SHOCK, Vol. 32, No. 5, pp. 554–562, 2009

IN VIVO IL-18 SUPPLEMENTATION AMELIORATES LETHAL ACUTE LUNG INJURY IN BURN-PRIMED ENDOTOXEMIC MICE: A NOVEL ANTI-INFLAMMATORY ROLE OF IL-18

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Received 23 Dec 2008; first review completed 14 Jan 2009; accepted in final form 20 Jan 2009

ABSTRACT—Previously, we have found that a prior burn insult induces lethal acute lung injury (ALI) and overproduction of proinflammatory cytokines after LPS challenge in mice. The current study was aimed to determine the role of IL-18 in burn-induced LPS hypersensitivity. Except sham group, mice were subjected to a 15% total body surface area full-thickness burn and either untreated or treated with IL-18 alone, IL-18 + anti–IL-10 antibody or IL-18 + isotype immunoglobulin G. LPS was intravenously administered to all mice on the 11th day, and the mice were killed at the indicated time point, or survival was examined. We additionally examined cytokine production by splenic cells *in vitro* for the elucidation of immunologic mechanisms. Unexpectedly, the liver IL-18 decreased transiently after burn injury, and *in vivo* IL-18 supplementation improved survival and ameliorated ALI, as well as reducing the lung contents of all cytokines examined, except IL-10. Neutralization of IL-10 cancelled the protective effect of IL-18. In splenic macrophages obtained from burned mice, the production of macrophage inflammatory protein 2 (MIP-2), TNF-α, and IL-10 was augmented, whereas *in vivo* IL-18 supplementation decreased MIP-2 production, but increased IL-10 production. Furthermore, a physiological concentration of IL-18 directly attenuated MIP-2 production by splenic cells *in vitro*. Burn injury induces LPS hypersensitivity through augmented production of proinflammatory cytokines by systemic macrophages. IL-18 supplementation is protective for LPS-induced lethal ALI through the direct anti-inflammatory effect on macrophages as well as by *in vivo* acceleration of IL-10 production, and could thus be an effective prophylactic strategy against septic complications in critically ill patients.

KEYWORDS—Two-hit phenomenon, priming, systemic inflammatory response syndrome, M2 macrophage interleukin-10

INTRODUCTION

Patients with critical conditions, such as severe burns, multiple trauma, and hemorrhagic shock, become susceptible to secondary insults, including sepsis, and may develop septic shock and/or multiple organ dysfunction syndrome, especially acute lung injury (ALI)/acute respiratory distress syndrome (1). Multiple organ dysfunction syndrome was not merely derived from the direct injurious effects of insults to these organs, and LPS administration after burn injury induced progressive hypoxia in a sheep model (2). Furthermore, 38% of the severely burned patients developed lung failure even without inhalation injury (3). These complications are major determinants of prognosis at the later stages, and new therapeutic strategies based on novel pathophysiological mechanisms are currently being sought. Hyperresponsiveness to secondary stimuli after an initial insult is known as the "two-hit" phenomenon and has been confirmed in several experimental models (1, 4, 5). Previously, we have developed a murine model of burn-primed lethal septic ALI and showed that a prior burn insult induced sustained and exaggerated production of TNF-α and macrophage inflammatory protein 2 (MIP-2) in response to an LPS challenge (6). We further demonstrated that two independent cytokine synthesis inhibitors ameliorated ALI and improved survival, and concluded that inflammatory cytokines play pivotal roles in inducing lethal endotoxemic ALI after burn injury (6). However, the mechanisms responsible for the exaggerated cytokine response after burn injury were not determined.

IL-18 was initially identified as a key mediator in *Propionibacterium acnes*–induced hypersensitivity to LPS (7, 8), and its diverse immunomodulating functions have recently been clarified. IL-18 induces interferon-γ (IFN-γ) and TNF-α in the presence of IL-12, and was initially considered to be a T_H1 or proinflammatory cytokine. *In vivo* IL-18 neutralization protects wild-type mice from the effects of LPS challenge (9), and IL-18 knockout mice are resistant to LPS (10). Furthermore, exogenous IL-18 protects mice from lethal *Cryptococcus* infection (11). Thus, IL-18 seems to have *in vivo* immunostimulatory and anti-infection activities. On the other hand, although it was recently revealed that IL-18 can also induce T_H2 cytokines, such as IL-4, IL-10, and IL-13, under specific conditions, especially when IL-12 is absent (8, 12, 13), the anti-inflammatory functions of IL-18 have not been elucidated.

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This study was supported by a grant-in-aid from the Ministry of Health, Labor and Welfare of Japan (grant no. 14370364) and by the Keio Gijuku Fukuzawa Memorial Fund for the Advancement of Education and Research.
DOI: 10.1097/SHK.0b013e31819e2db6

We initially speculated that the production of IL-18 is increased after burn injury, thus contributing to LPS hypersensitivity, and examined the sequential changes in IL-18 after burn injury in a murine model. Unexpectedly, however, IL-18 decreased transiently after burn injury, and thus we hypothesized that IL-18 could play anti-inflammatory functions at its physiological concentrations, and the decrease in IL-18 after burn injury is responsible for the induction of LPS hypersensitivity in the present study. We found that IL-18 supplementation *in vivo* rescued mice from lethal endotoxemic ALI after burn injury, as well as induced anti-inflammatory functions *in vivo* and *in vitro*, partly through the production of IL-10. Furthermore, we also examined the involvement of IL-10.

MATERIALS AND METHODS

Murine model of burn injury and sequential endotoxemia

Animal care and all procedures were approved by the Laboratory Animal Care and Use Committee of the School of Medicine, Keio University. Seven-week-old male Balb/c mice (Charles River Laboratories Japan, Inc, Yokohama, Japan), weighing 23 to 28 g, were caged and supplied with water and food *ad libitum* for approximately 1 week.

Our animal experimental protocols were summarized in Figure 1. The first series of experiments was performed to determine the time course of IL-18 and IL-12 in the lung and liver with or without burn injury (Fig. 1A). The mice were divided into sham and burn groups, and burn injury was applied as previously described (6). Briefly, the dorsum of the mice was dehaired with electrical clippers and depilatory cream under pentobarbital anesthesia (50 mg/kg) 1 day before the experiment. On the following day (day 1), each mouse was placed on a plastic template under effective ether anesthesia, and the shaved area was exposed to hot steam for 5 s to produce a full-thickness burn covering approximately 15% of the total body surface area (TBSA). Mice in the sham group were similarly anesthetized and shaved, but not exposed to hot steam. All mice were immediately resuscitated with 4 mL of sterile LPS-free isotonic sodium chloride solution intraperitoneally. The animals were killed on days 1, 3, 7, and 11 after injury (n = 5–6 on each day in each group).

The second series of experiments was planned to determine the effect of IL-18 supplementation on survival at 72 h after LPS administration, and the mice were divided into sham, burn, and IL-18 groups, and burn injury was applied to burn and IL-18 groups (Fig. 1B). On the 11th day after sham burn or burn injury, 3 mg/kg LPS (*Escherichia coli* 0111:B4; Sigma Chemical, St Louis, Mo) in 10 mL/kg isotonic sodium chloride solution was administered i.v. to all animals. In the IL-18 group, 0.2 μg of recombinant mouse IL-18 (MBL Co Ltd, Nagoya, Japan) was administered every other day for 10 days after burn injury. Before performing the experiments, we had carried out several preliminary studies to determine the optimal dosage and time point of IL-18 administration. This experimental protocol was also used to determine lung histopathology, microvascular permeability, and cytokine content after LPS administration. Furthermore, to assess the effect of burn injury and IL-18 administration *in vivo* on cytokine production, certain mice were killed on day 11 before LPS challenge, and spleen was removed for *in vitro* analysis.

Finally, we examined the effect of IL-10 neutralization after IL-18 supplementation on LPS-induced lethality to assess the contribution of IL-10 to the protective effect of IL-18 (Fig. 1C). In this experiment, mice were divided into five groups, including the three groups in Figure 1B. In another two groups, mice were subjected to burn injury, supplemented with IL-18, and then divided into two groups, one of which received 1 mg of rat antimouse IL-10 (JES5-2A5, Biolegend Inc, San Diego, Calif), and the other, isotype control immunoglobulin G ([IgG] Biolegend Inc) by intraperitoneal injection. Thirty minutes later, LPS was administered i.v. to all groups.

Sample preparation for lung histopathology, tissue cytokine analysis, and spleen cell culture

At the indicated time point, the mice were anesthetized with an intraperitoneal injection of pentobarbital, exsanguinated by cardiac puncture with

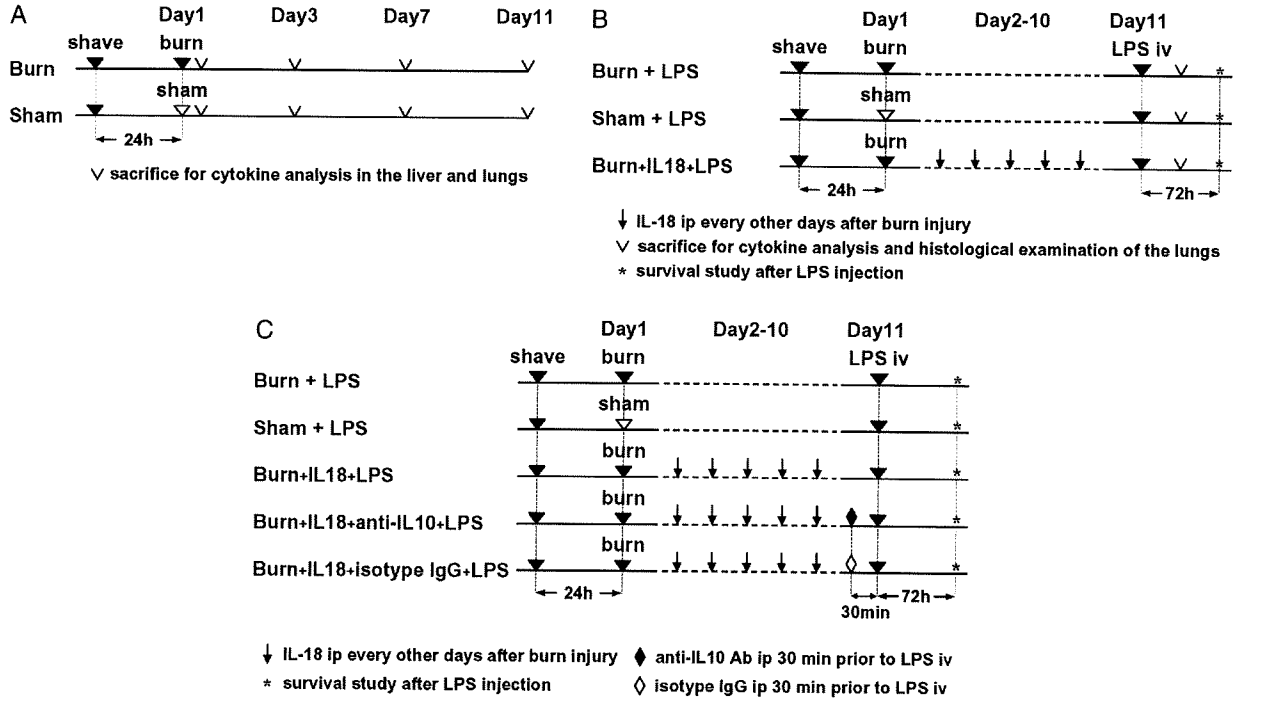

FIG. 1. **Illustration of *in vivo* experimental protocols.** A, The first protocol was aimed to determine the time course of IL-18 and IL-12 in the lung and liver after burn injury, and mice were divided into sham and burn groups. A 15% TBSA full-thickness burn was applied to burn group. The animals were killed on days 1, 3, 7, and 11. B, The second protocol was planned to determine the effect of burn injury and IL-18 supplementation on lethal LPS-induced ALI. After LPS administration, 72-h survival was observed, or lung histopathology, microvascular permeability, and cytokines content were examined. Mice were divided into sham, burn, and IL-18 groups, and the burn injury was applied to burn and IL-18 groups. In IL-18 group, 0.2 μg of mouse IL-18 was administered every other day for 10 days. On day 11, 3 mg/kg LPS was administered i.v. to all animals. Certain mice were killed, and spleen was removed on day 11 before LPS challenge, to assess *in vitro* cytokines production after burn injury or IL-18 administration. C, The third protocol was designed to examine the effect of IL-10 neutralization after IL-18 supplementation on LPS-induced lethality. Mice were divided into five groups, including the three groups in B. In another two groups, mice were subjected to burn injury, supplemented with IL-18 and then divided into two groups, one of which received 1 mg of rat antimouse IL-10, and the other isotype, control IgG by intraperitoneal injection 30 min before LPS administration.

a heparinized syringe, and the lungs, liver, and spleen were resected aseptically. The whole right lung was used for cytokine determination. The left upper lobe of the lung was sliced, fixed with buffered 4% paraformaldehyde solution, and subsequently stained with hematoxylin and eosin. The liver and the left lower lung were kept frozen until analysis. For cytokine analysis, the lung or liver was sliced, suspended in 10 or 50 times their weight of phosphate buffer solution (PBS), homogenized, and centrifuged, and the supernatants were collected. We determined the cytokine levels in the supernatants of liver and lungs by a specific sandwich enzyme-linked immunosorbent assay (ELISA) for each cytokine.

