

# Nitric oxide inhibits T cell adhesion and migration by down-regulation of $\beta$ 1-integrin expression in immunologically liver-injured mice

Yang Sun, Jianli Liu, Feng Qian, Qiang Xu \*

State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, 22 Han Kou Road, Nanjing 210093, China

Received 8 April 2005; received in revised form 16 June 2005; accepted 23 September 2005

## Abstract

Our previous study has reported that nitric oxide (NO) exerts a protective role in immunologically liver-injured mice induced by delayed-type hypersensitivity to picryl chloride. To explore the mechanism of the protection, we have now examined the effect of NO on T cell adhesion and migration. First, we isolated hepatocytes and nonparenchymal cells from the liver-injured mice and separated the nonparenchymal cells into Kupffer cell-enriched and lymphocyte-enriched populations. When these hepatocytes or the fractions of nonparenchymal cells were co-cultured with spleen T cells of the liver-injured mice in a Transwell system, the adhesive potential of the T cells was significantly inhibited in the presence of hepatocytes or the Kupffer cell-enriched population but not the lymphocyte-enriched population of nonparenchymal cells. This effect was dependent on NO production. The NO synthase inhibitor  $N_G$ -monomethyl-L-arginine (L-NMMA) could reverse this inhibition of cell adhesion and also decrease NO production. To confirm this effect of NO on T cells, we further examined the role of exogenous or endogenous NO on the adhesive activity of the Jurkat T cell line. As a result, the NO donor, *S*-nitroso-*N*-acetyl penicillamine (SNAP) caused a dose-dependent inhibition of the adhesion of Jurkat T cells. Furthermore, the binding ability of Jurkat T cells to collagen decreased gradually after co-incubation with macrophages stimulated by LPS+IFN- $\gamma$ , an effect which correlated well with the increasing NO level in the medium. Such opposite changes in cell adhesion and in NO production were also markedly reversed by L-NMMA. Moreover, treatment with SNAP reduced adhesion, transmigration, matrix metalloproteinase-9 production and  $\beta$ 1-integrin expression of spleen T cells of the liver-injured mice. Taken together, these findings suggest that NO can function as a down-regulator of T cell mobility, which might be one of the mechanisms by which NO exerts its protective effect in T cell-mediated liver injury.

© 2005 Elsevier B.V. All rights reserved.

**Keywords:** Nitric oxide; Liver injury; Delayed-type hypersensitivity; Adhesion; Migration

## 1. Introduction

Nitric oxide (NO), a reactive intermediate molecule derived from molecular oxygen and the guanidino nitrogen of L-arginine in a reaction catalyzed by NO

synthase, is involved in a variety of biological functions [1]. In the immune system, NO not only acts in a toxic fashion, participating in host defense against tumors or parasitic infections, but also functions as a regulatory molecule of the immune response [2–4]. Administration of NO donors has shown to inhibit the progress of Th1-type immune responses [5]. Mice lacking inducible NO synthase developed stronger experimental au-

\* Corresponding author. Tel./fax: +86 25 8359 7620.

E-mail address: molpharm@163.com (Q. Xu).

toimmune encephalomyelitis responses [6]. In addition, the high level of NO produced by activated macrophages or NO donors could inhibit the proliferation of activated T cells [7], suppress the secretion of cytokines [8] and counteract the apoptosis of myocardial cells [9]. All these findings suggested that NO might function as an important immunosuppressive factor against T cell immunity.

In T cell-mediated immune response such as delayed-type hypersensitivity, which is characterized by an infiltration of lymphocytes and macrophages, T cells and macrophages could counter-regulate each other's function by the secretion of various cytokines such as IFN- $\gamma$ , TNF- $\alpha$  and NO [10]. The co-existence of T cells and macrophages in the same inflammatory site makes it possible that macrophage-derived NO served as a modulator of T cell mobility and infiltration in vivo. It is well known that the infiltration of T cells to the inflammatory site is crucial for the development of T cell-mediated diseases and the suppression of T lymphocyte adhesion and migration may lead to an amelioration of inflammation [11]. So, it is believed that the NO-induced down-regulation of cell adhesion and migration might represent an important feedback to prevent excessive immune responses, which would otherwise result in serious and overwhelming inflammatory injury. In fact, the role of NO in suppressing leukocyte mobility has been extensively reported in T lymphocytes [12,13] and neutrophils [14,15].

Previously, we have established the liver-injury model induced by a delayed-type hypersensitivity (DTH) mechanism to picryl chloride, in which the hepatocyte damage was caused by liver-infiltrating T lymphocytes that migrate from peripheral lymphoid organs into the tissue [16–18]. We have also demonstrated that a large amount of NO was produced in the liver in the latter phase of the injury, and which then protected the hepatocytes from the immunological damage [19]. However, the detailed mechanism of this protection by NO is still unknown. Given the importance of T cell mobility in DTH, we thus examined the effect of NO on T cell adhesion and migration and its relevance in the animal model of DTH liver injury in this study.

## 2. Material and methods

### 2.1. Animals

Female BALB/c mice (SPF), aged 6–8 weeks (18–22 g), were obtained from the Laboratory Animal Center of Shanghai (Shanghai, China). They were maintained with free access to

pellet food (Jiangsu Cooperation Medicinal and Pharmaceutical Company, Nanjing, China) and water in plastic cages at  $21 \pm 2$  °C and kept on a 12 h light/dark cycle. Animal welfare and experimental procedures were carried out strictly in accordance with the guide for the care and use of laboratory animals (National Research Council of USA, 1996) and the related ethical regulations of our university. All efforts were made to minimize suffering and to reduce the number of animals used.

### 2.2. Cell line

Human leukemia Jurkat cell line was maintained in RPMI 1640 medium supplemented with 100 U/mL of penicillin, 100  $\mu$ g/mL of streptomycin and 10% fetal calf serum (FCS) under a humidified 5% (v/v) CO<sub>2</sub> atmosphere at 37 °C.

### 2.3. Drugs and reagents

Picryl chloride (PCI, Tokyo Kasei Industry, Tokyo, Japan); collagenase (182 U/mg) (Wako Pure Chemical Industries Ltd., Osaka, Japan); type I collagen (Collaborative Biomedical Products, USA); bovine serum albumin (BSA, sigma); phorbol 12,13-dibutyrate (PDBu, Wako Pure Chemical Industries Ltd., Japan); *S*-nitroso-*N*-acetyl penicillamine (SNAP, RBI Research Biochemicals International, Natick, MA); *N*<sub>G</sub>-monomethyl-L-arginine (L-NMMA, ABR Affinity Bioreagents, Golden, CO); lipopolysaccharide (LPS, from *E. coli*, Sigma); RPMI 1640 (Gibco, BRL); thioglycollate (Sigma); recombinant mouse IFN- $\gamma$  (Peprotech, USA); M-MLV Reverse Transcriptase (Promega, USA), acrylamide and bis-acrylamide (Shanghai Sangon Biotechnical Ltd. Co., Shanghai, China); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Sigma); gelatin and Coomassie brilliant blue R-250 (Sigma); crystal violet (Shanghai Yuanhang reagent factory, Shanghai, China); mouse T cell enrichment column (R&D system, USA); Transwell cell co-culture chamber (Costar Corp., MA); Polyvinyl pyrrolidone-free (PVPF) filter (Nucleopore Corp, CA); anti-mouse CD3-FITC (Biolegend, USA), anti-mouse CD11b-FITC, anti-mouse CD29 (Integrin  $\beta$ 1 chain) monoclonal antibody and PE-conjugated goat-anti-rat Ig antibody (BD Pharmingen); 96-well culture plates (Nunc).

