ELSEVIER

European Journal of Pharmacology 446 (2002) 177–185

ejp

www.elsevier.com/locate/ejphar

A novel anti-fibrotic agent pirfenidone suppresses tumor necrosis factor-α at the translational level

Hitoshi Nakazato, Hisashi Oku*, Shoji Yamane, Yuji Tsuruta, Ryuji Suzuki

Department of Immunology, Shionogi Discovery Research Laboratories, Shionogi & Co. Ltd., 2-5-1, Mishima, Settsu, Osaka 566-0022, Japan

Received 26 April 2002; accepted 3 May 2002

Abstract

A new experimental drug pirfenidone (5-methyl-1-phenyl-2-1*H*-pyridine-2-one) has been reported to have beneficial effects for the treatment of certain fibrotic diseases. Here, we studied the anti-inflammatory activities of pirfenidone by investigating the mechanism of its inhibitory effect on cytokine production. In RAW264.7 cells, a murine macrophage-like cell line, pirfenidone suppressed the proinflammatory cytokine tumor necrosis factor-α (TNF-α) by a translational mechanism, which was independent of activation of the mitogen-activated protain kinase (MAPK) 2, p38 MAP kinase, and c-*Jun* N-terminal kinase (JNK). In the murine endotoxin shock model, pirfenidone potently inhibited the production of the proinflammatory cytokines, TNF-α, interferon-γ, and interleukin-6, but enhanced the production of the anti-inflammatory cytokine, interleukin-10. The in vivo model also showed that pirfenidone suppressed the cytokine production by a translational mechanism, though interleukin-10 transcription was activated by pirfenidone. These findings show that pirfenidone inhibits the production of the proinflammatory cytokine selectively at the translational level. Therefore, cytokine inhibitory activities play an important role in the anti-inflammatory activities of pirfenidone. Coupled with the fact that this inhibitory effect is selective, translational, and not for total protein synthesis, this drug may have a clinical effect on inflammation and fibrosis with very low toxicity.

Keywords: Pirfenidone; Inflammation; Cytokine; TNF-α (tumor necrosis factor-α); Translation; MAPK2 (mitogen-activated protein) kinase 2

1. Introduction

In the primary immune response to infection or injury, macrophages synthesize proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6, and interferon-γ, which play important roles in promoting inflammatory processes (van Deuren et al., 1992; Bendtzen, 1988). The proinflammatory cytokines are highly expressed in the injured tissues and induced when macrophages are exposed to Gram-negative bacterial endotoxins (van Deuren et al., 1992; Bendtzen, 1988; Blackwell and Christman, 1996; Ozmen et al., 1994). Therefore, cytokine synthesis inhibitors prevent the immune response to invasive stimuli.

The proinflammatory cytokines are involved in the pathogenesis of fibrosis. The series of events can be divided into three stages: (1) the inflammatory process (Piguet et al., 1989, 1990, 1993); (2) tissue injury; and (3) the subsequent restoration process of woundhealing (Piguet et al., 1989; Khalil et al., 1996; Yoshida et al., 1995). The inflammatory process occurs with the production of proinflammatory cytokines, such as TNF-α. TNF-α was highly expressed in the lungs of mice, which had received bleomycin (Piguet et al., 1989). Therefore, the cytokine synthesis inhibitors have anti-fiborosis activity.

Pirfenidone (5-methyl-1-phenyl-2-(1*H*)-pyridone) is currently being evaluated as an anti-fibrotic agent. Pirfenidone can prevent formation of experimentally induced fibrotic lesions, and reduce or remove excessive fibrotic lesions or scar tissue (Iyer et al., 1995; Shimizu et al., 1996). In the progression of fibrosis, the proinflammatory cytokines continuously cause tissue inflammation and overexpression of collagen occurs due to an excessive response of repairing injured tissue (Zhang and Phan, 1996; van den Blink et al., 2000; Selman et al., 2001). Previous studies showed that pirfenidone downregulates the proinflammatory cytokine expression, fibroblast proliferation, and collagen matrix synthesis (Cain et al., 1998; Gurujeyalakshmi et al., 1999). Therefore, studies of the cytokine inhibition mechanism by pirfenidone are important for clarifying the effects of this

* Corresponding author. Tel.: +81-6-6382-2612; fax: +81-6-6382-2598.
E-mail address: hisashi.oku@shionogi.co.jp (H. Oku).

0014-2999/02/$ - see front matter
PII: S0014-2999(02)01758-2

drug on the mechanisms of immune system and fibrosis. However, the detailed mechanism remains to be elucidated.

In the present study, we show that the suppression of proinflamatory cytokines by lipopolysaccharide is specific, translational, and independent of mitogen-activated protein kinase (MAPK) 2, p38 MAP kinase and c-*Jun* N-terminal kinase (JNK). Because of the posttranscriptional inhibition, pirfenidone inhibits cytokine production even when cytokine transcription becomes fully activated by lipopolysaccharide. In the in vivo application of pirfenidone, cytokine inhibition is posttranscriptional and independent of MAPK2 as in vitro, and it can enhance the production of the protective interleukin-10.

2. Materials and methods

2.1. Animals and reagents

Female C57BL/6 mice (7–11 weeks) were purchased from Charles River Japan (Osaka, Japan). Lipopolysaccharide from *Escherichia coli* strain O55:B5 prepared by Boivon's method was obtained from Difco Laboratories (Detroit, MI). D-Galactosamine hydrochrolyde was purchased from Tokyo Chemicals (Tokyo, Japan). Actinomycin D was obtained from Sigma (St. Louis, MO). Multigel and immobilon polyvinylidene difluoride (PVDF) transfer membrane were purchased from Daiichi Pure Chemicals (Tokyo, Japan). RPMI 164O was purchased from Nikken (Kyoto, Japan). Fetal calf serum was obtained from Hyclone (Logan, UT). Penicillin–streptomycin was obtained from Life Technologies (Grand Island, NY).

All experiments using animals were followed according to Animal Care and Use Committee of Shionogi (Shionogi).