Evans blue lung microvascular permeability assay

Alterations of lung vascular permeability at 12 h after LPS administration were investigated by tissue accumulation of Evans blue as previously described (14). Evans blue avidly binds to serum albumin, and its tissue distribution was used as a marker for the transcapillary flux of macromolecules in ALI. Mice in the sham, burn, and IL-18 groups were administered 20 mg/kg Evans blue (Sigma-Aldrich Co) i.v. 120 min before termination of the experiment. After exsanguination, lungs were removed and kept frozen at −80°C. The right lungs were weighed and homogenized in 1 mL PBS, and Evans blue was extracted from lung homogenates by incubating samples in 2 mL of formamide at 60°C for 14 to 18 h. The supernatant was separated by centrifugation at 10,000*g* for 30 min. The concentration of Evans blue in lung homogenate supernatants was quantified by a dual wavelength spectrophotometric method at absorptions of 620 and 740 nm, which allows for correction of contaminating heme pigments.

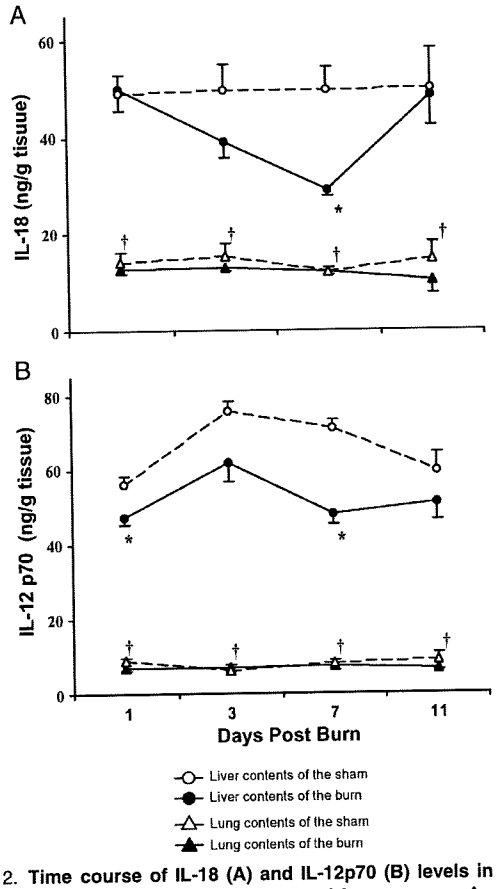

FIG. 2. **Time course of IL-18 (A) and IL-12p70 (B) levels in the liver and lung tissues of mice in the sham and burn groups.** As shown in Figure 1A, mice in the burn group were subjected to 15% TBSA burn injury, killed on days 1, 3, 7, and 11 after injury, and IL-18 and IL-12p70 were determined by ELISA. The levels of both IL-18 and IL-12 in the liver were significantly greater than those in the lungs throughout the course of observation. In the liver, the IL-18 level in the burn group was significantly lower than that in the sham group on day 7. The liver content of IL-12 was also lower in burn mice than in sham mice. In contrast, there were no significant differences in the lung IL-18 and IL-12 contents between the two groups. The values are expressed as mean ± SEM (n = 9–10 in each group at a different time point). Results are representative of at least two independent experiments performed in triplicate. $^{*}P < 0.05$ vs. liver level in sham group, $^{\dagger}P < 0.05$ vs. liver level in burn group on the same day.

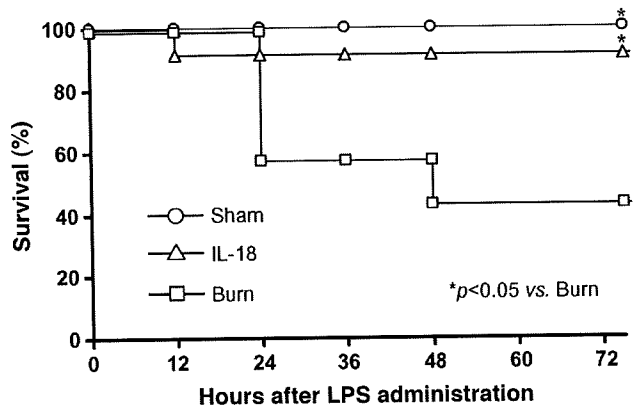

FIG. 3. **Cumulative survival after LPS challenge in the sham, burn, and IL-18 groups.** IL-18 significantly improved the survival of mice in the burn group after secondary endotoxemia. As shown in Figure 1B, mice were intraperitoneally injected with 200 ng IL-18 on days 2, 4, 6, 8, and 10 after burn injury. On day 11, 3 mg/kg LPS was administered, and the mice were observed for the next 72 h. Pretreatment with IL-18 significantly improved the survival of the mice. n = 10 to 14 in each group. Results are representative of two independent experiments. $^{*}P < 0.05$ by the log-rank test, as compared with the burn group.

Separation of murine spleen cells for in vitro *culture*

Spleens were resected aseptically from sham, burn, or IL-18 groups, and gently minced in RPMI-1640 supplemented with 5% fetal calf serum (FCS). For *in vitro* IL-18 treatment, spleens were obtained from untreated mice. Cells were then filtered with a nylon mesh strainer and incubated with 0.84% Tris-buffered ammonium chloride to induce hemolysis of any contaminating red blood cells. After centrifugation, the spleen cells were resuspended in RPMI-1640/5% FCS, counted, and diluted to yield a final concentration of 1.0×10^6 cells/mL.

Cytokine secretion by splenic cells in vitro

As most cytokines were undetectable in the blood even after burn injury, we examined the capacity of splenic cells harvested from burn and sham mice on the 11th day after insult (Fig. 1B) to produce cytokines *in vitro* in response to LPS and anti-CD3 antibody (Ab) to assess the changes in spleen cell immunologic phenotype. We adopted LPS as a specific stimulus for macrophages/monocytes and anti-CD3 Ab as a stimulus for T lymphocytes. As a preliminary experiment, we incubated spleen cells with several concentrations of LPS and anti-CD3 Ab and found that 10 μg/mL LPS and 1 μg/mL monoclonal antimouse CD3 Ab induced maximal cytokine production (data not shown). We also predetermined an optimal incubation period, 24 or 48 h, for each cytokine. In this experiment, spleen cells were suspended in RPMI-1640 supplemented with 5% FCS and cultured with either 10 μg/mL LPS or 1 μg/mL monoclonal antimouse CD3 Ab (clone I45-2C11, BD Biosciences) at 37°C in the presence of 5% carbon dioxide for 24 or 48 h for assessment of macrophage and lymphocyte function in the spleen, respectively.

In addition, to assess the immunoregulatory functions of IL-18 at physiological and pathologically low concentrations, we examined the secretion of MIP-2 and IL-10 by spleen cells in response to LPS. We harvested spleen cells from untreated mice, divided them into three groups, preincubated the cells with three different concentrations of IL-18 for 9 days, and finally stimulated them with 10 μg/mL LPS. The first and second groups were preincubated in RPMI-1640/5% FCS containing 100 pg/mL or 30 pg/mL IL-18. The third group was incubated with 30 pg/mL IL-18 for the initial 7 days and then with 100 pg/mL IL-18 for the next 2 days.

After each experiment, supernatants were collected by centrifugation and kept frozen at −80°C until analysis.

Cytokine assays by ELISA and RT-PCR

For analysis of cytokines and albumin in plasma, the supernatants from organ homogenates, or cultured splenocytes, we used specific sandwich ELI SA kits for each. The ELISA kits for mouse TNF-α, IFN-γ, IL-4, IL-6, IL-10, IL-12 p70, and IL-18 were purchased from BD Biosciences; for mouse MIP-2, from Wako Junyaku (Tokyo, Japan); and for mouse albumin, from Bethyl, Inc (Montgomery, Tex).

Levels of TNF-α, MIP-2, and IL-10 messenger RNA (mRNA) in lung tissue were also analyzed by real-time reverse transcription–polymerase chain reaction (RT-PCR), as previously described (6). Briefly, total RNA was extracted from each lung tissue sample using an RNAeasy kit (Qiagen, Germany), and single-stranded cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Rockville, Md) with an oligo(dT)$_{20}$ primer. Gene expression was measured by quantitative PCR with a dual-labeled fluorogenic probe and a 7700 Prism sequence detector (Applied Biosystems, Foster City, Calif).

Statistical analysis

Cytokine levels were compared by ANOVA, followed by Scheffe's comparison test. Cumulative survival within 72 h among the groups was compared by the log-rank test. The level of significance was accepted as $P < 0.05$. All data were expressed as means ± SEM, unless otherwise specified.

RESULTS

Unexpected temporal decrease in liver IL-18 levels after burn injury

We examined the levels of IL-18 in the plasma, lung, and liver of mice with burn injury. The plasma level of IL-18 was below the lower detection limit (25.43 pg/mL) on days 1, 3, 7, and 11 in burned mice, whereas plasma IL-18 was detectable in 3 out of 20 sham mice (130 pg/mL or lower, data not shown). As shown in Figure 2A, liver IL-18 levels in burned mice were significantly lower than those in sham mice on day 7. In contrast, no significant differences in lung IL-18 levels were observed between the two groups.

We also measured IL-12p70 levels in lung and liver. Liver IL-12 levels were significantly lower in the burn group than in the sham group on days 1 and 7 (Fig. 2B). The IL-18 and IL-12 levels in the liver were significantly higher than those in the lungs at all time points and in all groups (Fig. 2, A and B). These findings demonstrated that burn injury attenuated the production of T_H1 cytokines in the liver. We also obtained preliminary findings that the levels of plasma IL-18 at days 4 to 7 tended to be lower than those at day 1 and day 7 after burn injury, suggesting the activation of common pathological pathways in mice and humans (data not shown).

Protection from lethal ALI after LPS administration in mice with burns by in vivo *IL-18 supplementation*

Figure 3 shows the cumulative survival within 72 h after LPS challenge in each group. The survival rate at 72 h was 100% in the sham group, 43% in the burn group, and 92% in the IL-18 group. Mortality was significantly higher in the burn group than in the other two groups, indicating that IL-18 supplementation improved the survival.

