mandatory for tissue invasion but, rather, that this receptor increased the potential virulence of *S. pneumoniae* in the respiratory tract. As such, the role of the PAFR in primary (20) and secondary (this study) pneumococcal pneumonia does not seem to differ significantly.

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Cardiovascular, Pulmonary and Renal Pathology

Attenuation of Folic Acid-Induced Renal Inflammatory Injury in Platelet-Activating Factor Receptor-Deficient Mice

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Platelet-activating factor (PAF), a potent lipid mediator with various biological activities, plays an important role in inflammation by recruiting leukocytes. In this study we used platelet-activating factor receptor (PAFR)-deficient mice to elucidate the role of PAF in inflammatory renal injury induced by folic acid administration. PAFR-deficient mice showed significant amelioration of renal dysfunction and pathological findings such as acute tubular damage with neutrophil infiltration, lipid peroxidation observed with antibody to 4-hydroxy-2-hexenal (day 2), and interstitial fibrosis with macrophage infiltration associated with expression of monocyte chemoattractant protein-1 and tumor necrosis factor-α in the kidney (day 14). Acute tubular damage was attenuated by neutrophil depletion using a monoclonal antibody (RB6-8C5), demonstrating the contribution of neutrophils to acute phase injury. Macrophage infiltration was also decreased when treatment with a PAF antagonist (WEB2086) was started after acute phase. *In vitro* chemotaxis assay using a Boyden chamber demonstrated that PAF exhibits a strong chemotactic activity for macrophages. These results indicate that PAF is involved in pathogenesis of folic acid-induced renal injury by activating neutrophils in acute phase and macrophages in chronic interstitial fibrosis. Inhibiting the PAF pathway might be therapeutic to kidney injury from inflammatory cells. *(Am J Pathol 2006, 168:1413–1424; DOI: 10.2353/ajpath.2006.050634)*

Inflammation is an important component of renal injury, in both acute renal failure[1,2] and chronic renal damage that accompanies interstitial fibrosis.[3,4] The infiltrating inflammatory cells contribute to renal damage through generation of reactive oxygen species (ROS), further recruitment of leukocytes, and production of proinflammatory and profibrotic cytokines. Therefore, the mechanism of directing circulating leukocytes to the kidney and maintaining them there is a potential target for therapeutic intervention for kidney diseases.

Platelet-activating factor (PAF; 1-O-alkyl-2-acethl-*sn*-glycero-3-phosphocholine) is a potent proinflammatory phospholipid mediator with various biological effects such as activating platelets, neutrophils, eosinophils, and macrophages. PAF binds to a G-protein-coupled seven transmembrane receptor, PAF receptor (PAFR); PAF plays an important role in the pathophysiology of inflammatory conditions.[5,6] Synthesis of PAF has been demonstrated not only in blood cells but in the kidney under physiological conditions,[7] ischemia reperfusion injury,[8] and clipped kidney.[9] Treatment with several PAF antagonists has been reported in acute renal failure[10] and chronic renal failure animal models.[11,12] However, problems exist regarding the lack of specificity of PAF antagonists.[13–16] Therefore, we have developed genetically engineered PAFR-deficient (PAFR-KO) mice to confirm the role of PAF *in vivo*.[17] The resultant PAFR-KO mice exhibit attenuated symptoms in several animal models such as chemical lung injury,[18] bronchial asthma,[19] and sponge-induced subcutaneous granuloma formation.[20] It

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has been demonstrated that infiltrating inflammatory cells play important roles in these animal models.

The folic acid (FA)-induced renal injury model has been applied recently for evaluation of epithelial regeneration and interstitial fibrosis.[21–24] Intraperitoneal administration of FA with sodium bicarbonate induced renal injury that showed acute tubular necrosis with transient elevations of blood urea nitrogen (BUN) and serum creatinine (Cr) followed by development of interstitial fibrosis with macrophage infiltration.[21,25] Tubular obstructions by FA crystals and direct toxic effect of FA to tubular epithelial cells presumably cause renal damage,[26] but the precise mechanism of injury remains unclear. Inflammatory cell infiltrations were remarkable in injured kidneys of this model.

Here we examined the role of PAF in inflammatory renal injury induced by FA administration. Using PAFR-KO mice, we show attenuation of renal injury not only in cases of acute tubular damage but also in cases of development of chronic interstitial fibrosis *in vivo*. In addition, we evaluated the effects of neutrophil depletion and PAF antagonist treatment after the acute phase to confirm the contribution of leukocytes to FA-induced renal injury. We also demonstrate the role of PAF as a strong chemoattractant for macrophages *in vitro*, indicating that PAF blockade can prevent progression of inflammatory damage in kidney.

Materials and Methods

Reagents

Folic acid was obtained from Sigma Chemical Co. (St. Louis, MO) and WEB2086 was a generous gift from Boehringer (Ingelheim, Germany). We used a mouse antibody to 4-hydroxy-2-hexenal (HHE)-modified protein, in which the aldehyde HHE is derived from the oxidative process of polyunsaturated fatty acid, obtained from NOF Corp. (Tokyo, Japan) and a mouse anti-smooth muscle α actin (α-SMA) monoclonal antibody (1A4) (DakoCytomation Co. Ltd., Glostrup, Denmark) for immunohistochemical and Western blot analyses. We used a rat anti-mouse neutrophil antibody (Caltag Laboratories, Burlingame, CA), a rat anti-mouse F4/80 macrophage antigen antibody (MCA497R) (Serotec, Raleigh, NC), and a goat anti-mouse MCP-1/JE antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for immunohistochemical analyses. The monoclonal antibody RB6-8C5 was used for neutrophil depletion. It is a rat IgG2b that selectively binds to and depletes mature neutrophils but not lymphocytes or macrophages.[27–31] The hybridoma that secretes this antibody was a gift from Dr. R. Coffman (DNAX Research Inc., Palo Alto, CA); RB6-8C5 from hybridoma culture supernatants was purified through ammonium sulfate precipitation. All other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) unless otherwise specified.

Animal Model

We established PAFR-KO mice as described previously.[17] The PAFR-KO mice and the corresponding wild-type (PAFR-WT) mice were backcrossed for 10 generations onto a C57BL/6N genetic background. Mice were allowed food and water *ad libitum*, and experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals [Department of Health, Education and Welfare Publication No. (NIH) 86-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD]. Male and female mice used for this study were 9 to 11 weeks of age, weighing 18 to 25 g. Within each experimental group, the sex ratio, age, and weight of animals did not differ significantly. The animals were anesthetized with diethyl ether and administered FA by intraperitoneal injection at the dose of 250 mg/kg in vehicle (0.5 ml of 0.3 mmol/L $NaHCO_3$) or given vehicle alone. The dose of FA and $NaHCO_3$ was critical for induction of severe renal damage and was determined by preliminary studies. Blood was drawn from the tail vein of each animal, and the levels of BUN and Cr were measured 3 days before and at 2, 7, and 14 days after FA administration by the urease-indophenol method with Urea N B (Wako Pure Chemical Industries Ltd.) and the colorimetric method based on hydrogen peroxide measurement with Nescoat VLII CRE (Alfresa Pharma Corp., Osaka, Japan), respectively. Kidneys were removed 2 and 14 days after FA administration and fixed in 10% buffered formalin for staining with hematoxylin and eosin, Masson's trichrome, and immunochemical staining, except for mouse neutrophil detection for which methyl Carnoy's solution was used. Parts of kidneys were snap-frozen for immunohistochemical testing of MCP-1; they were otherwise used for Western blot analysis and real-time polymerase chain reaction (PCR) assay.

For neutrophil depletion experiments, PAFR-WT mice were treated with 50 μg of monoclonal antibody RB6-8C5. Injection of FA was performed the following day, and the animals were sacrificed 2 days after FA administration. Injection of 25 μg of monoclonal antibody RB6-8C5 was reported to cause significant neutrophil depletion from the subsequent day up to 3 days.[32–34] Blood collected at sacrifice was used for flow cytometry analyses to confirm neutrophil depletion. Mice that were untreated with RB6-8C5 served as control animals. To clarify the contribution of PAF for macrophage recruitment after initial damage, we intraperitoneally injected PAF antagonist (WEB2086) into PAFR-WT mice every day from day 2 to day 14 at the dose of 5 mg/kg and examined renal function (days 2, 7, and 14), macrophage infiltration, and interstitial fibrosis (day 14).

Flow Cytometry Analysis

Heparinized blood was incubated with anti-Gr-1 antibodies conjugated to fluorescein isothiocyanate (BD Pharmingen, San Diego, CA), and then Flowcount fluorospheres (Beckman Coulter Inc., Villepinte, France) were added. After red blood cell lysis with NH_4Cl buffer, cell suspen-

sion was run on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA), and the absolute count of Gr-1-positive cells was calculated using standardized Flowcount beads.

Morphological Evaluation of Kidneys

The area of the interstitial fibrosis in the cortex was evaluated with Masson's trichrome staining using a computer-aided evaluation program (AIS version 4.0; Fuji Photo Film Co. Ltd., Tokyo, Japan). Viewed at ×400 magnification, 10 randomly selected nonoverlapping fields from the cortical region were analyzed. The fibrotic areas stained in blue were depicted in digital images; then the percentage of the fibrotic area was calculated relative to the entire field area (percentage area). Glomeruli and large vessels were not included in the microscopic fields for image analysis. The degree of neutrophil and macrophage infiltration was measured as the average number of positive staining cells per field. The number of positively stained cells was determined in five randomly selected nonoverlapping fields at ×200 magnification in each section of the individual mouse renal cortex. Scores of respective kidneys as well as scores of each animal were averaged.

Western Blot Analysis

Western blot analyses were performed as described previously.[35] Briefly, harvested kidneys were homogenized on ice in a radioimmunoprecipitation assay buffer with protease inhibitors. The lysates were separated electrophoretically on a 10% polyacrylamide gel. After transferring proteins from the gel to a nitrocellulose membrane, Western blot analyses were performed using HHE or α-SMA antibody. After incubation with horseradish peroxidase-conjugated appropriate second antibody, chemiluminescent signal detection (ECL Plus; Amersham Biosciences Corp., Uppsala, Sweden) was performed using a cooled charge-coupled device camera system (LAS-1000; Fuji Photo Film Co. Ltd.). The membrane was incubated at 50°C for 30 minutes in a stripping buffer to remove all probes. Then it was reprobed with the antibody to α-tubulin (Santa Cruz Biotechnology Inc.). Densitometric analyses of bands compared with the density of α-tubulin were performed using National Institutes of Health Image software, version 1.62.

Immunohistochemical Analysis

Immunohistochemical staining of 2-μm paraffin sections was performed using indirect immunohistochemical techniques. Biotin-free immunohistochemical staining using horseradish peroxidase-conjugated polymer system was conducted according to the manufacturer's protocols (Histofine Mouse Stain kit and Histofine Simple Stain mouse MAX-PO (rat) kit; Nichirei Corp., Tokyo, Japan). The deparaffinized sections were preincubated with 0.3% hydrogen peroxide for 15 minutes and incubated with primary antibodies overnight at 4°C, followed by polymer-conjugated anti-mouse IgG. Protease K treatment was necessary for anti-F4/80 antibody; autoclaving in 10 mmol/L citrate-buffer (pH 6.0) was necessary for anti-HHE and anti-α-SMA antibody. For neutrophil staining, fixation with methyl Carnoy's solution was indispensable.[36] Diaminobenzidine tetrahydrochloride (Nichirei Corp.) was used for the substrate-chromogen reaction followed by counter staining with hematoxylin.

We used frozen sections of kidney to determine the immunohistochemistry of MCP-1, as described previously[37] but with modifications. With 8-μm cryostat sections fixed in acetone and goat anti-mouse MCP-1 antibody (Santa Cruz Biotechnology Inc.), a Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) and diaminobenzidine tetrahydrochloride were applied in accordance with the manufacturers' instructions. Sections were counterstained with methyl green (Vector Laboratories Inc.).

Real-Time PCR Assay for TNF-α and MCP-1 Expression

Harvested kidneys were homogenized with a homogenizer (Ultra-Turrax T8; IKA Labortechnik, Staufen, Germany) in Trizol total RNA isolation reagent (Invitrogen Corp., Carlsbad, CA). Total RNA was isolated by the acid guanidinium isothiocyanate-phenol-chloroform extraction method according to the manufacturer's protocols and treated with DNase I to remove potential contamination of DNA (DNA-free; Ambion Inc., Austin, TX). The mRNA was then reverse-transcribed to cDNA using MLV reverse transcriptase (RiverTra Ace; Toyobo Co. Ltd., Osaka, Japan) with random hexamers. Transcripts encoding tumor necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1 were measured using TaqMan real-time quantitative PCR with Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using TaqMan universal PCR master mix (Applied Biosystems) according to the manufacturer's specifications. The TaqMan probes and primers for TNF-α (assay identification number Mm00443258_m1) and MCP-1 (assay identification number Mm00441242_m1) were assay-on-demand gene expression products (Applied Biosystems). The PCR reaction conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. To normalize for variance in loaded cDNA, 18S ribosomal RNA were amplified in a separate tube. Amplification data were analyzed using Applied Biosystems' Prism sequence detection software version 2.1 (Applied Biosystems). Standard curves were prepared for each gene and the 18S ribosomal RNA in each experiment to normalize the relative expression of the genes of interest to the 18S ribosomal RNA control.