2.2. RAW264.7 cell culture

RAW264.7 murine macrophage-like cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in culture as described previously (Jue et al., 1990; Cohen et al., 1996). The confluent cells in 96-well plates were treated with lipopolysaccharide (1 μg/ml) in the absence or presence of pirfenidone. Next, after washing with phosphate buffered saline (PBS), the cells were lysed by cell lysis buffer (New England Biolabs, Beverly, MA). Cytokines in conditioning media and cell lysates were measured by enzyme-linked immuno-solvent assay (ELISA) as described below. Cell viability was measured by Cell counting kit (Dohjin Chemicals, Japan) after simulation with lipopolysaccharide.

2.3. Cytokine assays

TNF-α, interleukin-1, interleukin-6, interleukin-10, and interferon-γ concentrations in the supernatants and cell lysates were determined by ELISA kits purchased from Genzyme Techne (Minneapolis, MN). All ELISA kits were utilized as described by the manufacturers.

2.4. Metabolic labeling

RAW264.7 cells were cultured with lipopolysaccharide (1 μg/ml) in the absence or presence of pirfenidone for 2 h, then incubated for another 30 min in methionine-free media. After 1 h labeling with [^{35}S]methionine (Amersham Pharmacia Biotech, Buckinghamshire, UK), the cells were lysed by cell lysis buffer after washing with PBS. The metabolically labeled conditioning media and cell lysates were collected by centrifugation and precleared with Protein G Sepharose FF (Amersham Pharmacia Biotech). Subsequently, TNF-α proteins were immunoprecipitated with rat monoclonal anti-mouse antibody (BD PharMingen, San Diego, CA) and Protein G Sepharose FF. Immune precipitates were separated with Multigel-15/25. The gels were then amplified, vacuum dried, and exposed to X-ray film at −70 °C. For measurement of the pirfenidone effect on protein synthesis, the total labelled cell lysates were treated with hot trichloroacetic acid and counted by liquid scintillation counter.

2.5. Northern blot analysis

RAW264.7 cells were cultured with lipopolysaccharide (1 μg/ml) in the absence or presence of pirfenidone for 4 h. Next, total cellular RNA was isolated from cells with an RNeasy mini kit (QIAGEN, Hilden, Germany). The 2-μg samples of RNA were electrophorased through 1% agarose gel and transferred to a nylon membrane. For standardization, ribosomal RNAs were detected by soaking the blot in methylene blue solution. Hybridization in 0.5 M sodium phosphate pH 7.2, 1% bovine serum albumin (BSA), 1 mM EDTA, and 7% sodium dodecyl sulfate (SDS) at 45 °C and washing in 2 × standard saline citrate (SSC) and 0.1% SDS at 25 °C were done with TNF-α probe. RT-PCR amplimer sets for mouse TNF-α (Clontech, Palo Alto, CA) were used to prepare probe for TNF-α probe.

2.6. TNF-α transcript stability

RAW264.7 cells were cultured with lipopolysaccharide (1 μg/ml) in the absence or presence of pirfenidone for 4 h. Total RNA was isolated from parallel cultures before and at various times after transcriptional arrest with 5 μg/ml actinomycin D. The relative levels of TNF-α transcripts in total RNA from each sample were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, as described below.

2.7. RT-PCR analysis

Total RNA was reverse transcribed by using First-strand synthesis system for RT-PCR kit (Life Technologies) and amplified by ExTaq DNA polymerase (Takara, Kyoto,

Japan). The thermo-cycle conditions were 95 °C for 2 min, 60 °C for 1 min, and 72 °C for 1 min for denaturing, annealing, and extension. RT-PCR amplimer sets for mouse TNF-α, interleukin-6, interferon-γ, and interleukin-10 (Clontech) were used for PCR. The PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide fluorescence.

2.8. MAPK2 assay

A total of 1×10^6 RAW264.7 cells in six-well plates were starved in medium containing 0.1% bovine serum albumin for 16 h. The cells were then pretreated with pirfenidone (300 μg/ml) and, 3 h later, cultured with lipopolysaccharide (1 μg/ml) for 30 min. After washing with PBS, the cells were lysed using 200 μl cell lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin). Protein concentrations were determined using a BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL). For determination of MAPK2, c-*Jun* N-terminal kinase and p38 MAP kinase activities, SAPK/c-*Jun* kinase Assay Kit and SAPK2α/p38/RK Assay Kit (Upstate Biotechnology, Lake Placid, NY) were used. Each sample was separated on Multigel-10/20. The gels were then vacuum dried and exposed to X-ray film at −70 °C.

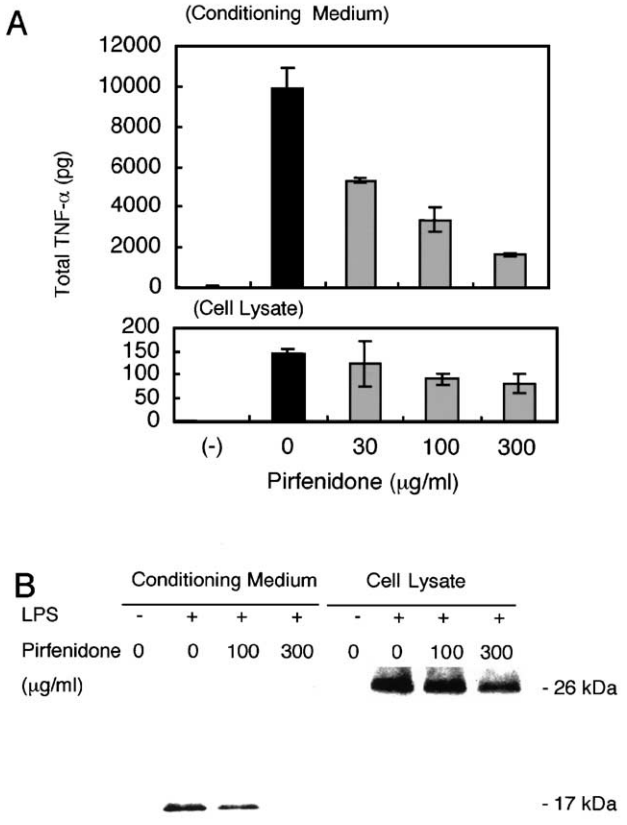

Fig. 1. Effect of pirfenidone on TNF-α secretion and expression in lipopolysaccharide-stimulated RAW264.7 cells. (A) RAW264.7 cells were treated with lipopolysaccharide (1 μg/ml) in the absence or presence of pirfenidone at indicated concentrations for 8 h. TNF-α in conditioning medium and cell lysate was measured by ELISA. Data shown are mean±SD ($n=3$). (B) RAW264.7 were treated with lipopolysaccharide (1 μg/ml) in the absence or presence of pirfenidone at indicated concentrations for 2.5 h. After being labeled with [^{35}S]methionine for 1 h, conditioning medium and cell lysate were immunoprecipitated with anti-TNF Ab. Samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorography. LPS: lipopolysaccharide.

