

TNF- α promotes LPA₁- and LPA₃-mediated recruitment of leukocytes in vivo through CXCR2 ligand chemokines^[S]

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Abstract Lysophosphatidic acid (LPA) is a bioactive lysophospholipid present in low concentrations in serum and biological fluids but in high concentrations at sites of inflammation. LPA evokes a variety of cellular responses via binding to and activation of its specific G protein-coupled receptors (GPCR), namely LPA₁₋₆. Even though LPA is a chemoattractant for inflammatory cells in vitro, such a role for LPA in vivo remains largely unexplored. In the present study, we used the murine air pouch model to study LPA-mediated leukocyte recruitment in vivo using selective LPA receptor agonist/antagonist and LPA₃-deficient mice. We report that 1) LPA injection into the air pouch induced leukocyte recruitment and that both LPA₁ and LPA₃ were involved in this process; 2) LPA stimulated the release of the pro-inflammatory chemokines keratinocyte-derived chemokine (KC) and interferon-inducible protein-10 (IP-10) in the air pouch; 3) tumor necrosis factor- α (TNF- α) injected into the air pouch prior to LPA strongly potentiated LPA-mediated secretion of cytokines/chemokines, including KC, IL-6, and IP-10, which preceded the enhanced leukocyte influx; and 4) blocking CXCR2 significantly reduced leukocyte infiltration. We suggest that LPA, via LPA₁ and LPA₃ receptors, may play a significant role in inducing and/or sustaining the massive infiltration of leukocytes during inflammation.—Zhao, C., A. Sardella, J. Chun, P. E. Poubelle, M. J. Fernandes, and S. G. Bourgoïn. TNF- α promotes LPA₁- and LPA₃-mediated recruitment of leukocytes in vivo through CXCR2 ligand chemokines. *J. Lipid Res.* 2011. 52: 1307–1318.

Supplementary key words inflammation • murine air pouch model • LPA receptor agonist/antagonist • LPA receptor deficient mice • CXC chemokines

Lysophosphatidic acid (LPA) is a simple lipid with a single fatty acyl chain, a glycerol backbone, and a free phosphate group (1). Despite the simplicity of its structure, LPA has been implicated in various physiological (cell growth, differentiation, migration, and survival) and pathological (angiogenesis, cancer, and autoimmunity) situations. LPA is present in micromolar concentrations in serum and biological fluids and in higher concentrations at sites of inflammation and tumor (2). LPA evokes a variety of cellular responses via binding to and activation of its specific G protein-coupled receptors (GPCR) (3). So far, five and possibly six GPCRs have been identified as specific LPA receptors, namely, LPA₁₋₆ (4). Two additional GPCRs, GPR87 (5) and P2Y10 (6), have been suggested to be activated by LPA.

LPA has been shown to be a chemoattractant for human inflammatory cells in in vitro studies. Chettibi et al. showed that LPA was a powerful stimulator of human neutrophil polarization and motility (7). Similarly, Fischer et al. described chemoattractive effects of LPA toward human PMNs under agarose (8). LPA also causes an increase in binding of monocytes and neutrophils to human aortic endothelial cells (9). Emerging evidence suggests a role for autotaxin (ATX)-LPA in facilitating lymphocyte migration into lymphoid and chronically inflamed nonlymphoid tissues (10, 11). The study of Kanda et al. demonstrated that LPA, locally generated by ATX in high endothelial venules, facilitates lymphocyte transendothelial migration

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Abbreviations: ATX, autotaxin; COX-2, cyclooxygenase-2; GPCR, G protein-coupled receptor; IL, interleukin; IP-10, interferon- γ -inducible protein-10; KC, keratinocyte-derived chemokine; KO, knock out; LPA, lysophosphatidic acid; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2; PGE₂, prostaglandin E₂; RA, rheumatoid arthritis; TNF- α , tumor necrosis factor- α ; WT, wild type.

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via stimulating chemokinesis rather than chemotaxis (10). The role of LPA in the recruitment of leukocytes to inflammatory sites in vivo, however, remains elusive.

Accumulating evidence suggests that LPA-induced cell migration may be mediated, in part, by chemokines. We recently reported that LPA induces the expression of a potent chemoattractant, interleukin (IL)-8 (12). Chemokines are small chemoattractant peptides that play key roles in the accumulation of inflammatory cells at the site of inflammation. Some chemokines, particularly CXC chemokines containing the ELR motif (including CXCL1, 2, 3, 5, 6, 7, and 8) are crucial for neutrophil recruitment via binding to and activation of their receptors CXCR1 or CXCR2 (13, 14). In the mouse, IL-8/CXCL8 and a functional homolog of CXCR1 are absent, and keratinocyte-derived chemokine (KC, Gro α , CXCL1) and macrophage inflammatory protein-2 (MIP-2) appear to be the major ELR CXC chemokines expressed at sites of tissue inflammation following injury and/or infection (15–19). It is assumed that neutrophil migration in this species is mediated mainly by KC and MIP-2 via binding to their sole receptor CXCR2 (20, 21). LPA was shown to regulate the expression of cyclooxygenase-2 (COX-2) and the production of prostaglandin E₂ (PGE₂) in studies using LPA₃-deficient mice (22). Additionally, LPA has been shown to induce the synthesis of PGE₂, cytokines and chemokines in synovocytes isolated from patients with rheumatoid arthritis (RA) (12, 23), a chronic inflammatory disease characterized by extensive leukocyte infiltration into the affected joint space (24).

In the present study, we employed an in vivo experimental model of inflammation, the murine air pouch model, to objectively evaluate the effect of LPA on leukocyte recruitment in vivo. The LPA receptor dependency and the mechanisms of LPA-induced leukocyte recruitment in vivo were investigated using both a pharmacological (LPA receptor agonists/antagonists) and a genetic (LPA₃^{-/-} mice) approach. We demonstrate that both LPA₁ and LPA₃ mediate the recruitment of leukocytes into the air pouch through a mechanism that is dependent on LPA-mediated chemokine synthesis and exacerbated by the pro-inflammatory cytokine TNF- α .

MATERIALS AND METHODS

Reagents

1-Oleoyl-sn-glycero-3-phosphate (LPA) was purchased from Sigma (St. Louis, MO). The LPA_{1/3} specific receptor antagonist (S)-phosphoric acid mono- (2-octadec-9-enoylamino-3-[4-(pyridine-2-ylmethoxy)-phenyl]propyl) ester (VPC32183) was obtained from Avanti Polar Lipid Inc. (Alabaster, AL). The specific LPA₂ agonist, dodecylphosphate, and LPA₃ agonist, L-sn-1-O-oleoyl-2-methyl-glycerol-3-phosphothionate (2S-OMPT), were obtained from Biomol (Plymouth Meeting, PA) and Echelon Biosciences Inc. (Salt Lake City, UT), respectively. Murine TNF- α was from PeproTech Inc. (Rocky Hill, NJ). CXCR2 antagonist SB225002 was from Calbiochem (San Diego, CA). Antibodies against KC (rat IgG_{2A}, clone 124014), MIP-2 (rat IgG_{2B}, clone 40605), CXCR2 (rat IgG_{2A}, clone 242216), and rat IgG_{2A} isotype

control (clone 54447) were purchased from R&D Systems Inc. (Minneapolis, MN). KC/MIP-2 ELISA kit and Proteome Profiler™ Mouse Cytokine Array Panel A were purchased from R&D Systems. The mouse Cytokine/Chemokine Luminex Multiplex Immunoassay kit was from Millipore Corporation (St. Charles, MO).

Mice

LPA₃ receptor knock out (KO) mice (LPA₃^{-/-}) were previously generated (22). Mice were subjected to genotyping for LPA₃ alleles as described previously (22, 25). Female Balb/c (wild-type) mice 6–8 weeks old (from Charles River, St.-Colomban, Canada) were used as background controls. All animal experimental procedures were approved by the Animal Care Committee at CHUL Research Center, Laval University and conformed to National Institutes of Health (NIH) guidelines and public law.