Histopathologic examination of the lung at 12 h after endotoxemia showed that LPS administration induced severe alveolar wall thickening and increased neutrophil accumulation, compatible with ALI, in the burn group (Fig. 4B), but not in the sham group (Fig. 4A). In the IL-18 group, the findings characteristic of ALI were attenuated (Fig. 4C). Moreover, lung albumin permeability as determined by Evans blue dye extravasation technique was significantly higher in the burn group than in the sham group and were significantly attenuated in the IL-18 group, which may also support the attenuation of lung injury by IL-18 supplementation (Fig. 4D).

Unique regulation of IL-10 after burn injury and IL-18 supplementation in vivo

We then determined the levels of various cytokines in lung homogenates at 12 h after *in* vivo LPS administration to assess local cytokine production *in vivo*. The cytokines examined were TNF-α, MIP-2, and IL-6 (proinflammatory

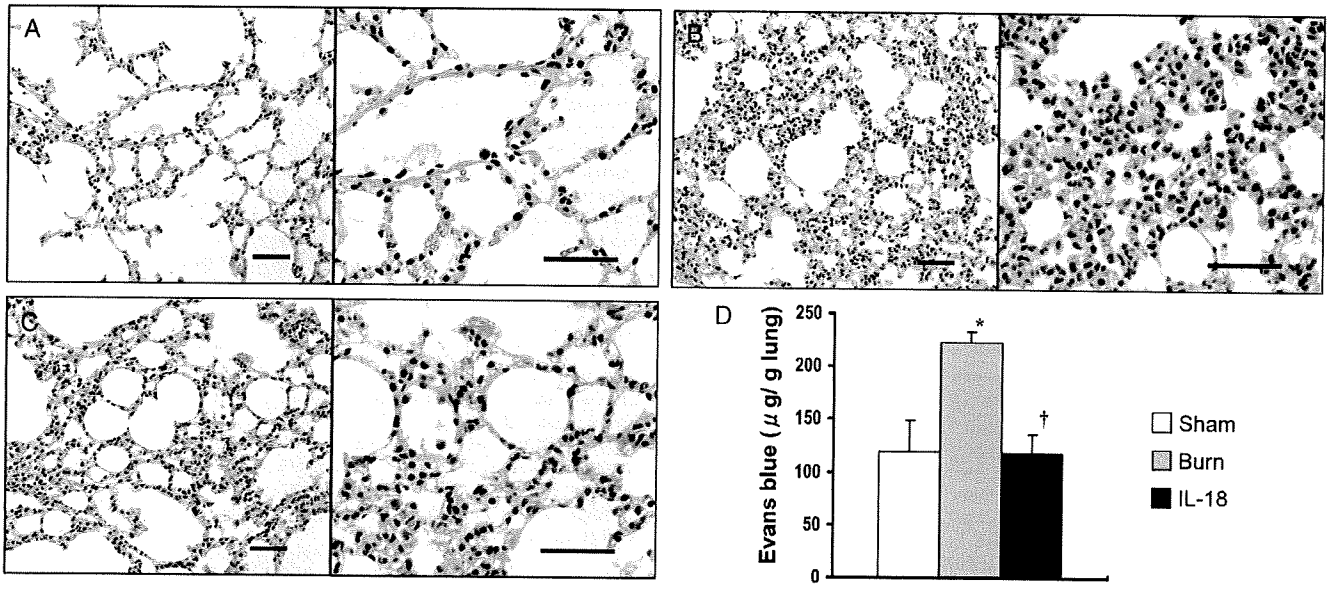

FIG. 4. **Lung histopathology and microvascular permeability at 12 h after LPS administration in the sham (A), burn (B), and IL-18 (C) groups, stained with hematoxylin and eosin.** Mice were killed after the experimental protocol shown in Figure 1B. The LPS administration induced severe alveolar wall thickening and increased neutrophil accumulation, compatible with ALI, in the burn group (B), but not in the sham group (A). In the IL-18 group, the findings characteristic of ALI were attenuated (C). Figures are representative of five independent experiments. Bars represent 50 μm. In addition, lung microvascular permeability was assessed by Evans blue extravasation technique (D). Lung vascular permeability was significantly higher in the burn group than those in the sham and IL-18 groups. The values were expressed as mean ± SEM (n = 3 per group). *$P < 0.05$ vs. sham group, †$P < 0.05$ vs. burn group.

cytokines), IFN-γ and IL-12 (T_H1 cytokines), and IL-4 and IL-10 (T_H2 cytokines). Levels of TNF-α, MIP-2, IL-6, IFN-γ, IL-12, IL-4, and IL-10 were significantly higher in the burn group than in the sham group (Fig. 5). In the IL-18 group, levels of all cytokines except IL-10 were significantly lower than those in the burn group, suggesting unique regulation of IL-10 production after burn injury and IL-18 supplementation. We also measured levels of TNF-α, MIP-2, and IL-10 mRNA expression in the lung by real-time RT-PCR. The TNF-α/glyceraldehyde-3-phosphate dehydrogenase mRNA ratios were 1.73 ± 0.31 in the sham group, 3.12 ± 0.50 in the burn group, and 1.73 ± 0.12 in the IL-18 group, being similar to the results obtained by ELISA.

Suppression of cytokine production by splenic T lymphocytes after burn injury and lack of effect of IL-18 supplementation

We next examined the capacity of spleen cells to produce various cytokines *in vitro*. As shown in Figure 6, the production of all cytokines in response to anti-CD3 Ab was significantly lower in the burn group than in the sham group, indicating that burn injury induced immunosuppression of lymphocytes. Cytokine production was not restored in the IL-18 group, suggesting that lymphocytes were not the major effector cells of exogenously administered IL-18.

Effects of burn injury and IL-18 on cytokine production by splenic macrophages

We also assessed the function of splenic macrophages/monocytes in response to LPS. The pattern and quantity of cytokines produced after LPS stimulation differed from those after CD3 stimulation. The production of TNF-α, MIP-2, and IL-10 was significantly higher in the burn group than in the sham group (Fig. 7, A, B, and E). In contrast, the production of IFN-γ was markedly lower in the burn group than in the sham group (Fig. 7C), and IL-4 was undetectable in both groups (Fig. 7D). In the IL-18 group, the production of MIP-2 was significantly lower than in the burn group (Fig. 7B). The production of TNF-α, IFN-γ, and IL-4 in the IL-18 group did not differ from that in the other two groups (Fig. 7, A, C, and D). In contrast to these changes, the production of IL-10 was significantly higher in the IL-18 group than in the burn group (Fig. 7E), compatible with *in vivo* lung IL-10 production (Fig. 5G).

Downregulation of MIP-2, but not IL10, production in vitro *by preincubation with physiological levels of IL-18*

To elucidate the *in vivo* effect of IL-18 supplementation on cytokine production, we examined the effect of preincubation with physiological (60, 100 pg/mL) and pathologically low (30 pg/mL) concentrations of IL-18 on MIP-2 and IL-10 production by LPS-stimulated splenic macrophages. The production of MIP-2 in the 30 pg/mL IL-18 group was significantly higher than that in the 30- to 100-pg/mL and 100-pg/mL groups (Fig. 8A). The production of MIP-2 in the 30- to 100-pg/mL group was significantly higher than that in the 100-pg/mL group, suggesting a parallel decrease of MIP-2 production with IL-18 concentration. In contrast, there was no significant difference in IL-10 production among the three groups, although there was a tendency for production to be

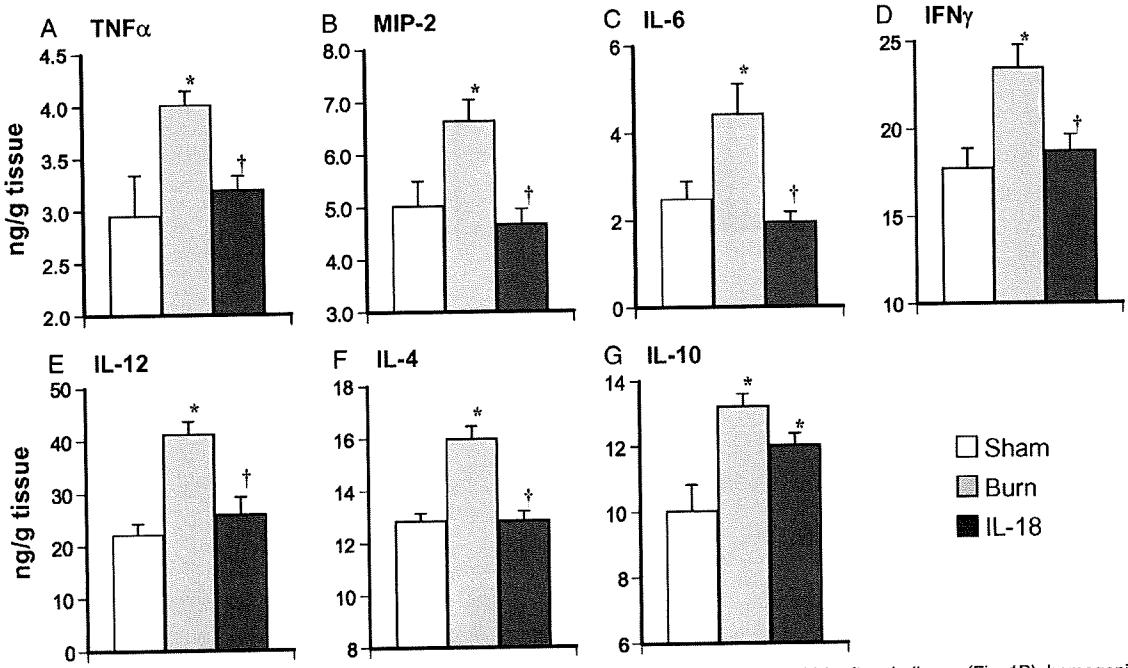

FIG. 5. **Cytokine contents in the lung at 12 h after LPS challenge *in vivo*.** The lungs were resected at 12 h after challenge (Fig. 1B), homogenized in 10 or 50 times their weight of PBS, and the supernatants were assayed for the following cytokines by ELISA: TNF-α (A), MIP-2 (B), IL-6 (C), IFN-γ (D), IL-12p70 (E), IL-4 (F), and IL-10 (G). Levels of TNF-α, MIP-2, IL-6, IFN-γ, IL-12, IL-4, and IL-10 were significantly higher in the burn group than in the sham group. In the IL-18 group, levels of all cytokines except IL-10 were significantly lower than those in the burn group, suggesting unique regulation of IL-10 production after burn injury and IL-18 supplementation. The values are expressed as mean ± SEM (n = 9–10 in each group). Results are representative of at least two independent experiments performed in triplicate. $^*P < 0.05$ vs. sham group, $^\dagger P < 0.05$ vs. burn group.

decreased in the 100-pg/mL group as compared with the other groups (Fig. 8B).

Participation of IL-10 in the in vivo *rescue effect of IL-18 supplementation after burn injury*

To clarify the participation of IL-10 in the *in vivo* rescue effect of IL-18 from lethal endotoxemia after burn injury, we examined the effect of IL-10 neutralization on host survival. As shown in Figure 9, IL-10 neutralization significantly decreased the survival after LPS challenge ($P < 0.05$), suggesting that IL-10 at least partly mediates the IL-18–induced protection against lethal endotoxemia after burn injury.

