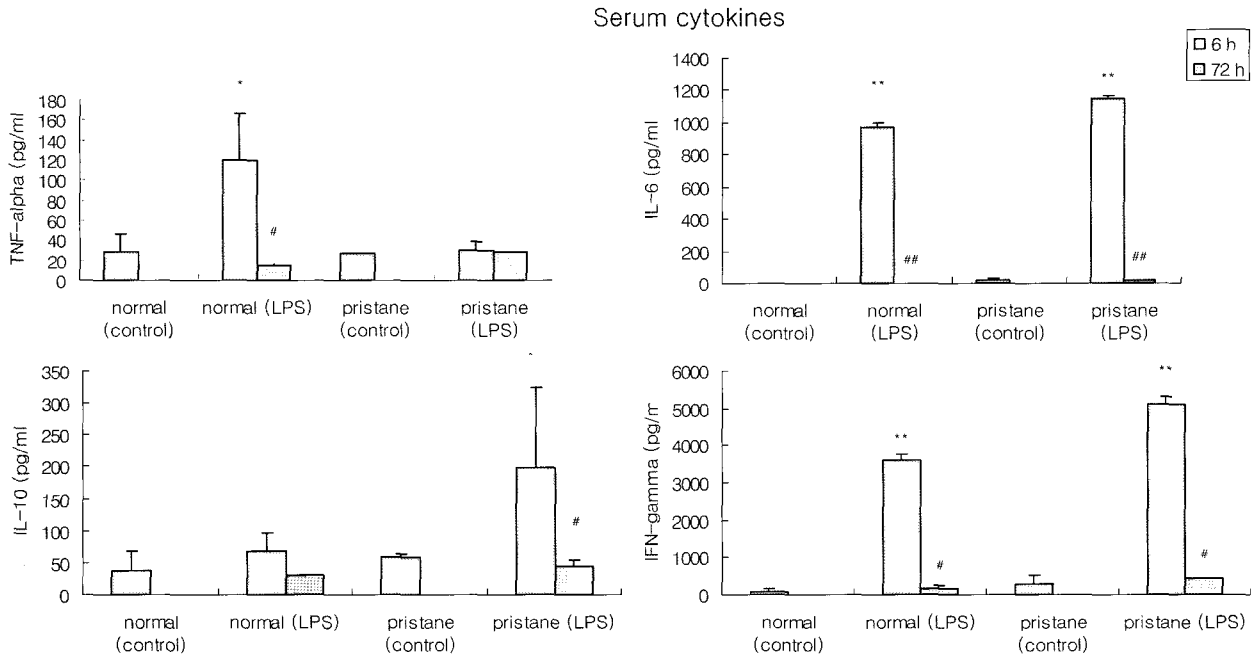

Fig. 2. Effect of LPS on the production of serum cytokines in pristane-primed mice. Bloods were harvested from hearts under anesthetics 6 h and 72 h after *i.p.* LPS injection or PBS in pristane-primed or normal mice. The sera were collected and the concentrations of serum cytokines were measured at 450 nm using ELISA. Each value represents the mean ± S.E. Other legends and methods are the same as in Fig. 1. * ($p<0.05$) and ** ($p<0.01$): Significantly different from the value in each PBS-treated control. # ($p< 0.05$) and ## ($p<0.01$): Significantly different from the value 6 h after LPS injection in each LPS-exposed group.