Measurement of PAF Levels in Kidney

Harvested kidneys were frozen immediately with liquid nitrogen at days 2 and 14. The PAF levels in kidney were measured by reverse-phase high performance liquid chromatography-electrospray ionization-tandem mass

spectrometry technique, as described in precedent studies.[38,39]

Migration Assay

Elicited peritoneal macrophages were harvested from PAFR-KO and PAFR-WT mice 3 days after injection of 4% thioglycolate. Peritoneal exudate cells were washed with phosphate-buffered saline and resuspended in RPMI 1640 medium supplemented with 0.1% bovine serum albumin. Cell migration was evaluated using a 96-well Boyden chamber as described previously, with minor modifications.[40] The PAF at concentrations of 0, 1, and 10 nmol/L in 300 μl of RPMI 1640 containing 0.1% bovine serum albumin was added to the lower wells of a chemotaxis chamber (Neuro Probe, Inc., Gaithersburg, MD). A polycarbonate filter (8-μm-pore size; Neuro Probe, Inc.) was layered onto the wells; 65 μl of cell suspension (4.0×10^6/ml) were applied to the upper wells. The chamber was incubated at 37°C in a humidified atmosphere in the presence of 5% CO_2 for 2 hours. At the end of incubation, the filter was removed, fixed with methanol, and stained with Diff-Quick (Sysmex Corp., Hyogo, Japan). Cells on the upper side of the filter were wiped away. The number of cells that had migrated to the lower side was determined by measuring optical densities at 595 nm using a 96-well microplate reader (model 3550; Bio-Rad Laboratories Inc., Hercules, CA). Each experiment was performed in triplicate.

Statistical Analysis

Results of statistical analyses are expressed as means ± SEM. Differences among groups at the same time point were examined using Student's *t*-test. Differences among experimental groups at different time point were confirmed by one-way analysis of variance followed by the Tukey-Kramer test for individual comparison of group means. These calculations were performed using StatView-J5.0 (SAS Institute Inc., Cary, NC). The null hypothesis was rejected when *P* was <0.05.

Results

Attenuation of Renal Dysfunction in PAFR-KO Mice and Efficacy of PAFR Antagonist

Administration of FA with sodium bicarbonate induced transient elevation of BUN and Cr at 48 hours after injection followed by subsequent renal dysfunction accompanied with interstitial fibrosis. Basal levels of BUN and Cr in PAFR-WT and PAFR-KO mice were similar [PAFR-WT: BUN 28.1 ± 0.8 mg/dl, Cr 0.25 ± 0.01 mg/dl (n = 16) versus PAFR-KO: BUN 27.8 ± 0.9 mg/dl, Cr 0.24 ± 0.01 mg/dl (n = 16)]. The measurement at 48 hours after FA injection showed statistically significant elevation of BUN and Cr compared with baseline in both PAFR-WT and PAFR-KO mice (PAFR-WT, $P < 0.001$; PAFR-KO, $P < 0.005$), and the levels of

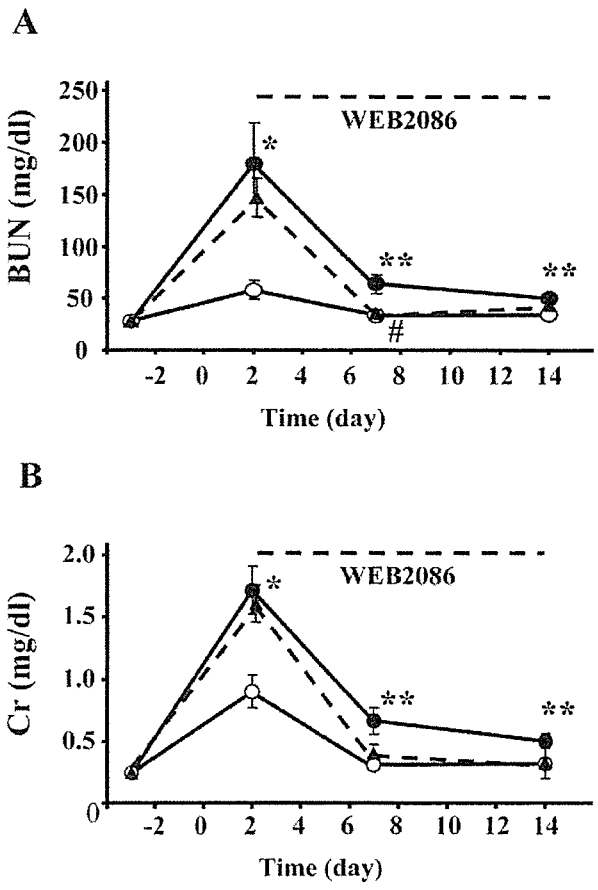

Figure 1. Time course of BUN (**A**) and Cr (**B**) levels in PAFR-KO and PAFR-WT mice subjected to FA administration. **Filled circles** indicate PAFR-WT mice, **open circles** indicate PAFR-KO mice, and **filled triangles** indicate WEB2086-treated PAFR-WT mice. WEB2086 was injected every day from days 2 to 14. The numbers of animals were 16 at days −3 and 2, 8 at days 7 and 14 in PAFR-WT and PAFR-KO mice, and 5 in WEB2086-treated PAFR-WT mice. $^*P < 0.005$, $^{**}P < 0.05$ (PAFR-WT versus PAFR-KO). $^{\#}P < 0.05$ (PAFR-WT versus WEB2086-treated PAFR-WT).

BUN and Cr in PAFR-WT mice were significantly higher compared with levels in PAFR-KO mice [PAFR-WT: BUN 180.0 ± 39.0 mg/dl, Cr 1.71 ± 0.20 mg/dl (n = 16) versus PAFR-KO: BUN 58.2 ± 8.9 mg/dl, Cr 0.90 ± 0.13 mg/dl (n = 16); $P < 0.005$] (Figure 1). The significant differences were also valid at days 7 and 14 [PAFR-WT: BUN 63.7 ± 9.4 mg/dl, Cr 0.66 ± 0.11 mg/dl (n = 8) versus PAFR-KO: BUN 33.1 ± 3.3 mg/dl, Cr 0.31 ± 0.04 mg/dl (n = 8); $P < 0.05$, at day 7] [PAFR-WT: BUN 50.2 ± 5.2 mg/dl, Cr 0.50 ± 0.06 mg/dl (n = 8) versus PAFR-KO: BUN 33.8 ± 3.3 mg/dl, Cr 0.31 ± 0.04 mg/dl (n = 8); $P < 0.05$, at day 14]. Treatment with WEB2086 after acute phase from day 2 to day 14 also showed partial protective effects for renal dysfunction [day 2: BUN 146.9 ± 18.7 mg/dl, Cr 1.60 ± 0.15 mg/dl; day 7: BUN 35.5 ± 3.6 mg/dl, Cr 0.39 ± 0.07 mg/dl; day 14: BUN 43.4 ± 3.9 mg/dl, Cr 0.32 ± 0.12 mg/dl (n = 5)]. In addition, there was a significant difference between WEB2086-treated and untreated PAFR-WT mice in BUN level at day 7 ($P < 0.05$) (Figure 1).

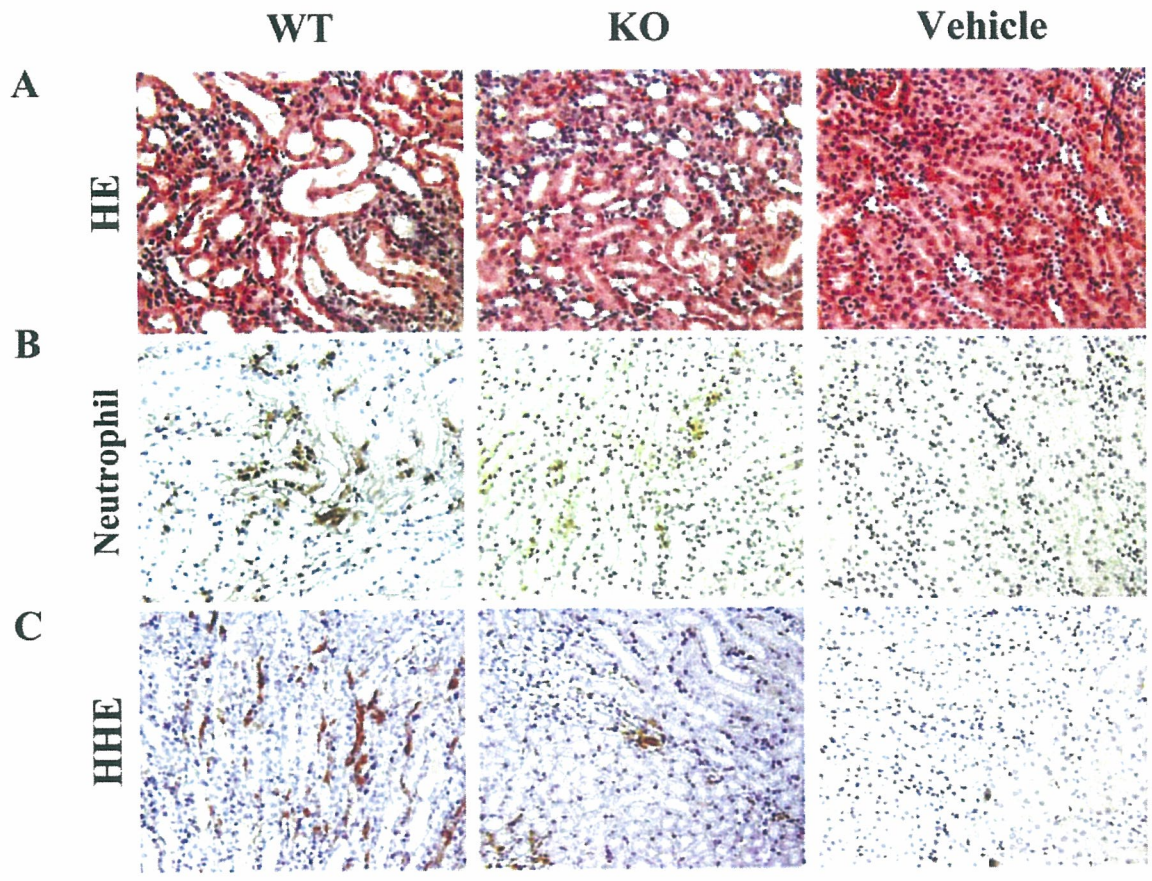

Figure 2. Histological findings at day 2 stained with H&E (**A**), immunohistochemistry using antibody to neutrophil (**B**), and antibody to HHE-modified proteins (**C**). Vehicle-treated PAFR-KO and PAFR-WT showed identical results. Therefore, only PAFR-WT mice are shown. Original magnifications, ×200.

Neutrophil and ROS Production in Acute Tubular Damage

In addition to attenuation of renal dysfunction, morphological analyses demonstrated that acute tubular necrosis was significantly milder in PAFR-KO mice than in PAFR-WT mice at 48 hours after FA administration (Figure 2A). Immunohistochemical staining with anti-neutrophil antibody showed that infiltrating neutrophils decreased significantly in PAFR-KO mice compared with PAFR-WT mice at day 2 [PAFR-WT: 27.5 ± 2.3/×200 field (n = 4) versus PAFR-KO: 6.9 ± 1.0/×200 field (n = 4); P < 0.001] and were detected predominantly around the damaged tubules (Figure 2B and Figure 3).

Through propagation of lipid peroxidation, ROS will damage tissue. Antibody to HHE, produced in the peroxidative metabolism of ω-3 polyunsaturated fatty acids in cell membranes, is a specific marker to detect lipid peroxidation.[35] Immunohistochemical analyses dominantly detected HHE-modified proteins around peritubular capillaries of damaged kidney tissues (Figure 2C). Lipid peroxidation was also quantified in kidney homogenates by Western blot analysis. In PAFR-WT mice, kidney homogenates showed a higher intensity of immunoreactive bands of HHE-modified proteins compared with those from PAFR-KO mice (Figure 4).

To evaluate the contribution of neutrophils for acute phase of FA-induced renal injury at day 2, we also injected FA into PAFR-WT mice pretreated with neutrophil depletion antibody. Neutrophil depletion was confirmed through flow cytometry analyses (Figure 5A). These ani-

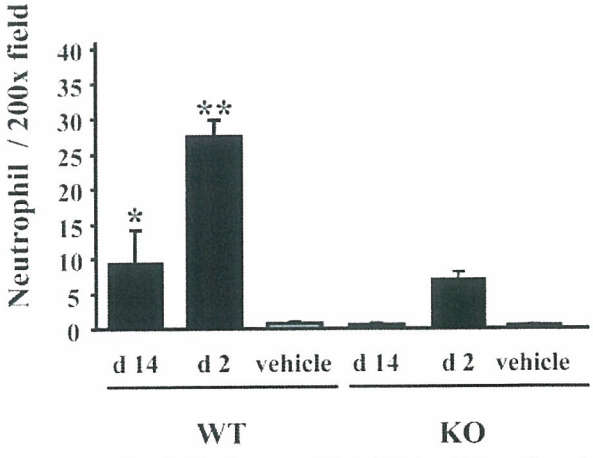

Figure 3. Number of infiltrating neutrophils in FA-injured kidney. Five animals were in the FA group; four received the vehicle. *P < 0.005 versus PAFR-KO mice at day 14 (d 14). **P < 0.00005 versus PAFR-KO mice at day 2 (d 2).