The air pouch model

Air pouches were raised on the dorsum of mice by subcutaneous injection of 3 ml sterile air on days 0 and 3 as previously described (26). Before the injection of air, mice were briefly anesthetized with isoflurane. On day 7, LPA or LPA receptor agonist/antagonist in 1 ml of the diluent (PBS + 0.1% BSA) were injected into air pouches. In some experiments, TNF- α was injected into air pouches 16 h prior to stimulation with LPA or LPA receptor agonist. To test a systemic effect of LPA receptor antagonism on LPA-induced leukocyte recruitment, LPA_{1/3} antagonist VPC32183 was intravenously injected 15 min prior to LPA injection into the air pouch. To define the role of CXCR2 and CXCL1 chemokines in LPA-induced leukocyte recruitment, the CXCR2 antagonist SB225002 and blocking antibodies against KC, MIP-2, and CXCR2 were intravenously injected 15 min prior to LPA injection into the air pouch. At specific times, mice were euthanized by asphyxiation using CO₂. The air pouch exudates were collected by washing once with 1 ml of PBS-5 mM EDTA, and then twice with 2 ml of PBS-5 mM EDTA. After 5 min centrifugation at 1,200 rpm, cell pellets were resuspended, stained with acetic blue, and the leukocytes were counted. Fifty to seventy-five thousand cells were centrifuged onto microscope slides at 500 rpm for 5 min using a cytospin centrifuge. The slides were then air dried and stained with Diff-Quik (Sigma, St. Louis, MO) to allow quantification of leukocyte subpopulations. For the purpose of cytokine/chemokine assays, air pouches were rinsed twice with 0.5 ml of cold PBS. The exudates were then centrifuged at 1,200 rpm for 5 min at 4°C, and supernatants were kept at -80°C for later cytokine/chemokine assays.

Real-time PCR

Total RNA from the air pouch wall tissue was isolated using Trizol (Gibco, Burlington, VT) and reverse-transcribed into cDNA with Superscript II (Invitrogen Life Technology, Carlsbad, CA). Quantitative real-time PCR was conducted using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma, St. Louis, MO). Amplification conditions were as follows: 30 cycles at 95°C (denaturation, 20 s), 60°C (annealing, 20 s), and 72°C (extension, 20 s). Mouse primer sequences used in real-time PCR were as follows: GAPDH, sense, 5'-AAC-TTT-GGC-ATT-GTA-GAA-GG-3', antisense, 5'-ACA-CAT-TGG-GGT-TAG-GAA-CA-3'; KC, sense, 5'-CTG-GGA-TTC-ACC-TCA-AGA-ACA-TCC-3', antisense, 5'-TGT-ATA-GTG-TTG-TCA-GAA-GCC-AGC-G-3'; LPA₁, sense, 5'-TCT-TCT-GGG-CCA-TTT-TCA-AC-3', antisense, 5'-TGC-CTG-AAG-GTG-GCG-CTC-AT-3'; LPA₃, sense, 5'-GCT-CCC-ATG-AAG-CTA-ATG-AAG-ACA-3', antisense, 5'-AGG-CCG-TCC-AGC-AGC-AGA-3'.

Assessment of cytokine/chemokine secretion in the air pouch

Proteome Profiler™ Mouse Antibody Array Panel A. The air pouches of WT mice (three mice per treatment group) were injected with TNF- α or its diluent (H₂O) 16 h prior to LPA injection. Expression of multiple cytokines/chemokines in response to LPA, TNF- α , and TNF- α /LPA were assayed using the Proteome Profiler™ Mouse Antibody Array Panel A, following the recommended protocol from R&D Systems. The air pouch exudates (1 ml/mouse) from each treatment group (three mice) were pooled, mixed with reconstituted Cytokine Array Panel A Detection Antibody Cocktail, added to the array membranes, and incubated at 4°C overnight. The array was then incubated with streptavidin-horseradish peroxidase followed by chemiluminescent detection. Each pair of duplicate spots in the film represents a cytokine/chemokine.

KC ELISA. The air pouches of WT mice (five mice per treatment group) were treated without or with TNF- α for 16 h prior to LPA injection. Aliquots (100 μ l) of the air pouch exudates (five mice per group) obtained at different time points after LPA injection were analyzed for the level of KC by ELISA according to the manufacturer's instructions (R&D Systems). Samples were monitored in duplicate. Optical densities were determined using a SoftMaxPro40 plate reader at 450 nm. The results were compared with a standard curve that was generated using known concentrations (pg/ml) of KC and were expressed in pg/ml.

MIP-2 ELISA. The air pouches of WT mice (five mice per treatment group) were treated without or with TNF- α for 16 h prior to LPA or OMPT injection of 1.5-2 h. Aliquots (100 μ l) of the air pouch exudates were analyzed for the level of MIP-2 by ELISA according to the manufacturer's instructions (R&D Systems). Every sample from each mouse was monitored in duplicate. The results were compared with a standard curve that was generated using known concentrations of MIP-2. The dynamic range of the MIP-2 ELISA is 15.625-1,000 pg/ml.

Multiplex immunoassay. The air pouches of WT mice (five mice per treatment group) were treated without or with TNF- α for 16 h prior to LPA injection. Inflammatory chemokines in the air pouch lavages were quantified using a Luminex multiplex immunoassay according to the manufacturer's instructions (Millipore Corporation). The mouse cytokine/chemokine multiplex immunoassay was used for the simultaneous measurement of mouse IL-1 β , IL-6, KC, MIP-2, and interferon-inducible protein-10 (IP-10). The dynamic range of the Multiplex Immunoassay is 3.2-10,000 pg/ml.

Statistical analysis

Unless otherwise stated, experiments were performed with 5-6 mice/treatment group, and results are expressed as mean \pm SE or as representative studies. All statistical analyses were performed using Prism 4.0 software. Statistical significance of the difference between samples of two different treatments was determined by *t*-test (two-tailed *P* value). For the dose response and time course studies, statistical significance between control and treated (dose response experiments) and between samples treated at 0 h with those treated at indicated time points (time course experiments) was determined by one-way ANOVA, Dunnett's multiple comparison test. Difference between treatments of wild-type, LPA₃^{+/-}, and LPA₃^{-/-} mice was compared using two-way ANOVA, Bonferroni post test. Multiple comparisons in the same experiment were made using one-way ANOVA, Bonferroni multiple comparison test. *P* values less than 0.05 were considered statistically significant.

RESULTS

LPA recruits leukocytes to the air pouch in a dose- and time-dependent manner

To examine whether LPA is able to recruit leukocytes in vivo, 1, 3, or 6 μ g of LPA in 1 ml of the diluent (equivalent to 2 μ M, 6 μ M, or 12 μ M, respectively) was injected into air pouches raised on WT mice. The choice of concentrations of LPA was based on the results of a previous in vitro study using human synovial fibroblasts (12). Leukocyte accumulation in the air pouch was measured 6 h later. Injection of LPA resulted in a dose-dependent accumulation of leukocytes in air pouches. Significantly increased leukocyte counts were detected in air pouches containing 3 μ g and 6 μ g of LPA (*P* < 0.01 for LPA versus control) (Fig. 1A). A dose of 3 μ g LPA was used for all further experiments. A time course experiment with 3 μ g LPA revealed that an increase in the number of migrated leukocytes started to be detectable 2 h post-LPA injection, reached the maximal level after 6 h, and declined thereafter (Fig. 1B). Staining of the recruited leukocytes with Diff-Quik revealed that the predominant subpopulation of LPA-recruited leukocytes at 6 h post-LPA injection was neutrophils (72.90 \pm 0.03% of the total leukocytes, *n* = 6) (Fig. 1C) and a few monocytes (supplemental Fig. 1).

LPA₁ and LPA₃ are involved in LPA-induced leukocyte recruitment into the air pouch

To examine the potential involvement of LPA receptor(s) in LPA-induced leukocyte recruitment, a genetic (using LPA₃ receptor-deficient mice) and pharmacological (using LPA receptor agonists/antagonists) approach was used, either alone or in combination. Because no difference was observed between WT and LPA₃^{+/-} mice regarding the number of leukocytes recruited to the air pouch in response to LPA (*P* = 0.1186 for WT versus LPA₃^{+/-} for control group, and *P* = 0.5034 for WT versus LPA₃^{+/-} for LPA group) (Fig. 2A, left panel) and OMPT (*P* = 0.9803 for WT versus LPA₃^{+/-} for control group, and *P* = 0.9493 for WT versus LPA₃^{+/-} for OMPT group) (Fig. 2D), either WT or LPA₃^{+/-} mice were used as controls throughout the study.