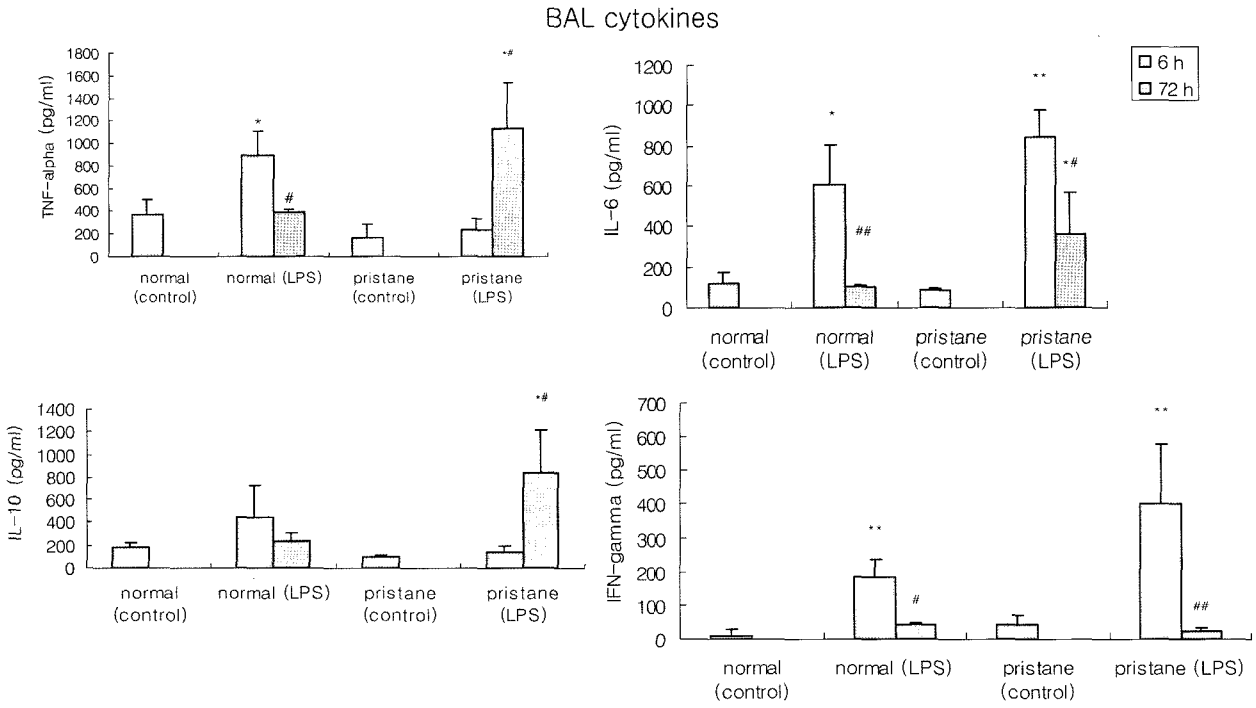

Fig. 3. Effect of LPS on the production of BAL cytokines in pristane-primed mice. The lungs were removed under anesthetics 6 h or 72 h after *i.p.* LPS or PBS injection in pristane-primed or normal mice. BAL fluids were performed twice in a total volume of 1 mL of PBS through an intratracheal polyethylene tube attached to a 1 mL-syringe and centrifuged. The BAL fluids were collected and the concentrations of serum cytokines were measured at 450 nm using ELISA. Each value represents the mean ± S.E. Other legends and methods are the same as in Fig. 1. * ($p<0.05$) and ** ($p<0.01$): Significantly different from the value in each PBS-treated control. # ($p< 0.05$) and ## ($p<0.01$): Significantly different from the value 6 h after LPS injection in each LPS-exposed group.