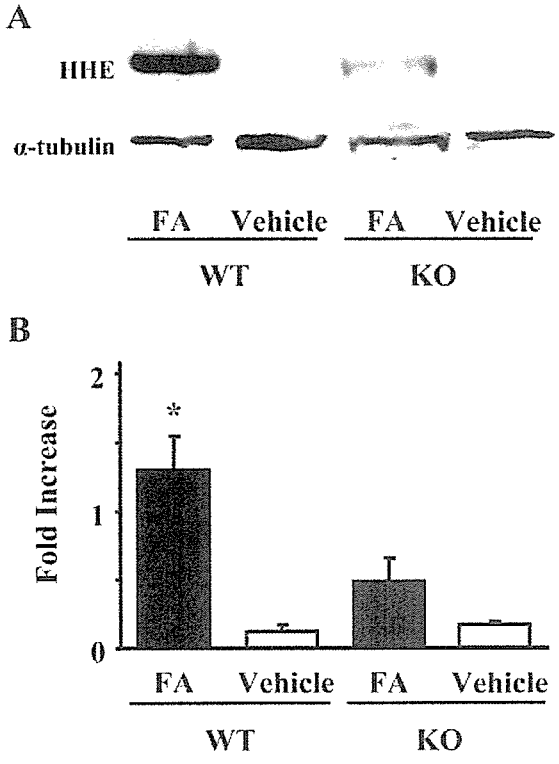

Figure 4. Western blot analysis of HHE-modified protein expression in kidney homogenates at day 2. A representative image is shown in **A** (**top**). **B:** The histogram depicts the relative density of bands in comparison with α-tubulin. Five animals were in the FA group; three received the vehicle. *$P <$ 0.05 versus PAFR-KO mice with FA administration.

mals showed remarkable amelioration of renal dysfunction [control: BUN 133.5 ± 21.1 mg/dl, Cr 1.54 ± 0.09 mg/dl (n = 5) versus neutrophil depletion: BUN 35.3 ± 2.4 mg/dl, Cr 0.47 ± 0.16 mg/dl (n = 5); $P < 0.005$] (Figure 5, B and C).

Development of Interstitial Fibrosis and Macrophage Infiltration

The FA-induced renal injury model showed development of segmental interstitial fibrotic lesions at day 14. Figure 6A shows that interstitial fibrotic areas stained in blue by Masson's trichrome staining were more prominent in kidneys of PAFR-WT mice than in those of PAFR-KO mice. Quantitative analyses demonstrated that the fibrotic area in PAFR-WT mice was significantly larger than PAFR-KO mice [PAFR-WT: 5.4 ± 0.7% (n = 5) versus PAFR-KO: 2.1 ± 0.7% (n = 5); $P < 0.05$] (Figure 7). Immunohistochemical and Western blot analyses with anti-α-SMA antibody were performed to detect interstitial myofibroblasts. Positive staining of α-SMA was found in the identical site of fibrotic area stained blue with Masson's trichrome staining (Figure 6B) and seen in vascular smooth muscle cells (data not shown). Kidney homogenates of PAFR-WT mice were shown by Western blot analysis to have a higher intensity of immunoreactive bands of α-SMA than those from PAFR-KO mice (Figure 8).

Macrophage infiltration was evaluated using immunostaining for F4/80, a specific macrophage antigen. Infil-

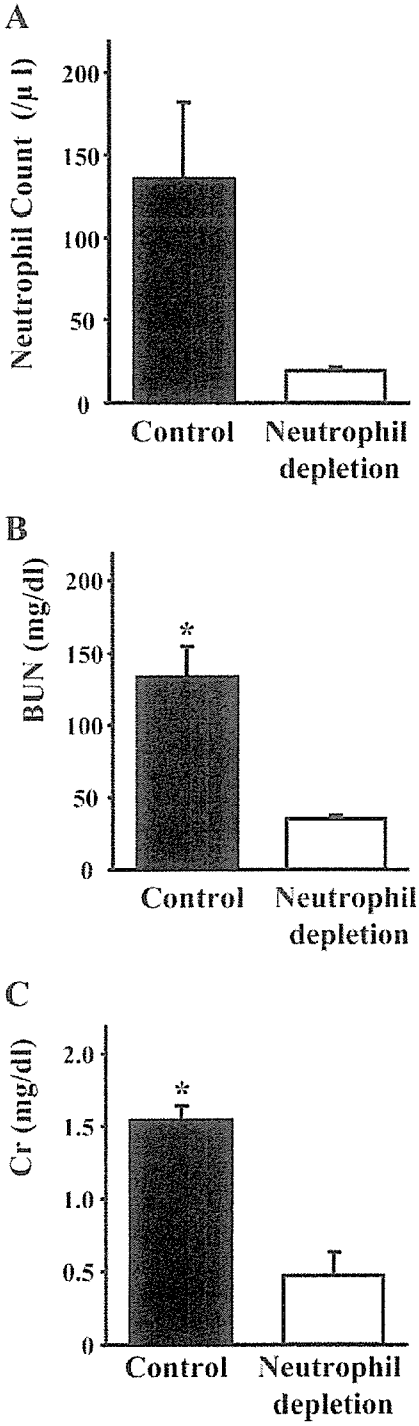

Figure 5. Effects of neutrophil depletion on acute phase of FA-induced renal injury. Injection of a monoclonal neutrophil depletion antibody (RB6-8C5) at day −1 caused a significant decrease of neutrophil count in blood of PAFR-WT mice at day 2. **A:** Renal dysfunction at day 2 was attenuated with treated PAFR-WT mice compared with untreated PAFR-WT mice (**B** and **C**). *$P < 0.005$ versus untreated PAFR-WT mice. Five animals were in each group.

trated F4/80-positive macrophages were detected in the peritubular interstitium around fibrotic areas in kidneys harvested 14 days after FA administration (Figure 6C). The number of infiltrated F4/80-positive macrophages at

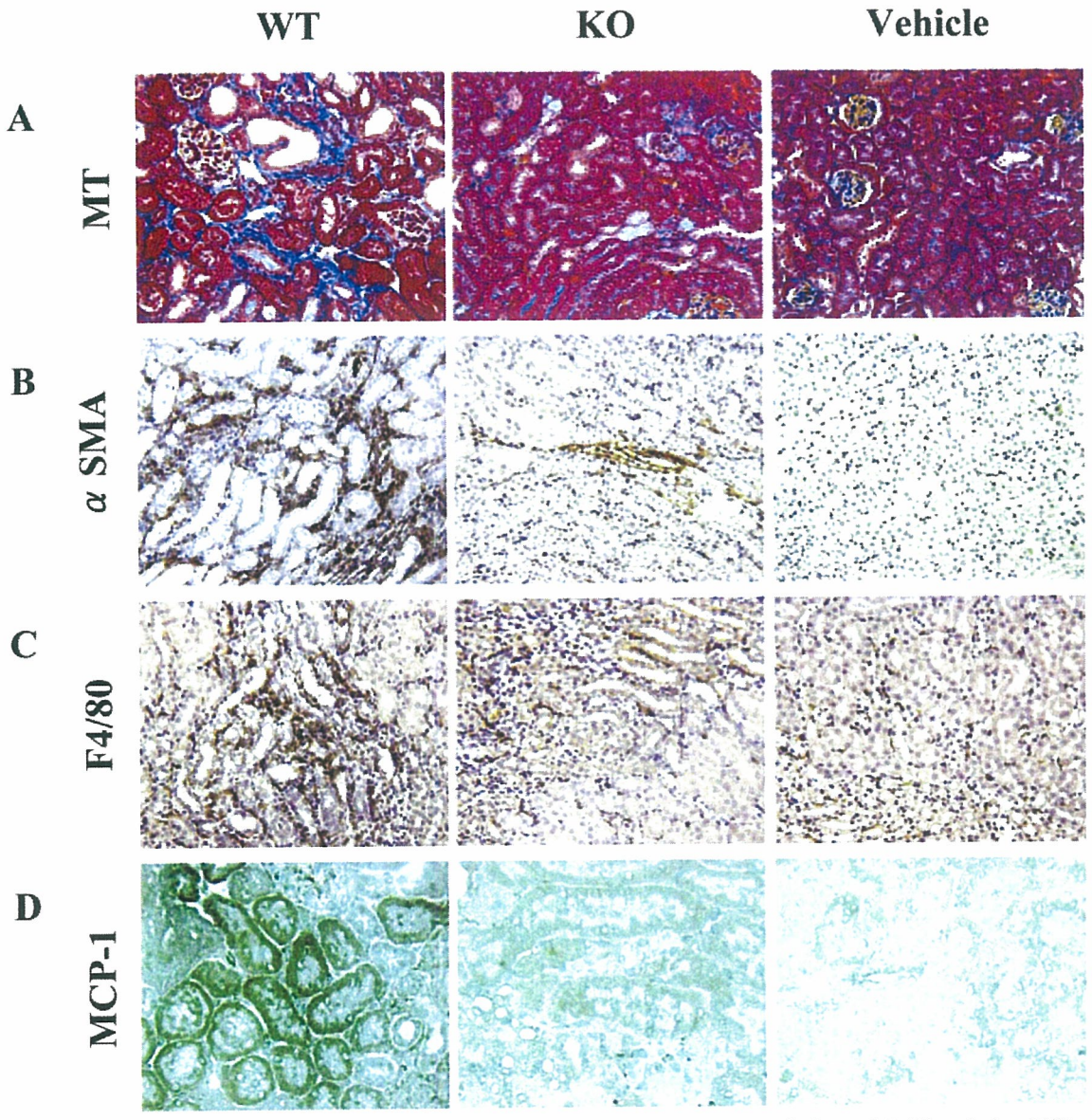

Figure 6. Histological findings at day 14 stained with Masson's trichrome (**A**), immunohistochemistry using antibody to α-SMA (**B**), antibody to F4/80-positive macrophage (**C**), and immunohistochemical analyses using antibody to MCP-1 with frozen sections. Vehicle-treated PAFR-KO and PAFR-WT showed identical results. Therefore, only PAFR-WT mice results are shown. Original magnifications: ×100 (**A**); ×200 (**B**, **C**); ×400 (**D**).

day 14 in PAFR-WT mice was significantly higher than that in PAFR-KO mice [PAFR-WT: 97.5 ± 11.9/×200 field (n = 5) vs. PAFR-KO: 32.1 ± 8.6/×200 field (n = 5); $P <$ 0.05] (Figure 9). In contrast, the numbers of F4/80-positive cells in kidneys harvested at 48 hours after FA administration did not differ among groups [PAFR-WT: 28.5 ± 6.7/×200 field (n = 5) versus PAFR-KO: 18.7 ± 2.0/×200 field (n = 5); P = 0.21].

The PAFR-WT mice treated with WEB2086 after acute phase every day (from day 2 to day 14) showed significantly decreased interstitial fibrosis and macrophage infiltration at day 14 compared with untreated PAFR-WT mice [fibrotic area: 3.5 ± 0.3% (n = 5); $P <$ 0.05 versus untreated PAFR-WT mice and macrophage counts: 66.0 ± 6.3/×200 field (n = 5); $P <$ 0.05 versus untreated PAFR-WT mice] (Figures 7 and 9).

TNF-α and MCP-1 Expression in FA-Induced Renal Injury

We examined TNF-α and MCP-1 mRNA expression using TaqMan real-time PCR assay to confirm the contribution of macrophages for FA-induced renal injury. Decreased expression of both TNF-α and MCP-1 were shown in the kidney of PAFR-KO mice compared with PAFR-WT mice (Figure 10). Furthermore, increased expression of these cytokines at day 14 was found only in PAFR-WT mice.

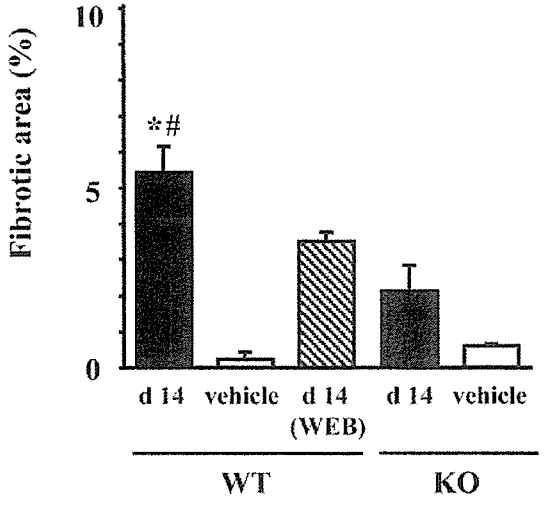

Figure 7. The fibrotic area in the cortex from the kidney at day 14 (d 14) was calculated using a computer-aided evaluation program. Five animals were in the FA group; four received the vehicle. $^{*}P < 0.05$ versus PAFR-KO mice. $^{*}P < 0.05$ versus PAFR-KO mice with FA administration. $^{\#}P < 0.05$ versus WEB2086-treated PAFR-WT mice.