### 2.4. Liver injury evoked by PCI-induced DTH (PCI-DTH)

Mice were sensitized twice by painting 0.1 mL of 1% PCI in ethanol on the skin of their abdomens over an interval of 5 days. Five days after the 2nd sensitization, they were injected with 10  $\mu$ L of 0.2% PCI in olive oil into the liver. Liver injury was triggered and reached a peak at 12–18 h after the antigen challenge as shown in our previous report [16]. The initial studies demonstrated that olive oil used as the vehicle did not cause liver damage in the used dosage. Two controls, olive oil challenge to PCI-sensitized mice and PCI challenge to naive mice, were also used and no damage was observed. Spleen T cells at 6 h and liver nonparenchymal cells and hepatocytes at

0 and 24 h after the challenge were isolated for further culture *in vitro*.

## 2.5. Cell preparation

### 2.5.1. Spleen T cells

Spleen was removed under sterile conditions from PCI-DTH liver-injured mice 6 h after antigen challenge and the

cells were dissociated in 5 mL RPMI 1640 medium containing 10% FCS. After centrifugation at 200 g for 5 min, 0.17 M Tris (hydroxymethyl aminomethane)–0.75% NH<sub>4</sub>Cl solution was added to remove erythrocytes. After washing twice with RPMI 1640 medium, the cells were found to be about 98% viable, as estimated by trypan blue exclusion. T cells were purified with a mouse T cell enrichment column according to the instruction of the kit so that about 88% lymphocytes were

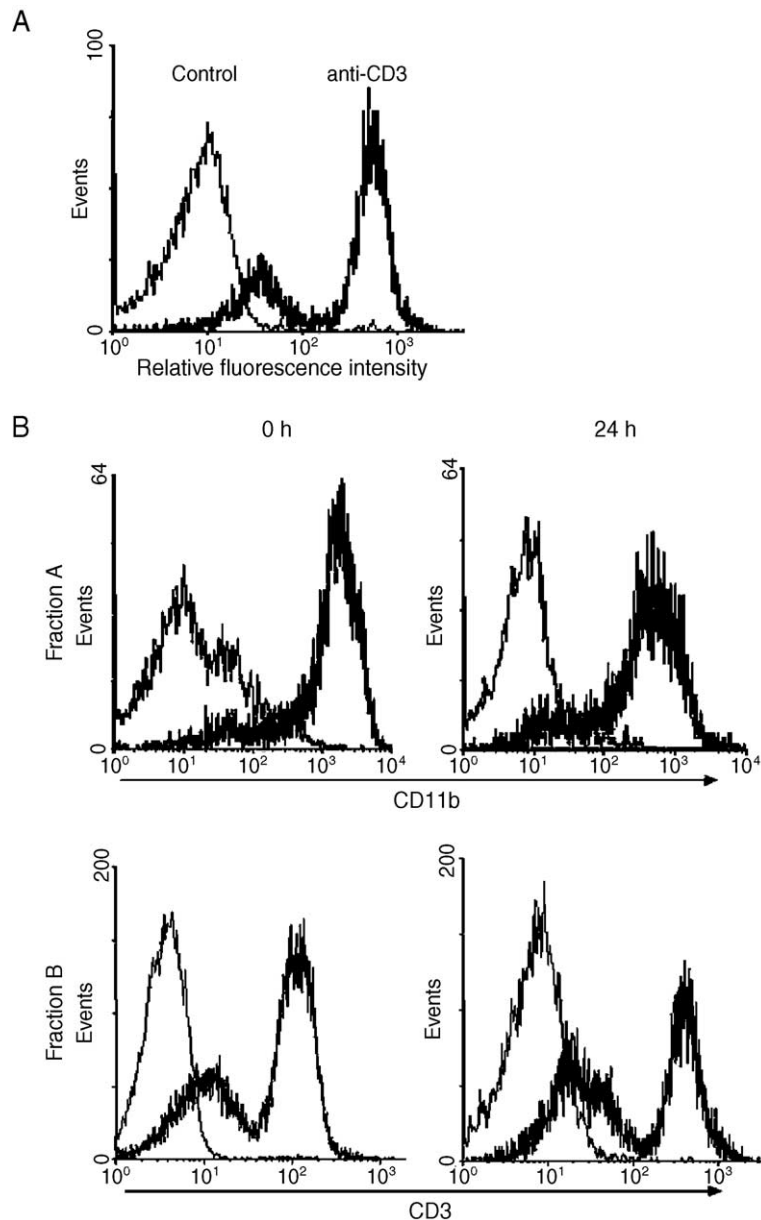


Fig. 1. Representative flow cytometric results of purified spleen T cells (A) and fractions of the nonparenchymal cells (B). After purification using the mouse T cell enrichment column, about 88% of lymphocytes were CD3-positive T cells. In some cases, the nonparenchymal cells were separated into two fractions. Then, Fraction A was confirmed to be about 88–94% of Kupffer cells and Fraction B was the lymphocyte-enriched population with 70–78% of CD3<sup>+</sup> T cells. The figures shown here are the representatives of three different experiments.

CD3-positive T cells as assessed by flow cytometric analysis (Fig. 1A).

### 2.5.2. Liver nonparenchymal cells and hepatocytes

Liver nonparenchymal cells and hepatocytes were isolated from liver-injured mice at 0 and 24 h by the modified two-step perfusion method [17]. In brief, the livers of the mice were first perfused in situ via the portal vein with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free Hank's balanced salt solution (HBSS) supplemented with 0.5 mM EGTA and 25 mM HEPES at 37 °C until the blood in the organ was completely removed. Then, the buffer was replaced with 0.1% collagenase solution in HBSS (containing 4 mM  $\text{CaCl}_2$  and 0.8 mM  $\text{MgSO}_4$ ). After a few minutes of perfusion, the liver was excised rapidly from the body cavity and dispersed into cold HBSS. The cell suspension generated was filtered through a 100 gauze mesh. By differential centrifugation at 50 g for 2 min, parenchymal cells were recovered in the cell pellet. After washing twice to remove dead cells and debris, the hepatocytes were resuspended in RPMI 1640 medium containing 10% fetal calf serum (FCS) and found to be 85% to 92% viable by trypan blue dye exclusion. The supernatant obtained after isolating the above parenchymal cells was used for preparation of nonparenchymal cells by centrifugation at 300 g for 10 min. In some cases, the nonparenchymal cell suspensions were overlaid on Percoll gradient solutions consisting of 25%, 50% and 80% Percoll and then centrifuged at 600 g for 20 min. The resulting layers which appeared between 25% and 50% or between 50% and 80% of Percoll were named as Fraction A and Fraction B, respectively. Then, Fraction A was confirmed to be about 88–94% of Kupffer cells and Fraction B was the lymphocyte-enriched populations with 70–78% of  $\text{CD3}^+$  T cells as assessed by flow cytometric assay (Fig. 1B). Cells in the different layers were collected and washed three times with RPMI 1640 medium. The viability of cells in either of the layers was more than 98%. The hepatocytes, nonparenchymal cells and their fractions were used immediately for culture.