The number of leukocytes recruited into air pouches increased in a time-dependent manner (Fig. 1B). A significant accumulation of leukocytes in the air pouches of WT or LPA₃^{+/-} mice was observed 6 h after the injection of LPA (Fig. 2A, left and right panels). This effect, however, was attenuated in LPA₃^{-/-} mice by 46% (*P* < 0.05) (Fig. 2A). When LPA was injected into air pouches together with the LPA_{1/3} receptor antagonist VPC32183 (10 μ M), LPA-induced leukocyte recruitment was almost completely blocked in both LPA₃^{+/-} (93% decrease; *P* < 0.01) and in LPA₃^{-/-} mice (98% decrease; *P* < 0.05) (Fig. 2A, right panel). Similar to the air pouch administration of VPC32183, intravenous injection (0.5 mg/kg) of this compound also significantly attenuated leukocyte recruitment into air pouches by LPA in both WT (70% decrease; *P* < 0.05) and LPA₃^{-/-} mice (86% decrease; *P* < 0.05) (Fig. 2B). To further confirm the role of the LPA₃ receptor in

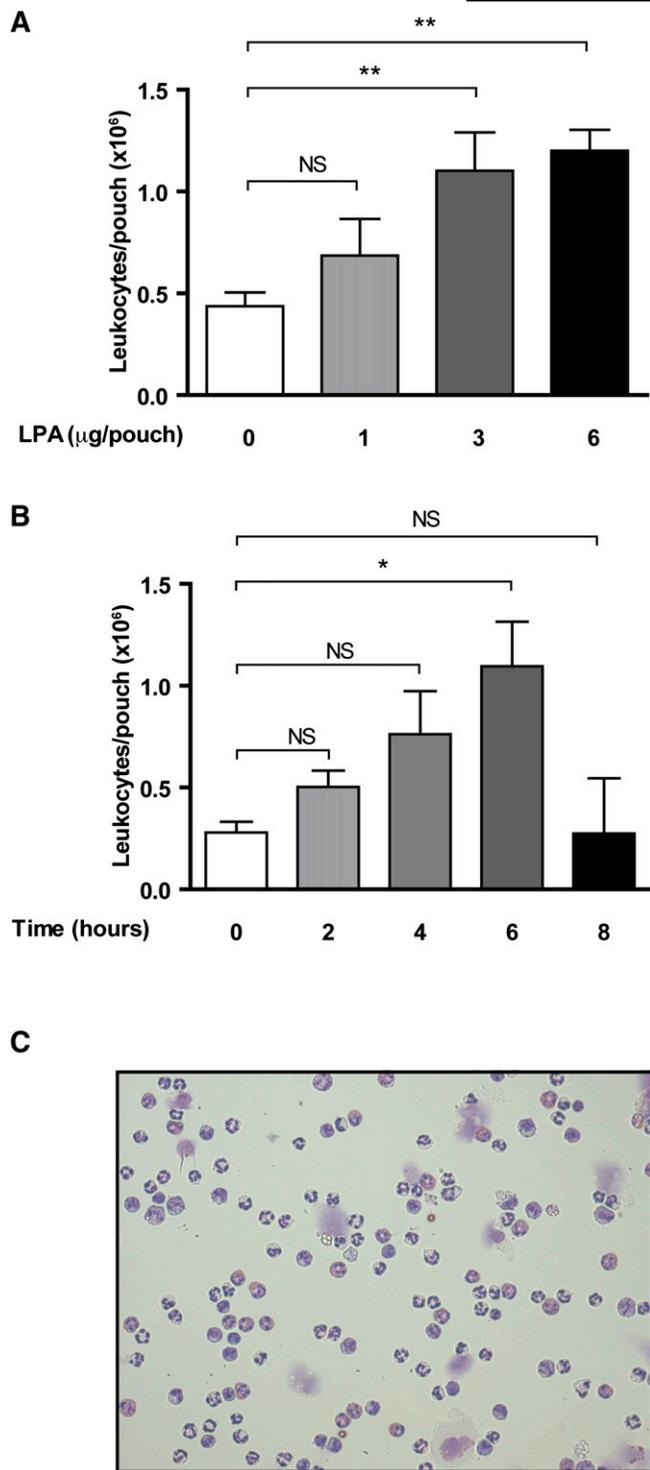


Fig. 1. Effect of LPA on leukocyte recruitment into the air pouch. Air pouches were raised in the dorsal skin of WT mice as described in Materials and Methods. (A) On day 7, 1 ml of PBS containing various amounts of LPA (0, 1, 3, and 6 μg) was injected into the air pouches of WT mice. At 6 h postinjection, the air pouch was rinsed with PBS, and the leukocytes in the air pouch exudate were counted. (B) One ml of PBS containing 3 μg LPA was injected into the air pouches of WT mice. At various time points (0, 2, 4, 6, and 8 h), air pouch exudates were collected, and leukocytes were counted. Data represent the mean ± SE of at least six mice per group. **P* < 0.05; ***P* < 0.01. (C) Microscopy of Diff-Quik-stained leukocytes recruited into the air pouch at 6 h post-LPA injection. The image is representative of six different mice (200×).

LPA-mediated leukocyte recruitment, the LPA₃ receptor agonist OMPT (1, 3, or 6 μg in 1 ml of the diluent that are equivalent to 2 μM, 6 μM, or 12 μM) was injected into air pouches raised on WT mice (Fig. 2C). The choice of concentrations of OMPT was based on the results from a previous in vitro study using human synovial fibroblasts (12). A significant increase in leukocyte counts was detected at 1 μg/pouch of OMPT, and the maximum amount of migrated cells was seen at 3 μg/pouch of OMPT (*P* < 0.01 for OMPT 3 μg/pouch versus control group). On the other hand, the LPA₂ receptor agonist dodecylphosphate had no effect on leukocyte recruitment according to a dose-response experiment using concentrations ranging 1-6 μg/pouch (data not shown). At 3 μg/pouch of OMPT, a similar accumulation of leukocytes was observed in the air pouches of WT and LPA₃^{+/-} mice (*P* < 0.01 for OMPT versus control). In contrast to leukocytes recruited by LPA in LPA₃^{-/-} mice where LPA-induced leukocyte recruitment was only partially attenuated (Fig. 2A, B), leukocyte recruitment by OMPT was completely blocked in LPA₃^{-/-} mice (Fig. 2D). The data suggest that both LPA₁ and LPA₃ are involved in LPA-mediated leukocyte recruitment in vivo.

TNF-α potentiates LPA- and OMPT-induced leukocyte recruitment into the air pouch

To determine whether LPA-induced leukocyte recruitment is modulated by cytokines typical of an inflammatory environment, air pouches raised on WT mice were injected with 50 ng/pouch of TNF-α 16 h prior to injecting LPA into the air pouches. As shown in Fig. 3A, TNF-α itself had no significant effect on leukocyte accumulation compared with nontreated control. TNF-α injection into the murine air pouch resulted in a transient recruitment of leukocytes with maximal accumulation at 2-4 h postinjection, and returned to baseline by 8 h postinjection (27). TNF-α-recruited leukocytes in the air pouch might have gone through the apoptotic process (28, 29) after 16 h of treatment. Pretreatment with TNF-α, however, strongly enhanced LPA-induced leukocyte recruitment. The number of leukocytes recruited in TNF-α-pretreated air pouches by LPA increased by 147% compared with unprimed air pouches (*P* < 0.01). We then compared the effect of TNF-α-priming on LPA- and OMPT-induced leukocyte recruitment between LPA₃^{+/-} and LPA₃^{-/-} mice (Fig. 3B, C). In LPA₃^{-/-} mice, LPA- and OMPT-mediated leukocyte accumulation in TNF-α-primed air pouches was reduced by 25% (*P* < 0.05) (Fig. 3B) and 76% (*P* < 0.001) (Fig. 3C), respectively, compared with LPA₃^{+/-} mice. In TNF-α-pretreated air pouches, the LPA₃ receptor antagonist VPC32183 (20 μM) decreased LPA-induced leukocyte recruitment by 83.9% (*P* < 0.01) (Fig. 3D). Quantitative real-time PCR showed that the mRNA expression of both LPA₁ and LPA₃ in the air pouch tissues was greatly enhanced by TNF-α after 16 h (*P* < 0.05) (Fig. 3E).