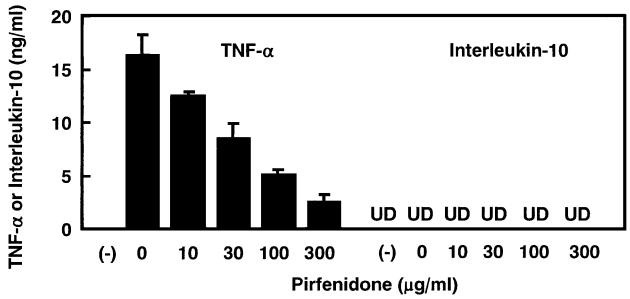

Fig. 2. Interleukin-10 was much less induced by lipopolysaccharide than TNF-α in RAW264.7 cells, and pirfenidone did not enhance interleukin-10 induction. RAW264.7 cells were treated with lipopolysaccharide (1 μg/ml) in the absence or presence of pirfenidone at indicated concentrations for 8 h. TNF-α and interleukin-10 in conditioning medium was measured by ELISA. Data shown are mean±SD ($n=3$). UD: underdetectable limit.

2.9. Western blot analysis

Cell lysates were prepared from 1×10^6 RAW264.7 cells in six-well plates as described above. A total of 150 μl cell lysates were concentrated by Microcon-10 (Millipore, Bedford, MA), added to Laemmli buffer and boiled for 5 min. The sample was separated using Multigel-12.5, and transferred to a PVDF membrane. The membrane was probed with anti-phospho-activation transcription factor (ATF2) or anti-phospho-c-*Jun* (Upstate), the substrate of p38 MAP kinase and JNK, and subsequently incubated with a secondary anti-rabbit antibody conjugated to horse radish peroxidase (New England Biolabs). Bands were detected using

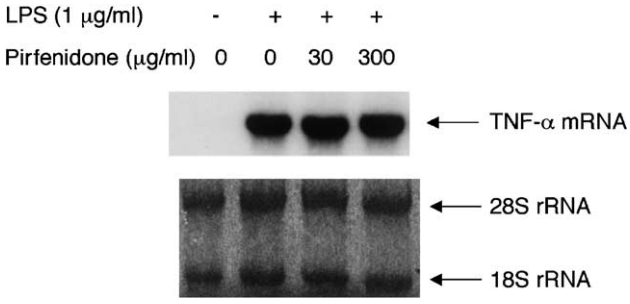

Fig. 3. Lack of effect of pirfenidone on TNF-α mRNA induction by lipopolysaccharide in RAW 264.7 cells. RAW264.7 cells were cultured with lipopolysaccharide (1 μg/ml) in the absence or presence of pirfenidone for 4 h and total cellular RNA was isolated. Two-microgram samples of RNA were electrophorased, standardized by ribosomal RNAs detection, and used for Northern blot analysis with TNF-α probe as Materials and methods. LPS: lipopolysaccharide.

ECL Western Blotting Detection Reagent (Amersham Pharmacia Biotech).

2.10. Experimentally induced endotoxic shock and protocols

C57BL/6 mice received oral administration of pirfenidone. After 10 min, murine endotoxin shock was induced by intraperitoneal injection with 50 μg/kg lipopolysaccharide and 250 mg/kg D-galactosamine hydrochrolyde. Each mouse was sacrificed at various time points after lipopolysaccharide treatment and the plasma was harvested. All plasma were stored at −80 °C until cytokine assays. Plasma cytokine levels were measured by ELISA, as described above. For determination of the transcription level of each inflammatory cytokine, spleens were removed from individual mice at the times indicated, and total spleen RNA was isolated using TRIzol reagent (Life Technologies). Cytokine expressions were determined by RT-PCR analysis, as described above. All experiments using animals were followed according to Animal Care and Use Committee of Shionogi (Shionogi).

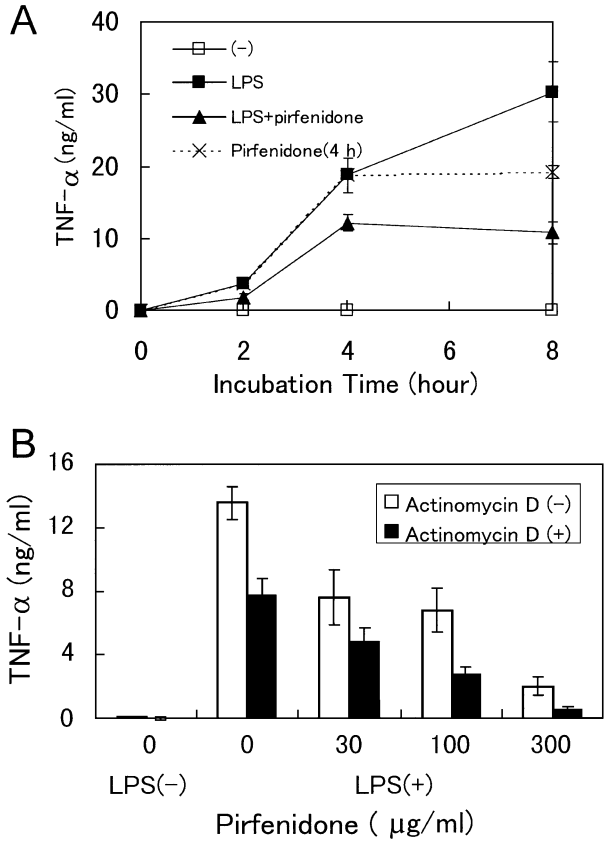

Fig. 4. Posttranscriptional suppressive effect of pirfenidone on TNF-α production. (A) Effect of pirfenidone on TNF-α production, when added to RAW264.7 cells at the same time or after lipopolysaccharide. RAW264.7 cells were incubated without (closed square) or with 300 μg/ml pirfenidone either at the same time (closed triangle) or 4 h (x) after 1 μg/ml lipopolysaccharide treatment. Control cells were not treated with lipopolysaccharide (open square). TNF-α in conditioning medium was measured by ELISA. Each value represents mean±SD ($n=3$). (B) Effect of pirfenidone on TNF-α production after transcriptional arrest by actinomycin D. RAW264.7 cells were treated only with lipoplysaccharide (1 μg/ml) for 4 h, and conditioning medium was changed for new medium containing lipopolysaccharide, pirfenidone, and actinomycin D (5 μg/ml). TNF-α in conditioning medium at 2 h after the medium change was measured by ELISA. Each value represents mean±SD ($n=3$). LPS: lipopolysaccharide.