These results indicate that macrophage accumulation to the injured kidney was amplified even after acute tubular damage at day 2. In addition, immunohistochemical analysis at day 14 revealed that MCP-1 protein was expressed dominantly in the proximal tubular epithelium (Figure 6D).

PAF Levels in Kidney

To clarify the presence of PAF and PAFR pathway in FA-induced renal injury, we examined the levels of PAF in the kidney. The renal PAF levels were virtually the same in normal kidney of PAFR-KO and PAFR-WT mice [PAFR-WT: 0.96 ± 0.15 pg/mg tissue (n = 4) versus PAFR-KO: 1.16 ± 0.39 pg/mg tissue (n = 4)] and increased by administration of FA, especially at day 14 in PAFR-WT mice [PAFR-WT at day 14, 11.08 ± 3.38 pg/mg tissue (n = 4) versus PAFR-WT normal kidney; $P < 0.05$] (Figure 11). The PAF levels in PAFR-WT mice were higher than those in PAFR-KO mice, especially at day 14, but those differences were not statistically significant.

PAF-Induced Chemotaxis in Macrophage

Infiltration of F4/80-positive macrophages in interstitial fibrosis of the kidney was attenuated in PAFR-KO mice; PAF is also known as a potent chemoattractant for leukocytes. For that reason, we examined the direct chemotactic activity of macrophages to PAF using a Boyden chamber system. Macrophages isolated from PAFR-WT mice showed a remarkable chemotactic activity to PAF at concentrations of 1 and 10 nmol/L. On the other hand, PAF induced no chemotaxis in macrophages isolated from PAFR-KO mice (Figure 12).

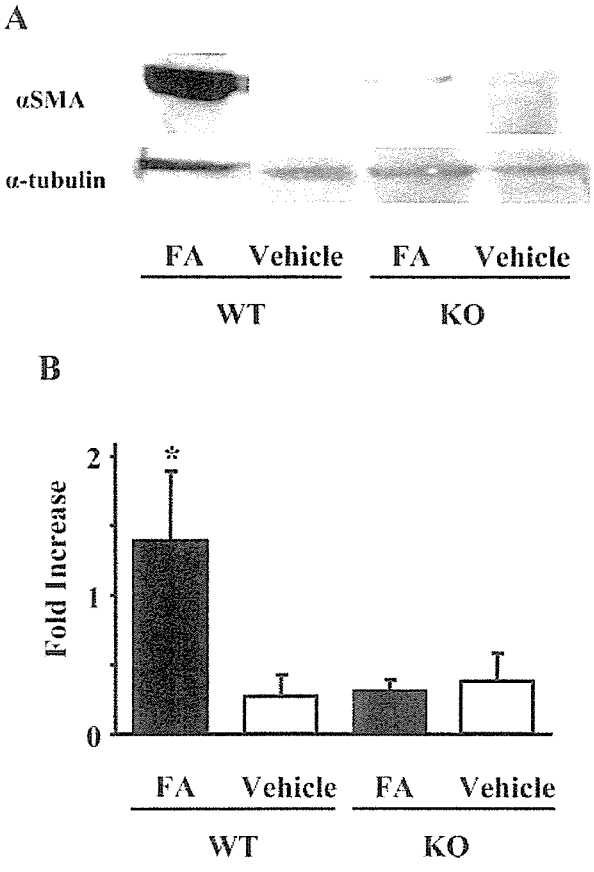

Figure 8. Western blot analysis of α-SMA expression in kidney homogenates at day 14. A representative image is depicted in **A (top)**. **B:** The histogram illustrates the relative density of bands in comparison with α-tubulin. Five animals were in the FA group; four received the vehicle. $^{*}P < 0.05$ versus PAFR-KO mice with FA administration.

Discussion

This study demonstrated that FA-induced renal injury comprises an acute and a subsequent chronic phase. Infiltration of ROS-producing neutrophils in acute tubular damage and macrophages in chronic interstitial fibrosis was attenuated significantly in PAFR-KO mice in comparison with PAFR-WT mice. Moreover, treatments of PAFR-WT mice with neutrophil depletion antibody and PAFR antagonist, which were started after the acute phase, also showed significant amelioration of acute tubular damage and macrophage infiltration and interstitial fibrosis in the chronic phase. Therefore, our data indicate that leukocytes play a crucial role both in the acute phase and the chronic phase of this animal model and that PAF is involved in pathogenesis of FA-induced inflammatory renal injury. To our knowledge, this study is the first to demonstrate the attenuation of renal injury in PAFR-KO mice, although several precedent studies have shown the effects of PAFR antagonists on renal disease in animal models.[10–12] However, those studies using PAFR antagonists often include problems related to drug specificity. Some PAFR antagonists inhibit the effect of histamine through interaction with its G protein-coupled receptor[13,15]; other antagonists inhibit the activity of PAF

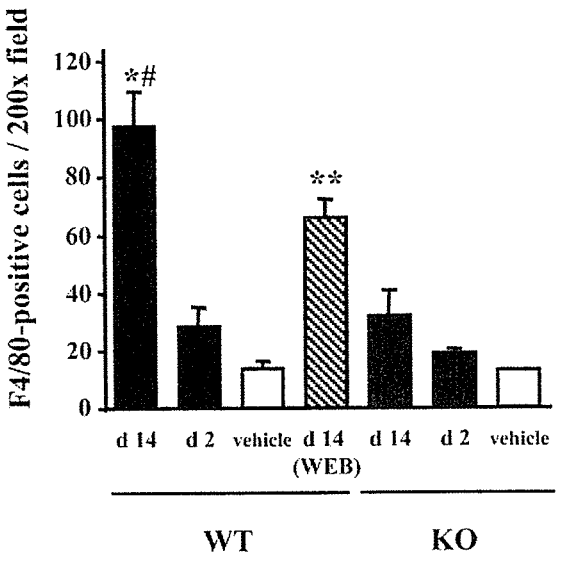

Figure 9. Number of infiltrating F4/80-positive cells in the tubulointerstitium with FA-induced renal injury. Five animals were in the FA group; four received the vehicle. $^{*}P < 0.005$ versus PAFR-KO mice at day 14 (d 14). $^{\#}P < 0.05$ versus WEB2086-treated PAFR-WT mice at day 14. $^{**}P < 0.05$ versus PAFR-WT mice at day 14.

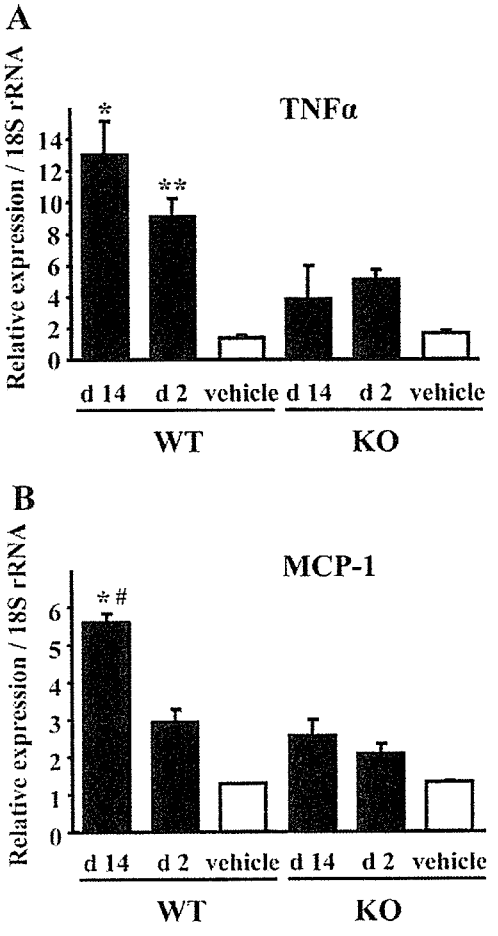

Figure 10. Quantitative analyses of expression of TNF-α (**A**) and MCP-1 (**B**) using real-time PCR. Eight animals were in the FA group; three received the vehicle. **A:** TNF-α expression was higher in PAFR-WT mice, both at days 2 and 14. $^{*}P < 0.005$ versus PAFR-KO mice at day 14 (d 14), $^{**}P < 0.05$ versus PAFR-KO mice at day 2 (d 2). **B:** MCP-1 expression was higher in PAFR-WT mice than in PAFR-KO mice at day 14. $^{*}P < 0.05$ versus PAFR-KO mice at day 14. Moreover, MCP-1 expression increased in PAFR-WT mice at day 14 greater than that at day 2. $^{\#}P < 0.05$ versus PAFR-WT mice at day 2.

acetylhydrolase, which inactivates PAF.[14,16] The present study demonstrates the pathological role of PAF in FA-induced renal injury using genetically engineered mice in which PAFR signaling was decreased congenitally by disrupting the PAFR gene. Several reports have used PAFR-KO mice to elucidate the role of PAF in various pathophysiologies. The PAFR-KO mice exhibited attenuation in animal models in which inflammatory injuries were crucial: chemical lung injury,[18] bronchial asthma,[19] and sponge-induced subcutaneous granuloma formation.[20] On the other hand, PAFR-KO mice showed exacerbation of infection models such as pulmonary gram-negative bacteria infection[41] and cardiac *Trypanosoma* infection.[42] Those reports, along with the present study, indicate that PAF causes both beneficial and deleterious effects by activating leukocytes in different circumstances.

The neutrophil infiltration and lipid peroxidation of HHE in kidney at day 2 in PAFR-KO mice were significantly lower than those of PAFR-WT mice in this study. Stimulation of neutrophils with PAF *in vitro* engenders numerous cellular responses including chemotaxis,[43–45] degranulation,[46] and ROS production.[43,47] Priming neutrophils with PAF enhances ROS production by NADPH oxidase, which catalyzes superoxide production.[48] Through *in vivo* analysis with ischemia-reperfusion kidney injury, Kelly and colleagues[49] showed that PAFR antagonist reduced neutrophil infiltration. Lloberas and colleagues[50] showed that antioxidant treatment with vitamin C decreased PAF release from kidney and neutrophil infiltration to kidney. Moreover, neutrophil depletion by a monoclonal antibody RB6-8C5 significantly ameliorated FA-induced acute tubular damage in this study. These findings indicate that ROS produced by activated infiltrating neutrophils play an important role in FA-induced acute tubular damage. On the other hand, incomplete recovery of FA-induced renal injury in PAFR-KO mice and better improvement in neutrophil-depleted PAFR-WT mice than PAFR-KO mice, despite the lack of statistical difference between them, indicates that additional factors related to neutrophil activation might contribute to FA-induced injury.

Recruitment and activation of macrophages and lymphocytes to the kidney play a central role in a final pathway of most injuries to chronic kidney damage. Therefore, regulating these immunocompetent cells might be a potential target for therapeutic intervention for kidney disease.[4] The PAFR-KO mice showed significant amelioration of FA-induced renal injury that developed to remarkable interstitial fibrosis with macrophage infiltration accompanied by the increase in the expression of TNF-α and MCP-1. Contribution of TNF-α for interstitial fibrosis was demonstrated in unilateral ureter obstruction, another renal fibrosis model, with TNF-α receptor knockout mice.[51] In PAFR-KO mice, reduced macrophage infiltration number and lack of a PAF receptor pathway might engender decreased TNF-α expression in the kidney and

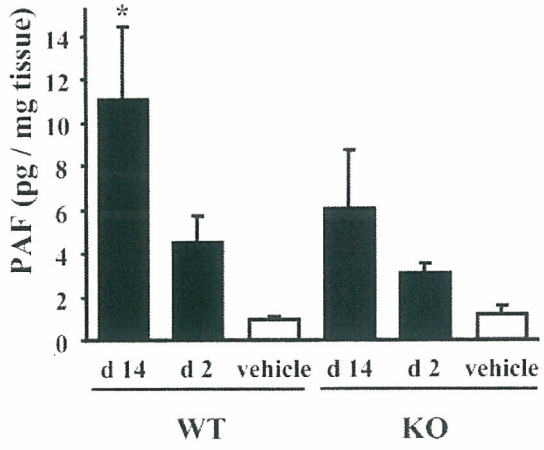

Figure 11. PAF level in FA-injured kidney. Four animals were in each group. $^{*}P < 0.05$ versus PAFR-WT mice receiving the vehicle.