Release of cytokines/chemokines in response to LPA precedes leukocyte recruitment

Since LPA induces cytokine/chemokine synthesis (12), the following experiments were designed to analyze

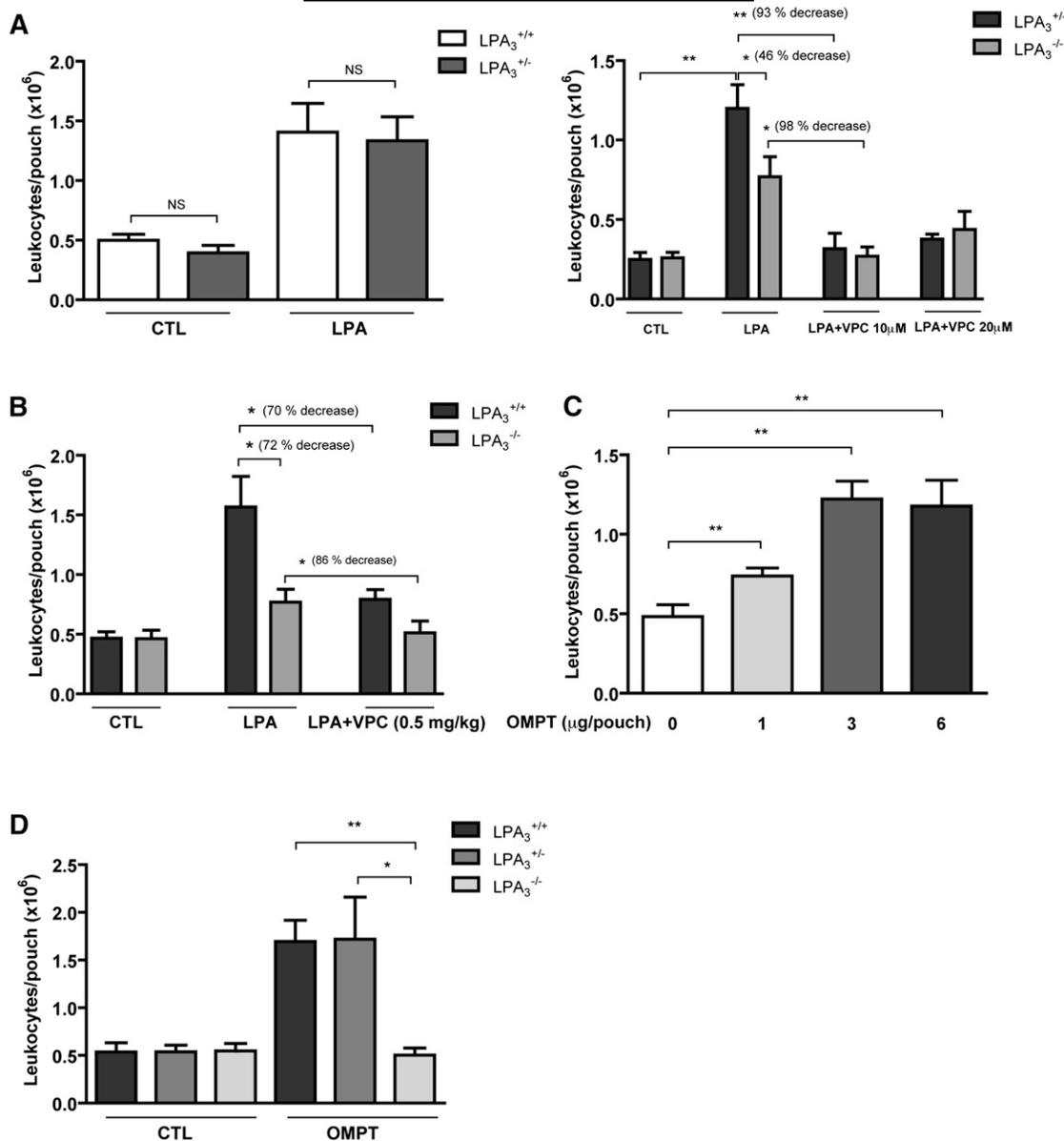


Fig. 2. Involvement of LPA receptor(s) in LPA-induced leukocyte recruitment into the air pouch. (A, B) One ml of PBS containing 3 μ g LPA was injected into the air pouches of WT or LPA₃^{-/-} mice in the absence/presence of the LPA_{1/3} receptor antagonist VPC32183 [10 μ M and 20 μ M, (A, right panel)] or 15 min after VPC32183 intravenous injection [0.5 mg/kg (B)]. The LPA diluent solution (PBS + 0.1% BSA) was used as a control (CTL). (C) One ml of PBS containing LPA₃ receptor agonist OMPT (0, 1, 3, and 6 μ g) was injected into the air pouch in WT mice. The OMPT diluent solution (H₂O) was used as a control (CTL). (D) One ml of PBS containing 3 μ g OMPT was injected into the air pouches of WT (dark columns), LPA₃^{+/-} (gray columns), and LPA₃^{-/-} mice (light-gray columns). Six h after LPA or OMPT injection, the air pouches were rinsed, and the leukocytes in the air pouch exudates were counted. Data represent the mean \pm SE of at least five mice per group. **P* < 0.05; ***P* < 0.01.

whether cytokines or chemokines secreted in air pouches play a role in the migration of leukocytes toward LPA in TNF- α -pretreated air pouches. All experiments described in this section were performed with WT mice. Since we observed that neutrophils are the predominant leukocytes in exudates of air pouches injected with LPA and TNF- α and that neutrophil migration in the mouse is mediated mainly by KC and MIP-2 via binding to their sole receptor CXCR2 (20, 21), the involvement of these two chemokines in LPA-mediated leukocyte recruitment was first examined. As shown in **Fig. 4**, intravenous injection of the CXCR2 antagonist SB225002 (**Fig. 4A, B**) and a neutraliz-

ing CXCR2 antibody (**Fig. 4C**) 15 min prior to LPA administration into the air pouch significantly decreased LPA-mediated leukocyte recruitment in both TNF- α -pretreated (**Fig. 4B, C**) and TNF- α -unpretreated air pouches (**Fig. 4A, C**). In TNF- α -unpretreated air pouches, SB225002 administration at 0.3 mg/kg, the highest concentration tested, decreased LPA-induced infiltration of leukocytes by 81.3% (*P* < 0.05) (**Fig. 4A**), whereas intravenous injection of the anti-CXCR2 Ab decreased LPA-induced infiltration of leukocytes by 80% (*P* < 0.001) (**Fig. 4C**). Similarly, in TNF- α -pretreated air pouch, SB225002 inhibited LPA-mediated leukocyte recruitment (up to