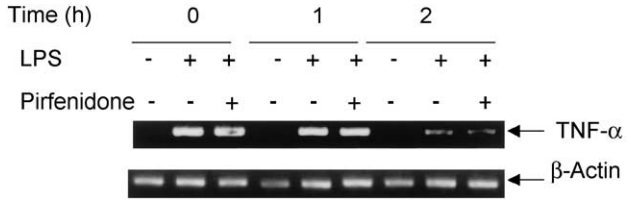

Fig. 5. Lack of effect of pirfenidone on TNF-α transcript stability in lipopolysaccharide-stimulated RAW264.7 cells. RAW264.7 cells were cultured with lipopolysaccharide (1 μg/ml) in the absence or presence of 300 μg/ml pirfenidone for 4 h and then treated with 5 μg/ml actinomycin D to inhibit new mRNA synthesis. At the times indicated, total cellular RNA was isolated and analyzed for TNF-α mRNA by RT-PCR method. LPS: lipopolysaccharide.

3. Results

3.1. Pirfenidone suppression of TNF-α is translational

To investigate the mechanism of pirfenidone action on cytokine synthesis, we used the murine macrophage-like cell line RAW264.7. First, we determined whether pirfeni-

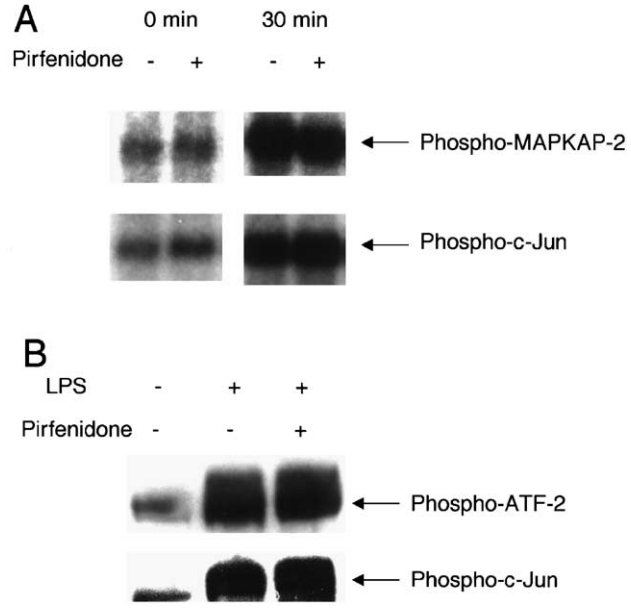

Fig. 6. Lack of effect of pirfenidone on MAPK2 pathway in lipopolysaccharide-stimulated RAW264.7 cells. RAW264.7 cells were starved for 16 h and pretreated with 300 μg/ml pirfenidone for 3 h prior to a 30 min stimulation with 1 μg/ml lipopolysaccharide. The cells were lysed, and an equal amount of each sample was analyzed for (A) MAPK2 activity and (B) phosporylation of the MAPK2 substrates as Materials and methods. LPS: lipopolysaccharide.

done inhibits TNF-α secretion or expression when RAW264.7 cells were exposed to lipopolysaccharide for 8 h. Exposure of RAW264.7 cells to pirfenidone significantly suppressed the levels of both cell-associated and secreted TNF-α by ELISA (Fig. 1A). But pirfenidone did not affect RAW264.7 cell viability (data not shown).

We then examined the effect of pirfenidone on TNF-α synthesis by metabolically labeling lipopolysaccharide-stimulated RAW264.7 cells with [^{35}S]methionine for 1 h in the absence or presence of pirfenidone. The levels of synthesized pro-TNF-α and mature protein were monitored by immunoprecipitation analysis of cell lysates and conditioning medium. As shown in Fig. 1B, 26 kDa pro-TNF-α measured in the cell lysates was inhibited by pirfenidone in the same manner as 17 kDa mature-TNF-α in the conditioning medium. Pirfenidone did not affect total ^{35}S-labelled protein synthesis (data not shown). These results provide direct evidence that the mechanism of pirfenidone action occurs via inhibition of cytokine synthesis, and not by suppressing cytokine release or processing.

We also investigated whether anti-inflammatory cytokine interleukin-10 expression is enhanced by pirfenidone in RAW264.7 cells. Interleukin-10 was undetectable level in lipopolysaccharide-treated RAW264.7 cells. As shown in Fig. 2, pirfenidone was not able to enhance interleukin-10 expression.

Next, we determined whether pirfenidone inhibits the TNF-α transcription. Northern blots of total RNA extracted from RAW264.7 cells treated concomitantly with lipopolysaccharide and pirfenidone showed that pirfenidone had no effect on the level of TNF-α mRNA (Fig. 3). So pirfenidone did not affect lipopolysaccharide-stimulated TNF-α transcription. This finding was confirmed in experiments, in which pirfenidone was added to the cells at 4 h after the lipopolysaccharide challenge when TNF-α transcription was fully activated, and was able to suppress TNF-α production at 8 h after the lipopolysaccharide challenge (Fig. 4A). The level of TNF-α mRNA at 2 h after the pirfenidone addition at 4 h after the lipopolysaccharide challenge was same as that at 6 h after the lipopolysaccharide challenge (data not shown). In addition, pirfenidone suppressed lipopolysaccharide-induced TNF-α production even after transcriptional arrest by actinomycin D added at the time when TNF-α transcription was fully activated by lipopolysacchar-