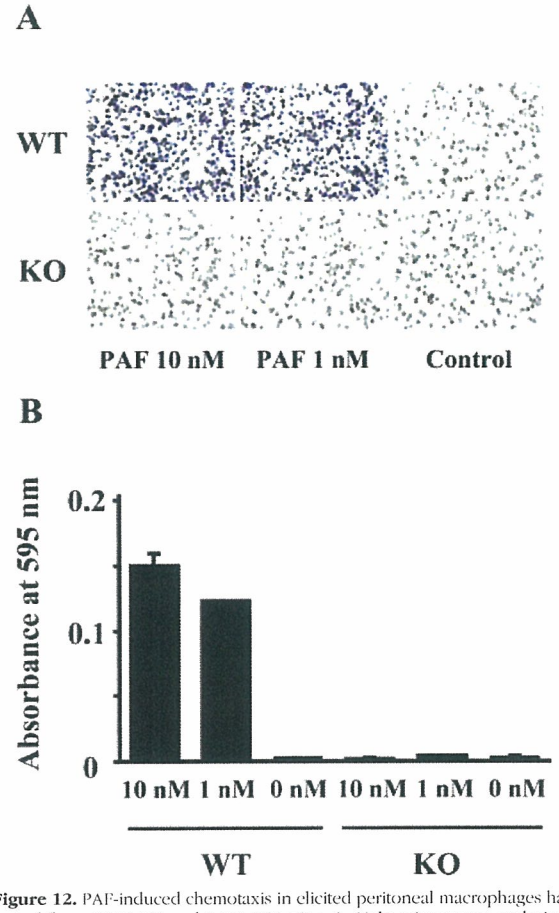

Figure 12. PAF-induced chemotaxis in elicited peritoneal macrophages harvested from PAFR-KO and PAFR-WT mice. **A:** Light microscope analysis of the migration toward PAF in the Boyden chamber assay. **B:** Quantitative analysis with measured absorbance at 595 nm. Each experiment was performed in triplicate. Original magnifications, ×200.

subsequent interstitial fibrosis. MCP-1 is presumed to play a potent role in macrophage chemotaxis and activation. Decreased expression of MCP-1 in PAFR-KO mice was in accordance with attenuated macrophage infiltration. MCP-1 production has been reported in tubular epithelial cells in unilateral ureter obstruction model[52] and cultured renal proximal tubular epithelial cells.[53] We also found MCP-1 production in tubular epithelial cells by immunohistochemistry that was decreased in PAFR-KO mice. Furthermore, PAF activates nuclear factor-κB,[54] which is critical for production of MCP-1 in tubular epithelial cells.[55] Expression of PAFR has been demonstrated in microdissected rat tubular cells[56] and tubular cell line LLC-PK1.[57] Taken together, it is quite plausible that PAF causes MCP-1 production in renal tubular cells. Indeed, Jocks and colleagues[58] showed MCP-1 induction by PAF in glomerular immune injury model with isolated perfused rat kidney. Beaudeux and colleagues[59] showed that PAF stimulated MCP-1 release in monocytes isolated from human peripheral blood. Further examination is required to clarify the role of PAF receptor pathway in renal tubular cells in this model.

We further examined the effect of blocking the PAF receptor pathway on macrophage recruitment with PAFR antagonist WEB2086 *in vivo*. It is noteworthy that treatment with WEB2086, even after acute tubular injury, also attenuated macrophage infiltration and interstitial fibrosis. Because we showed the chemotaxis activity of PAF to macrophages by Boyden chamber chemotaxis assay, PAF is likely to have a direct effect on the recruitment of macrophages to the injured kidney. However, renal functions and macrophage infiltration were not improved by WEB2086 to the degree of those of PAFR-KO mice. This might be because the degree of initial injury was so severe that WEB2086 could not improve renal function. Alternatively, WEB2086 could not suppress the PAF receptor pathway completely in this injury because of unfavorable pharmacokinetics and pharmacodynamics. Although several reports have described the effects of a single injection of WEB2086 at the dose of 5 to 10 mg/kg on mouse inflammation models,[60,61] the optimal administration dose for chronic animal models and renal dysfunction models remains unclear. Further evaluation is required for effects of WEB2086 on FA-induced renal injury.

We measured PAF levels in the kidney and found higher PAF levels in the kidneys of PAFR-WT mice than in those of PAFR-KO mice. This result indicated that there was an additional portion of PAF produced by oxidative stress secondary to initial injury and this component was decreased in PAFR-KO mice. We also found higher PAF levels at the chronic phase (day 14) than in the acute phase (day 2), especially in PAFR-WT mice. Changes of PAF levels in the kidney were similar to those of macrophage counts and MCP-1 expression. Therefore, it is plausible that PAF produced by initial injury enhanced macrophage infiltration to the kidney through MCP-1 expression and that the recruited macrophages produced PAF in the kidney. Our data indicate a vicious cycle of an amplifying effect of macrophage recruitment that is exacerbated by the PAF pathway.

The present study demonstrates the involvement of PAF in FA-induced renal inflammatory injury. Blockade of the PAF receptor pathway significantly attenuates both acute tubular damage and subsequent chronic development of interstitial fibrosis by reducing infiltrating leuko-

cytes. Such a blockade suggests a potent therapeutic approach to kidney injury caused by inflammatory cells.

Acknowledgments

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Immunomodulation by n-3- versus n-6-rich lipid emulsions in murine acute lung injury—Role of platelet-activating factor receptor

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Objective: **Cytokines, platelet-activating factor (PAF), and eicosanoids control local and systemic inflammation. Conventional soybean oil-based lipid emulsions used for parenteral nutrition may aggravate the leukocyte inflammatory response or adhesion to the vessel wall. Fish oil-based lipid emulsions, in contrast, may exert an anti-inflammatory effect.**

Design: **We investigated the impact of lipid emulsions on leukocyte invasion, protein leakage, and cytokines in two murine models of acute inflammation.**

Setting: **Research laboratory of a university hospital.**

Subjects: **Wild-type mice and PAF-receptor knockout mice.**

Interventions: **Mice received an infusion of normal saline, fish oil- or soybean oil-based lipid emulsions before lipopolysaccharide challenge.**

Measurements and Main Results: **Preinfusion with soybean oil resulted in increased leukocyte invasion, myeloperoxidase activity, and protein leakage and exaggerated release of tumor necrosis factor (TNF)-α as well as macrophage inflammatory protein (MIP)-2 into the alveolar space after intratracheal lipopolysaccharide challenge. In contrast, preinfusion with fish oil reduced leukocyte invasion, myeloperoxidase activity, protein leakage, and TNF-α as well as MIP-2 generation. Corresponding profiles were found in plasma following intraperitoneal lipopolysaccharide application: Soybean oil increased but fish oil decreased the TNF-α and MIP-2 formation. When PAF-receptor-deficient mice were challenged with lipopolysaccharide, leukocyte invasion, lung tissue myeloperoxidase, cytokine generation, and alveolar protein leakage corresponded to those observed in wild-type animals. Fish oil and soybean oil lost their diverging effects on leukocyte transmigration, myeloperoxidase activity, leakage response, and cytokine generation in these knockout mice. Similarly, the differential impact of both lipid emulsions on these lipopolysaccharide-provoked changes was suppressed after pretreating animals with a PAF-receptor antagonist.**

Conclusions: **Fish oil- vs. soybean oil-based lipid infusions exert anti- vs. proinflammatory effects in murine models of acute inflammation. The PAF/PAF-receptor-linked signaling appears to be a prerequisite for this differential profile. (Crit Care Med 2007; 35:544–554)**

Key Words: platelet-activating factor; fish oil; lipid emulsions; inflammation; sepsis; acute lung injury

Acute lung injury, the systemic inflammatory response syndrome, and sepsis are common in intensive care patients (1, 2). At early time points, all three entities are associated with an excessive inflammatory response (3), but in later stages of systemic inflammatory response syndrome or sepsis, a reduced immune response may be detected, termed compensatory anti-inflammatory response syndrome (4). Lipids, lipid mediators, and inflammation are closely interrelated (3, 5). The generation of pro- and anti-inflammatory as well as vasoactive eicosanoids (such as prostaglandin E_2, prostaglandin I_2, and thromboxane A_2) is coupled to the generation of free arachidonic acid from phospholipids. In the context of lipids and inflammation, eicosanoids represent a major focus of interest due to their strong proinflammatory and anti-inflammatory potencies (3). Among the n-6 fatty acids in the Western diet and current nutritional regimes applied in intensive care units, linoleic acid is the most prominent fatty acid, giving rise to its elongation and desaturation product and eicosanoid precursor arachidonic acid. The n-3 fatty acids, including eicosapentaenoic acid and docosahexaenoic acid, make up an appreciable part of the fat in cold-water fish and seal meat. Eicosapentaenoic acid-derived 5-series leukotrienes generated by the 5-lipoxygenase and the cyclooxygenase product thromboxane A_3 possess markedly reduced inflammatory and vasomotor potencies compared with the arachidonic acid-derived lipid mediators and may even exert antagonistic functions (6).

In addition to acting as a precursor for eicosanoid formation, n-3 vis-à-vis n-6 fatty acid incorporation into membrane

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(phospho)-lipid pools was suggested to influence lipid-related intracellular signaling events (7). Subclasses of phosphatidylcholine such as the platelet-activating factor (PAF)-precursor pool, as well as phosphatidylinositol and sphingomyelin pools, may be particularly relevant in this respect. Next, gene transcription is modulated as nuclear translocation of nuclear factor-κB and is inhibited by n-3 fatty acids involving signaling by plasma membrane translocation and activation of protein kinase C (8). Furthermore, research work has linked a specific genetic background to the reduction in proinflammatory cytokine generation in volunteers (9).

PAF is a major lipid mediator derived in two steps from phosphatidylcholine yielding free arachidonic acid and the active mediator (10). Furthermore, PAF-like molecules may be generated by oxidative attack on unsaturated fatty acids in phosphatidylcholine, which may aggravate sepsis and tissue injury (11). PAF promotes adhesion of leukocytes to endothelial cells, and application of PAF-receptor antagonists (PAF-RA) ameliorates features of sepsis and shock in experimental models (12), but effects on mortality in septic patients were not reported in clinical phase III studies (13, 14). Mice carrying a targeted disruption of the PAF-receptor (PAF-R) gene exhibit an ameliorated response to an acid-aspiration lung injury model; however, they remain sensitive to lipopolysaccharide (LPS) and endotoxin-induced shock (15).

Different groups reported a major influence of nutrition including n-3 fatty acids on morbidity of intensive care patients. In the adult respiratory distress syndrome, a tailored nutrition with eicosapentaenoic acid, γ-linoleic acid, and antioxidants was reported to improve oxygenation, reduce length of mechanical ventilation, decrease incidence of new organ failures, and shorten length of stay in the intensive care unit (16). Using fish oil supplements, however, several days to weeks are required to effectively influence the fatty acid composition of membrane (phospho)-lipids and thereby the lipid mediator profile in humans (17). In contrast, when administering a fish oil-based lipid emulsion via the intravenous route in volunteers or septic patients, we recently demonstrated rapid changes in cell membrane fatty acid composition and leukocyte functions (18, 19).

In the present study, we developed a murine model suitable for continuous long-term intravenous lipid infusions and subsequently submitted mice to intratracheal or intraperitoneal LPS injection. In addition to analyzing systemic cytokine generation, we focused on compartmentalized inflammatory events such as cytokine appearance in the bronchoalveolar space and recruitment of leukocytes to the alveolar compartment.

MATERIALS AND METHODS

Reagents. Lipoven 10% (soybean oil, or SO) and Omegaven 10% (fish oil, or FO) were purchased from Fresenius-Kabi (Bad Homburg, Germany). Analysis of fatty acid composition of the lipid emulsions is given in Table 1. Chemicals of highest purity were obtained from Merck (Darmstadt, Germany). LPS (O111:B4) from *Escherichia coli* was from Sigma-Aldrich (Dreisenhofen, Germany). The PAF antagonist BN52021 originated from Biomol (Hamburg, Germany).

Animals. Local government authorities and university officials responsible for animal protection approved the study. Parent and offspring PAF-R −/− mice on the BALB/c background and wild-type animals (WT) were kept under standard conditions with a 12-hr day/night cycle under specific pathogen-free conditions. Animals 8–12 wks old (18–21 g weight) were used for experiments. For implantation of a jugular vein catheter, mice were anesthetized by an intraperitoneal injection of a 1:1 mixture of xylazine at 80–100 mg per kilogram of body weight (Bayer, Leverkusen, Germany)/ketamine (Pharmacia & Upjohn, Erlangen, Germany). When animals were anesthetized and spontaneously breathing, points of incisions were shaved and disinfected. A silicon catheter (Braun, Melsungen, Germany) was inserted into the left jugular vein and tied.

Table 1. Fatty acid composition of the soybean oil (SO)-based and fish oil (FO)-based lipid-emulsion (g/L)

Fatty Acid	SO	FO
C14:0	—	4.9
C16:0	12.4	10.7
C16:1n-7	—	8.2
C18:0	5.0	2.4
C18:1n-9	24.1	12.3
C18:2n-6	52.2	3.7
C18:3n-3	8.2	1.3
C20:4n-6	—	2.6
C20:5n-3	—	18.8
C22:5n-3	—	2.8
C22:6n-3	—	16.5
Others	—	16.1

The SO-based emulsion (Lipoven) and the FO-based lipid emulsion (Omegaven) were manufactured with identical techniques and additives. Repetitive gas chromatographic controls of both lipid emulsions revealed <0.3% free eicosapentaenoic acid or arachidonic acid as related to the esterified amounts of these fatty acids.