### 2.5.3. Murine macrophages

Macrophages were harvested by peritoneal lavage from mice 3 days after i.p. injection with 1 mL of sterile 3% thioglycollate. The cells were washed and resuspended in RPMI 1640 medium supplemented with 10% FCS and seeded in 24-well plates at a final concentration of  $2 \times 10^6$  cells per well. Following 4-h incubation at 37 °C, nonadherent cells were removed by washing and adherent monolayers were used for further culture [20].

### 2.6. Transwell cell co-culture system

Transwell cell culture chambers (Costar 3422, Cambridge, MA) were used as previously reported [18]. Polyvinyl pyrrolidone-free filters with a 3  $\mu\text{m}$  pore size were attached to the bottom of the chamber. They were then placed as the inside compartment on the 24-well plate (Falcon 3847, Becton Dickinson) to form a Transwell cell co-culture system. Jurkat

cells ( $1 \times 10^5$ /well) or spleen T cells ( $5 \times 10^5$ /well) were seeded in the inside compartment, and the nonparenchymal cells and its fractions ( $2 \times 10^6$ /well), hepatocytes ( $2 \times 10^5$ /well), or macrophages ( $2 \times 10^6$ /well) were added in the outside compartment. The medium could freely permeate between the two compartments.

### 2.7. Nitrite determination

NO release from the cells was measured as nitrite accumulation in the medium using a standard Griess reaction [21]. Briefly, 100  $\mu\text{L}$  of supernatant was incubated with an equal volume of Griess reagent (a combination of equal amounts of 0.2% naphthyl ethylenediamine dihydrochloride in water and 2% sulfanilamide in 5%  $\text{H}_3\text{PO}_4$ ) at room temperature for 10 min. Then, absorbance at 540 nm was measured by an ELISA reader (Sunrise Remote/Touch Screen, TECAN, Austria) and nitrite concentrations were calculated by a  $\text{NaNO}_2$  standard curve.

### 2.8. Adhesion assay

Adhesion assay was performed according to the report [22] with some modifications. Briefly, a flat-bottom 96-well microplate was coated with 50  $\mu\text{L}$  solution containing type I collagen (50  $\mu\text{g}/\text{mL}$ ) and left at 4 °C overnight. Nonspecific binding sites were blocked with 0.2% BSA for 2 h at room temperature followed by washing three times with phosphate buffer solution. The cells were suspended in RPMI 1640 medium and spleen T cells ( $5 \times 10^5$ ) or Jurkat cells ( $1 \times 10^5$ ) were added to each well. The cells were incubated at 37 °C for 1 h with or without PDBu (100 ng/mL) and the non-adherent cells were removed by washing three times with RPMI 1640 medium. Then, cells were fixed with methanol/acetone (1:1) and stained with 0.5% crystal violet in 20% methanol. Unbound dye was removed in tap water and the plate was dried in air. Bound dye was extracted with 1% SDS. The absorbance of the samples was measured at 592 nm. The wells which were fixed and stained without previous washing were regarded as the absorbance of the total cells. The results were expressed as the mean percentage of total cells from triplicate wells and the experiments were repeated three times. Spleen cells from control animals were subjected to the same assay procedures in parallel. Specificity of cell adhesion assays was corroborated using BSA as substratum.

### 2.9. Migration assay

Migration assay was performed according to the report [23] with some modifications. Briefly, polyvinyl pyrrolidone-free filters with a 3.0  $\mu\text{m}$  pore size were attached to the bottom of a Transwell chamber and coated with 50  $\mu\text{L}$  solution of type I collagen (50  $\mu\text{g}/\text{mL}$ ) overnight at room temperature. They were then placed on the 24-well plate to form a Transwell co-culture system. SNAP diluted in 1 mL medium at

various concentrations, or medium alone, was added to the lower chamber. T cells isolated from the liver injury model were added to the upper chamber. After incubation for 8 h in 5% CO<sub>2</sub> at 37 °C, the inserts were removed and 50 µL of MTT solution (50 µg/mL) were added to the lower chamber for a further 4 h culture. The supernatant was aspirated carefully and 200 µL of dimethylsulphoxide were added to dissolve any precipitate. The absorbance was read at 540 nm. The OD values represent the cell number that migrated from the upper chamber into the lower chamber.

### 2.10. Gelatin zymography assay

Analysis by zymography on gelatin gel allows detection of enzymatic activity of the secreted collagenases MMP-9 [24]. Briefly, spleen T cells isolated from various treated mice were suspended in serum-free RPMI 1640 medium at a density of  $5 \times 10^5$ /well and incubated at 37 °C in 5% CO<sub>2</sub> for 24 h. Spleen T cells from control animals were subjected to the same assay procedures in parallel. Twenty microlitres of the supernatants were mixed with 10 µL sample buffer (62.5 mM Tris-HCl containing 10% glycerol, 0.00125% bromophenol blue and 12% sodium dodecyl sulfate (SDS)) without reducing agent, and were subjected to SDS-PAGE in 5% polyacrylamide gels that were copolymerized with 2 mg/mL of gelatin at 4 °C for 1 h. After electrophoresis, the gels were washed twice in the rinsing buffer (50 mM Tris-HCl containing 2.5% Triton X-100, 5 mM CaCl<sub>2</sub>, 1 µM ZnCl<sub>2</sub>, 0.05% NaN<sub>3</sub>) for 1 h at room temperature to remove SDS. Then, they were incubated for 36 h at 37 °C in the incubation buffer (50 mM Tris-HCl containing 5 mM CaCl<sub>2</sub>, 1 µM ZnCl<sub>2</sub>, 0.05% NaN<sub>3</sub>). The gels were stained with 0.1% Coomassie brilliant blue R250 for 30 min, and destained for 8 h in a solution of 10% acetic acid and 10% isopropanol. The proteolytic activity was evidenced as clear bands (zones of gelatin degradation) against the blue background of stained gelatin.

### 2.11. Reverse transcriptase-polymerase chain reaction (RT-PCR) [25]

Total RNA was extracted from spleen T cells using Tripure reagent (Roche) as described by the manufacturer. Single-stranded cDNA was synthesized from 2 µg of total RNA by reverse transcription using 0.5 µg primer of oligo(dT)<sub>18</sub>. Following cDNA synthesis, amplification was performed using the following primers (Genebase, Shanghai, China): beta-actin 5'-ACATCTGCTGGAAGGTGGAC and 3'-GGA-CCCATTGACCACCATGG, MMP-9 5'-CAGCCAACTA-TGACCAGGAT and 3'-TATTTCTGCTGTATCTGCCGT. PCR cycle conditions were: 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min for 30 cycles. After amplification, PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide dyeing. The relative expressions were quantified densitometrically using LabWorks 4.0 software, and calculated according to the reference bands of beta-actin.