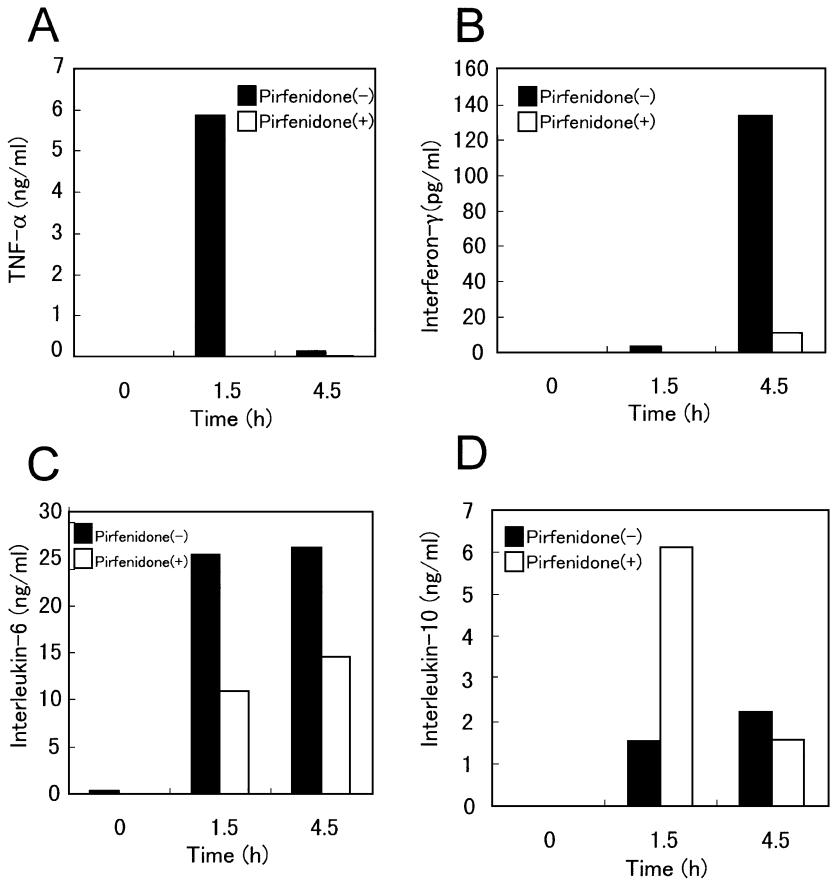

Fig. 7. Pirfenidone suppression of TNF-α (A), interferon-γ (B), and interleukin-6 (C) production but enhancement of interleukin-10 (D) production in lipopolysaccharide-injected mice. Female C57BL/6 mice were pretreated with 500 mg/kg pirfenidone 10 min before intraperitoneal injection of 50 μg/kg lipopolysaccharide and 250 mg/kg D-galactosamine. Plasma from two mice were used for cytokine production analysis by ELISA. All cytokine determinations are representative two independent experiments.

ide (Fig. 4B). Therefore, the effect of pirfenidone on TNF-α production is posttranscriptional.

To determine whether pirfenidone affects posttranscriptional events by reducing the stability of mRNA, we compared the decay profiles of TNF-α mRNA in lipopolysaccharide-stimulated RAW264.7 cells incubated with and without pirfenidone. As shown in Fig. 5, the decay patterns of TNF-α mRNA in both pirfenidone-treated and untreated RAW264.7 cells were very similar. Therefore, pirfenidone had no effect on the mRNA stability.

3.2. The effect of pirfenidone is independent of MAPK2

The MAPK2s, p38 MAPK and c-*Jun* N-terminal kinase (JNK), regulate lipopolysaccharide-stimulated cytokine translation (Lee et al., 1994; Swantek et al., 1997; Andersson and Sundler, 2000). We tested whether the posttranscriptional effect of pirfenidone is due to interference with MAPK2 pathways. Fig. 6A shows that the activation of p38 and JNK by lipopolysaccharide was not influenced by pretreatment with pirfenidone. The data was the same when pirfenidone was added at the time of lipopolysaccharide addition (data not shown). It is reported that the peak activity occurs at approximately 30 min poststimulation by lipopolysaccharide (Swantek et al., 1997).

We also determined whether pirfenidone has an effect on the phosphorylation of ATF-2 and c-*Jun*, the substrate of p38 and JNK, by lipopolysaccharide. Fig. 6B shows that pirfenidone did not inhibit phophorylation of ATF-2 and c-*Jun* in RAW264.7 cells. The phosphorylation of ATF-2 and c-*Jun* in both lipopolysaccharide and pirfenidone-treated RAW264.7 cells decreased same as that in only lipopolysaccharide-treated cells from 30 min until 7 h after lipopolysaccharide treatment (data not shown). Therefore, the pirfenidone posttranscriptional effect on cytokine production is independent of the MAPK2 pathway.

3.3. The effect of pirfenidone on cytokine production is posttranscriptional

Based on the above in vitro data, we surmised that pirfenidone can also have a posttranscriptional effect on cytokine production in vivo.

First, we determined whether pirfenidone could regulate cytokine production in serum of lipopolysaccharide-injected mice. We administered pirfenidone (250 mg/kg) orally to mice, followed by an intraperitoneal injection of lipoporysaccharide (50 μg/kg) and 250 mg/kg D-galactosamine hydrochrolyde 10 min later, and determined the serum levels of the different cytokines at 1.5 and 4.5 h after the lipopolysaccharide challenge. As shown in Fig. 7, pirfenidone suppressed serum levels of TNF-α, interferon-γ, and interleukin-6, but the suppression of interleukin-6 production was weak. In contrast, under parallel experimental conditions, anti-inflammatory interleukin-10 production was enhanced by pirfenidone.