The catheter was tunneled to the neck of the animal and connected to an osmotic minipump (Alzet, Cupertino, CA) filled with NaCl 0.9% situated in an external device tied to the back allowing easy access and exchange of pumps without anesthesia.

Murine Model of Acute Lung Injury. Mice were anesthetized with xylazine/ketamine, a small catheter was inserted in the trachea, and LPS (0, 1, or 10 μg in 50 μL of normal saline/mouse) was instilled. Twenty-four hours after LPS application, mice were killed by an overdose of isoflurane (Abbot, Wiesbaden, Germany), and bronchoalveolar lavage (BAL) was performed as described (20). An additional BAL was performed after 4 hrs in mice receiving 10 μg of LPS as a precaution since the escalation in LPS dose could have increased mortality in the model. However, even after receiving the higher dose of LPS, all animals survived the observation period. Alveolar-recruited leukocytes recovered from lungs of LPS-challenged and control mice were counted in a counting chamber. Differentiation of leukocytes in blinded fashion was done on differential cell counts of Pappenheim-stained cytocentrifuge preparations, using overall morphologic criteria, including differences in cell size and shape of nuclei. Protein in bronchoalveolar lavage was determined according to Lowry et al (21).

Model of Intraperitoneal Inflammation. Mice were anesthetized with xylazine/ketamine, and LPS (2 μg/mouse) or vehicle was injected intraperitoneally. For cytokine measurements from plasma and white blood cell count in peripheral blood, mice were exsanguinated 2 hrs after LPS treatment in deep anesthesia.

Enzyme-Linked Immunosorbent Assay. Cytokine enzyme-linked immunosorbent assays for tumor necrosis factor (TNF)-α and macrophage inflammatory protein (MIP)-2 were performed according to the manufacturer's (R&D, Wiesbaden, Germany) instructions.

Myeloperoxidase Assay. Lung myeloperoxidase (MPO) was determined as an index of tissue neutrophil accumulation 24 hrs after LPS challenge as described (22). After weighing of lung stored at −80°C, the frozen lung was homogenized, sonicated, and centrifuged at 25,000 × *g*. MPO activity was calculated from change in absorbance (460 nm) resulting from decomposition of H_2O_2 in the presence of o-dianisidine.

Wet-to-Dry Ratio. To assess pulmonary edema, determination of lung wet weight was performed after removal of extraneous bronchial and cardiac structures as described (23). To measure dry weight, lungs were incubated in a drying oven a week at 80°C and then reweighed.

Peripheral White Blood Cell Counts. White blood count of peripheral blood was measured as previously described (24).

Experimental Protocol. Seven days after central venous catheter implantation in mice, exchange of pumps was performed. Then, 200 μL per day of either SO, FO, or 0.9% NaCl was infused over 3 days with the mice being al-

lowed access to water and chow *ad libitum*. The amount of lipids infused is equivalent to 1.0 g/kg/day. However, the energy expenditure of mice is nearly three times higher that that of humans. Therefore, the infused lipids were considered to be close to lower limits of recommended amount of lipids in parenteral nutrition. While receiving infusions, mice were subjected to low-dose unfractionated heparin injected subcutaneously. In experiments with WT mice treated with PAF-RA, 10 mg/kg of body weight BN52021 (Biomol, Hamburg, Germany) was injected into the tail vein 30 mins before intratracheal LPS application.

Statistics. Data are given as the mean ± SEM. Two-way analysis of variance was performed to test for differences between different infusion groups and mice strains (WT, PAF-R −/−, PAF-RA). *Post hoc* analysis was carried out using Student-Newman-Keuls' test. As data of protein in lavage and leukocytes in BAL (1 μg, 24 hrs) were not normally distributed, log-transformation was performed. Wet-to-dry ratios between unstimulated and stimulated groups were compared using Student's *t*-test. Probability (p) values <.05 were considered to indicate statistical significance. Analysis was carried out using SigmaStat.

RESULTS

Effect of Lipid Emulsions on Alveolar Leukocyte Recruitment and Wet-to-Dry Ratio in LPS-Induced Acute Lung Injury. Without LPS challenge, we found 0.10 ± 0.01 × 10^6 leukocytes in the BAL fluid without significant variation between NaCl and lipid infusion groups. After stimulation of WT mice with 1 μg of LPS, leukocytes migrated into the alveolar space, with their numbers in BAL fluid rising to 1.09 ± 0.08 × 10^6 cells after 24 hrs (Fig. 1*A*). Preinfusion of SO massively increased leukocyte recruitment by nearly 100% ($p < .01$ vs. NaCl). In contrast, in mice receiving FO, leukocytes were significantly reduced to <60% ($p < .01$ vs. SO and NaCl). When using 10

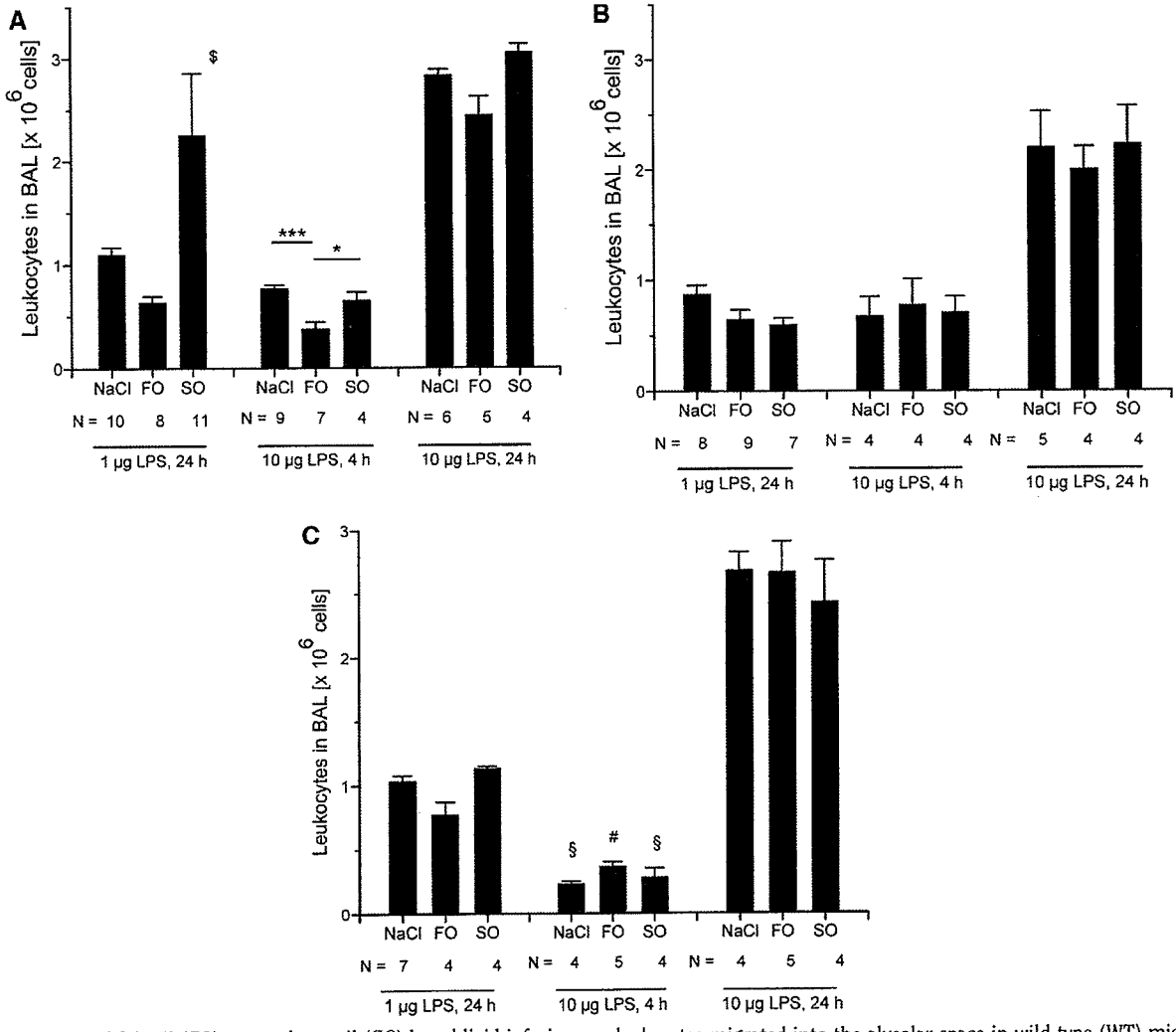

Figure 1. Impact of fish oil (*FO*)- vs. soybean oil (*SO*)-based lipid infusions on leukocytes migrated into the alveolar space in wild-type (WT) mice, mice lacking the platelet-activating factor-receptor (PAF-R −/−), and WT mice treated with platelet-activating factor receptor antagonist (PAF-RA) in a model of acute lung injury. WT mice (*A*), PAF-R −/− mice (*B*), and WT mice treated with a PAF-RA (*C*) were infused with saline (*NaCl*) or FO- or SO-based lipid emulsions, followed by application of 1 or 10 μg of endotoxin (lipopolysaccharide, LPS) intratracheally 4 or 24 hrs before performing a bronchoalveolar lavage (*BAL*). Total lavage leukocyte counts are given. Only in WT mice, a significant difference of SO- vs. FO-based lipid emulsions, and of saline (control) vs. FO-based lipid emulsion, respectively, was detectable ($\$p < .01$ all groups differed significantly from each other; $*p < .05$ FO vs. SO; $***p < .001$ FO vs. control). Animals exposed to 10 μg of LPS for 4 hrs in the PAF-RA groups exhibited a reduction of leukocyte numbers ($\S p < .05$ vs. WT and PAF-R −/−; $\#p < .05$ vs. PAF-R −/−). Data are given as mean ± SEM. Numbers of animals per group are detailed below columns. Error bars are missing when falling into symbol.

μg of LPS in the normal saline group, we found 0.76 ± 0.04 × 10^6 leukocytes after 4 hrs rising to 2.83 ± 0.06 × 10^6 leukocytes after 24 hrs in the BAL. After 4 hrs, infusion of FO-based lipid emulsions induced a significant reduction of transmigrated leukocytes by nearly 50% ($p < .001$ vs. NaCl and $p < .05$ vs. SO), whereas SO had no impact on leukocyte invasion compared with NaCl. After 24 hrs in mice receiving SO, leukocyte invasion was comparable to mice receiving NaCl, whereas infusions of FO-based lipids led to a small reduction.

Without LPS challenge, differential count of leukocytes in BAL was 2.5 ± 0.8% granulocytes, 97.0 ± 0.9% monocytes/macrophages, and 0.5 ± 0.3% lymphocytes without effect of lipid emulsions on this feature. At 24 hrs after challenge with 1 μg of LPS, 78.1 ± 3.2% granulocytes, 2.0 ± 0.4% lymphocytes, and 20.2 ± 3.1% monocytes/macrophages were detected in the BAL of WT mice receiving NaCl. This profile of predominant neutrophil invasion was not changed by infusion of lipids or LPS dose.

Wet-to-dry ratio was determined to evaluate degree of lung injury. Without LPS, wet-to-dry ratio was 4.62 ± 0.07 in control animals with both lipid infusions exhibiting no further impact. After instillation of 10 μg of LPS in mice receiving normal saline, wet-to-dry ratio increased to 5.12 ± 0.09 after 24 hrs ($p < .01$ vs. control) whereas both lipid emulsions did not modulate this feature and did not differ significantly from the NaCl group.

Significance of PAF-R for Recruitment of Leukocytes Into the Alveolar Space and Its Modulation by Lipid Emulsions. Bronchoalveolar lavage performed 4 or 24 hrs after intratracheal LPS challenge with 1 or 10 μg of LPS in PAF-R −/− mice submitted to infusion of normal saline (control) indicated a similar response as encountered in corresponding WT animals. Quantity (Fig. 1*B*) and cell differentiation of recruited leukocytes were not different in both strains. The diverging effect of preinfused lipid emulsions on alveolar recruitment of leukocytes was, however, fully lost in PAF-R −/− mice. In both lipid infusion groups, numbers of recruited leukocytes were slightly but not statistically significantly different compared with normal saline control mice. This finding was irrespective of LPS dose or time of lavage.

To confirm our results in PAF-R −/− mice, we treated WT mice with the PAF-RA BN52021. WT mice were infused for 3 days with normal saline (NaCl control) or with FO- or SO-based lipid emulsions followed by application of 1 or 10 μg/mouse LPS intratracheally 4 or 24 hrs before performing a BAL (Fig. 1*C*). A dose of 10 mg/kg BN52021 was injected into the tail vein 30 mins before LPS application. In mice treated with normal saline, the absolute leukocyte numbers and differential count in BAL did not differ from WT or PAF-R −/− mice 24 hrs after LPS challenge. However, 4 hrs after instillation of 10 μg of LPS, we found a strong reduction of leukocytes in all PAF-RA groups. The reduction was significant in animals receiving NaCl or SO compared with respective WT and PAF-R −/− groups ($p < .05$ for each comparison). In animals receiving FO, leukocytes were significantly lower compared with the FO-PAF-R −/− group ($p < .05$) but comparable to WT mice infused with FO-based emulsions.