### 2.12. Flow cytometric analysis

Expression of adhesion molecules on the surface of T lymphocytes was detected using flow cytometry. Purified T cells were incubated with the NO donor SNAP at 37 °C for 8 h. Then, the cells were washed twice with cold PBS and incubated with a certain concentration of β1-integrin monoclonal antibody or suitable isotype control at 4 °C for 30 min. After centrifugation (300 g, 5 min) and removal of the supernatant, cells were incubated with phycoerythrin (PE)-conjugated secondary antibody (30 min, 4 °C in dark). The cells were then fixed in 0.5 mL 1% paraformaldehyde (10

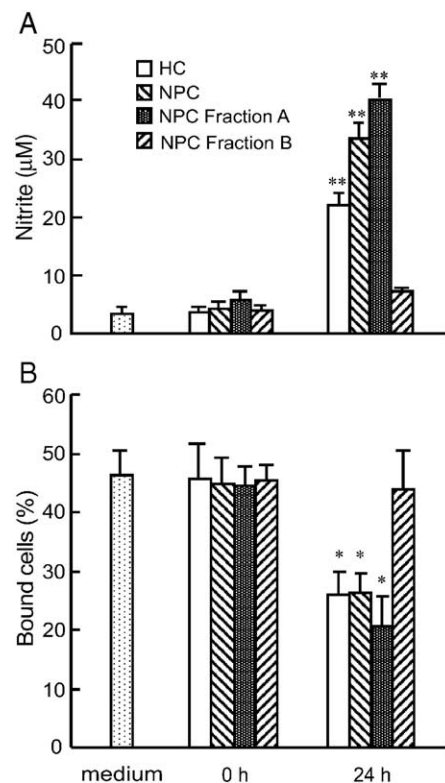


Fig. 2. Production of NO and inhibition of spleen T cell adhesion to type I collagen by nonparenchymal cells (NPC) and hepatocytes (HC) from PCI-DTH liver-injured mice. NPC, their fractions and HC were isolated from PCI-DTH liver-injured mice at 24 h after antigen challenge (24 h) or from mice which had been formerly twice sensitized but not challenged (0 h). Spleen T cells were isolated and purified from the liver-injured mice at 6 h after antigen challenge. Then, these cells were subjected to the Transwell system. After incubation at 37 °C for 12 h, the production of NO in the supernatant of the outer chamber was determined by the Griess reaction (A). At the same time, spleen T cells were harvested and transferred to type I collagen coated plates and cell adhesion induced by PDBu was tested as described in Material and methods (B). Data were expressed as the mean  $\pm$  s.d. of three separate experiments and each assay was performed in triplicate sets. \* $P < 0.05$ , \*\* $P < 0.01$  vs 0 h.

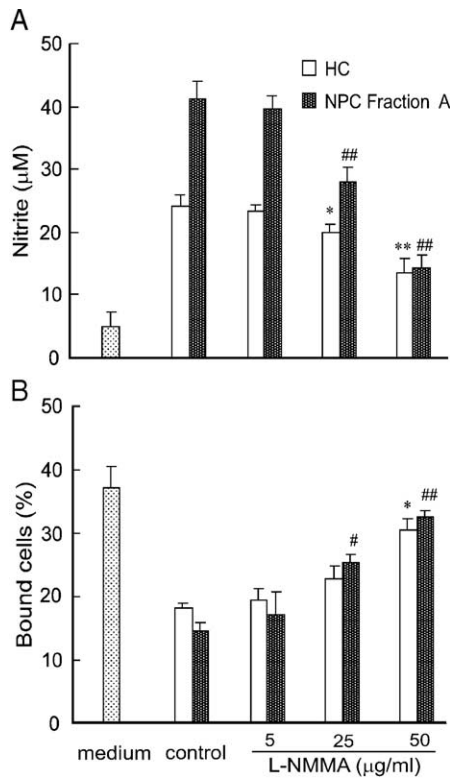


Fig. 3. NO synthase inhibitor L-NMMA reduced NO production (A) and reversed the inhibition of spleen T cell adhesion (B) by 24 h Fraction A of nonparenchymal cells (NPC) and hepatocytes (HC) in the Transwell system. Fraction A of nonparenchymal cells and hepatocytes were isolated at 24 h and seeded into the outer chamber in the presence of L-NMMA. Spleen T cells isolated at 6 h were seeded into the inside chamber of the Transwell system. After incubation at 37 °C for 12 h, T cells were harvested and transferred to a collagen coated plate and cell adhesion induced by PDBu was tested. At the same time, the production of NO in the supernatant of the outer chamber was determined by the Griess reaction. Data were expressed as the mean ± s.d. of three separate experiments and each assay was performed in triplicate sets. #*P*<0.05, ##*P*<0.01 vs control for Fraction A of nonparenchymal cells; \**P*<0.05, \*\**P*<0.01 vs control for hepatocytes.

min, 4 °C) after washing twice with the PBS/0.1% BSA buffer. Cells were analyzed on a FACSCalibur (Becton-Dickinson). The fluorescence intensity of each cell was compared with that of the isotype control.

2.13. Statistical analysis

Results were expressed as mean ± s.d. of three independent experiments and each experiment includes triplicate sets. Results were statistically evaluated by Student’s *t* test when only two value sets were compared, and one-way ANOVA followed by Dunnett’s test when the data involved three or more groups. *P*<0.05 was considered to be significant.

3. Results

3.1. Both nonparenchymal cells and hepatocytes from PCI-DTH liver-injured mice produced large amounts of NO and caused inhibition of T cell adhesion that could be reversed by L-NMMA

Nonparenchymal cells and hepatocytes were isolated from PCI-DTH liver-injured mice at 0 or 24 h after antigen

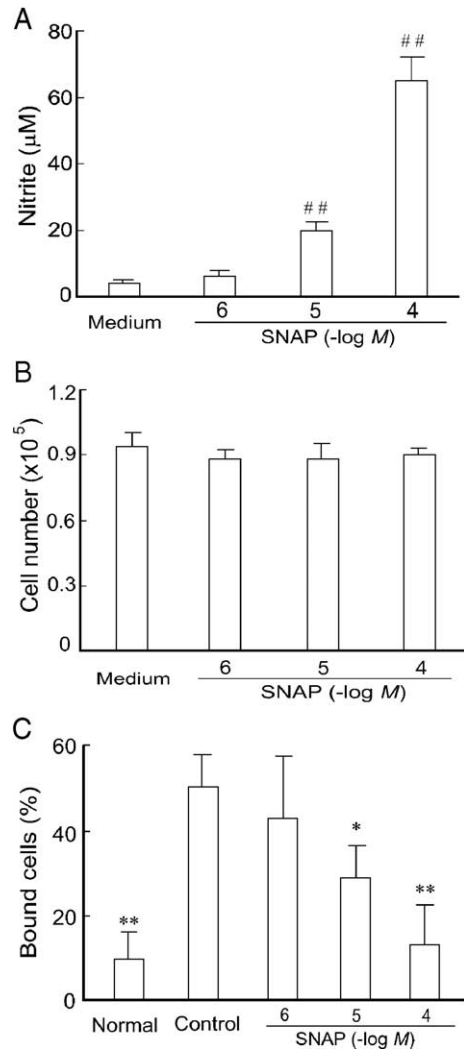


Fig. 4. Effect of NO donor SNAP on the viability and adhesion of Jurkat cells. Jurkat cells were pretreated with various concentrations of SNAP at 37 °C for 8 h. After the incubation, the supernatants were collected to determine nitrite levels (A) and the cells were counted to evaluate the viability (B). In addition, T cells were washed after the pretreatment with SNAP and added to wells coated with type I collagen and cell adhesion was determined (C). Data were expressed as the mean ± s.d. of three separate experiments and each assay was performed in triplicate sets. ##*P*<0.01 vs medium; \**P*<0.05, \*\**P*<0.01 vs control.