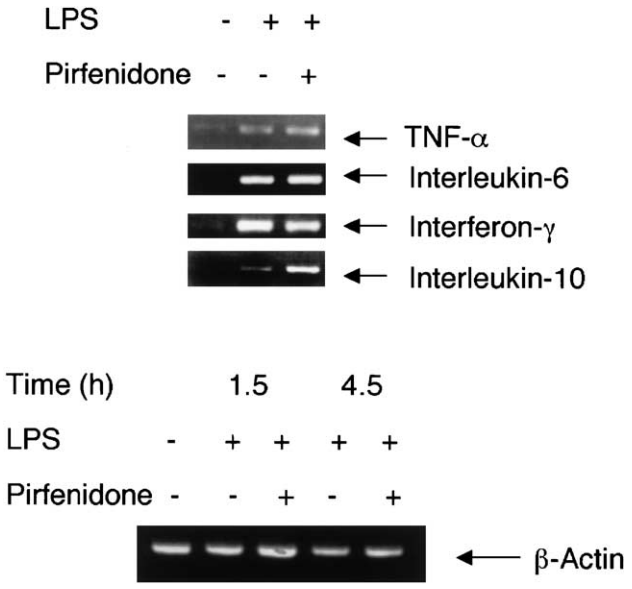

Fig. 8. Pirfenidone does not suppress TNF-α, interferon-γ, and interleukin-6 mRNA expression but enhances interleukin-10 mRNA expression in spleen from lipopolysaccharide-injected mice. Female C57BL/6 mice were pretreated with 500 mg/kg pirfenidone 10 min before intraperitoneal injection of 50 μg/kg lipopolysaccharide and 250 mg/kg D-galactosamine. Spleen was isolated 1.5 h after lipopolysaccharide for TNF-α, interleukin-6, and interleukin-10, 4.5 h after lipopolysaccharide for interferon-γ, and used for RT-PCR analysis of cytokine mRNA expression. LPS: lipopolysaccharide.

Next, we determined the cytokine mRNA levels in spleen at 1.5 and 4.5 h after the lipopolysaccharide challenge. Similar to its in vitro effect, pirfenidone had no effect on the levels of TNF-α, interferon-γ, and interleukin-6 (Fig. 8). On the other hand, pirfenidone enhanced the interleukin-10 mRNA level. Taken together, though pirfenidone upregulates the transcriptional level of the anti-inflammatory cytokine interleukin-10, Pirfenidone also downregulates the posttranscriptional level of the preinflammatory cytokine production in vivo.

4. Discussion

In the primary immune response, the inflammation is regulated by cytokine induction and function. The proinflammatory cytokines, such as TNF-α, interferon-γ, and interleukin-6, are highly expressed in the inflammatory tissue site, and induced when macrophages are exposed to Gram-negative bacterial endotoxins (van Deuren et al., 1992; Bendtzen, 1988; Blackwell and Christman, 1996; Ozmen et al., 1994). On the other hand, the anti-inflammatory cytokines, such as interleukin-10, suppress induction of the proinflammatory cytokines (Fiorentino et al., 1991; Howard et al., 1993). Therefore, cytokine synthesis inhibitors have anti-inflammatory effects and can be used clinically as anti-inflammatory drugs (McNiff et al., 1995; Takahashi et al., 1993; Karin, 1998).

Pirfenidone, a small molecular weight molecule (m.w. of 185.2), is known to be a broad spectrum anti-fibrotic agent. It has been developed for clinical use against idiopathic pulmonary fibrosis, which is extremely difficult to treat. Pirfenidone can safely arrest further progression of existing fibrotic lesions, reduce or remove excessive fibrotic lesions or scar tissue, and prevent the development of fibrotic lesions (Iyer et al., 1995; Shimizu et al., 1996). Pirfenidone is also reported to regulate cytokine synthesis. Pirfenidone suppresses not only growth factors, such as platelet-derived growth factor (PDGF) (Gurujeyalakshmi et al., 1999) and transforming growth factor (TGF)-β (Iyer et al., 1999), that increase the fibrotic lesion, but also proinflammatory cytokines, such as TNF-α and interleukin-6 (Cain et al., 1998). It is reported that TNF-α, one of the proinflammatory cytokines, is expressed in the lung of mice that receive bleomycin and plays an important role in primary fibrosis formation (Piguet et al., 1989, 1990, 1993). The process of the fibrosis formation can be divided into three stages: (1) the inflammatory process, (2) tissue injury, and (3) a subsequent restoration process of woundhealing (Khalil et al., 1996; Yoshida et al., 1995). Therefore, the suppressive effect of pirfenidone on TNF-α production is one of the important factors of the anti-fibrotic pirfenidone action.

In our current study, pirfenidone selectively suppressed cytokine induction by lipopolysaccharide, but not the transcription and mRNA stability. Therefore, the suppressive effect of pirfenidone on cytokine synthesis is posttranscriptional. From in vitro data, pirfenidone suppressed induction of both 26 kDa pro-TNF-α in cells and 17 kDa mature-TNF-α in medium. TNF-α coverting enzyme is known as a pro-TNF-α processing enzyme. However, because pirfenidone suppressed intracellular pro-TNF-α, the suppression is not dependent on TNF-α coverting enzyme but translational.

MAPK2s, p38 MAPK, and c-*Jun* N-terminal kinase (JNK) are know to regulate cytokine production at the post-transcriptional level. (Lee et al., 1994; Swantek et al., 1997; Andersson and Sundler, 2000). Indeed, it is reported that the p38 MAPK inhibitors, SB203580 (4-(3-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1*H*-imidazole) (Andersson and Sundler, 2000), SB202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl) 1*H*-imidazole) (Manthey et al., 1998), and CNI-1493 (*N*,*N*-bis[3,5-bis[1-(aminoiminomethyl)hydrazonoethyl]phenyl]decanediamide tetrahydrochloride) (Cohen et al., 1996) suppress cytokine production at the posttranscriptional level. However, pirfenidone did not affect both activation of MAPK2 and phosphorylation of the substrates of MAPK2 in RAW264.7 cells. Thus, cytokine suppression of pirfenidone may be independent of MAPK2. As molecules that suppress cytokine production at the translational level, inosine (Hasko et al., 2000a) and spermine (Zhang et al., 1997; Hasko et al., 2000b) are known, and both have anti-inflammatory effects. It is reported that an adenine and uridine (AU)-rich element (ARE), UUAUUUAUU in the 3′ -untranslated region (3′ -UTR) of cytokine transcripts is an important determinant of the posttranscriptional regulation of cytokine production (Akashi et al., 1994; Lagnado et al., 1994; DeMaria and Brewer, 1996). ARE has been regarded as a regulator of cytokine mRNA stability, and it is reported that AUF1 protein binds the ARE domain and makes cytokine mRNA unstable (Demaria and Brewer, 1996). However, as pirfenidone did not suppress TNF-α mRNA stability, its suppressive effect may not be related to these proteins. A recent study has shown that TIA-I binds to ARE in TNF-α transcripts 3′ -UTR as a translational silencer, and does not regulate the transcript stability (Piecyk et al., 2000). Pirfenidone may regulate activity of translational silencers such as TIA-I at the translational level.

