

Stimulation of Ly-6G on neutrophils in LPS-primed mice induces platelet-activating factor (PAF)-mediated anaphylaxis-like shock

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ABSTRACT

Previously, two anti-Ly-6G mAb—RB6-8C5 and 1A8—have been used to deplete neutrophils in mice and to clarify their involvement in immune responses. During the course of experiments on neutrophil depletion, we noticed that i.v. injection of RB6-8C5 or 1A8 induced anaphylaxis-like shock in mice pretreated i.v. with LPS. Signs of shock, such as hypothermia, appeared within a few minutes, and the mice died of shock within 20 min of the antibody injection. In vivo experiments, including depletion of various cell types, indicated that neutrophils and macrophages (but not platelets, basophils, or mast cells) are involved in the shock. Experiments using various drugs and gene-targeted mice demonstrated that PAF is the central mediator of the shock. Optimal LPS priming required at least 1 h, and the priming was associated with neutrophil accumulation within pulmonary and hepatic blood vessels. Consistently, following 1A8 injection into LPS-pretreated mice, the mRNA for LysoPAFAT (a PAF biosynthetic enzyme) was markedly up-regulated in neutrophils accumulated in the lung but not in macrophages. These results suggest that (1) stimulation of Ly-6G on LPS-primed neutrophils induces PAF-mediated anaphylaxis-like shock in mice, (2) neutrophils are primed by LPS during and/or after their accumulation in lung and liver to rapidly induce LysoPAFAT, and (3) macrophages may play a pivotal role in the priming phase and/or in the challenge phase by unknown mechanisms. These findings may be related to adult respiratory distress syndrome, although the natural ligand for Ly-6G remains to be identified. *J. Leukoc. Biol.* 91: 000–000; 2012.

Introduction

Neutrophils are essential effector cells within the innate immune system. To clarify their functions, neutrophil depletion by means of RB6-8C5 mAb has been used in various murine disease models [1–3]. RB6-8C5 was originally reported to recognize Gr-1, a surface antigen that was believed to be present only on mature granulocytes [4]. Subsequent studies revealed that Gr-1 antigen belongs to the Ly-6 gene family, that Ly-6G is the major antigen detected by RB6-8C5, and that this antigen is expressed on neutrophils [5]. However, RB6-8C5 also binds to Ly-6C, which is expressed on neutrophils, DCs, and subpopulations of lymphocytes and monocytes [6]. Thus, in vivo administration of RB6-8C5 might deplete not only neutrophils but also other Gr-1⁺ cells. In this context, Daley et al. [6] proposed the use of an Ly-6G-specific mAb, 1A8, to deplete neutrophils.

The *Ly-6* locus on mouse chromosome 15 comprises a family of Ly-6 proteins, including Ly-6C and Ly-6G molecules [7, 8]. The mouse Ly-6 molecules are GPI-anchored, cell-surface glycoproteins. Despite their use as hematopoietic differentiation markers, little is known about their physiological functions, although it has been suggested that some Ly-6 molecules may be involved in cell signaling and cell adhesion [7, 8].

PAF, a potent phospholipid mediator with diverse biological activities, induces such phenomena as platelet activation, airway constriction, hypotension, and vascular permeation [9]. In addition to its role as a physiological mediator, PAF plays a dominant role as a mediator of anaphylaxis [10]. PAF, which acts by binding to a G protein-coupled seven-transmembrane receptor [9, 11], is synthesized by a variety of cells (including neutrophils, eosinophils, monocytes, macrophages, mast cells, and endothelial cells) via two distinct pathways—de novo and remodeling pathways [9, 12]. The latter is regulated by extracellular signals and plays a critical role in stimulus-coupled PAF biosynthesis [12]. The initiation of this remodeling pathway requires membrane-phospholipid hydrolysis by PLA₂. This enzyme supplies LysoPAF, a direct precursor of PAF, and the