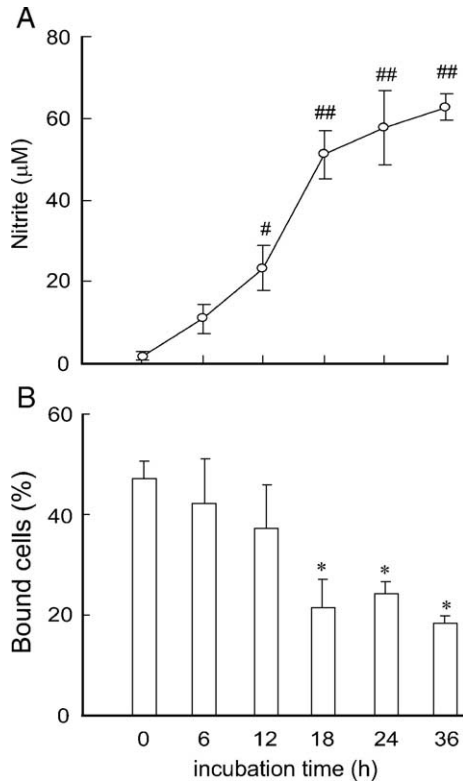


Fig. 5. Production of NO (A) and inhibition of the adhesion of Jurkat T cells to type I collagen (B) by activated peritoneal macrophages in the Transwell cell culture system. Macrophages were seeded into the outer chamber in the presence of LPS (1 µg/mL)+IFN-γ(200 U/mL) and Jurkat cells were seeded into the inside chamber. After incubation at 37 °C for the indicated times, Jurkat cells were harvested and the cell adhesion was tested. At the same time, the production of NO in the supernatant of the outer chamber was determined by the Griess reaction. Data were expressed as the mean ± s.d. of three separated experiments and each assay was performed in triplicate sets. #*P*<0.05, ##*P*<0.01 vs 0 h for NO production; \**P*<0.05 vs 0 h for cell adhesion.

challenge and co-cultured with spleen T cells isolated from the mice at 6 h after antigen challenge in a Transwell system. The spleen T cells were confirmed to be about 88% of CD3<sup>+</sup> T cells (Fig. 1A), while Fractions A and B of the nonparenchymal cells were confirmed to be about 88–94% of Kupffer cells and 70–78% of CD3<sup>+</sup> T cells, respectively (Fig. 1B). The liver cells at 0 h did not influence the adhesion of spleen T cells. However, those at 24 h significantly inhibited spleen T cells adhesion to collagen. At the same time, high levels of NO were observed in the 24 h liver cells, including total nonparenchymal cells, their Fraction A and hepatocytes, while little NO was detected in the 0 h cells. However, Fraction B of the nonparenchymal cells did not inhibit the T cell adhesion and did not produce NO (Fig. 2A and B). The NO synthase inhibitor L-NMMA dose-dependently inhibited the production of NO but restored the adhesion of spleen T cells suppressed by either the Fraction A of nonparenchymal cells or hepatocytes at 24 h (Fig. 3).

### 3.2. NO donor SNAP inhibited the adhesion of Jurkat T cells to collagen

SNAP, an NO donor, released NO spontaneously in solution in a dose-dependent manner (Fig. 4A). It was noted that SNAP had no cytotoxic effect on Jurkat T cells at the used concentrations (Fig. 4B). Jurkat cells, a classic T cell line, were co-cultured with various concentrations of SNAP for 8 h and the cell attachment to type I collagen was triggered by the PKC activator, PDBu. The result showed that the binding of T cells to collagen stimulated by PDBu was suppressed in a dose-dependent manner after treatment with the NO donor (Fig. 4C).

### 3.3. Activated macrophages inhibited the adhesion of co-cultured Jurkat T cells to collagen and the NO synthase inhibitor L-NMMA reversed the inhibition

In the Transwell co-culture system, peritoneal macrophages were added into the lower chamber in the presence

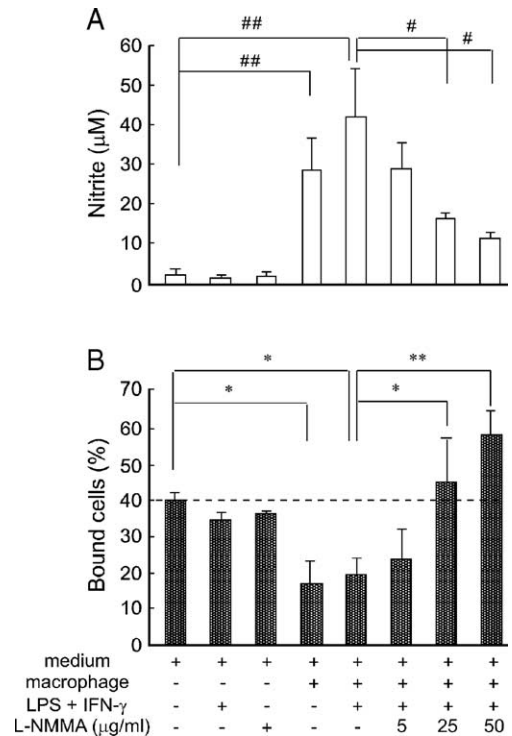


Fig. 6. NO synthase inhibitor L-NMMA decreased NO production (A) and reversed the inhibition of Jurkat T cell adhesion (B) by activated peritoneal macrophages in the Transwell system. Macrophages ( $2 \times 10^6$ /well) were seeded into the outer chamber in the presence of LPS (1 µg/mL)+IFN-γ (200 U/mL) or LPS+IFN-γ+L-NMMA. Jurkat cells were added to the inside chamber and incubated at 37 °C for 24 h. Then, Jurkat cells were harvested and the cell adhesion was tested. In addition, the production of NO in the supernatant of the outer chamber was determined. Data were expressed as the mean ± s.d. of three separate experiments and each assay was performed in triplicate sets. #*P*<0.05, ##*P*<0.01 for NO production by macrophages; \**P*<0.05, \*\**P*<0.01 for adhesion of Jurkat T cells.

of LPS+IFN- $\gamma$ , and Jurkat T cells were seeded into the upper chamber. After co-culture for 0, 6, 12, 18, 24 and 36 h, respectively, NO production in the supernatant and the adhesive ability of T cells were measured. As shown in Fig. 5A, NO production in the medium increased rapidly after incubation and was near to maximum after 18 h. Correspondingly, the adhesive capability to collagen of T cells decreased time-dependently and reached significant inhibition after 18 h (Fig. 5B). Thus, we next examined the effect of 18 h co-culture and the influence of the NO synthase inhibitor L-NMMA. When only LPS+IFN- $\gamma$  or L-NMMA or medium alone was added to the lower chamber, the NO level in the supernatant was almost undetectable, and T cells in the upper chamber were found to have a similar high adhesive ability. In this system, a large amount of NO production was found to be due to the addition of peritoneal macrophages and such production was further increased in the co-presence of LPS+IFN- $\gamma$  (Fig. 6A).

Meanwhile, the adhesive ability of T cells to collagen was significantly inhibited compared with medium alone. The presence of L-NMMA during the stimulation dose-dependently inhibited the production of NO and enhanced the adhesive ability of T cells (Fig. 6B).