Pirfenidone suppressed proinflammatory cytokine production, but on the other hand, enhanced that of the anti-inflammatory cytokine interleukin-10. Inosine, which suppresses cytokine production at the posttranscriptional level, also enhances interleukin-10 production in vivo (Hasko et al., 2000a). In case of cytokine suppression by pirfenidone in vivo, there may be a similar mechanism of cytokine regulation for both inosine and pirfenidone. Though pirfenidone suppressed proinflammatory cytokine at the post-transcriptional level, pirfenidone enhanced interleukin-10 at the transcriptional level. It is reported that no relevant contribution from Rel, C/EBP, or AP-1 binding sites, which regulate most proinflammatory cytokine promoters, was observed in the interleukin-10 promoter (Brightbill et al., 2000). Therefore, pirfenidone may regulate other transcription factors (e.g. Sp1). Interleukin-10 suppresses the serum level of TNF-α and lethal shock in endotoxin-challenged mouse (Howard et al., 1993). It is reported that introduction of the interleukin-10 gene into mice was reported to inhibit bleomycin-induced lung injury in vivo (Arai et al., 2000). Therefore, interleukin-10 may have an anti-inflammatory effect and an anti-fibrotic effect. However, interleukin-10 was much less induced by lipopolysaccharide than TNF-α and pirfenidone did not enhance interleukin-10 production in RAW264.7 cells though pirfenidone suppressed lipopolysaccharide-stimulated TNF-α induction. It suggests that the mechanism of pirfenidone action is unlikely to occur through interleukin-10 production or that the mechanism of pirfenidone action is both cell and tissue dependent. It is reported that sodium butyrate is able to inhibit proinflammatory cytokine interleukin-12 but enhance interleukin-10 in vitro model (Selman et al., 2001). But pirfenidone does not have mechanism like this in RAW264.7 cells.

In the endotoxic shock model, transcription of proinflammatory cytokines is already activated. The TNF-α mRNA level is reported to increase in the bleomycin fibrosis model (Piguet et al., 1989). Pirfenidone suppressed TNF-α induction even after transcription became fully activated. Consequently, the posttranscriptional effect of pirfenidone on cytokine production is one of the reasons that this drug can be used clinically as an anti-fibrotic drug.

In conclusion, pirfenidone suppressed the proinflammatory cytokine TNF-α and interferon-γ selectively at the posttranscriptional level, and while it enhanced the anti-

inflamatory cytokine interleukin-10. In the in vitro model, TNF-α production was suppressed at the translational level and even after transcription became fully activated. These findings suggest that pirfenidone has not only an anti-fibrotic effect but also an anti-inflammatory effect via cytokine regulation and offers a clinical effect with few side effects because it acts on not transcription but post-transcription.

Acknowledgements

The authors thank Dr. K. Shirai and Dr. T. Sakata of Shionogi for their useful suggestions and expert technical assistance.

References

Akashi, M., Shaw, G., Hachiya, M., Elstner, E., Suzuki, G., Koeffler, P., 1994. Number and location of AUUUA motifs: role in regulating transiently expressed RNAs. Blood 83, 3182–3187.

Andersson, K., Sundler, R., 2000. Signalling to translational activation of tumour necrosis factor-alpha expression in human THP-1 cells. Cytokine 12, 1784–1787.

Arai, T., Abe, K., Matsuoka, H., Yoshida, M., Mori, M., Goya, S., Kida, H., Nishino, K., Osaki, T., Tachibana, I., Kaneda, Y., Hayashi, S., 2000. Introduction of the interleukin-10 gene into mice inhibited bleomycin-induced lung injury in vivo. Am. J. Physiol., Lung Cell. Mol. Physiol. 278, L914–L922.

Bendtzen, K., 1988. Interleukin 1, interleukin 6 and tumor necrosis factor in infection, inflammation and immunity. Immunol. Lett. 19, 183–191.

Blackwell, T.S., Christman, J.W., 1996. Sepsis and cytokines: current status. Brit. J. Anesthesia 77, 110–117.

Brightbill, H.D., Plevy, S.E., Modlin, R.L., Smale, S.T., 2000. A prominent role for Sp1 during lipopolysaccharide-mediated induction of the IL-10 promotor in macrophages. J. Immunol. 164, 1940–1951.

Cain, W.C., Stuart, R.W., Lefkowitz, D.L., Starnes, J.D., Margolin, S., Lefkowitz, S.S., 1998. Inhibition of tumor necrosis factor and subsequent endotoxin shock by pirfenidone. Int. J. Immunopharmacol. 20, 685–695.

Cohen, P.S., Nakshatri, H., Dennis, J., Caragine, T., Bianchi, M., Cerami, A., Tracey, K.L., 1996. CNI-1493 inhibits monocyte/macrophage tumor necrosis factor by suppression of translation efficiency. Proc. Natl. Acad. Sci. USA 93, 3967–3971.

DeMaria, C.T., Brewer, G., 1996. AUF1 binding affinity to A+U-rich elements correlates with rapid mRNA degradation. J. Biol. Chem. 271, 12179–12184.

Fiorentino, D.F., Zlotnik, A., Mosmann, T.R., Howard, M., O'Garra, A., 1991. Interleukin-10 inhibits cytokine: production by activated macrophages. J. Immunol. 147, 3815–3822.

Gurujeyalakshmi, G., Hollinger, M.A., Giri, S.N., 1999. Pirfenidone inhibits PDGF isoforms in bleomycin hamster model of lung fibrosis at the translational level. Am. J. Physiol. 276, L311–L318.

Hasko, G., Kuhel, D.G., Nemeth, Z.H., Mabley, J.G., Stachlewitz, R.F., Virag, L., Lohinai, Z., Southan, G.J., Salzman, A.L., Szabo, C., 2000a. Inosine inhibits inflammatory cytokine production by a posttranscriptional mechanism and protects against endotoxin-induced shock. J. Immunol. 164, 1013–1019.

Hasko, G., Kuhel, D.G., Marton, A., Nemeth, Z.H., Deitch, E.A., Szabo, C., 2000b. Spermine differentially regulates the production of interleukin-12 p40 and interleukin-10 and suppresses the release of the T helper 1 cytokine interferon-γ. Shock 14, 144–149.