Abbreviations: Clo-lip=clodronate-liposomes, H1R=histamine receptor type 1, HDC=histidine decarboxylase, KO=knockout, LysoPAF=1-*O*-alkyl-*sn*-glycero-3-phosphocholine, LysoPAFAT=acetyl-CoA:1-*O*-alkyl-*sn*-glycero-3-phosphocholine acetyltransferase, LysoPAFAT/LPCAT2=acetyl-CoA:1-*O*-alkyl-*sn*-glycero-3-phosphocholine acetyltransferase/lysophosphatidylcholine acyltransferase 2, NAC=*N*-acetyl-L-cysteine, PAF=platelet-activating factor, PBS-lip=PBS-liposomes, TMB=3,3',5,5'-tetramethylbenzidine, WBC=white blood cell

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LysoPAF is then converted to PAF by LysoPAFAT [12]. Among the many different types of PLA₂, cytosolic PLA₂ α is essential for producing PAF through the remodeling pathway [13]. Recently, Shindou et al. [14] identified the LysoPAFAT gene, and they designated the relevant enzyme LysoPAFAT/LP-CAT2.

During the course of experiments on neutrophil depletion, we noticed that i.v. injection of RB6-8C5 or 1A8 induced anaphylaxis-like shock in mice pretreated with LPS. Shock signs began within a few minutes, and the mice died of shock within 20 min of the antibody injection. These findings tempted us to hypothesize that such events, in which neutrophils play important roles, may occur in patients developing infectious diseases. Thus, in the present study, we examined the mechanism underlying the shock induced by a combination of LPS and a given anti-Ly-6G mAb. Using various drugs and mice, we demonstrated that the shock is mediated by PAF, neutrophils, and macrophages.

MATERIALS AND METHODS

Animals

BALB/c mice were provided by the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine (Sendai, Japan), whereas C57BL/6, C3H/HeN, C3H/HeJ, and WBB6F1-W/W^u mice were purchased from SLC (Shizuoka, Japan). BALB/c IL-1 KO mice (deficient in IL-1 α and IL-1 β), BALB/c TNF- α KO mice, and BALB/c IL-1 plus TNF- α KO mice were established from original IL-1 α KO, IL-1 β KO, and TNF- α KO mice [15, 16]. C57BL/6 HDC KO mice [17, 18], C57BL/6 H1R KO mice [19], and C57BL/6 TLR2 KO mice [20] were established as described previously. C57BL/6 PAFR KO mice were provided by the RIKEN BioResource Center through the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology (Japan) [10]. Male BALB/c mice (8–12 weeks old) were used in the present study, unless indicated otherwise. All procedures complied with the Guidelines for the Care and Use of Laboratory Animals in Tohoku University.

Reagents

An anti-Gr-1 mAb (RB6-8C5, rat IgG2b) and an anti-mouse platelet mAb (Pm-1, rat IgG) were purified from the ascites fluid of the respective hybridoma cells [1, 21]. Although the molecular antigen for Pm-1 has not been identified, Pm-1 has been shown to deplete platelets in mice [22, 23]. An anti-CD200R mAb (Ba103, rat IgG2b) was kindly provided by Dr. Hajime Karasuyama (Tokyo Medical and Dental University, Tokyo, Japan) [24]. Other agents used and their suppliers were as follows: anti-Ly-6G mAb (1A8, rat IgG2a, BioLegend, San Diego, CA, USA); anti-Ly-6C mAb (ER-MP20, rat IgG2a, GenWay Biotech, San Diego, CA, USA); isotype control mAb (RTK4530, rat IgG2b, BioLegend); control rat IgG (Rockland Immunochemicals, Gilbertsville, PA, USA); LPS from *Escherichia coli* O55:B5, prepared by a phenol extraction method (Sigma-Aldrich, St. Louis, MO, USA); synthetic *E. coli*-type lipid A (compound 506, Peptide Institute, Osaka, Japan); PAFR antagonist, WEB-2086, 4-(2-chlorophenyl)-9-methyl-2-(3-morpholino-3-oxo-propyl)-6H-thieno[3,2-f] [1, 2, 4]triazolo[4,3- α] [1, 4]diazepine (Enzo Life Sciences, Farmingdale, NY, USA); antioxidant NAC (Wako Pure Chemical Industries, Osaka, Japan); free radical scavenger, edaravone, 3-methyl-1-phenyl-2-pyrazolin-5-one (Mitsubishi Wellpharma, Osaka, Japan); NOS inhibitor L-NAME (Sigma-Aldrich); and glucocorticoid anti-inflammatory agent, dexamethasone 21-phosphate disodium salt (Sigma-Aldrich). All of the mAb and reagents used were dissolved in sterile saline. Experimental protocols are described in the text or in the legend to the figure relating to each experiment.