DISCUSSION

To address the mechanisms involved in the exaggerated response to endotoxemia after burn injury, we examined the role of IL-18 in a mouse model of burn injury. Contrary to our initial speculation, the levels of IL-18 in the liver decreased transiently after burn injury, and similar results were observed in the plasma of burned patients. Thus, in the present study, we hypothesized that a physiological concentration of IL-18 could be anti-inflammatory, and the temporal decrease in IL-18 was responsible for the burn-induced LPS hypersensitivity. We observed that *in vivo* IL-18 supplementation significantly improved survival, attenuated ALI, and decreased the lung levels of all cytokines examined, except for IL-10. *In vitro* studies of splenic macrophages revealed that production of proinflammatory cytokines, such as MIP-2 and TNF-α, as well as T_H2 cytokine, IL-10, was augmented after burn injury, and that *in vivo* IL-18 supplementation decreased the production of MIP-2 as well as TNF-α, and accelerated the production of IL-10. Furthermore, IL-18 at a physiological concentration directly attenuated the production of MIP-2, but did not induce the production of IL-10 by LPS-stimulated spleen cells. Finally, neutralization of IL-10 cancelled the rescue effect of IL-18 supplementation *in vivo*. Taken together, we speculated that IL-18 supplementation demonstrated the protective role through the direct anti-inflammatory function on macrophages as well as by *in vivo* acceleration of IL-10 production, and would thus be applicable for prophylaxis against LPS hypersensitivity in critically ill patients.

In vivo *protective role of IL-18 against LPS-induced ALI after burn injury*

Our present study clearly demonstrated that IL-18 functions as an anti-inflammatory cytokine and protects mice from burn-primed lethal endotoxemic ALI. Several reports have indicated *in vivo* protective roles of IL-18 in various types of microbial infection (11, 15–19). In our present model, we used LPS as a secondary stimulus after burn injury and showed that IL-18 had a protective effect against ALI and also attenuated acute inflammation. In addition, we showed that slight changes in IL-18 concentration directly affected the

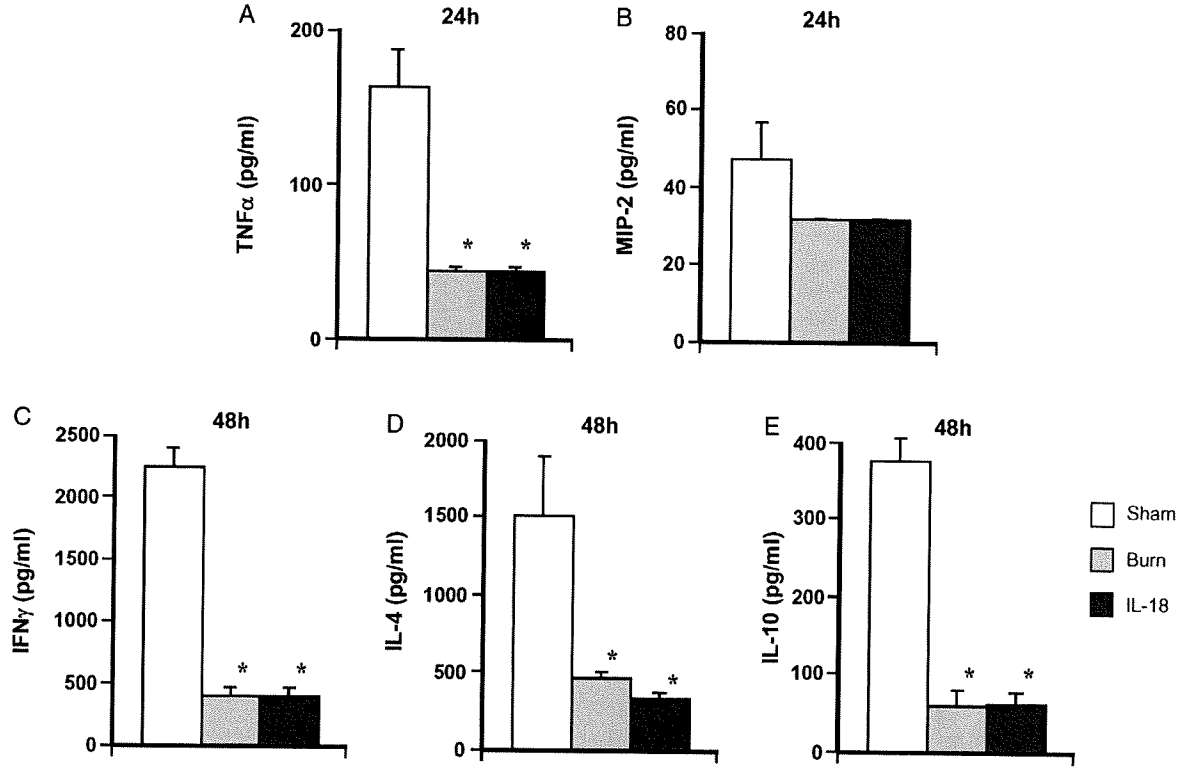

FIG. 6. **Anti-CD3 Ab–induced cytokine production by spleen cells *in vitro*.** On day 11 after injury, the spleen was resected (Fig. 1B), and the spleen cells were plated on 6-well plates and stimulated with 1 μg/mL antimouse CD3 Ab for 24 or 48 h. After culture, the following cytokines in the supernatant were assayed by ELISA: TNF-α (A), MIP-2 (B), IFN-γ (C), IL-4 (D), IL-10 (E). The production of all cytokines in response to anti-CD3 Ab was significantly lower in the burn group than in the sham group, indicating that burn injury abrogated the immunoresponse of lymphocytes. Cytokine production was not restored in the IL-18 group. The values are expressed as mean ± SEM (n = 9–10 in each group). Results are representative of at least two independent experiments performed in triplicate. *$P < 0.05$ vs. sham group.

production of proinflammatory cytokines by macrophages *in vitro*, thus suggesting a major role of IL-18 in determining macrophage phenotypes under various critical conditions.

We speculated that the protective effect of IL-18 against burn-primed endotoxemia *in vivo* was mediated by the attenuation of ALI because pathological features compatible with ALI, including severe alveolar wall thickening and increased neutrophil accumulation, were observed after LPS administration and were significantly attenuated in mice that received IL-18 supplementation. In addition, LPS induced an increase of proinflammatory as well as T_H1 cytokines, which was attenuated by IL-18 supplementation in the lungs, thus further supporting our speculation. Although T_H2 cytokines were also increased in the lungs after burn injury, their concentration may have been insufficient to suppress the production of proinflammatory and T_H1 cytokines.

A protective role of IL-18 against sepsis after burn injury has been reported previously by Kinoshita et al. (20–22), but the immunologic phenotype induced by IL-18 was different from that observed in the current study. They observed that a similar dose of exogenous IL-18 augmented the T_H1 function of liver mononuclear cells using a murine model involving 20% TBSA burn injury and secondary monomicrobial or polymicrobial infection on day 7. In contrast, in our model, LPS was administered on day 11 after 15% TBSA burn injury, and the opposite immunologic phenotype was observed in splenic macrophages. This discrepancy between the two experiments after IL-18 supplementation might have been caused by the difference in the period between the initial and secondary insults or the difference in the mouse species used. The difference in burn size may also have been contributory because the immune function of patients has been shown to be dependent on the size of the surface burn (23). There was also a discrepancy in the levels of IL-12 p70 or IL-18 after burn injury between the previous study and ours (24–28) possibly because of similar reasons.

Macrophages are responsible for in vivo *and* in vitro *protective roles of IL-18 supplementation*

In our experiments, *in vivo* IL-18 supplementation after burn injury diminished the production of MIP-2 and augmented that of IL-10 by spleen cells in response to LPS. Furthermore, *in vitro* preincubation with a physiological concentration of IL-18 also directly inhibited the production of MIP-2 by LPS-stimulated splenic cells, confirming the direct anti-inflammatory function of IL-18. Because macrophages are most abundant and can produce all of the cytokines examined in the present study, we speculate that they were the major source of the cytokines produced in response to LPS.

In addition, changes in immunologic phenotype may also have been derived from a change in macrophage phenotype. Macrophage subtypes similar to T_H1 and T_H2 lymphocytes have recently been recognized and termed M1 and M2 (29, 30). M1 macrophages preferentially produce TNF-α, IL-1, and

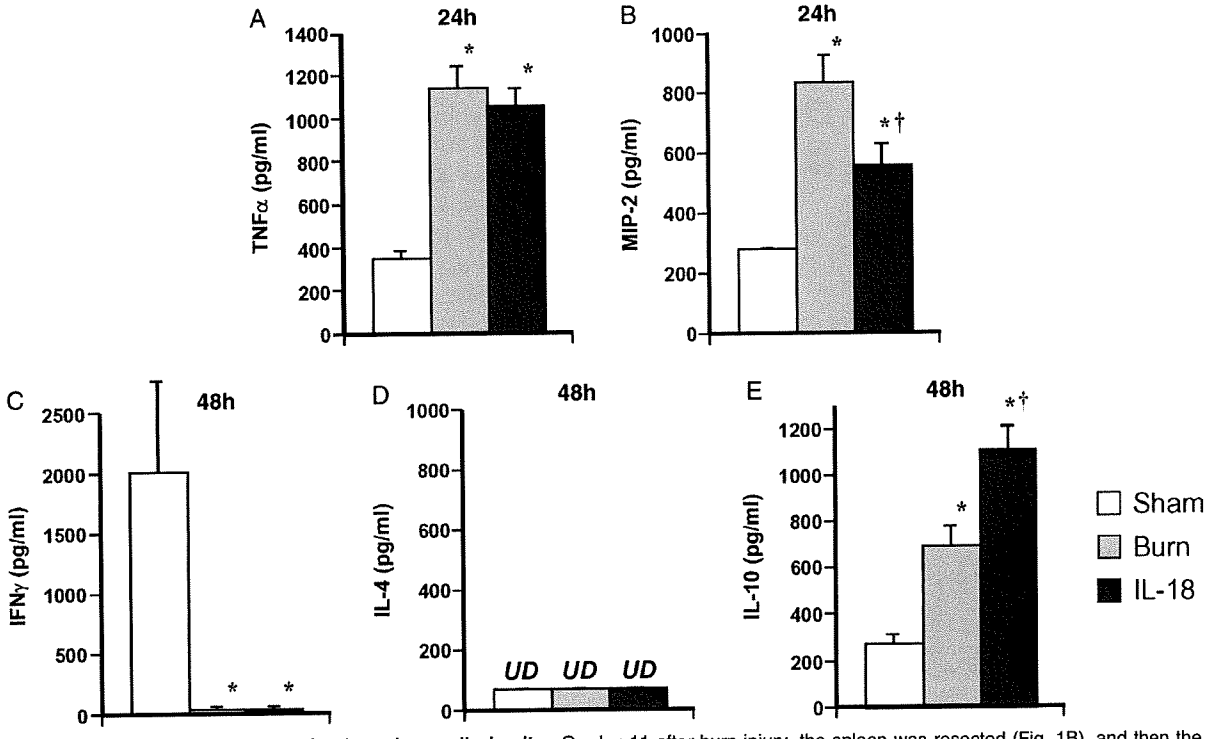

FIG. 7. **LPS-induced cytokine production by spleen cells *in vitro*.** On day 11 after burn injury, the spleen was resected (Fig. 1B), and then the spleen cells were stimulated with 10 μg/mL LPS for 24 or 48 h. After culture, the following cytokines in the supernatant were assayed by ELISA: TNF-α (A), MIP-2 (B), IFN-γ (C), IL-4 (D), IL-10 (E). The production of TNF-α, MIP-2, and IL-10 was significantly higher in the burn group than in the sham group (A, B, E). In contrast, the production of IFN-γ was markedly and significantly lower in the burn group than in the sham group (C), and IL-4 was undetectable (UD) in both groups (D). In the IL-18 group, the production of MIP-2 was significantly lower than in the burn group. The production of TNF-α, IFN-γ, and IL-4 in the IL-18 group did not differ from that in the other two groups (A–D). In contrast to these changes, the production of IL-10 was significantly higher in the IL-18 group than in the burn group (E). The values are expressed as mean ± SEM (n = 9–10 in each group). Results are representative of at least two independent experiments performed in triplicate. $^*P < 0.05$ vs. sham group, $^\dagger P < 0.05$ vs. burn group.