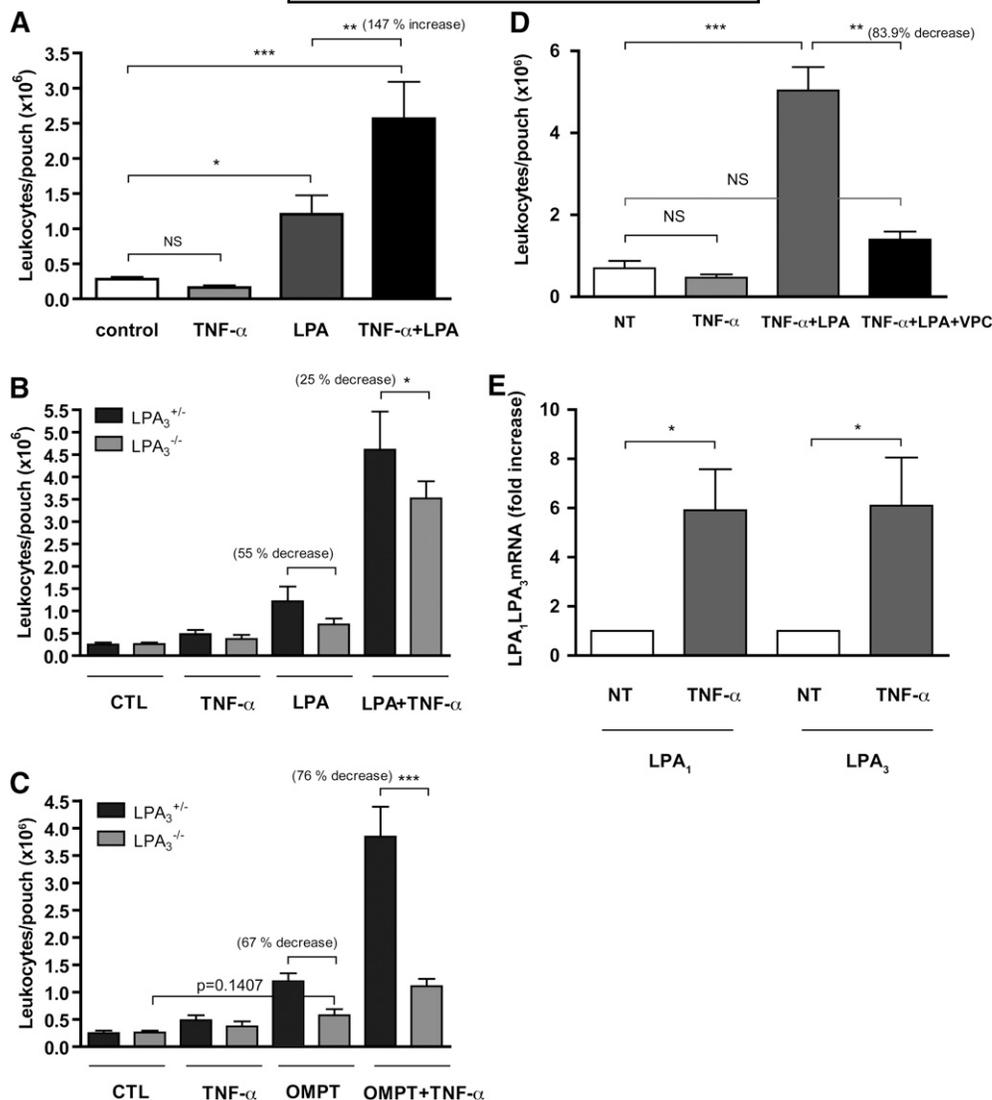


Fig. 3. Effect of TNF- α pretreatment on LPA- and OMPT-induced leukocyte recruitment into the air pouch. At day 6 of air pouch establishment, 50 ng of TNF- α in 1 ml PBS was injected into the air pouch 16 h prior to LPA (A, B, D) and OMPT (C) air pouch injection in WT (A, D) or LPA₃^{+/-} and LPA₃^{-/-} mice (B, C). At 16 h after TNF- α administration, 3 μ M LPA was injected into the air pouches (A–D) in the absence (A–C) or presence (D) of 20 μ M VPC32183. At 6 h after LPA or OMPT injection, the air pouch was rinsed, and the leukocytes in the air pouch exudates were counted. Data represent the mean \pm SE of five mice per group. * P < 0.05; ** P < 0.01; *** P < 0.001. The levels of LPA₁ and LPA₃ mRNA in air pouch wall tissues were measured by real-time PCR (E). The Ct values for LPA₁ and LPA₃ without TNF treatment are 18.57 ± 0.5317 ($n = 6$) and 23.16 ± 0.2290 ($n = 6$), respectively. Data are expressed as fold increase over nontreated controls after normalization to GAPDH.

61%) in a concentration-dependent manner ($P < 0.05$) (Fig. 4B), whereas the anti-CXCR2 Ab decreased LPA-induced leukocyte recruitment by 60% ($P < 0.05$) (Fig. 4C). Administration of neutralizing Abs against KC decreased by 81.4% LPA-induced neutrophil recruitment ($P < 0.05$) (Fig. 4D). In contrast, the MIP-2 blocking antibody had no significant effect on LPA-induced leukocyte recruitment into the air pouch ($P = 0.4885$) (Fig. 4D).

A qualitative mouse cytokine/chemokine antibody array assay, in which a broad panel of cytokine and chemokine expression in air pouch exudate fluid can be examined, was then performed. Expression of six cytokines/chemokines was induced by LPA compared with nontreated control,

including BLC (CXCL13/BCA-1), IL-1 β , IL-6, IL-16, KC, and MIP-2 (Fig. 5A, C and supplemental Fig. II). The expression of triggering receptor expressed on myeloid cells 1 (TREM-1), an activating receptor involved in inflammatory disease, was also induced by LPA (Fig. 5A, C). When air pouches were primed with TNF- α , LPA induced a broader and more intensive expression of inflammation markers. In addition to BLC (CXCL13/BCA-1), IL-1 β , IL-6, MIP-2, and TREM-1, it also enhanced the expression of I-309 (CCL1/TCA-3), IL-1ra, IP-10, and KC (Fig. 5B, C).

The next series of experiments was performed to accurately quantify the expression of cytokines and chemokines. Since we did not observe an effect of the anti-MIP-2

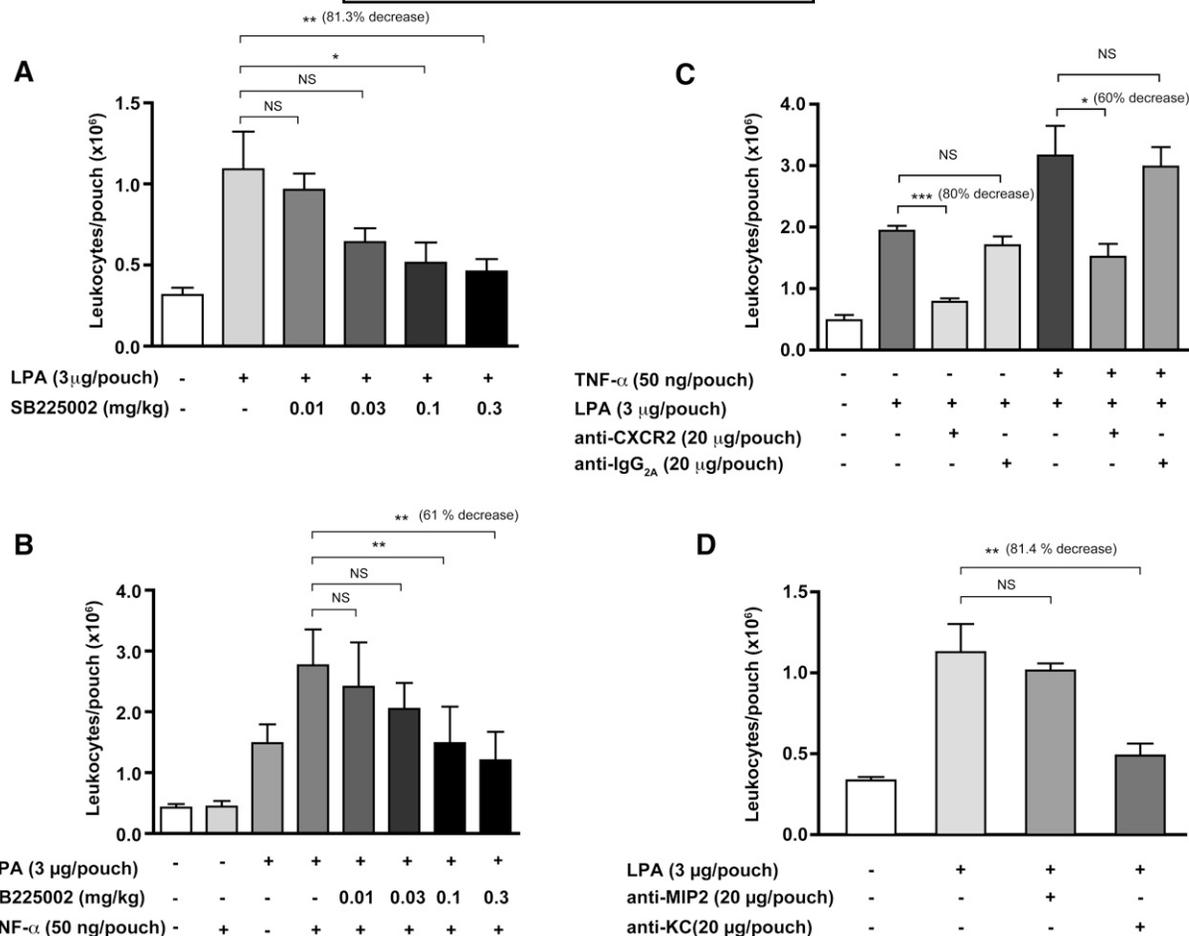


Fig. 4. Effect of blocking CXCR2 and CXCL1 chemokines on LPA-mediated leukocyte recruitment. One ml of PBS containing 3 μg LPA was injected into TNF-α-unprimed (A–D) and TNF-α-primed (50 ng/pouch, 16 h; B, C) air pouches with or without prior intravenous injection of CXCR2 antagonist SB225002 (at indicated doses, A, B), of anti-CXCR2 antibody or an isotype control IgG_{2A} (20 μg/mouse, C) and of anti-KC, anti-MIP-2 (20 μg/mouse, D) in WT mice. At 6 h postinjection of LPA, the air pouch was rinsed, and the leukocytes in the air pouch exudates were counted. Data represent the mean ± SE of three to five mice per group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