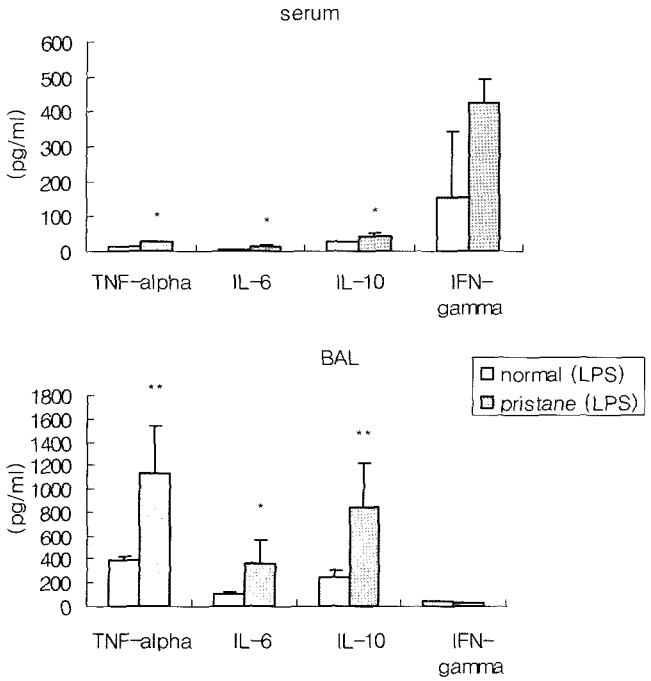

Fig. 4. Comparison of the *in vivo* late production of cytokines in LPS-exposed pristane-primed mice and LPS-exposed normal mice. Bloods and BAL fluids were harvested 72 h after *i.p.* LPS injection or PBS in pristane-primed or normal mice. The sera and BAL fluids were collected and the concentrations of cytokines were measured at 450 nm using ELISA. Each value represents the mean ± S.E. Other legends and methods are the same as in Fig. 3. Each value represents the mean ± S.E. * ($p<0.05$) and ** ($p<0.01$): Significantly different from the value 72 h after LPS injection in each LPS-exposed normal group.

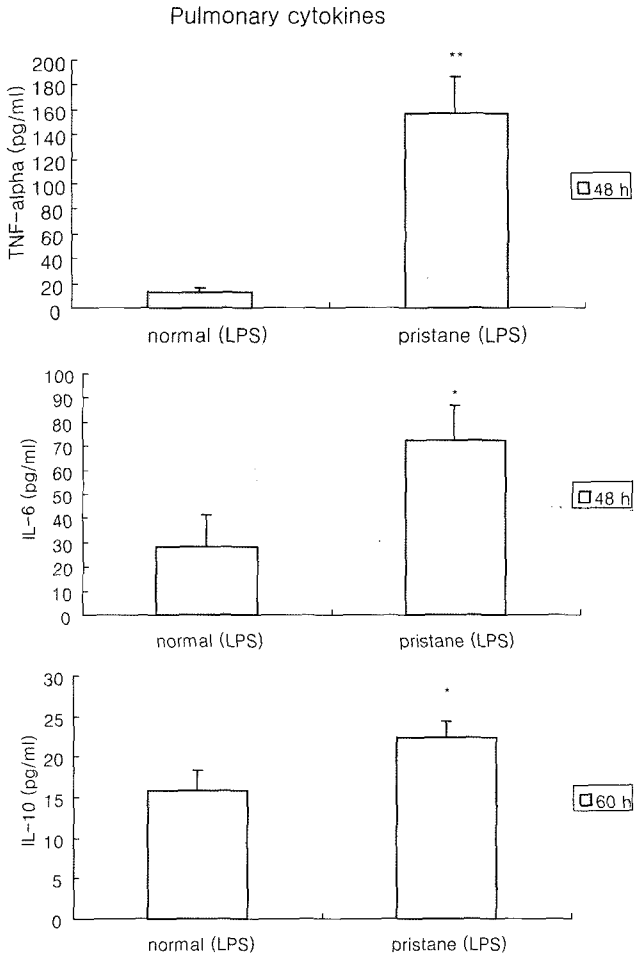

Fig. 5. Effect of LPS on the *in vitro* production of cytokines by lung cells from LPS-exposed pristane-primed mice. Lungs were removed under anesthetics from LPS-exposed pristane-primed or LPS-exposed normal mice 24 h after *i.p.* LPS injection. The lungs were perfused with 10 mL of PBS, minced, and incubated in RPMI 1640 medium containing 670 U/mL collagenase IV for 45 min at 37°C. The lung cell suspensions were obtained by filtration through a nylon mesh of 140 μm diameter. Erythrocytes were removed by red blood lysis buffer. Each lung cells (1×10^6 cells/mL) in complement-RPMI supplemented was cultured for 48 h or 60 h in the presence of 10 μg/mL of LPS or vehicle. The concentrations of cytokines were measured at 450 nm using ELISA. Each value represents the mean ± S.E. Other legends and methods are the same as in Fig. 1. * ($p<0.05$) and ** ($p<0.01$): Significantly different from the value in LPS-exposed normal mice.

enhanced 72 h in LPS-exposed pristane-primed mice compared to LPS-exposed normal mice.