As in PAF-R −/− mice, FO or SO no longer modulated the amount of alveolar recruited leukocytes after treatment with a PAF-RA. This was consistent irrespective of time of lavage and dose of LPS. In particular, in contrast to WT mice, SO-based lipid emulsions lost their capacity to double alveolar leukocyte transmigration.

LPS-Induced Accumulation of Neutrophils in Lung Tissue. MPO activity was measured before and 24 hrs after LPS challenge to assess neutrophil accumulation in lung tissue. In lungs of WT mice without LPS exposure, MPO activity was 0.9 ± 0.3 units/g without significant effect of lipid emulsions (Table 2). After instillation of 10 μg of LPS, MPO increased to 6.6 ± 0.7 units/g in control animals. After infusion of SO-based lipid emulsions, MPO was nearly two-fold higher compared with the FO and normal saline groups ($p < .05$ for each comparison). After application of 1 μg of LPS intratracheally, we found the same trend for MPO determination in WT animals, but results failed to reach the level of significance. The diverging effect of preinfused lipid emulsions on neutrophil accumulation in lung tissue was, however, lost in PAF-R −/− mice and after application of the PAF-RA in mice receiving 10 μg of LPS.

LPS-Induced Alveolar Protein Leakage. To examine the impact of lipids on lung injury, we determined protein in the BAL as marker for vascular leakage. In WT animals without LPS challenge, protein concentration was determined as 32 ± 3 μg/mL without significant modulation by lipid emulsions (Table 3). After intratracheal challenge with 1 μg or 10 μg of LPS, protein in BAL increased to 273 ± 16 μg/mL or 551 ± 34 μg/mL, respectively, after 24 hrs in WT animals. Infusion of FO significantly decreased alveolar protein leakage after 1 μg ($p < .01$ vs. NaCl and SO) and 10 μg of LPS, whereas after SO infusion protein concentrations slightly increased. Due to the marked reduction of protein in BAL after FO after 1 μg of LPS, FO-WT animals differed significantly from FO-PAF-R −/− mice ($p < .05$).

Generation of MIP-2 After LPS Instillation. We then examined the impact of lipid emulsions on intra-alveolar cytokine generation in our model. Without LPS stimulation, MIP-2 concentration in BAL

Table 2. Myeloperoxidase activity in lung homogenates (units/g of tissue)

	Without LPS	1 μg of LPS (24 Hrs)	10 μg of LPS (24 Hrs)
Wild-type			
NaCl	0.9 ± 0.3 (n = 4)	3.3 ± 0.7 (n = 4)	6.6 ± 0.7 (n = 8)
FO	1.0 ± 0.4 (n = 4)	3.4 ± 0.4 (n = 4)	6.8 ± 1.4 (n = 4)
SO	0.9 ± 0.4 (n = 4)	5.1 ± 0.9 (n = 4)	11.3 ± 0.9 (n = 4)[a]
PAF-R −/−			
NaCl	0.8 ± 0.4 (n = 4)	ND	9.1 ± 1.0 (n = 4)
FO	0.9 ± 0.4 (n = 4)	ND	9.4 ± 1.4 (n = 4)
SO	0.9 ± 0.3 (n = 4)	ND	7.7 ± 0.5 (n = 4)
PAF-RA			
NaCl	0.9 ± 0.4 (n = 4)	ND	9.5 ± 1.5 (n = 4)
FO	0.8 ± 0.4 (n = 4)	ND	8.3 ± 0.4 (n = 4)
SO	0.9 ± 0.5 (n = 4)	ND	9.6 ± 1.6 (n = 4)

LPS, lipopolysaccharide; NaCl, saline; FO, fish oil; SO, soybean oil; PAF-R −/−, mice lacking the platelet-activating factor receptor; ND, not done; PAF-RA, platelet-activating factor receptor antagonist.

[a]$p < .05$ for comparison with NaCl and FO. Wild-type mice, PAF-R −/− mice, and wild-type mice treated with a PAF-RA were infused for 3 days with NaCl (control) or with FO- or SO-based lipid emulsions, followed by stimulation with 1 or 10 μg of endotoxin (LPS) intratracheally for 24 hrs.

Table 3. Protein concentration in bronchoalveolar lavage (μg/mL)

	Without LPS	1 μg of LPS (24 Hrs)	10 μg of LPS (24 Hrs)
Wild-type			
NaCl	32 ± 3 (n = 4)	273 ± 16 (n = 21)	551 ± 34 (n = 4)
FO	33 ± 4 (n = 4)	148 ± 21 (n = 6)[a,b]	416 ± 21 (n = 5)[c]
SO	31 ± 5 (n = 5)	313 ± 40 (n = 11)	641 ± 11 (n = 4)
PAF-R −/−			
NaCl	31 ± 4 (n = 4)	347 ± 36 (n = 11)	382 ± 89 (n = 6)
FO	33 ± 5 (n = 4)	338 ± 69 (n = 9)	462 ± 43 (n = 4)
SO	32 ± 5 (n = 4)	304 ± 67 (n = 7)	654 ± 115 (n = 5)
PAF-RA			
NaCl	33 ± 4 (n = 4)	325 ± 40 (n = 4)	552 ± 86 (n = 4)
FO	33 ± 3 (n = 4)	253 ± 50 (n = 4)	424 ± 102 (n = 6)
SO	32 ± 4 (n = 4)	331 ± 70 (n = 4)	527 ± 119 (n = 6)

LPS, lipopolysaccharide; NaCl, saline; FO, fish oil; SO, soybean oil; PAF-R −/−, mice lacking the platelet-activating factor receptor; PAF-RA, platelet-activating factor receptor antagonist.

[a]Within WT, $p < .01$ FO vs. NaCl and SO; [b]within FO infusion groups, $p < .05$ WT vs. PAF-R−/−; [c]within WT, $p < .05$ FO vs. SO. Wild-type mice (WT), PAF-R −/− mice, and WT mice treated with a PAF-RA were infused for 3 days with NaCl (control) or with FO-, or SO-based lipid emulsions, followed by stimulation with 1 or 10 μg of endotoxin (LPS) intratracheally for 24 hrs.

was below detection limit regardless of infusion used or mouse line examined. At 24 hrs after challenge with 1 μg of LPS, we found an increase in lavage MIP-2 concentration to 112 ± 6 pg/mL in mice infused with normal saline (Fig. 2*A*). In mice receiving SO, MIP-2 was similar compared with the NaCl group. However, in mice receiving FO, MIP-2 concentration was significantly reduced ($p < .001$ vs. NaCl and $p < .05$ vs. SO). When using 10 μg of LPS in the normal saline group, we found a tremendous increase in MIP-2 after 4 hrs to 1225 ± 96 pg/mL and a decline to 165 ± 18 pg/mL after 24 hrs. After 4 hrs, infusion of FO-based lipid emulsions induced a small reduction of MIP-2, but preinfusion with SO provoked a significant increase of nearly 25% compared with the NaCl group ($p < .05$ vs. FO or NaCl). After 24 hrs in mice receiving SO, MIP-2 concentration remained massively elevated (741 ± 73 pg/mL, $p < .001$ vs. NaCL or FO) and was reduced to 140 ± 18 pg/mL in the FO group.

In PAF-R −/− mice receiving normal saline, MIP-2 levels 24 hrs after challenge with 1 or 10 μg of LPS were comparable to WT animals. The MIP-2 concentrations in both lipid infusion groups did not differ significantly from each other and from saline control irrespective of time of lavage or dose of LPS (Fig. 2*B*). After 4 hrs in mice receiving 10 μg of LPS, MIP-2 concentrations were significantly higher compared with corresponding WT animals in the NaCl and FO groups ($p < .05$) but did not differ from the animals receiving SO.

To confirm our results in PAF-R −/− mice, we treated WT mice with the PAF-RA BN52021. At 24 hrs after challenge with 1 μg of LPS, PAF-RA pretreatment exhibited a reduction of MIP-2 down to about one third compared with WT and PAF-R −/− mice receiving normal saline (Fig. 2*C*, $p < .05$). This reduction by PAF-RA pretreatment was also found in mice receiving either lipid emulsion compared with WT and PAF-R −/− animals ($p < .05$).

Using 10 μg of LPS in the PAF-RA group, MIP-2 concentrations after 4 and 24 hrs were similar to the concentrations detected in WT and PAF-R −/− mice receiving NaCl. In contrast to WT animals, but similar to PAF-R −/−, application of PAF-RA abolished differential impact of FO vs. SO lipid infusions on intra-alveolar MIP-2 concentrations irrespective of dose of LPS or time of lavage. Due to the massive increase after infusion of SO-based lipid emulsions, WT animals differed significantly from PAF-R −/− and PAF-RA groups ($p < .01$).

TNF-α Concentration in BAL After LPS Challenge. Next, we examined impact of lipid emulsions on TNF-α concentration in BAL. Without LPS stimulation, TNF-α was undetectable in BAL in all groups irrespective of mouse line and infusion used. At 24 hrs after challenge with 1 μg of LPS, we found an increase in TNF-α concentration to 162 ± 12 pg/mL in mice infused with normal saline (Fig. 3*A*). In mice receiving SO, a similar TNF-α concentration was detected. In contrast, infusion of FO resulted in a significant reduction of TNF-α ($p < .05$ vs. NaCl and SO). After challenge with 10 μg of LPS in mice receiving normal saline, TNF-α concentration massively rose to 1496 ± 92 pg/mL after 4 hrs and to 564 ± 126 pg/mL after 24 hrs. After 4 hrs, infusion of SO led to a further small increase. In contrast, in the FO group, TNF-α was significantly reduced by nearly 25% compared with the NaCL group ($p < .05$ vs. NaCl and SO). However, in the BAL performed after 24 hrs, TNF-α concentration was not significantly modulated by lipid emulsions.

In PAF-R −/− mice receiving NaCl infusions, TNF-α was slightly lower after LPS challenge as compared with WT animals (Fig. 3*B*). TNF-α concentrations in the FO or SO group did not differ significantly from each other and from saline control irrespective of time of lavage or dose of LPS.

To confirm our results in PAF-R −/− mice, we treated WT mice with the PAF-RA BN52021. At 24 hrs after challenge with 1 μg of LPS, TNF-α concentration in PAF-RA-pretreated mice receiving NaCl was 100 ± 12 pg/mL, exhibiting a significant reduction by nearly 40% compared with saline-infused WT mice ($p < .05$, Fig. 3*C*). When instilling 10 μg of LPS, TNF-α concentrations after 4 and 24 hrs were similar to concentrations determined in WT and PAF-R −/− mice receiving normal saline. In contrast to WT animals, but similar to PAF-R −/−, injection of PAF-RA inhibited the differential impact of FO and SO lipid infusions on intra-alveolar TNF-α concentrations irrespective of dose of LPS or time of lavage.

After 4 hrs in mice stimulated with 10 μg of LPS, TNF-α concentrations were increased in all infusion groups receiving PAF-RA. In mice infused with FO, the PAF-RA mice differed significantly from the WT and PAF-R −/− animals ($p < .05$). As TNF-α was also increased in WT mice after SO infusion, the PAF-RA SO group and the WT SO group differed significantly from the PAF-R −/− mice ($p < .05$).

Cytokine Concentrations in Plasma After Intraperitoneal LPS Instillation. Next, we asked if lipid emulsions would have similar effects in a model of abdominal inflammation. In pilot experiments, we determined that TNF-α concentration in plasma peaked at 2 hrs after intra-abdominal LPS instillation in our model. Using the previous infusion setting, we challenged mice with 2 μg LPS intraperitoneally 2 hrs before kill and bleeding.