antibody on LPA-induced leukocyte recruitment (Fig. 4D) or MIP-2 secretion in response to LPA or OMPT (ELISA, data not shown), we focused on KC. Although not significant, a trend for increased secretion of KC was detected in LPA-treated versus control mice. The potential increase in KC secretion was further investigated at the mRNA level using real-time PCR in addition to ELISA in TNF-α-primed air pouches. A significant increase of KC mRNA expression and secretion was observed in TNF-α-primed air pouches (Fig. 6). The increase in KC secretion began at 1 h and reached a maximum at 2 h after LPA injection (1719 ± 542.20 pg/ml; *P* < 0.001 for 2 h versus nontreated control), after which it declined (Fig. 6A). Enhanced expression of KC mRNA was also detected in TNF-α-primed air pouches (Fig. 6B) compared with control air pouch tissues, which increased further at 1–2 h post-LPA injection (Fig. 6C). Thus, the peak of KC secretion preceded that of neutrophil recruitment into the air pouch.

Luminex technology was then used to analyze the secretion of multiple cytokines/chemokines induced by LPA and OMPT with or without TNF-α-priming in the air pouch (Fig. 7). The expression of KC, IL-6, and to a lesser degree, IP-10 was observed in response to LPA and OMPT

in TNF-α-primed air pouches. The release of KC (Fig. 7A), IL-6 (Fig. 7B) and IP-10 (Fig. 7C) in response to LPA and OMPT was strongly enhanced (in the case of KC and IL-6) by TNF-α or additive (in the case of IP-10) to that of TNF-α. Particularly, TNF-α priming alone induced a small release of KC (230.6 ± 17.08 pg/ml) when monitored after 16 h, which was comparable to that induced by LPA (197.3 ± 17.75 pg/ml) and OMPT (371.1 ± 31.83 pg/ml) and slightly higher than that of the control group (41.11 ± 3.08 pg/ml). Priming with TNF-α for 16 h, however, increased LPA- or OMPT-induced KC release 14.04-fold (*P* < 0.001) and 5.18-fold (*P* < 0.01), respectively (Fig. 7A). TNF-α priming alone, LPA, and OMPT alone did not induce significant amount of IL-6 secretion compared with nontreated control. However, LPA- and OMPT-mediated IL-6 secretion increased 10.65-fold (*P* < 0.01) and 6.51-fold (*P* < 0.05), respectively, in TNF-α-primed air pouches compared with that in unprimed air pouches (Fig. 7B). Among the five cytokines/chemokines tested, IL-1β was not detected in response to LPA or OMPT in control and TNF-α-primed air pouches, suggesting that the amount of this cytokine in the air pouch lavages was below the threshold detection limit of the multiplex immunoassay. Although

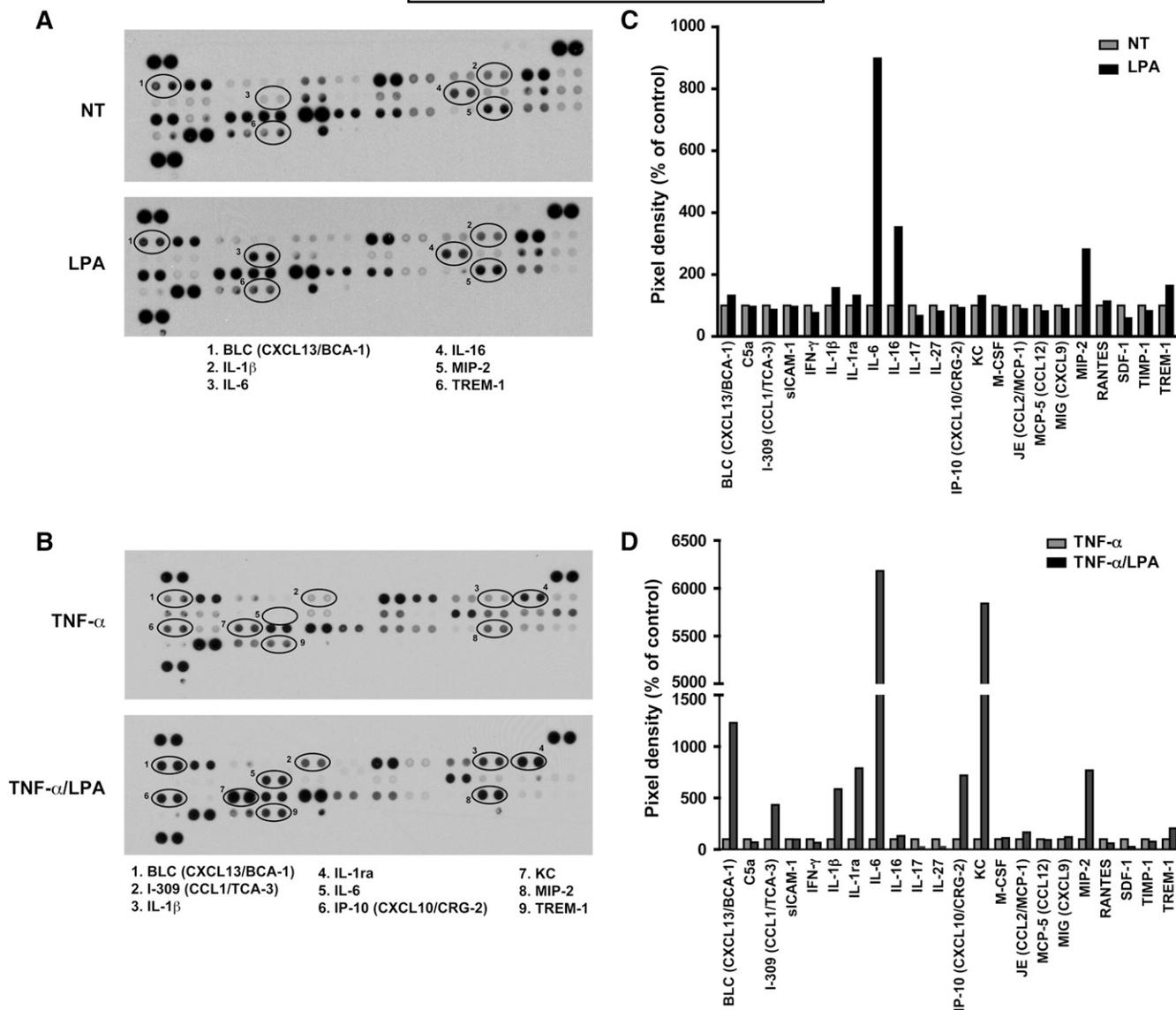


Fig. 5. Effect of LPA and TNF- α pretreatment on the secretion of cytokines/chemokines in air pouches. LPA (3 μ g) in 1 ml PBS was injected into the air pouch with or without TNF- α pretreatment for 16 h in WT mice. At 2 h after LPA injection, air pouch exudates were collected, and cytokine/chemokine secretion was analyzed using Proteome ProfilerTM Mouse Antibody Array Panel A as described in Material and Methods. (A, B) Circled pairs of duplicate spots represent one cytokine/chemokine, the secretion of which was upregulated. Data from (A) and (B) are quantified as pixel density and presented as percentage of nontreated control (C) and TNF- α -treated (D) air pouches, respectively.

some mice seemed to respond to LPA and OMPT (data not shown), on average, no significant increase in the amounts of MIP-2 was detected in our samples using either the Luminex assay or the MIP-2 ELISA. The involvement of this cytokine in LPA-induced leukocyte recruitment needs further investigation.