Effect of LPS on the *in vitro* production of cytokines by lung cells from LPS-exposed pristane-primed mice

Lungs were removed under anesthetics from LPS-exposed pristane-primed or LPS-exposed normal mice 24 h after *i.p.* LPS injection. We cultured lung cells obtained from LPS-exposed pristane-primed or LPS-exposed normal mice with 10 ug/mL of LPS for 48 h for TNF-α and IL-6 assay, and 60 h for IL-10 at 37°C, 5% CO_2 incubation. Our observations showed that the *in vitro* production of pulmonary TNF-α, IL-6 and IL-10 were significantly augmented in LPS-exposed pristane-primed mice compared to LPS-exposed normal mice (Fig. 5).

DISCUSSION

Chronic, inflammatory lupus-like disease, which is induced by pristane, is characterized by increased production of proinflammatory cytokines and multiple organ injuries including lungs and kidneys (Shacter, *et al.*, 1992; Balomenos *et al.*, 1998; Dean *et al.*, 2000). The delayed excess of proinflammatory cytokines after infections may play an important role in inflammatory processes leading to persistent lung injury in chronic systemic inflammation. We focused on effect of LPS on the early and late pulmonary vascular permeability and proinflammatory cytokine production in pristane-primed mice compared with normal mice. In this study, we observed that LPS induced late overproduction of pulmonary TNF-α, IL-6, and IL-10 in pristane-primed mice compared with normal

mice, suggesting that LPS may trigger to persistently develop lung injury in chronic, inflammatory lupus-like pristane-primed mice.

Systemic inflammation promotes multiple organ failure through the induction of diffuse microvascular leak (Anderson and Harken, 1990). Altered pulmonary vascular permeability is induced by stimulated neutrophils and results in lung damage (Tanita *et al.*, 1999). We measured effect of LPS on the pulmonary vascular permeability 6 h and 72 h after *i.p.* LPS injection in pristane-primed mice compared with normal mice. In this study (Fig. 1), our observations are supporting previous studies that pulmonary vascular permeability is upregulated in lupus compared with normal model (Karim *et al.*, 2002), and that LPS markedly induces the increased lung vascular permeability at early phase of acute systemic inflammation and then the first peaks sharply decline, leading to rapid recovery to near normal state (Welsh *et al.*, 1989). However, our results were shown that LPS didn't more trigger the upregulation of pulmonary vascular permeability 6 h and 72 h in pristane-primed mice. Therefore, these results suggest that persistently upregulation of pulmonary vascular permeability may maintain in pristane-primed mice without influence of LPS.

LPS induces lung injury through overproduction of proinflammatory cytokines or imbalance between pro- and anti- inflammatory cytokines in plasma and BAL fluids in sepsis (Martin, 1997; Bauer *et al.*, 2000). Upregulation of gene expression of TNF-α and IL-6 in plasma, BAL fluids and the whole organs increases with the degree of lung injury on the early inflammatory responses in endotoxemia (Ulich *et al.*, 1991; Suter *et al.*, 1992; DiCosmo *et al.*, 1994). Also, persistently elevated proinflammatory cytokines including BAL TNF-α, IL-1β, and IL-6 contribute to continuous lung injury process (Meduri *et al.*, 1995a; Meduri *et al.*, 1995b). As shown in Fig.2 and Fig.3, our results showed that LPS greatly induced increased production of serum and BAL TNF-α, IL-6 and IFN-γ at early phase in normal mice and then the first peaks remarkably declined, indicating that LPS is a transiently potent inducer of acute lung injury in normal state.