Howard, M., Muchamuel, S., Andrade, S., Menon, S., 1993. Interleukin 10 protects mice from lethal endotoxemia. J. Exp. Med. 177, 1205–1208.

Iyer, S.N., Wild, J.S., Schiedt, M.J., Hyde, D.M., Margolin, S.B., Giri, S., 1995. Dietary intake of pirfenidone ameliorates bleomycin induced lung fibrosis in hamsters. J. Lab. Clin. Med. 125, 779–785.

Iyer, S.N., Gurujeyalakshmi, G., Giri, S.N., 1999. Effects of pirfenidone on transforming growth factor-beta gene expression at the transcriptional level in bleomycin hamster model of lung fibrosis. J. Pharmacol. Exp. Ther. 291, 367–373.

Jue, D.M., Sherry, B., Luedke, C., Manogue, K.R., Cerami, A., 1990. Processing of newly synthesized cachectin/tumor necrosis factor in endotoxin-stimulated macrophages. Biochemistry 29, 8371–8377.

Karin, M., 1998. New twists in gene regulation by glucocorticoid receptor: is DNA binding dispensable? Cell 93, 487–490.

Khalil, N., O'Connor, R.N., Flanders, K.C., Unruh, H., 1996. TGF-β1, but TGF-β2 or TGF-β3, is differencially present in epithelial cells of advanced pulmonary fibrosis. Am. J. Respir. Cell. Mol. Biol. 14, 131.

Lagnado, C.A., Brown, C.Y., Goodall, G.J., 1994. AUUUA is not sufficient to promote poly(A) shortening and degradation of an mRNA: the functional sequence within AU-rich elements may be UUAUUUA(U/A)(U/A). Mol. Cell. Biol. 14, 7984–7995.

Lee, J.C., Laydon, J.T., McDonnell, P.C., Gallagher, T.F., Kumar, S., Green, D., McNulty, D., Blumenthat, M.J., Heys, J.R., Sandvatter, S.W., Strickler, J.E., McLaughlin, M.M., Siemens, I.R., Fischer, S.M., Livi, G.P., White, J.R., Adams, J.L., Young, P.R., 1994. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 372, 739–746.

Manthey, C.L., Wang, S.W., Kinney, S.D., Yao, Z., 1998. SB202190, a selective inhibitor of p38 mitogen-activated protein kinase, is a powerful regulator of LPS-induced mRNAs in monocytes. J. Leukoc. Biol. 64, 409–417.

McNiff, P.A., Laliberte, R.E., Svensson, L., Pazoles, C.J., Gabel, C.A., 1995. Inhibition of cytokine activation processes in vitro by tenidap, a novel anti-inflammatory agent. Cytokine 7, 196–208.

Ozmen, L., Pericin, M., Hakimi, J., Chizzonite, R.A., Wysocka, M., Trinchieri, G., Gately, M., Garotta, G., 1994. Interleukin 12 , interferon γ, and tumor necrosis factor α are the key cytokines of the generated Shwarzman reaction. J. Exp. Med. 180, 907–915.

Piecyk, M., Wax, S., Beck, A.R.P., Kedarsha, N., Gupta, M., Maritim, B., Chen, S., Gueydan, C., Kruys, V., Streuli, M., Anderson, P., 2000. TIA-1 is a translational silencer that selectively regulates the expression of TNF-α. EMBO J. 19, 4154–4163.

Piguet, P.F., Collart, M.A., Grau, G.E., Kapanci, Y., Vassalli, P., 1989. Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. J. Exp. Med. 170, 655–663.

Piguet, P.F., Collart, M.A., Grau, G.E., Sappino, A.P., Vassalli, P., 1990. Requirement of tumour necrosis factor for development of silica-induced pulmonary fibrosis. Nature 344, 245–247.

Piguet, P.F., Ribaux, C., Karpuz, V., Grau, G.E., Kapanci, Y., 1993. Expression and localization of tumor necrosis factor-alpha and its mRNA in idiopathic pulmonary fibrosis. Am. J. Pathol. 143, 651–655.

Selman, M., King, T.E., Pardo, A., 2001. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. Ann. Intern. Med. 134, 136–151.

Shimizu, F., Fukagawa, M., Kurokawa, K., Yamauchi, S., Margolin, S.B., 1996. Pirfenidone prevents the progression of irreversible glomerular sclerotic lesions. J. Am. Soc. Nephrol. 7, 1965.

Swantek, J.L., Cobb, M.H., Geppert, T.D., 1997. Jun N-terminal inase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-α) translation: glucocorticoids inhibit TNF-α translation by blocking JNK/SAPK. Mol. Cell Biol. 17, 6274–6282.

Takahashi, N., Brouckaert, P., Fiers, W., 1993. Cyclooxygenase inhibitors prevent the induction of tolerance to the toxic effects of tumor necrosis factor. J. Immunother. 14, 16–21.

van den Blink, B., Jansen, H.M., Peppelenbosch, M.P., 2000. Idiopathic

pulmonary fibrosis: molecular mechanisms and possible therapeutic strategies. Arch. Immunol. Ther. Exp. (Warsz) 48, 539–545.

van Deuren, M., Dofferhoff, A.S., van der Meer, J.W., 1992. Cytokines and the response to infections. J. Pathol. 168, 349–356.

Yoshida, M., Sakuma, J., Hayashi, S., Abe, K., Saito, I., Harada, S., Sakatani, M., Yamamoto, S., Matsumoto, N., Kaneda, Y., 1995. A histologically distinctive interstitial pneumonia induced by overexpression of the interleukin 6, transforming growth factor beta 1, or platelet-derived growth factor B gene. Proc. Natl. Acad. Sci. U. S. A. 92, 9570–9574.

Zhang, K., Phan, S.H., 1996. Cytokines and pulmonary fibrosis. Biol. Signals 5, 232–239.

Zhang, B.M., Caragine, T., Wang, H.C., Cohen, P.S., Botchkina, G., Soda, K., Bianchi, M., Ulrich, P., Cerami, A., Sherry, B., Tracey, K.J., 1997. Spermine inhibits proinflammatory cytokine synthesis in human mononuclear cells: a counterregulatory mechanism that restrains the immune response. J. Exp. Med. 185, 1759–1768.