IL-12, whereas M2 macrophages produce IL-10 and IL-1 receptor antagonists. Thus, although we did not confirm immunohistochemically, our present experiments seem to indicate that burn injury induces a predominance of the M1 phenotype, and that IL-18 supplementation reverses this phenotypic deviation and favors M2 polarization.

We also examined T lymphocyte cytokine profiles, but all of the cytokines produced by T lymphocytes were strongly inhibited after burn injury and were not restored by *in vivo* IL-18 supplementation. Therefore, we conclude that the anti-inflammatory function of IL-18 was not mediated by T lymphocytes.

In vivo *protective role of IL-18 is at least partly mediated by induction of IL-10*

In the present study, we found that the *in vivo* protective role of IL-18 against endotoxemic shock was associated with a significant decrease in the levels of lung cytokines, except for IL-10, and augmented production of IL-10 with decreased

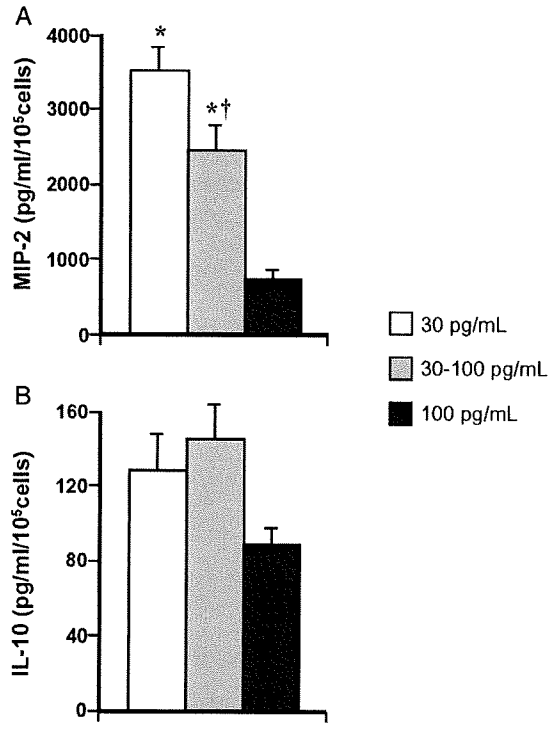

FIG. 8. **Effect of preincubation with IL-18, at three different concentrations, on MIP-2 and IL-10 production by LPS-stimulated splenic macrophages.** We harvested spleen cells from untreated mice, divided them into three groups, preincubated each cell group with the following dissimilar concentrations of IL-18 before stimulation with 10 μg/mL LPS: 100 pg/mL for 9 days, 30 pg/mL for 9 days, or 30 pg/mL for the initial 7 days and then with 100 pg/mL for the next 2 days. The levels of MIP-2 and IL-10 in the supernatant were assayed by ELISA. The production of MIP-2 in the 30 pg/mL IL-18 group was significantly higher than those in the 30- to 100-pg/mL and 100-pg/mL groups (A). The production of MIP-2 in the 30- to 100-pg/mL group was significantly higher than that in the 100-pg/mL group, suggesting a parallel decrease of MIP-2 production with IL-18 concentration. In contrast, there was no significant difference in IL-10 production among the three groups, although there was a tendency for a decrease in the 100 pg/mL group as compared with the other groups (B). The values are expressed as mean ± SEM (n = 9–10 in each group). Results are representative of at least two independent experiments performed in triplicate. $^{*}P < 0.05$ vs. 100 pg/mL IL-18 group, $^{\dagger}P < 0.05$ vs. 30-pg/mL IL-18 group.

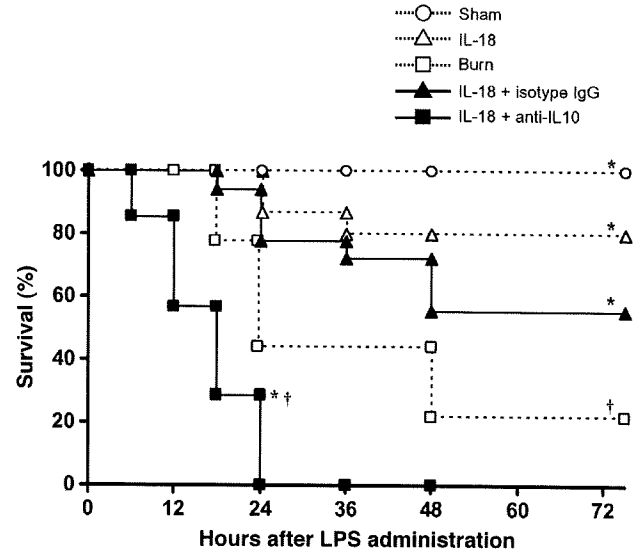

FIG. 9. **Effect of *in vivo* IL-10 neutralization on IL-18–induced protection against lethal endotoxemia.** The experiment was performed following the protocol shown in Figure 1C. IL-10 neutralization significantly decreased the survival after LPS challenge in IL-18–supplemented mice (n = 7–18 in each group). Results were representative of three independent experiments. $^{*}P < 0.05$ by log-rank test vs. the burn group, $^{\dagger}P < 0.05$ vs. the IL-18 + isotype IgG group.

production of MIP-2 *in vitro* by splenic macrophages. Because IL-10 is anti-inflammatory and protective for endotoxemia *in vivo* (31–33), we examined the effect of IL-10 neutralization to confirm the involvement of IL-10 and obtained a supportive result. Thus, we could speculate that the *in vivo* protective role of IL-18 was at least partly mediated by the systemically augmented production of IL-10. However, the mechanisms of the augmented IL-10 production afforded by IL-18 supplementation *in vivo* were not attributable to the direct effect of IL-18 on macrophages because the production of IL-10 was not induced by a physiological concentration of IL-18 *in vitro*. Augmented production of IL-10 *in vivo* after IL-18 supplementation has been reported in a mouse model of bleomycin-induced lung injury (34) and in tumor-bearing mice (35). Although both authors claimed that macrophages were essential for the enhanced IL-10 production, the precise mechanism of IL-18–induced augmentation of IL-10 production *in vivo* is still undetermined.

In conclusion, in addition to the previously known T_H1 and proinflammatory functions of IL-18, our present study demonstrated that IL-18 can also have novel anti-inflammatory functions at a physiological concentration. Because we have also observed a transient decrease of the plasma IL-18 level in patients with burn injury (data not shown), IL-18 supplementation may be applicable for prophylaxis against septic shock or multiple organ dysfunction syndrome in patients with a major burn injury or other severe insults. Further studies to elucidate the mechanisms of action of IL-18 and to confirm its efficacy for the treatment of various conditions will be mandatory.

ACKNOWLEDGMENTS

The authors thank Dr. Takayuki Shiomi (Department of Pathology, School of Medicine, Keio University) for taking histopathologic photographs and Ms. Yumiko Kamoshita and Ms. Jyunko Nishida for technical assistance. The authors also thank

Dr. Yotaro Shinozawa (Professor, Department of Emergency and Critical Care Medicine, Tohoku University Graduate School of Medicine) for his supervision.

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Macrophage derived chemokine (CCL22), thymus and activation-regulated chemokine (CCL17), and CCR4 in idiopathic pulmonary fibrosis

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Published: 29 August 2009

Respiratory Research 2009, **10**:80 doi:10.1186/1465-9921-10-80

Received: 21 March 2009
Accepted: 29 August 2009

This article is available from: http://respiratory-research.com/content/10/1/80

Abstract

Background: Idiopathic pulmonary fibrosis (IPF) is a chronically progressive interstitial lung disease of unknown etiology. Previously, we have demonstrated the selective upregulation of the macrophage-derived chemokine CCL22 and the thymus activation-regulated chemokine CCL17 among chemokines, in a rat model of radiation pneumonitis/pulmonary fibrosis and preliminarily observed an increase in bronchoalveolar (BAL) fluid CCL22 levels of IPF patients.

Methods: We examined the expression of CCR4, a specific receptor for CCL22 and CCL17, in bronchoalveolar lavage (BAL) fluid cells, as well as the levels of CCL22 and CCL17, to elucidate their pathophysiological roles in pulmonary fibrosis. We also studied their immunohistochemical localization.

Results: BAL fluid CCL22 and CCL17 levels were significantly higher in patients with IPF than those with collagen vascular diseases and healthy volunteers, and there was a significant correlation between the levels of CCL22 and CCL17 in patients with IPF. CCL22 levels in the BAL fluid did not correlate with the total cell numbers, alveolar lymphocytes, or macrophages in BAL fluid. However, the CCL22 levels significantly correlated with the numbers of CCR4-expressing alveolar macrophages. By immunohistochemical and immunofluorescence analysis, localization of CCL22 and CCR4 to CD68-positive alveolar macrophages as well as that of CCL17 to hyperplastic epithelial cells were shown. Clinically, CCL22 BAL fluid levels inversely correlated with DLco/VA values in IPF patients.

Conclusion: We speculated that locally overexpressed CCL22 may induce lung dysfunction through recruitment and activation of CCR4-positive alveolar macrophages.

Background

Idiopathic pulmonary fibrosis (IPF), also called usual interstitial pneumonia (UIP) on histological basis, is a chronically progressive interstitial lung disease of unknown etiology, characterized by diffuse interstitial inflammation, fibroblast proliferation with accelerated remodeling of extracellular matrix, and hyperplasia of type II epithelial cells. The prognosis for IPF patients is poor with a median survival of 3-5 years [1-3]. Although several agents such as glucocorticoids, immunosuppressants and pirfenidone, have been administered to IPF patients, less than 30% patients show objective evidence of improvement, and there is no established treatment that certainly improves their outcomes [2-4]. The key pathogenic mechanisms of pulmonary fibrosis are still ill defined, but it is speculated that the disintegration of inflammatory and structural cells, as well as disregulated production of bioactive mediators including cytokines, chemokines, and growth factors, contributes to its pathogenesis [1-3]. Thus, novel therapies based on a novel understanding of its pathophysiology are eagerly awaited.

The thymus and activation-regulated chemokine, CCL17, and the macrophage-derived chemokine CCL22 are members of the CC chemokine family, and CCR4 was identified as their specific receptor [5,6]. CCL17 and CCL22 have been recognized as Th2 chemokines, and their involvement in allergic diseases, such as atopic dermatitis, bronchial asthma and eosinophilic pneumonia has been revealed [7,8]. However, there is increasing evidence that these two chemokines are also involved in the pathophysiology of pulmonary fibrosis. Belperio et al. demonstrated that CCL17, CCL22 and CCR4 were overexpressed in a mice model of bleomycin-induced pulmonary fibrosis [9], and Pignatti et al. showed that CCR4 expression on bronchoalveolar lavage (BAL) fluid CD4 T cells were significantly elevated in IPF patients [10]. We have previously demonstrated the selective upregulation of CCL22 and CCL17 in a rat model of radiation pneumonitis/pulmonary fibrosis [11]. In this model, CCL22 and CCL17 were localized primarily to alveolar macrophages, whereas CCR4 was expressed by alveolar macrophages as well as lymphocytes. In addition, we observed elevated levels of CCL22 in BAL fluid of IPF patients by preliminary experiments. Thus, the current study was aimed to further elucidate the role of CCL22 and CCL17 in IPF. We determined CCL22 and CCL17 levels in BAL fluid using new sensitive ELISAs, and analyzed their correlation with clinical parameters. Furthermore, we analyzed CCR4 expression on BAL fluid cells and obtained supportive results that CCL22 and CCR4 contribute to the pathophysiology of IPF.

Materials and methods

Study Population

We studied 19 patients with IPF (18 males and 1 female, mean age 67.0 ± 1.9 years, SEM), 6 with sarcoidosis (3 males and 3 females, mean age 58.5 ± 23.2 years), and 9 with collagen vascular diseases associated with interstitial pneumonia (CVD-IP; 3 males and 6 females, mean age 59.4 ± 14.8 years), along with 6 non-smoking healthy volunteers without any medication in the previous six months (6 males, aged between 20 and 24 years). After obtaining informed consent from all patients and healthy volunteers, BAL was performed by a standard procedure. BAL total cell numbers were counted and differential cell counts were analyzed. The study was approved by the Ethical Committee of the School of Medicine, Keio University.