Upregulation of TNF-α, IL-6, IL-10, and IFN-γ observed in lupus contribute to active pathogenesis of lupus-like tissue injury and high mortality (Malide *et al.*, 1995; Balomenos *et al.*, 1998; Mihara *et al.*, 1998; Park *et al.*, 1998). As shown in Fig. 2 and Fig. 3, we observed that levels of serum and BAL TNF-α were not altered 6 h in LPS-exposed pristane-primed mice compared with pristane-primed controls, while levels of BAL TNF-α were greatly increased 72 h. It appears that downregulation of TNF-α is correlated to induction of decreased apoptosis during chronic T cell activation in autoimmunity and then upregulation of TNF-α is associated with active autoimmunity (Theofilopoulos and Lawson, 1999; Childs, 2006). Furthermore, higher BAL to plasma ratios for TNF-α, IL-1β, IL-6, and IL-8 correlates with lung disease activity and high mortality (Meduri *et al.*, 1995b) and BAL TNF-α plays a more important role in the development and progression of inflammation rather than serum TNF-α in patients with sepsis (Goodman *et al.*, 1996). Therefore, our results suggest that downregulation of circulating and pulmonary TNF-α at early phase in LPS-exposed pristine-primed mice may induce decreased apoptosis during chronic T cell activation and then up-regulation of BAL TNF-α may lead to late progression of active lung injury.

Our results also showed that production of proinflammatory IL-6 in plasma and BAL fluids was remarkably increased 6 h in LPS-exposed pristane-primed mice compared with pristane-primed controls, while levels of BAL IL-6 were greatly increased 72 h. Recent studies reported that pulmonary overexpression of IL-10 enhanced lung fibrosis, lung disease development or the susceptibility to pneumococcal pneumonia after influenza infection (van der Sluijs *et al.*, 2004; Barbarin *et al.*, 2005). Our data demonstrated that early overproduction of serum IL-10 was increased in LPS-exposed pristane-primed mice compared with pristane-primed controls, while late overproduction of BAL IL-10 was remarkably induced. In addition, our data showed that production of serum and BAL IFN-γ was remarkably increased 6 h in LPS-exposed pristane-primed mice compared with pristane-primed controls, while was not altered 72 h. Therefore, our data suggest LPS may trigger upregulation of lung inflammatory responses *via* late overproduction of BAL IL-6 and IL-10 in lupus-like pristine-primed mice. Also, the increase in IL-6 and IL-10 production may be a cause of defective T cell responses and act as potent stimulators of B cell proliferation leading to active pathogenesis of lung injury in lupus (Llorente *et al.*, 1995; Childs, 2006).

As shown in Fig. 4, we also observed that late production of serum and BAL TNF-α, IL-6 and IL-10 were significantly upregulated 72 h in LPS-exposed pristane-primed mice compared to those in LPS-exposed normal mice, indicating that pulmonary inflammatory responses may be more persistently active in LPS-exposed lupus-like diseases than those in LPS-exposed normal state. Therefore, these findings indicate that LPS may induce persistent progression of lung injury *via* late overproduction of BAL TNF-α, IL-6 and IL-10 in pristane-primed mice.

We measured the *in vitro* production of TNF-α, IL-6, and IL-10 by lung cells from LPS-exposed pristane-primed mice compared to those from LPS-exposed normal mice. Our observations showed that LPS significantly induced increased the *in vitro* production of TNF-α, IL-6, and IL-10 by lung cells in LPS-exposed pristane-primed mice compared to those in LPS-exposed normal mice (Fig. 5),

suggesting that LPS may induce active progression of pulmonary inflammation through overproduction of BAL TNF-α, IL-6 and IL-10 in chronic, inflammatory lupus-like syndrome.

In conclusion, our findings indicate that LPS may trigger persistent progression of lung injury through late overproduction of BAL TNF-α, IL-6, and IL-10 in lupus-like chronic inflammation disease compared to normal condition.

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