3.4. NO donor SNAP inhibited the adhesion, migration and MMP-9 production of spleen T cells purified from PCI-DTH liver-injured mice

Spleen T cells were purified from the liver-injured mice at 6 h after challenge and treated with SNAP for 8 h in vitro. The ability of the cells to adhere to collagen and to migrate through collagen-coated membranes was then examined. As shown in Fig. 7A and B, both adhesion and migration of spleen T cells were dose-dependently inhibited by SNAP. Moreover, SNAP dose-dependently inhibited MMP-9 activity

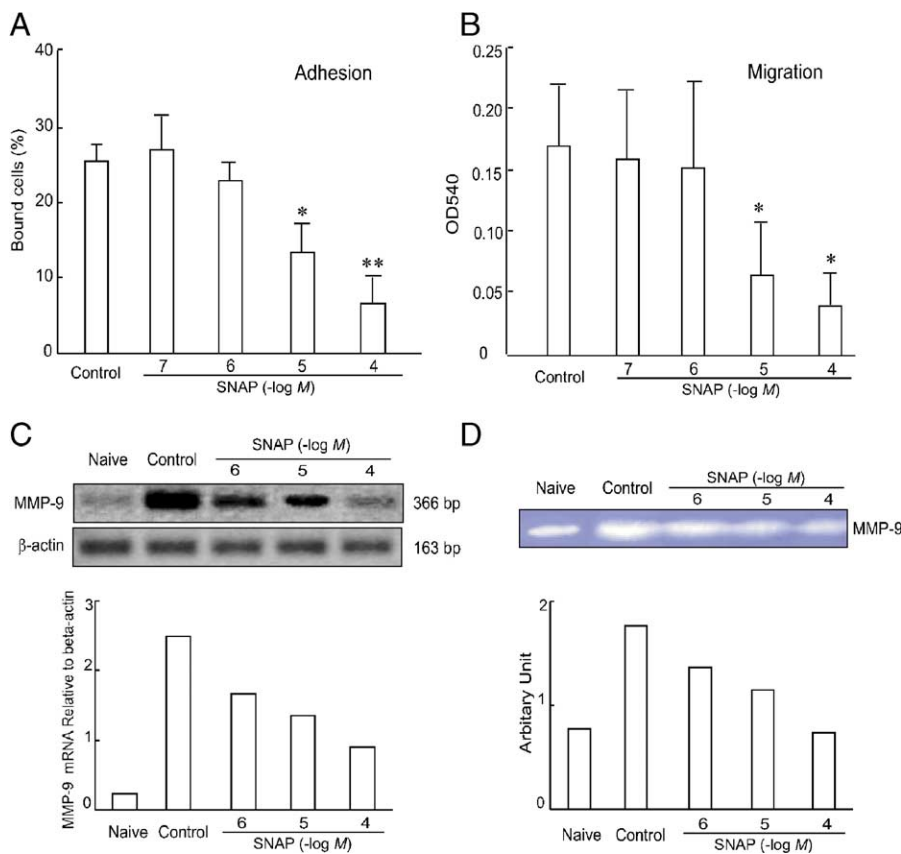


Fig. 7. Effect of NO donor SNAP on the adhesion (A) of, migration (B) of, and MMP-9 expression (C) and production (D) by spleen T cells purified from PCI-DTH liver-injured mice. Six hours after antigen challenge, spleen T cells were purified by mouse T cell enrichment columns and pretreated with various concentration of SNAP at 37 °C for 8 h. One part of the T cells was added to the inside chamber of the Transwell system for the migration assay and the remainder was transferred to a type I collagen coated plate for the adhesion assay. In addition, spleen T cells that had been pretreated with SNAP for 8 h were washed twice and incubated at 37 °C for 24 h. Then, the supernatant was used for the zymography assay and total RNA was extracted from the cells and used for RT-PCR. The bands of MMP-9 were semi-quantified densitometrically using LabWorks 4.0 software. The figure shown here is the representative of three different experiments. Naive: T cells from naive mice, Control: T cells from PCI-DTH liver-injured mice. Data were expressed as the mean  $\pm$  s.d. of three separated experiments and each assay was performed in triplicate sets. \* $P < 0.05$ , \*\* $P < 0.01$  vs control.

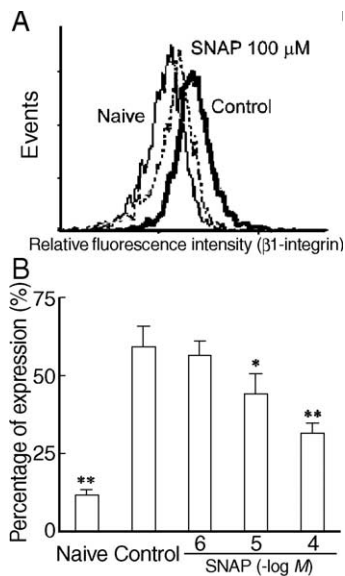


Fig. 8. Effect of NO donor SNAP on the  $\beta 1$ -integrin expression of spleen T cells purified from PCI-DTH liver-injured mice. Six hours after antigen challenge, spleen cells were isolated from the liver-injured mice and T cells were purified. After incubation with SNAP for 8 h, T cells were washed twice and  $\beta 1$ -integrin expression was measured by flow cytometry. A. A representative histogram is shown. B. The results are expressed as mean  $\pm$  s.d. of three different experiments. Naive: T cells from naive mice, Control: T cells from PCI-DTH liver-injured mice. \* $P < 0.05$ , \*\* $P < 0.01$  vs control.

in spleen T cells purified from PCI-DTH liver-injured mice at 6 h after antigen challenge at both the transcription (Fig. 7C) and translation (Fig. 7D) levels. It was noted that SNAP did not show any cytotoxicity to T cells when incubated at 37  $^{\circ}$ C for 8 h (data not shown).

### 3.5. NO donor SNAP decreased the expression of $\beta 1$ -integrin on spleen T cells purified from PCI-DTH liver-injured mice

Spleen T cells were purified from the liver-injured mice at 6 h after the challenge and treated with SNAP for 8 h in vitro. The expression of  $\beta 1$ -integrin was then measured by flow cytometry. As shown in Fig. 8, SNAP significantly decreased  $\beta 1$ -integrin expression on the spleen T cells in a dose-dependent manner.