DISCUSSION

In the present study, we investigated the role of LPA in leukocyte migration toward an *in vivo* inflammatory environment. We observed that injection of LPA resulted in the effective recruitment of a mixed inflammatory leukocyte population to murine air pouches and that both LPA₁ and LPA₃ receptors were involved in this process. More-

over, LPA-induced leukocyte accumulation in the air pouch was dependent upon the release of the pro-inflammatory chemokine KC. Interestingly, significantly elevated concentrations of KC were detected in the exudates of TNF- α -primed air pouches, which preceded LPA-induced leukocyte recruitment that was enhanced by TNF- α . To our knowledge, this is the first study to characterize the mechanism by which LPA may recruit leukocytes to an inflammatory environment *in vivo*.

Several lines of evidence support our finding that LPA is an efficient stimulator of leukocyte recruitment *in vivo*. Chettibi et al. reported that LPA stimulated neutrophil polarization and motility (7) and Fischer et al. showed that LPA induced directed cell migration (8). Our *in vivo* data also corroborate other studies in animal models of airway

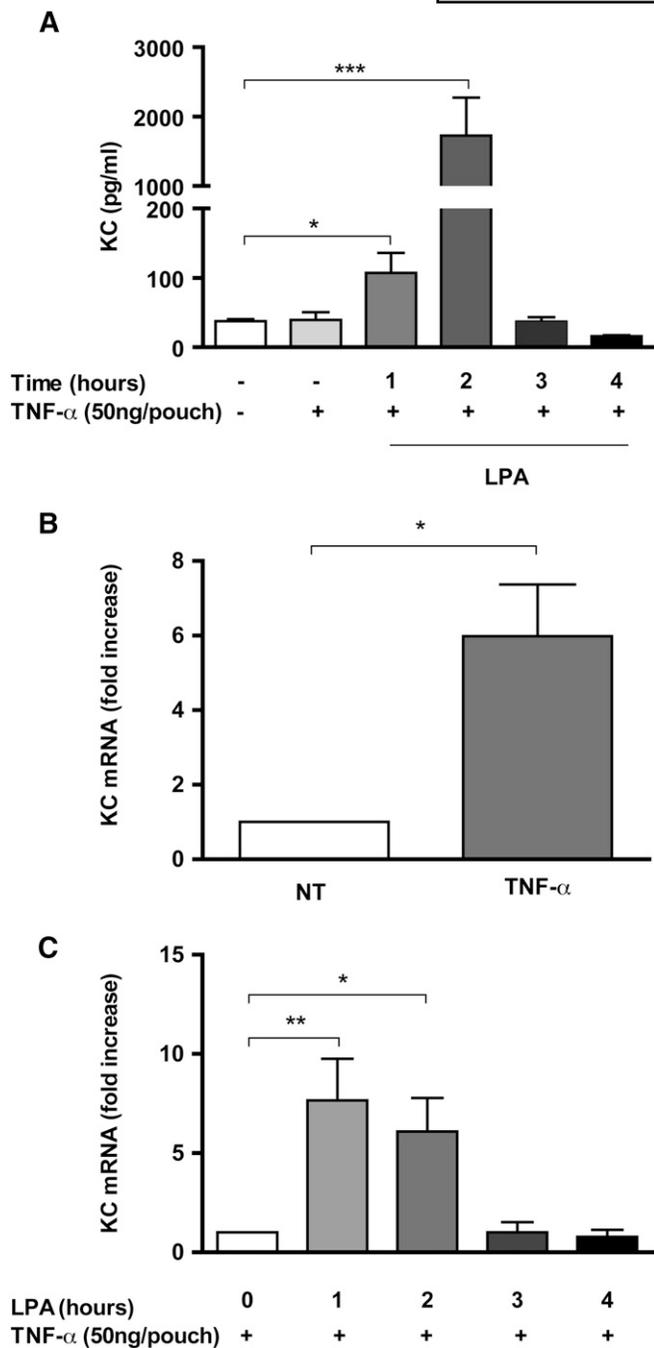


Fig. 6. Effect of LPA on KC secretion in the air pouch. LPA (3 μ g) in 1 ml PBS was injected into the air pouch with or without TNF- α pretreatment for 16 h in WT mice. (A) At indicated time points, air pouch exudates were collected, and KC secretion was analyzed by ELISA. (B, C) At indicated time points, total RNA was extracted from the air pouch wall tissues. KC mRNA was measured by real-time PCR. Data are expressed as fold increase over nontreated control after normalization to GAPDH. Data represent the mean \pm SE of five mice per group. * P < 0.05; ** P < 0.01; *** P < 0.001.

inflammation, showing that LPA enhances infiltration of inflammatory cells into the pulmonary airway possibly through a mechanism involving chemokine synthesis (30, 31).

Identification of receptors involved in LPA-induced recruitment of leukocytes in vivo is of therapeutic interest for inflammatory diseases. We provide direct evidence that

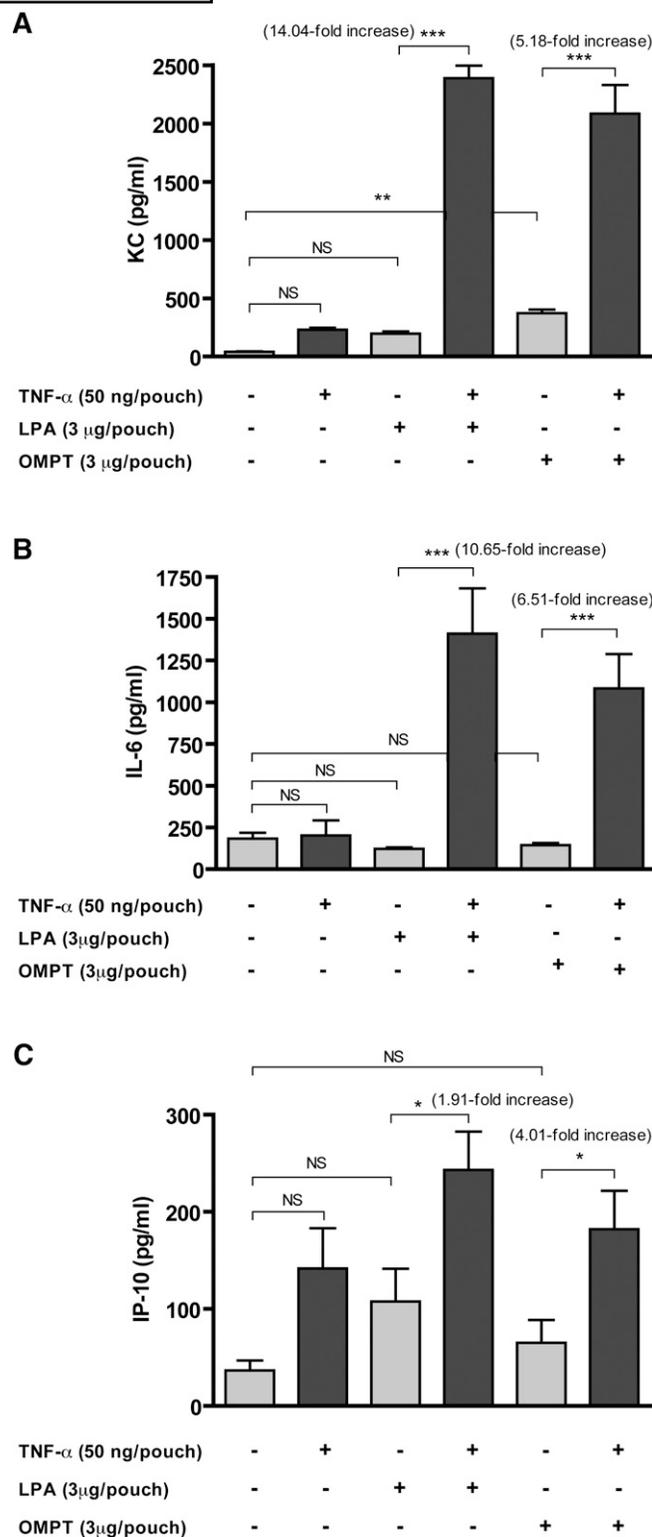


Fig. 7. Effect of LPA and OMPT on cytokine/chemokine secretion in the air pouch. LPA (3 μ g) in 1 ml PBS was injected into the air pouch with or without TNF- α pretreatment for 16 h in WT mice. At 2 h after LPA injection, air pouch exudates were collected, and cytokine/chemokine secretion was analyzed using a Luminex multiplex immunoassay as described in Materials and Methods. (A) KC secretion. (B) IL-6 secretion. (C) IP-10 secretion. Data represent the mean \pm SE of five mice per group. * P < 0.05; ** P < 0.01; *** P < 0.001.