IPF was diagnosed, according to the diagnostic criteria by American Thoracic Society (ATS)/European Respiratory Society (ERS), for cases that satisfied all four major criteria: (1) exclusion of other known causes of interstitial lung disease; (2) abnormal pulmonary function; (3) bibasilar reticular abnormalities with minimal ground-glass opacities on high resolution computed tomography (HRCT) scans; (4) transbronchial lung biopsy specimen or BAL fluid showing no features to support an alternative diagnosis [3]. In addition, at least three of the four minor criteria had to be fulfilled: (1) age>50 years; (2) insidious onset of otherwise unexplained dyspnea on exertion; (3) duration of illness >3 months; (4) bibasilar, inspiratory crackles. Open lung biopsy was performed in one IPF patient, and transbronchial lung biopsy (TBLB) in 11 patients without any atypical findings. No patients showed any atypical findings in BAL fluid cell analysis, nor symptoms or signs of respiratory tract infection, and none had been treated with corticosteroids or immunosuppressants. We excluded patients who showed massive lung honeycombing on chest X-rays or chest CT scans, and those with an acutely exacerbating clinical course.

Sarcoidosis was diagnosed from chest X-ray findings, BAL fluid differential cell counts, and histological findings from TBLB. Non-caseous granulomas were confirmed by TBLB in all patients.

CVD-IP was diagnosed according to the criteria of the American College of Rheumatology. Two patients with rheumatoid arthritis (RA), 1 with polymyositis (PM)/dermatomyositis (DM), 2 with mixed connective tissue disease (MCTD), 2 with systemic sclerosis (SSc), and 2 with Sjogren's syndrome (SjS) were included in the study.

Lung Function Tests and Lung Fibrosis Scores on Chest X-Rays

Spirometry was performed for all IPF and sarcoidosis patients and 8 patients with CVD-IP. Single-breath carbon monoxide diffusing capacity (DLco) was evaluated in 15 patients with IPF, 5 with sarcoidosis, and 5 with CVD-IP. PaO_2, $PaCO_2$, and alveolar-arterial oxygen gradient ($AaDO_2$) were evaluated in 16 patients with IPF. In addition, scores for pulmonary fibrosis were assigned from chest X-rays following a previously described method [12].

BAL Fluid CCL22 and CCL17 Analysis

CCL22 and CCL17 concentrations in BAL fluids were determined by sensitive sandwich ELISAs according to the manufacturer's protocols (GT Development Co., Seattle WA). The absorbance at 450 nm was determined on a microplate reader (SPECTRAFluor Plus, Tecan Co., Minneapolis, MN), and the concentrations were determined by interpolation of their absorbance from the standard curve. Each sample was tested in triplicate and the mean value was obtained. The detection limit for both CCL22 and CCL17 was 6.3 pg/ml.

Flow Cytometric Analysis of BAL Fluid Cell Subpopulations

For flow cytometric analysis, 5×10^5 BAL cells were suspended in 100 μl phosphate-buffered saline (PBS) and incubated with (FITC)-conjugated anti-human CD4 monoclonal antibody (cat. #551120, Becton, Dickinson, Franklin Lakes, NJ) and phycoerythrin-conjugated anti-human CCR4 monoclonal antibody (Becton, Dickinson) for 30 min. After incubation, the cells were washed twice with PBS, and analyzed using a flow cytometer following the previously established protocol (Epics XL•MC L, Beckman Coulter, Inc., Fullerton, CA) [13,14]. Alveolar macrophages were primarily identified on a forward and side scattergram, and we additionally used CD4 as a marker of alveolar macrophages as well as helper T lymphocytes to better eliminate contaminated neutrophils and debris. A weakly CD4-positive cell population was gated [15], and the expression of CCR4 was analyzed.

Histological and Immunohistochemical Examination

For histological and immunohistochemical analysis, we used lung tissue obtained through TBLB or open lung biopsy. The lung tissue was fixed with 10% formalin, embedded in paraffin, and the paraffin sections were stained with hematoxylin and eosin (HE). For immunohistochemistry, the sections were stained with specific goat polyclonal antibodies against human CCL22, CCL17 (Santa Cruz Biotechnology Inc, Santa Cruz, CA), CCR4 (Abcam, Cambridge, UK), or monoclonal antibody for human CD68 (KP1, Santa Cruz Biotechnology Inc) [16,17], using an indirect streptavidin-biotinylated complex method. We additionally performed immunofluorescence staining using Alexa-488- and Cy3-labeld secondary antibody to show the colocalization of CCL22, CCR4 and CD68. In these analyses, DAPI was used for the staining of nuclei.

Statistical Analysis

All data are presented as mean ± SEM. A one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test was applied to detect statistically significant differences among groups. Significant differences were accepted at $p < 0.05$.

Results

Patient Characteristics and BAL Fluid Analysis

Clinical characteristics as well as BAL fluid data of the patients are summarized in Tables 1 and 2. DLco/VA was significantly lower in patients with IPF than in those with CVD-IP. The total BAL fluid cell number was significantly higher in patients with CVD-IP than in the other groups. The percentage of BAL fluid macrophages was significantly lower in IPF, CVD-IP and sarcoidosis patients than in healthy volunteers, and it was significantly lower in CVD-IP patients than in IPF patients. Patients with sarcoidosis and CVD-IP showed a significantly increased percentage of BAL fluid lymphocytes than those with IPF and healthy volunteers. The percentage of BAL fluid neutrophils was significantly higher in patients with CVD-IP than in the other groups. The percentage of BAL fluid eosinophils was significantly higher in patients with IPF than those with sarcoidosis and healthy volunteers.

Table 1: Patient Characteristics and Lung Functions

	IPF	Sar	CVD-IP	HV
Male/female	18/1	3/3	3/6	6/0
Age (range)	67.0 ± 1.9 (48-83)	58.5 ± 23.2 (24-76)	59.4 ± 14.8 (33-76)	N.D. (20-24)
Smoker	16*†	6*	3	0
PaO_2/FIO_2	372 ± 9.2 (307-453)	419 ± 29 (319-529)	358 ± 23 (278-448)	N.D.
%VC	62 ± 4.6 (33-110)	101 ± 6.1# (83-120)	67 ± 6.6 (43-98)	N.D.
DLCO/VA	4.0 ± 0.2† (2.6-5.4)	4.8 ± 0.4 (4.1-6.3)	7.1 ± 1.9 (4.4-14.0)	N.D.

IPF, idiopathic pulmonary fibrosis; Sar, sarcoidosis; CVD-IP, collagen vascular disease with interstitial pneumonia; HV, healthy volunteers; N.D, not determined; DLco, single-breath carbon monoxide diffusing capacity; VA, alveolar ventilation per minute

Age data and lung function parameters are shown as mean ± SEM.

*$p < 0.001$ *v. s.* HV

§$p < 0.001$ *v. s.* CVD-IP, †$p < 0.005$ *v. s.* CVD-IP

#$p < 0.0005$ *vs.* IPF

Table 2: BAL Fluid Cell Characteristics

	IPF (n = 19)	Sar (n = 6)	CVD-IP (n = 8)	HV (n = 6)
Total cells (10^5/ml)	6.2 ± 0.8 (1.9-14.8)	4.9 ± 0.3 (4.0-6.0)	11.2 ± 3.1*#ℓ (1.1-27.9)	2.7 ± 0.5 (0.6-4.0)
Macrophage (%)	78.0 ± 2.6ℓ (60.2-97.0)	62.9 ± 10.8* (29.5-95.0)	44.0 ± 9.9"§ (5.5-74.5)	95.6 ± 0.3§$ (94.7-96.6)
Lymphocyte (%)	11.3 ± 2.1 (0-27.4)	34.6 ± 10.5*‡ (5.0-68.5)	33.8 ± 8.7*‡ (12.0-87.5)	3.1 ± 0.2 (2.6-4.0)
Neutrophil (%)	6.1 ± 1.4 (1.0-23.0)	1.7 ± 0.8 (0-4.0)	18.4 ± 8.6ℓ#ℓ (0-65.5)	1.1 ± 0.1 (0.7-1.6)
Eosinophil (%)	4.4 ± 1.1ℓ# (0-14.5)	0.5 ± 0.3 (0-1.9)	1.7 ± 0.9 (0-7.5)	0.2 ± 0.2 (0-0.9)
CD4/CD8	3.1 ± 0.6 (0.2-9.6)	11.2 ± 4.0&¥ (2.4-29.3)	1.8 ± 0.5 (0.4-3.9)	N.D.

IPF, idiopathic pulmonary fibrosis; Sar, sarcoidosis; CVD-IP, collagen vascular disease with interstitial pneumonia; HV, healthy volunteers; N.D., not determined
All data were shown as mean ± SEM.
"$p < 0.0001$ *v.s.* HV, *$p < 0.005$ *v.s.* HV, ℓ$p < 0.05$ *v.s.* HV
†$p < 0.0005$ *v.s.* CVD-IP, &$p < 0.005$ *v.s.* CVD-IP
$$p < 0.005$ *v.s.* Sar, #$p < 0.05$ *v.s.* Sar
§$p < 0.0001$ *v.s.* IPF, ¥$p < 0.001$ *v.s.* IPF, ‡$p < 0.005$ *v.s.* IPF, ℓ$p < 0.05$ *v.s.* IPF

BAL Fluid Chemokines, Cell Differentials and Subpopulations

CCL22 and CCL17 BAL fluid levels were significantly higher in patients with IPF than in those with CVD-IP and healthy volunteers (Fig 1A, B). CCL22 BAL fluid levels were significantly correlated with CCL17 levels in IPF patients (Fig 1C). We found no correlation of CCL22 and CCL17 with the total cell numbers and differential cell counts in BAL fluid.

To further elucidate the roles of these chemokines in recruiting cells to the lungs in fibrotic lung diseases, we analyzed CCR4-positive BAL fluid cell subpopulations by flow cytometry. CCL22 levels were significantly correlated with the total number of CCR4-positive BAL fluid cells in all patients examined. Furthermore, CCL22 levels were significantly correlated with the number of CCR4-positive alveolar macrophages (Fig 2A), but not with lymphocytes (Fig 2B). These correlations were not observed between these subpopulations and CCL17 BAL fluid levels. CCL22 levels in IPF patients were significantly correlated with the number of CCR4-positive alveolar macrophages ($R = 0.87$, $p < 0.001$) and CCR4-positive lymphocytes ($R = 0.75$, $p < 0.01$). In contrast, BAL fluid CCL17 levels did not correlate with CCR4-positive alveolar macrophages or lymphocytes in IPF patients.

Immunohistochemical Localization of CCL22, CCL17, and CCR4 in IPF

We also examined the localization of CCL22, CC17, and CCR4 by immunohistochemistry. A fraction of alveolar macrophages were positive for CCL22, whereas CCL17 was exclusively expressed by hyperplastic epithelial cells (Fig 3A, B). CCR4 also seemed to be weakly positive for a part of alveolar macrophages (Fig 3C). CD68, a specific marker of macrophages, was localized in the cells identical or similar to CCL22- and CCR4-positive cells (Fig 3D). There were very few lymphocytes, and CCR4-positive lymphocytes were barely found.

To further confirm the localization of CCL22 and CCR4 to alveolar macrophages, we used dual immunofluorescence staining technique. Localization of CCL22 and CCR4 to a fraction of CD68-positive alveolar macrophages was shown (Fig 4A, B). These observations suggested that alveolar macrophage-derived CCL22 as well as epithelial cell-derived CCL17 contribute to the recruitment and activation of CCR4-positive cells, which are probably alveolar macrophages in IPF patients.