## 4. Discussion

We have previously reported that NO might play a protective role in PCI-DTH liver-injured mice and demonstrated that NO produced by nonparenchymal cells and hepatocytes in the latter phase could alleviate the hepatocyte damage caused by infiltrating T lymphocytes from peripheral lymphoid organs [17–19]. However, the detailed mechanisms for the NO protection are still poorly understood. In the present study, we exam-

ined the effect of NO in the inflammatory site on peripheral T lymphocyte mobility in the PCI-DTH liver-injured mice to elucidate the protective role of NO. A Transwell system was used to evaluate the effect of NO released from nonparenchymal cells and hepatocytes on the adhesive activity of peripheral spleen T lymphocytes. Both nonparenchymal cells and hepatocytes at 24 h were found to produce higher levels of NO than those at 0 h. Meanwhile, the adhesive capacity of the spleen T cells at 6 h after the challenge, at which time-point we have previously demonstrated that T cells exhibit a strong mobility, and subsequently infiltrate into the liver [17,18], was significantly inhibited when co-cultured with nonparenchymal cells and hepatocytes at 24 h but not with those at 0 h in the Transwell system (Fig. 2B). The NO synthase inhibitor L-NMMA inhibited the production of NO by nonparenchymal cells and hepatocytes at 24 h in a concentration-dependent manner and also reversed the suppression of spleen T cells adhesion (Fig. 3). Because the nonparenchymal cell population includes a variety of cell types, we further separated the nonparenchymal cells into two populations, a Kupffer cell-enriched population (Fraction A) and a lymphocyte-enriched population (Fraction B) to clarify which component of the nonparenchymal cells plays the major role inhibiting T cell adhesion. Compared with the inhibition on the cell binding activity of total nonparenchymal cells at 24 h, Fraction A significantly decreased the adhesion whereas Fraction B did not show any inhibitory effect. On the other hand, Fraction A at 24 h produced more NO than total nonparenchymal cells, while Fraction B produced very little NO (Fig. 2). These data suggested that Kupffer cell-derived NO from 24 h nonparenchymal cells acted as the major force to down-regulate T cell adhesion, while the lymphocyte population in the nonparenchymal cells hardly produced any NO and did not have such a function. Moreover, nonparenchymal cells and hepatocytes from PCI-DTH liver-injured mice have also been demonstrated to produce more NO upon Th1 cytokines stimulation in vitro [18]. Recently, growing evidence indicates that NO may serve as an important modulator of lymphocyte mobility including adhesion and migration [2,3,12,13,26]. These results implied that NO produced in the latter course of liver injury has the capacity of inhibiting peripheral T cell adhesion, while this adhesion to extracellular matrices such as collagen is crucial for T cells to infiltrate into the inflammatory locus in the DTH reaction [11].

To confirm such an inhibitory effect of NO, in the next experiment, we used exogenous and endogenous production of NO to validate its role in the adhesive

activity of Jurkat cells, a well-known T cell line. The *in vitro* treatment of Jurkat T cells with SNAP, which releases NO spontaneously in solution, caused a dose-dependent inhibition of cell adhesion to collagen (Fig. 4C). This result suggested that exogenous NO in this case could down-regulate the adhesive ability of T lymphocytes. Furthermore, to confirm the physiologic significance of this result, the effects of macrophage-derived NO, which is the main NO source in the immune response [27], on the adhesion of Jurkat T cells were examined in the Transwell co-culture system. As shown in Fig. 5, the collagen-binding ability of T cells in the upper chamber negatively correlated with the level of NO in the medium. That is to say, as NO production rose, the cell attachment to collagen triggered by PDBu decreased. Similarly, the cell adhesion was gradually recovered from the suppressed state with the decreased NO production by the NO synthase inhibitor L-NMMA (Fig. 6B). Macrophages are known to produce large amount of NO upon activation by LPS+IFN- $\gamma$  [28]. It was also reported that lymphocytes themselves could produce a certain level of NO under appropriate stimulation [29]. However, NO observed in our work seemed unlikely to derive from T cells because NO was almost undetectable when macrophages were absent (Fig. 6A). Moreover, the possibility that the used reagents themselves caused inhibition of cell attachment could also be ruled out based on the fact that cell adhesion was unaffected when LPS+IFN- $\gamma$  or L-NMMA was added alone to the lower chamber (Fig. 6B). All these findings suggested that NO produced by macrophages inhibited T cell adhesion. Therefore, it is reasonable to conclude that the suppression of T cell infiltration and mobility might underlie, at least in part, the protective effect of NO in PCI-DTH liver-injured mice. This supposition was further confirmed by the observation that the NO donor SNAP could inhibit the adhesion and transmigration (Fig. 7A and B), MMP-9 message RNA expression and protein activity (Fig. 7C and D) of peripheral T lymphocytes freshly purified at 6 h from the liver-injured mice in a dose-dependent manner. This result suggests that NO could down-regulate peripheral T lymphocyte mobility and infiltration into the liver and that this inhibition accounts for the amelioration of the liver injury.

Recent data indicate that the recruitment and infiltration of T lymphocytes in inflammation is regulated by the intricate interplay of adhesion and chemokine receptors [30]. Among them, integrin–collagen interactions are therefore of considerable importance in the pathogenesis of various inflammatory disorders. Cell adhesion to collagens is mediated by the integrins  $\alpha 1\beta 1$

and  $\alpha 2\beta 1$ , both of which bind a range of collagens including types I, IV and VI [31,32]. In the next experiment, therefore, we observed the effect of NO on the  $\beta 1$ -integrin expression to elucidate its inhibitory function on cell adhesion to collagen. Flow cytometric data revealed that SNAP significantly down-regulated  $\beta 1$ -integrin expression on activated peripheral spleen T cells after 8 h of culture (Fig. 8). This finding suggests that NO may inhibit T cell mobility in PCI-DTH liver-injured mice by down-regulating the  $\beta 1$ -integrin expression.

In summary, all the above findings suggested the involvement of NO in the regulation of T lymphocyte adhesion and migration in PCI-DTH liver-injured mice, which might be one of the mechanisms by which NO exerts its protective role in the liver injury. Overproduction of NO has been implicated in the process of many liver diseases or hepatic inflammation in both patients and animal models, including sepsis [14], endotoxemia [33], ischemia reperfusion [34] and liver regeneration [35]. Moreover, the protective role of NO was observed after partial hepatectomy [36], in the liver transplant [37] and carbon tetrachloride-induced liver damage [38]. Our present work may provide a new reference with regard to the mechanism of this protection and the therapeutic use of NO donors and NO synthase inhibitors.

## Acknowledgements

This study was supported by National Natural Science Foundation of China (No. 30230390) and Natural Science Foundation of Jiangsu Province (BK2003206).

## References

- [1] Conner EM, Grisham MB. Nitric oxide: biochemistry, physiology and pathophysiology. *Methods* 1995;7:3–13.
- [2] Holan V, Krulova M, Zajicova A, Pindjakova J. Nitric oxide as a regulatory and effector molecule in the immune system. *Mol Immunol* 2002;38:989–95.
- [3] Coleman JW. Nitric oxide in immunity and inflammation. *Int Immunopharmacol* 2001;1:1397–406.
- [4] Zidek Z, Masek K. Erratic behavior of nitric oxide within the immune system illustrative review of conflicting data and their immunopharmacological aspects. *Int J Immunopharmacol* 1998; 20:319–43.
- [5] Namazi MR. Nitric oxide donors as potential additions to anti-alopeia areata armamentarium. *Inflamm Res* 2003;52:227–9.
- [6] Kahl KG, Schmidt HH, Jung S, Sherman P, Toyka KV, Zielasek J. Experimental autoimmune encephalomyelitis in mice with a targeted deletion of the inducible nitric oxide synthase gene: increased T-helper 1 response. *Neurosci Lett* 2004;358:58–62.
- [7] Van der Veen RC, Dietlin TA, Gray JD, Gilmore W. Macrophage-derived nitric oxide inhibits the proliferation of activated