LPA-induced leukocyte recruitment was mimicked by the specific LPA₃ agonist OMPT in WT mice, indicating the involvement of this receptor in the process. This effect was, however, only partially attenuated in LPA₃^{-/-} mice, suggesting that receptor(s) other than LPA₃ might be involved. When LPA was co-injected with the LPA_{1/3} receptor antagonist VPC32183 into the air pouch, leukocyte accumulation in the lumen was almost totally abrogated in both LPA₃^{+/-} and in LPA₃^{-/-} mice. These findings clearly demonstrate that, in addition to LPA₃, LPA₁ was also involved in LPA-mediated leukocyte recruitment.

TNF- α is a key mediator of inflammation in the pathogenesis of inflammatory disease, such as RA, in which LPA may play a role (23) and was demonstrated in experimental models of arthritis in rodents (32). We, therefore, also examined the effect of LPA on leukocyte recruitment into murine air pouches injected with TNF- α . TNF- α -pretreatment of air pouches strongly enhanced LPA-induced leukocyte recruitment. The enhancing effect of TNF- α on leukocyte recruitment was also seen with LPA₃ receptor agonist OMPT. Since TNF- α upregulates LPA₃ receptor mRNA expression and correlates with the enhancement of LPA-mediated cytokine/chemokine secretion in RA synoviocytes (12), the potentiating effect of TNF- α -priming in an inflammatory cavity on LPA-mediated leukocyte recruitment *in vivo* makes this lysophospholipid even more relevant to the pathology of inflammatory diseases, such as RA.

We previously reported that, in human synoviocytes from RA patients, LPA stimulates the secretion of cytokines/chemokines and that this effect is enhanced by pretreatment with TNF- α (12). Note that sphingosine-1-phosphate (SIP), another bioactive lysophospholipid structurally related to LPA, has been recently identified as an important mediator of RA synoviocyte migration, survival, and cytokine/chemokine production (33). To further characterize the mechanism through which LPA recruits leukocytes *in vivo*, we evaluated the participation of CXCR2 ligands in LPA-mediated leukocyte infiltration in TNF- α -primed and unprimed air pouches. The interaction between CXCR2 and its cognate chemokine ligands, particularly KC, MIP-2, and IL-8, appears to play a critical role in controlling the recruitment of leukocytes into sites of acute inflammation in various model systems (reviewed in Ref. 34). Our data showed that blocking CXCR2 or a neutralizing antibody targeting KC significantly reduced LPA-mediated recruitment of leukocytes into TNF- α -unpretreated air pouches. Moreover, we provide direct evidence that the peak of KC secretion preceded LPA-mediated leukocyte recruitment in TNF- α -pretreated air pouches, since the synthesis of the chemokine peaked 2-4 h prior to leukocyte accumulation. This suggests that KC is responsible for the initiation of the leukocyte recruitment to the air pouch induced by LPA, a conclusion that is supported by other reports (20, 21).

The Proteome Profiler assay also revealed an increase in the expression of another CXCR2 ligand and neutrophil chemoattractant, MIP-2, after LPA and TNF- α /LPA treat-

ment. Our observation, however, that the anti-MIP-2 antibody did not effectively block LPA-induced leukocyte recruitment suggests a marginal role of this chemokine in LPA-induced leukocyte recruitment. We cannot exclude the possibility that the ND50 for the anti-MIP-2 antibody might be higher than that for the anti-KC antibody. The marginal role of MIP-2 in LPA-induced leukocyte recruitment in this experimental model is further supported by our inability to consistently observe an increase in MIP-2 expression by ELISA or using a Luminex approach. The amount of measurable MIP-2 is close to the detection limit of these assays. We did not consistently observe an increase in MIP-2 in the animals tested ($n = 5$). Further experimentation is required to address this issue. KC and MIP-2 genes might each exhibit a distinct temporal pattern of expression or kinetic profile following LPA stimulation, and this pattern of expression is a consequence of cell type-specific bias for individual chemokine gene expression. Indeed, Armstrong et al. recently reported that KC and MIP-2 are expressed in nonoverlapping cell populations at sites of surgical injury and suggested that this cell type-specific pattern of chemokine expression is independent of the stimulus used and is instead an inherent characteristic of the different cell types (i.e., stromal, endothelial, and epithelial cells favor KC, and myeloid leukocytes favor MIP-2) (35). A similar temporal pattern of KC and MIP-2 expression has been also demonstrated by other researchers and in different models (36-38).

Since CXCR2 neutralization did not completely block LPA-induced leukocyte recruitment in TNF- α -primed air pouches, our data suggest that other chemokines or inflammatory mediators could be involved at the beginning of the inflammatory response. Production of pro-inflammatory cytokine IL-6 and another CXC chemokine IP-10/CXCL10 by LPA in TNF- α -primed air pouches was also observed. The secretion of these two cytokines/chemokines was observed 4 h prior to leukocyte accumulation in the air pouch, suggesting their involvement in the beginning of the inflammatory response. IL-6 has been reported to stimulate the release of IL-8 and MCP-1 (39) and expression of adhesion molecules (40), resulting in recruitment of leukocytes to inflammatory sites (41). Of note, in air pouch tissues treated with TNF- α for 16 h, the levels of LPA₁ and LPA₃ mRNA were strongly enhanced compared with control air pouch tissues. Thus, we suggest that LPA acted on LPA₁ and LPA₃ to release CXCR2 ligands (e.g., KC) and other chemoattractant mediators (e.g., IL-6, IP-10, CXCL13, and TREM-1), which then mediated leukocyte migration into the air pouch. From our results we cannot, however, deduce which cell type in the air pouch lining tissue contributed to the expression of these cytokines/chemokines. Indeed, LPA induces a global expression of genes in mouse fibroblasts, including transcriptional factors, cytokines, chemokines, and metalloproteinase (42), to commit fibroblasts to create an inflammatory microenvironment that favors leukocyte accumulation.

Several chemokines or molecules induced by LPA have been identified in inflammatory diseases. In our qualitative chemokine analysis, we also observed that LPA with or

without TNF- α pretreatment triggered the expression of CXCL13 and TREM-1. CXCL13 is a homeostatic B cell chemoattractant (43) that is required for the normal organization of B cell follicles in secondary lymphoid organs (44) and for recruiting B cells in extralymphoid sites when overexpressed (45). CXCL13 mRNA and protein expression and spontaneous CXCL13 secretion were detected in RA synovial fluid T cells (46), and it was suggested that CXCL13 tissue expression may be critically involved in the process of ectopic lymphoid neogenesis, a phenomenon seen in 25-50% of RA patients, via recruitment of B cells into the synovium (47). TREM-1 is an immunoglobulin-like cell surface receptor mainly expressed on neutrophils and CD14 monocytes (48). TREM-1 expression is increased during both acute (49, 50) and chronic inflammation (51). Moreover, a soluble form of TREM-1 was detected in the blood sample of human patients with inflammatory disorders, such as sepsis (52), pneumonia (53), acute pancreatitis (54), and peptic ulcer disease (55). Very recently, Kuai et al. reported that TREM-1 expression is increased in the synovium of RA patients (56). The expression of an eosinophil-recruiting chemokine CCL1 (I-309), which has been implicated in inflammatory diseases, such as atopic dermatitis (57) and asthma (58), was also seen to be upregulated by LPA in TNF- α -primed air pouches. Quantitative analysis for these chemokines needs further investigation.

In summary, our study provides a definitive link between LPA and leukocyte accumulation in a murine inflammatory site in vivo. LPA mediates leukocyte recruitment via LPA₁ and LPA₃ receptors and through increased secretion of CXCL1 chemokines, such as KC, and possibly other pro-inflammatory cytokines, including IL-6 and IP-10, in the air pouch exudates. Since LPA, ATX (the enzyme that produces LPA), and lysophosphatidylcholine (LPC), the substrate of ATX, are abundant in RA synovial fluids (12, 23), we suggest that inflammatory cell accumulation in the RA synovium may be partly attributable to LPA. LPA may induce the recruitment of inflammatory cells to the synovial fluid of RA patients, either directly or indirectly, by stimulating inflammatory cytokine/chemokine secretion in the RA synovium. Our study contributes to a better understanding of the mechanisms involved in promoting inflammation and implies that both LPA and CXCR2 receptors may be effective targets for therapeutic intervention. **lib**

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