Correlation between BAL Fluid Chemokines and Clinical Parameters

We further examined the correlation between the BAL fluid chemokines and various clinical parameters, including serum lactate dehydrogenase, C-reactive protein, KL-6, and semi-quantitative scores of chest X-ray abnormalities

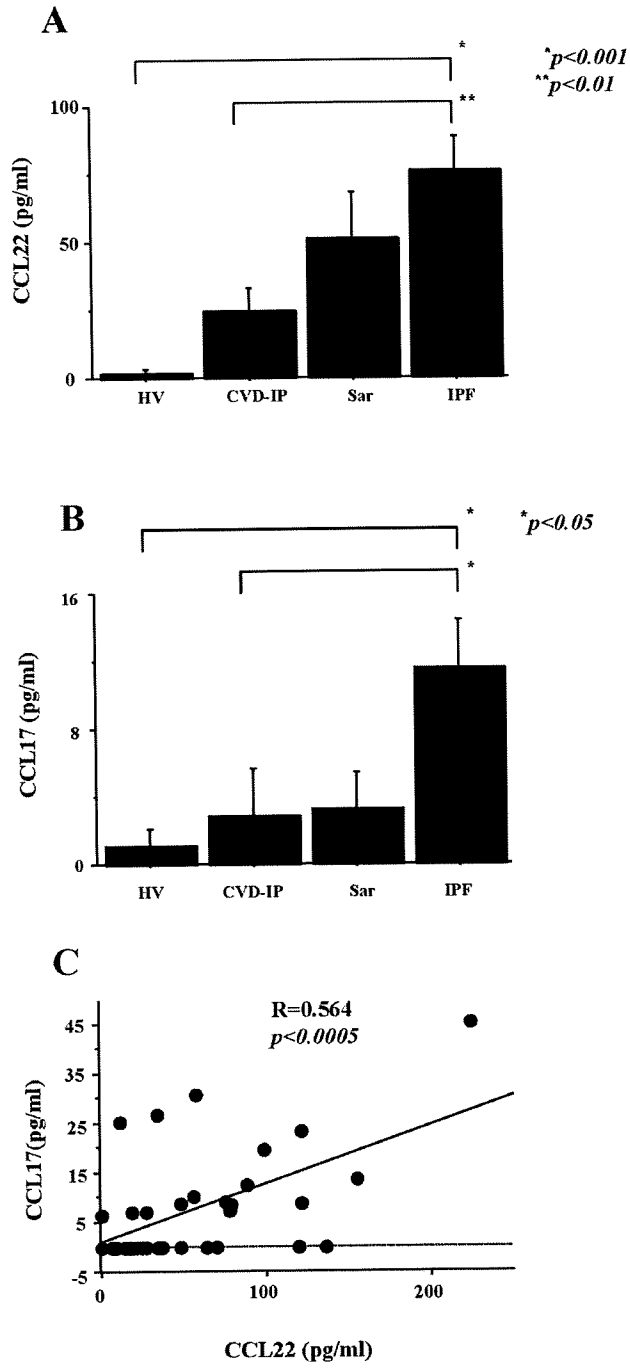

Figure 1
BAL fluid CCL22 and CCL17 in fibrotic lung diseases. BAL fluid levels of CCL22 and CCL17 were determined by sensitive ELISAs. CCL22 and CCL17 levels were significantly higher in patients with idiopathic pulmonary fibrosis (IPF) than in those with CVD-IP and healthy volunteers (A, B). In IPF patients, BAL fluid CCL22 levels correlated significantly with CCL17 levels (C). IPF, idiopathic pulmonary fibrosis; HV, healthy volunteers; CVD-IP, collagen vascular disease with interstitial pneumonia; Sar, sarcoidosis.

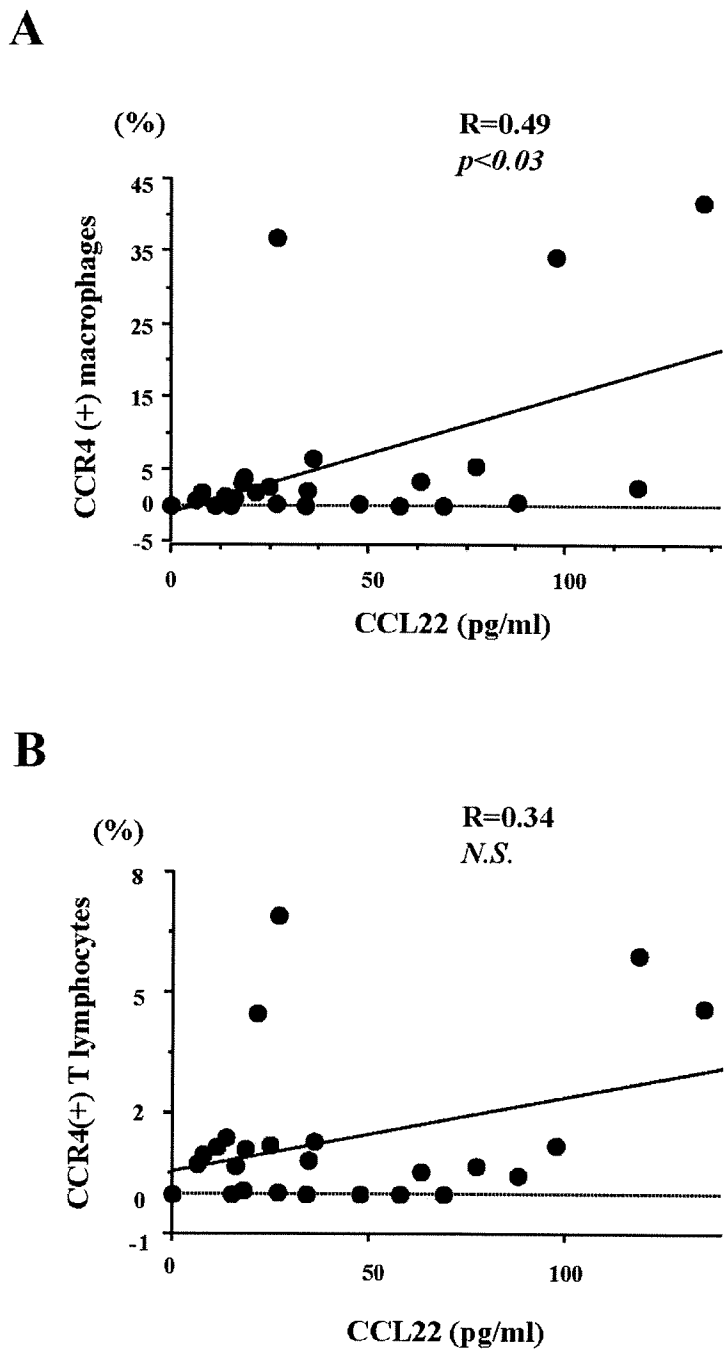

Figure 2
Correlations between BAL fluid CCL22 and CCR4-positive alveolar macrophages and lymphocytes in all patients examined. To further elucidate the roles of the chemokines in recruiting cells to the lungs in fibrotic lung diseases, we analyzed CCR4-positive BAL fluid cell subpopulations by flow cytometry in IPF. CCL22 levels significantly correlated with the number of CCR4-positive alveolar macrophages (A). CCL22 levels in IPF patients were significantly correlated with the number of CCR4-positive alveolar macrophages and lymphocytes. These correlations were not observed between these subpopulations and BAL fluid CCL17 levels.

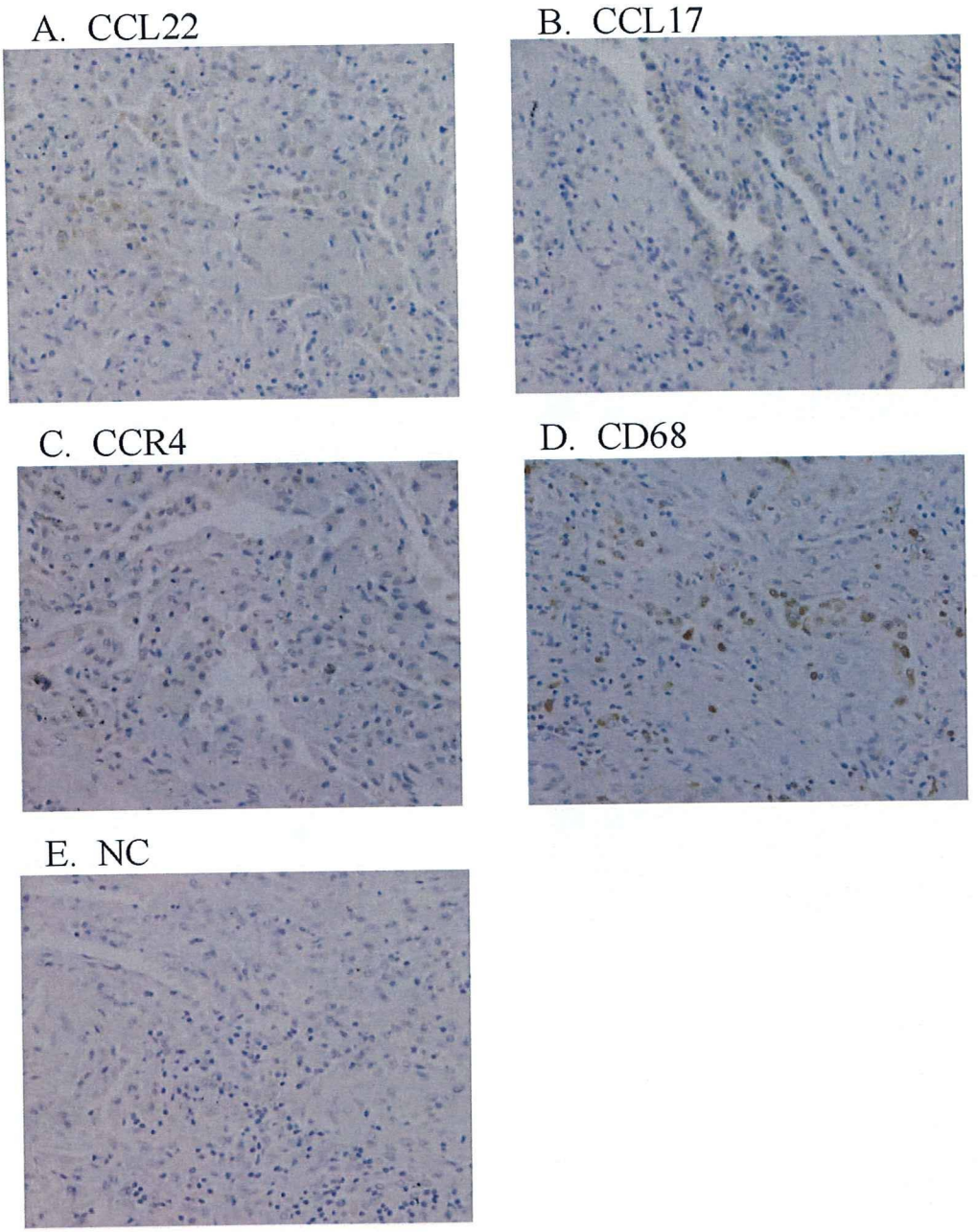

Figure 3
Lung immunohistochemical photomicrograph of CCL17, CCL22, CCR4, and CD68 in patients with idiopathic pulmonary fibrosis (IPF). We examined the localization of CCL17, CCL22, CCR4, and CD68 by immunohistochemistry. The sections were initially incubated with anti-CCL22 antibody (A), anti-CCL17 antibody (B), anti-CCR4 antibody (C), anti-CD68 antibody (D), or their diluent buffer (E), and then stained using an indirect streptavidin-biotinylated complex method. A fraction of the alveolar macrophages was positive for CCL22, whereas CCL17 was exclusively expressed by some hyperplastic epithelial cells (A, B). There were few alveolar macrophages which were weakly positive for CCR4 (C). The tissue distribution of alveolar macrophages was confirmed by their positivity for CD68 (D). In contrast, no lung cells were positively stained in negative control (NC) sections (E).