- T helper cells and is induced during antigenic stimulation of resting T cells. *Cell Immunol* 2000;199:43–9.
- [8] Bauer H, Jung T, Tsikas D, Stichtenoth DO, Frolich JC, Neumann C. Nitric oxide inhibits the secretion of T-helper 1- and T-helper 2-associated cytokines in activated human T cells. *Immunology* 1997;90:205–11.
- [9] Maejima Y, Adachi S, Ito H, Nobori K, Tamamori-Adachi M, Isobe M. Nitric oxide inhibits ischemia/reperfusion-induced myocardial apoptosis by modulating cyclin A-associated kinase activity. *Cardiovasc Res* 2003;59:308–20.
- [10] Fernandes JL, Mamoni RL, Orford JL, Garcia C, Selwyn AP, Coelho OR, et al. Increased Th1 activity in patients with coronary artery disease. *Cytokine* 2004;26:131–7.
- [11] de Fougerolles AR, Sprague AG, Nickerson-Nutter CL, Chi-Rosso G, Rennert PD, Gardner H, et al. Regulation of inflammation by collagen-binding integrins alpha1beta1 and alpha2beta1 in models of hypersensitivity and arthritis. *J Clin Invest* 2000;105:721–9.
- [12] Hokari R, Miura S, Fujimori H, Tsuzuki Y, Shigematsu T, Higuchi H, et al. Nitric oxide modulates T-lymphocyte migration in Peyer's patches and villous submucosa of rat small intestine. *Gastroenterology* 1998;115:618–27.
- [13] Staykova MA, Berven LA, Cowden WB, Willenborg DO, Crouch MF. Nitric oxide induces polarization of actin in encephalitogenic T cells and inhibits their in vitro trans-endothelial migration in a p70S6 kinase-independent manner. *FASEB J* 2003;17:1337–9.
- [14] Benjamim CF, Silva JS, Fortes ZB, Oliveira MA, Ferreira SH, Cunha FQ. Inhibition of leukocyte rolling by nitric oxide during sepsis leads to reduced migration of active microbicidal neutrophils. *Infect Immun* 2002;70:3602–10.
- [15] Secco DD, Paron JA, de Oliveira SH, Ferreira SH, Silva JS, Cunha FQ. Neutrophil migration in inflammation: nitric oxide inhibits rolling, adhesion and induces apoptosis. *Nitric Oxide* 2003;9:153–64.
- [16] Xu Q, Wang R, Jiang JY, Wu FH, Lu JF, Tan PK, et al. Liver injury model in mice induced by a cellular immunologic mechanism delayed-type hypersensitivity-induced liver injury to picryl chloride and phenotype of effector cell. *Cell Immunol* 1996;167:38–43.
- [17] Xu Q, Jiang JY, Cao JS, Wu FH, Fujii H, Saiki I. LEA-1/ICAM-1 interaction is essentially involved in the pathogenesis of delayed-type hypersensitivity-induced liver injury to picryl chloride. *Life Sci* 1998;62:1281–92.
- [18] Xu Q, Cao JS, Zhang XM. Liver-infiltrating T lymphocytes cause hepatocyte damage by releasing humoral factors via LFA-1/ICAM-1 interaction in immunological liver injury. *Inflamm Res* 2002;51:44–50.
- [19] Cao JS, Xu Q, Koda A. Protective involvement of nitric oxide in the liver injury induced by delayed-type hypersensitivity to picryl chloride. *Inflamm Res* 2000;49:578–83.
- [20] Kopydlowski KM, Salkowski CA, Cody MJ, van Rooijen N, Major J, Hamilton TA, et al. Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. *J Immunol* 1999;163:1537–44.
- [21] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [<sup>15</sup>N] nitrate in biological fluids. *Anal Biochem* 1982;126:131–8.
- [22] Franitza S, Hershkoviz R, Kam N, Lichtenstein N, Vaday GG, Alon R, et al. TNF- $\alpha$  associated with extracellular matrix fibronectin provides a stop signal for chemotactically migrating T cells. *J Immunol* 2000;165:2738–47.
- [23] Bonecchi R, Gianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type I helper cells (Th1s) and Th2s. *J Exp Med* 1998;187:129–34.
- [24] Torimura T, Ueno T, Kin M, Harada R, Nakamura T, Sakamoto M, et al. Laminin deposition to type IV collagen enhances heptotaxis, chemokinesis, and adhesion of hepatoma cells through  $\beta$ 1-integrins. *J Hepatol* 2001;35:245–53.
- [25] Mitsui G, Hirano T, Niwano Y, Mitsui K, Ohara O, Yanagihara S, et al. Effect of a topical steroid on gene expressions for chemokines in mice with contact hypersensitivity. *Int Immunopharmacol* 2004;4:57–69.
- [26] Ialenti A, Ianaro A, Maa P, Sautebin L, Di Rosa M. Nitric oxide inhibits leukocyte migration in carrageenan-induced rat pleurisy. *Inflamm Res* 2000;49:411–7.
- [27] Jeon SD, Lim JS, Moon CK. Carbofuran suppresses T-cell-mediated immune responses by the suppression of T-cell responsiveness, the differential inhibition of cytokine production, and NO production in macrophages. *Toxicol Lett* 2001;119:143–55.
- [28] Vadeveloo PK, Vairo G, Hertzog P, Kola I, Hamilton JA. Role of type I interferons during macrophage activation by lipopolysaccharide. *Cytokine* 2001;2:1639–46.
- [29] Gagnon C, Leblond FA, Filep JG. Peroxynitrite production by human neutrophils, monocytes and lymphocytes challenged with lipopolysaccharide. *FEBS Lett* 1998;431:107–10.
- [30] D'Ambrosio D, Iellem A, Colantonio L, Clissi B, Pardi R, Sinigaglia F. Localization of Th-cell subsets in inflammation: differential thresholds for extravasation of Th1 and Th2 cells. *Immunol Today* 2000;21:183–6.
- [31] White DJ, Puranen S, Johnson MS, Heino J. The collagen receptor subfamily of the integrins. *Int J Biochem Cell B* 2004;36:1405–10.
- [32] Tuckwell D, Humphries M. Integrin–collagen binding. *Semin Cell Dev Biol* 1996;7:649–57.
- [33] Wirthlin DJ, Cullen JJ, Spates ST, Conklin JL, Murray J, Caropreso DK, et al. Gastrointestinal transit during endotoxemia: the role of nitric oxide. *J Surg Res* 1996;60:307–11.
- [34] Schulz R, Kelm M, Heusch G. Nitric oxide in myocardial ischemia/reperfusion injury. *Cardiovasc Res* 2004;61:402–13.
- [35] Carnovale CE, Scapini C, Alvarez ML, Favre C, Monti J, Carrillo MC. Nitric oxide release and enhancement of lipid peroxidation in regenerating rat liver. *J Hepatol* 2000;32:798–804.
- [36] Sugawara Y, Kubota K, Ogura T, Esumi H, Inoue K, Takayama T, et al. Increased nitric oxide production in the liver in the perioperative period of partial hepatectomy with Pringle's maneuver. *J Hepatol* 1998;28:212–20.
- [37] Yagnik GP, Takahashi Y, Tsoulfas G, Reid K, Murase N, Geller DA. Blockade of the L-Arginine/NO synthase pathway worsens hepatic apoptosis and liver transplant preservation injury. *Hepatology* 2002;36:573–81.
- [38] Muriel P. Nitric oxide protection of rat liver from lipid peroxidation, collagen accumulation, and liver damage induced by carbon tetrachloride. *Biochem Pharmacol* 1998;56:773–9.