Before LPS challenge, leukocyte counts in peripheral blood were 6.6 ± 0.4 G/L

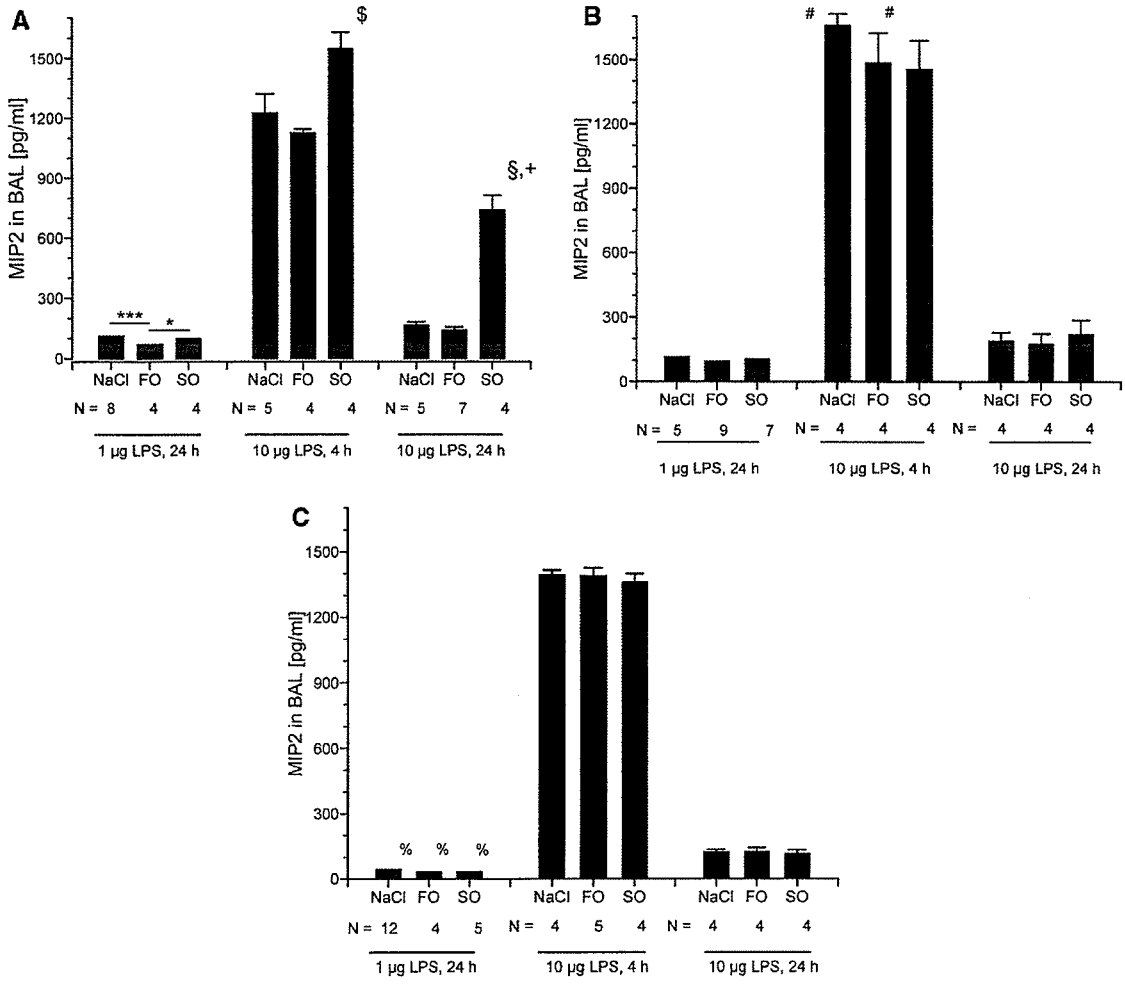

Figure 2. Impact of fish oil (*FO*)- vs. soybean oil (*SO*)-based lipid infusions on alveolar macrophage inflammatory protein (*MIP*)-2 generation in wild-type (WT) mice, mice lacking the platelet-activating factor-receptor (PAF-R −/−), and WT mice treated with platelet-activating factor receptor antagonist (PAF-RA) in a model of acute lung injury. WT mice (*A*), PAF-R −/− mice (*B*), and WT mice treated with a PAF-RA (*C*) were infused for 3 days with normal saline (control) or FO- or SO-based lipid emulsions, followed by stimulation with 1 or 10 µg of endotoxin (lipopolysaccharide, LPS) intratracheally 4 or 24 hrs before lavage. MIP-2 concentrations in bronchoalveolar lavage (*BAL*) were determined by enzyme-linked immunosorbent assay. Infusion of FO lipid emulsions resulted in decreased MIP-2 formation in WT mice (*$p < .05$ vs. SO; ***$p < .001$ vs. control). Infusion of SO resulted in increased MIP-2 generation compared with control and FO in both 10-µg groups ($\$p < .05$; $\S p < .001$). In PAF-R −/− or in WT mice treated with PAF-RA, no significant impact of lipid emulsions could be detected. MIP-2 concentrations in PAF-R −/− mice receiving 10 µg of LPS were higher after 4 hrs compared with corresponding WT controls ($\#p < .05$). After 24 hrs in WT-SO mice challenged with 10 µg of LPS, MIP-2 was significantly higher compared with the corresponding infusion groups of PAF-R −/− and PAF-RA mice ($+p < .01$). All infusion groups of PAF-RA mice receiving 1 µg of LPS were significantly lower than corresponding PAF-R −/− and WT groups ($\%p < .05$). Data are given as mean ± SEM. Numbers of animals per group are detailed below columns. Error bars are missing when falling into symbol.

(n = 5) without significant impact of the lipid emulsions. Two hours after intraperitoneal LPS challenge, 7.5 ± 0.8 × 10^9/L leukocytes were found in peripheral blood in mice receiving normal saline (n = 4). After FO infusion, leukocytes were slightly lower and SO slightly increased peripheral leukocytes; however, no significant difference was found.

Before LPS instillation in PAF-R −/− mice, leukocyte counts in peripheral blood were slightly higher compared with WT mice (7.2 ± 0.5 × 10^9/L, n = 4). Again, no significant impact of the lipid emulsions was found. After LPS injection into the peritoneal cavity, we detected 7.9 ± 1.6 × 10^9/L leukocytes in mice receiving NaCl. After FO or SO treatment, 8.5 ± 1.8 × 10^9/L and 7.1 ± 1.9 × 10^9/L leukocytes were measured (n = 4 each). However, no significant difference was found between infusion regimes.

Before LPS stimulation, no MIP-2 was detectable in plasma in either group. In WT controls, LPS challenge induced an increase to 9.0 ± 1.9 ng/mL. Infusion of FO-derived lipid emulsions resulted in a diminished MIP-2 serum concentration, whereas application of SO-based lipids increased MIP-2 levels significantly compared with the FO group ($p < .05$, Fig. 4*A*). In PAF-R −/−, LPS stimulation induced a three-fold increase in MIP-2 concentrations compared with WT in mice receiving NaCl ($p < .01$). This increase was also detected in both lipid infusion groups; however, differences between the infusion groups were abolished (Fig. 4*B*). The increase in MIP-2 was significant comparing PAF-R −/− mice with WT animals receiving FO ($p < .01$) but not in mice infused with SO.

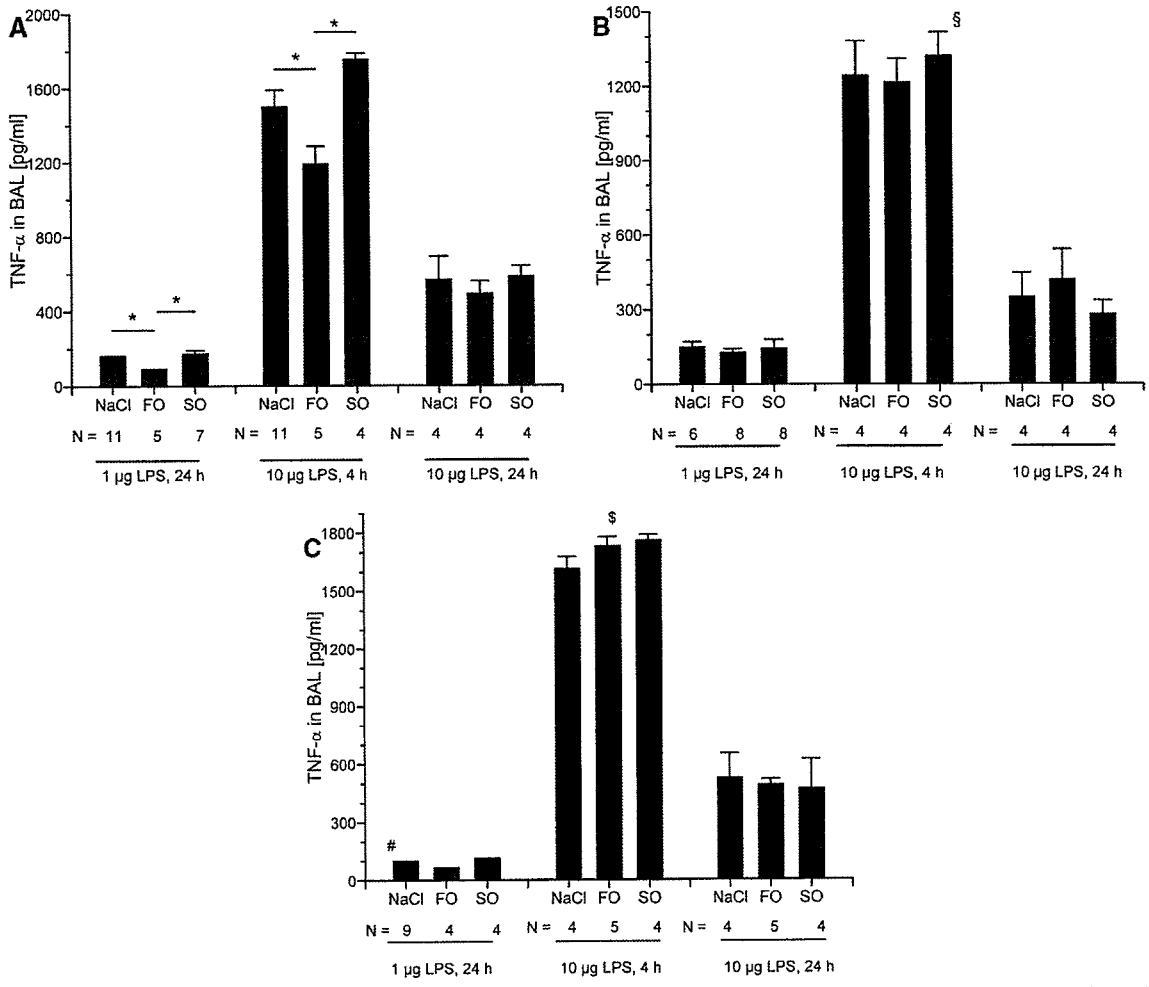

Figure 3. Impact of fish oil (*FO*)- vs. soybean oil (*SO*)-based lipid infusions on alveolar tumor necrosis factor (*TNF*)-α generation in wild-type (WT) mice, mice lacking the platelet-activating factor-receptor (PAF-R −/−), and WT mice treated with platelet-activating factor receptor antagonist (PAF-RA) in a model of acute lung injury. WT mice (*A*), PAF-R−/− mice (*B*), and WT mice treated with a PAF-RA (*C*) were infused for 3 days with saline (control) or FO- or SO-based lipid emulsions, followed by stimulation with 1 or 10 μg of endotoxin (lipopolysaccharide, LPS) intratracheally 4 or 24 hrs before lavage. TNF-α concentration in bronchoalveolar lavage was determined by enzyme-linked immunosorbent assay. Infusion of FO resulted in decreased TNF-α formation in WT mice (*$p < .05$ vs. SO and control). In PAF-R−/− mice or WT mice treated with PAF-RA, no significant impact of lipid emulsions could be detected. In PAF-RA mice challenged with 1 μg of LPS, TNF-α concentration of the NaCl group was lower compared with corresponding WT animals (#$p < .05$). After infusion of FO in PAF-RA mice, TNF-α concentration was higher compared with the WT-FO and PAF-R−/−-FO animals receiving 10 μg of LPS after 4 hrs ($$p < .05$). TNF-α concentration was lower in the PAF-R−/−-SO group after 10 μg of LPS and 4 hrs compared with the WT-SO and PAF-RA-SO animals (§$p < .05$). Data are given as mean ± SEM. Numbers of animals per group are detailed below columns. Error bars are missing when falling into symbol.

Before LPS challenge, we did not detect TNF-α in plasma in all groups. Following intraperitoneal LPS application, plasma TNF-α concentration rose to 303 ± 20 pg/mL in control animals. Infusion of SO-based lipids resulted in a significant increase of nearly 50% in TNF-α plasma concentration (Fig. 4*C*, $p < .01$ vs. control). After infusion of FO-derived lipids, TNF-α decreased significantly to 181 ± 12 pg/mL ($p < .05$ vs. control and $p < .001$ vs. SO). LPS challenge in PAF-R −/− mice infused with NaCl evoked a similar TNF-α concentration compared with WT controls (Fig. 4*D*). Again, no significant difference between the different infusion groups became detectable.

DISCUSSION

Using two different models of acute inflammation in mice, we have demonstrated that a 3-day course of lipid emulsion infusions is sufficient to modulate inflammatory responses induced by LPS. In contrast to SO-based lipid emulsions, FO-based lipid emulsions reduced pulmonary leukocyte invasion, protein leakage, and cytokine generation as well as cytokine appearance in the intravascular compartment. We present evidence that diverging effects of lipids emulsions are linked to PAF-R signaling. In mice carrying the disrupted PAF-R gene (PAF-R −/−), as well as in mice treated with a PAF-RA, the general response to endotoxin remained intact, but the differential response to FO vs. SO lipids was lost. A drawback of both models applied is the use of LPS in a single-hit model to induce inflammatory responses. Such a model is clearly different from clinical and experimental sepsis induced by bacterial infection.

Responses to endotoxin in PAF-R −/− mice were intact in our acute lung injury