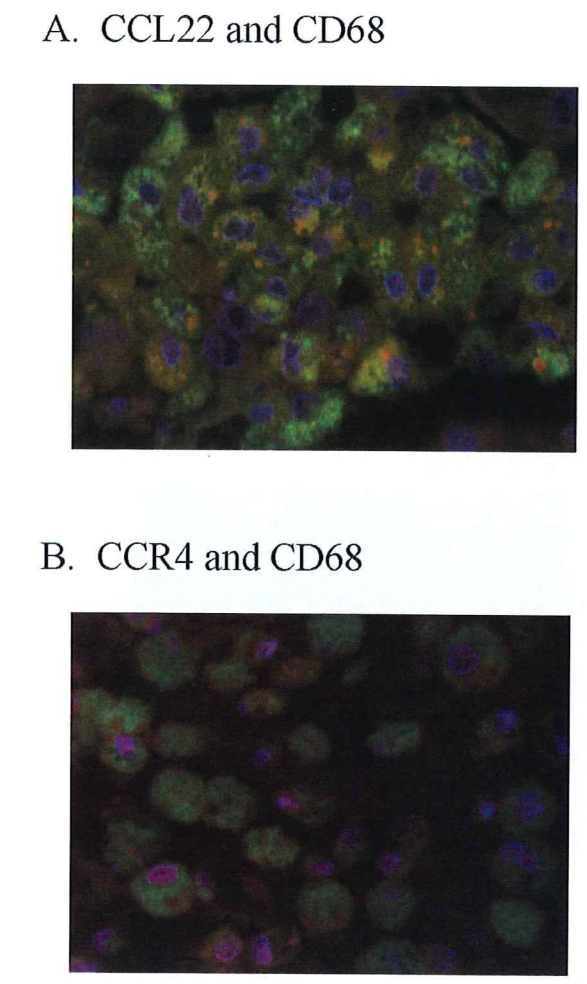

Figure 4
Lung immunofluorescence photomicrograph of CCL22 and CCR4 in patients with idiopathic pulmonary fibrosis (IPF). We examined the localization of CCL22 and CCR4 in CD68-positive alveolar macrophages by a dual immunofluorescence technique. A. Localization of CCL22 (red) to a certain fraction of CD68 (green) -positive alveolar macrophages was shown. B. Localization of CCR4 (red) to a small fraction of CD68 (green) -positive alveolar macrophages was shown. Nuclei were counterstained with DAPI (blue).

in IPF patients. We assessed the degree of radiographic abnormalities according to Watter's method [12]. Briefly, areas of abnormal shadows, presence of honeycombing, and the diameter of the main pulmonary artery were assessed by expert pulmonologists, and a semi-quantitative radiological score was calculated for each patient. However, we did not find any significant correlations between any of the clinical parameters examined and the CCL22 and CCL17 levels in BAL fluid.

We next examined the correlation of the BAL fluid chemokines with indices of lung function tests in IPF patients. An inverse correlation was observed between BAL fluid CCL22 levels and DLco/VA values (Fig 5). Although BAL fluid CCL17 also tended to correlate inversely with DLco/VA, no statistical significance was present. There were no significant correlations between the two BAL chemokines levels and other parameters of lung function, including %VC and PaO_2/FIO_2.

Discussion

In the present study, we examined the T-helper 2 (Th2) chemokines, CCL22, CCL17, and BAL fluid cells expressing CCR4, a specific receptor for these chemokines, to elucidate their pathophysiological roles in IPF patients. We also studied the localization of CCL22, CCL17, and CCR4 by immunohistochemistry. The levels of CCL22 and CCL17 in BAL fluid were significantly higher in patients with IPF than in those with CVD-IP and healthy volunteers, and there was a significant correlation between the levels of CCL22 and CCL17 in IPF. CCL22 levels in the BAL fluid did not correlated with total cell numbers, alveolar lymphocytes, and macrophages in the BAL fluid. However, the CCL22 levels were significantly correlated

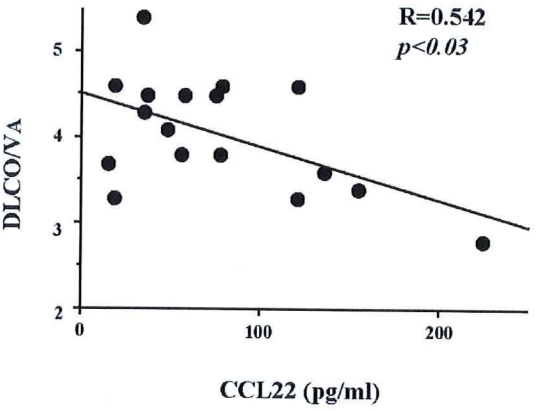

Figure 5
Correlation between BAL fluid CCL22 and lung diffusing capacity in idiopathic pulmonary fibrosis (IPF) patients. We examined the correlation of BAL fluid chemokines with indices of lung function tests in IPF patients. An inverse correlation was observed between BAL fluid CCL22 levels and DLco/VA values. Although BAL fluid CCL17 also tended to correlate inversely with DLco/VA, there was no statistical significance. DLco, single-breath carbon monoxide diffusing capacity; VA, alveolar ventilation per minute.

with the numbers of CCR4-expressing alveolar macrophages. By immunohistochemical analysis, localization of CCL22 and CCR4 to alveolar macrophages as well as that of CCL17 to hyperplastic epithelial cells were shown. Clinically, CCL22 levels in BAL fluid inversely correlated with DLco/VA values in IPF patients. Collectively, we speculated that locally overexpressed CCL22 may contribute to the induction of lung dysfunction mainly through recruitment of CCR4-positive alveolar macrophages.

Increased Production of CCL17and CCL22 in IPF

In our previous study, we showed that the production of CCL22 and CCL17 in rat radiation pneumonitis increased significantly, but CCL17 was undetectable in BAL fluid of IPF patients [11]. Previous reports found no significant increase in BAL fluid CCL17 [9,18]. Using a more sensitive ELISA kit in the current experiment, we confirmed significant increases in CCL17 and CCL22 BAL fluid levels in IPF patients as compared with those in CVD-IP patients and healthy volunteers. The levels of CCL17 were lower than those of CCL22 in 14 out of 16 patients examined, and there was a significant correlation between the two levels, suggesting a common stimulus or stimuli for their induction.

In our study, CCL17 was positive in hyperplastic epithelial cells. Our results regarding CCL17 were consistent with previous observations in IPF [9,19], and CCL17 detected in BAL fluid could be mainly derived from these cells. Bronchial epithelial cells are the major source of CCL17 under physiological and pathological conditions, including bronchial asthma [20], and CCL17 is inducible by various stimuli, such as TNF-alpha, interleukin (IL)-4, interferon-gamma, and TGF-beta [21,22]. Because overproduction of these cytokine has been shown previously, they also could be *in vivo* stimuli for CCL17 in IPF.

Our study revealed that immunoreactive CCL22 was predominantly localized to alveolar macrophages, whereas Marchal-Sommé et al reported that CCL22 was positive in hyperplastic epithelial cells, fibroblasts, and endothelial cells, but not in alveolar macrophages [19]. However, because our previous study showed the localization of CCL22 to alveolar macrophages in a rat radiation pneumonitis model [11], and the augmented production of CCL22 was shown in IPF [23], it is reasonable to speculate that alveolar macrophages are at least partly responsible for high levels of CCL22 in IPF. CCL22 is inducible in alveolar macrophages by IL-4, PGE_2, and TGF-beta [24]. Because overproduction of these mediators has been shown previously [25], they may be *in vivo* inducers of CCL22 in IPF.

Possible Contribution of Lung CCL22 to the Recruitment of CCR4-Positive Alveolar Macrophages

In the present study, we found that BAL fluid levels of CCL22 were significantly correlated with the number of CCR4-positive alveolar macrophages among all patients examined. CCL22 levels in IPF patients were significantly correlated with the number of CCR4-positive alveolar macrophages and lymphocytes. Thus, although the percentage of CCR4-positive cells was relatively small among alveolar macrophages, the results may indicate that locally overproduced CCL22, but not CCL17, contributes to the recruitment of alveolar macrophages, and to a lesser extent, alveolar lymphocytes to the lungs in IPF patients.

In animal models of pulmonary fibrosis, we have found CCR4 expressed on alveolar macrophages in rat radiation pneumonitis/pulmonary fibrosis, and Belperio et al. demonstrated predominant CCR4 expression on alveolar macrophages in mice bleomycin-induced pulmonary fibrosis [9]. Furthermore, Trujillo et al. recently demonstrated that bleomycin induced CCL17-dependent activation of CCR4 in alveolar macrophages using CCR4-deficient mice [26]. Thus, the CCL22-CCR4 axis may contribute to the activation of alveolar macrophages in pneumonitis and pulmonary fibrosis.

Inverse Correlation of BAL Fluid CCL22 with Lung Diffusing Capacity in IPF

Our current study demonstrated that CCL22 was inversely correlated with DLco/VA. Because DLco/VA is affected by both total surface area and thickness of alveolar walls, and these regions are the major targets of alveolar macrophage infiltration in IPF, the results may suggest that alveolar macrophage recruitment by CCL22 induces a dose-dependent decrease in DLco/VA. It is also possible that CCL22 or CCR4-positive alveolar macrophages are involved in the destruction of lung parenchyma in IPF.

Previously, Pignatti et al. demonstrated an increase in CCR4-positive alveolar T-lymphocytes and their inverse correlation with DLco in IPF [10]. In contrast, the increase of CCR4 expression on T-lymphocytes was relatively small and we did not find their significant correlation with the parameters of lung functions, including DLco in our study. The discrepancy between their and our results may be derived from the difference in disease stages or characteristics. All of our patients were in a stable stage, and we excluded the patients who showed massive lung honeycombing, or were treated with corticosteroids, whereas they did not exclude such patients. In addition, the CCR4-expressing alveolar macrophages, as well as BAL fluid CCL22 levels, were not examined in their study. Since we also found a significant correlation between BAL fluid CCL22 levels and CCR4-positive lymphocytes in IPF patients, it is possible to speculate that locally overpro-

duced CCL22 contributes to the recruitment of CCR4-positive alveolar macrophages, and to a lesser extent, to the recruitment of CCR4-positive alveolar T-lymphocytes.

Conclusion

CCL22 and CCL17 were both increased in BAL fluid of IPF patients and CCL22 levels in BAL fluid correlated proportionally with the numbers of CCR4-positive alveolar macrophages, and inversely with DLco/VA. CCL22 may contribute to the recruitment and activation of alveolar macrophages, and consequently to the destruction of lungs in patients with IPF.

List of Abbreviations

$AaDO_2$: alveolar-arterial oxygen gradient; BAL: bronchoalveolar lavage; CVD-IP: collagen vascular disease with interstitial pneumonia; ELISAs: enzyme-linked immunosorbent assay; DLco: single-breath carbon monoxide diffusing capacity; HV: healthy volunteers; IPF: idiopathic pulmonary fibrosis: N.D: not determined; Sar: sarcoidosis; TBLB: transbronchial lung biopsy; UIP: usual interstitial pneumonia; VA: alveolar ventilation per minute

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YY primarily collected and analyzed the data, with the help of TI and FS. This manuscript was prepared by YY under SF's instruction. TS was involved in pathological diagnosis and immunohistochemical analysis. SA contributed to FACS analysis and interpretation of data. This study was supported by the scientific fund for KY, AI, and SF.

Acknowledgements

We thank Kazuko Sano for conducting immunohistochemical analysis. This study was supported in part by Grants-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and the Keio Gijuku Fukuzawa Memorial Fund for the Advancement of Education.

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