Evaluation of shock

Rectal temperature was measured using a digital thermometer (TD-300, Shibaura Electronics, Saitama, Japan) just before the challenge and then every 10 min for 1 h. At the same time, mouse activity was assessed, and the maximal severity of rapid shock was scored as described elsewhere [25]; the scoring system was: 0 (no signs of shock), 1 (staggering), 2 (crawling and prostration), 3 (prostration and weak convulsions), 4 (prostration and strong convulsions), and 5 (death).

Blood cell counts and flow cytometry

Mice were decapitated, and peripheral blood was collected in test tubes containing EDTA-2Na (final concentration, 1 mg/ml blood). A 20- μ l aliquot of the blood was taken and subjected to the estimation of RBCs, WBCs, and platelets by means of a Celltac hematology analyzer (MEK-5258, Nihon Kohden, Tokyo, Japan). The rest of the blood was subjected to immunofluorescence analysis to determine the percentage of neutrophils among WBCs. RBCs were removed by hypotonic lysis, and the remaining cells were incubated with an anti-Gr-1 mAb (RB6-8C5), followed by FITC-labeled donkey anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Cells were analyzed using FACSCalibur with CellQuest software (BD Biosciences, San Jose, CA, USA). Cells were gated on WBCs by standard forward- and side-scatter properties, and the percentage of Gr-1^{high} cells among the WBCs was determined. Although there are Gr-1⁺ blood cells other than neutrophils, a high expression of Gr-1 is limited to neutrophils [6].

Cell sorting

Lungs were removed from decapitated mice and transferred into Petri dishes containing RPMI-1640 medium with 5% FCS and 4 mM EDTA. Then, the lungs were minced with scissors into pieces not exceeding 2 mm in diameter and mashed with the rubber end of a syringe plunger. The cells were filtered through a 70- μ m cell strainer (BD Biosciences), and RBCs were removed by hypotonic lysis. The remaining cells were incubated with an anti-Ly-6G mAb (1A8), followed by the above FITC-labeled donkey anti-rat IgG, and then incubated with R-PE-labeled anti-F4/80 mAb (CI: A3-1, Serotec, Oxford, UK). Neutrophils (Ly-6G^{high}), macrophages (Ly-6G⁻F4/80⁺), and other cells (Ly-6G⁻F4/80⁻) were sorted using FACSaria II with Diva software (BD Biosciences).

MPO activity assay

MPO activity can be used as a marker for tissue neutrophil content [26]. Left lungs, left liver lobes, and spleens were removed from decapitated mice and weighed. Each left liver lobe was cut into pieces, each weighing ~50 mg before being weighed. These tissue samples were frozen immediately on dry-ice and stored at -80°C. After thawing, samples were homogenized (using an HG 30 homogenizer, Hitachi, Tokyo, Japan) for 30 s in 2 ml 50 mM sodium phosphate buffer (pH 7.4) containing 10 mM N-ethylmaleimide. Tissue homogenates were centrifuged for 30 min at 10,000 g at 4°C. The resulting pellets were resuspended in 0.5% hexadecyltrimethylammonium bromide in 50 mM sodium phosphate buffer (pH 5.4; 1 ml/50 mg tissue), and they were subjected to sonication, three freeze/thaw cycles, and further sonication. Then, these suspensions were centrifuged for 30 min at 10,000 g at 4°C, and supernatants were collected and stored at -80°C for later use. MPO activity was determined by measuring the H₂O₂-dependent oxidation of TMB. Test samples and MPO standard (Alexis Biochemicals, San Diego, CA, USA) were added to a 96-well plate and reacted with 1.6 mM TMB and 0.3 mM H₂O₂ in 50 mM sodium phosphate buffer (pH 5.4) in the dark for 20 min at room temperature. Then, the reaction was stopped by the addition of 1 M phosphoric acid. The absorbance of each sample or standard was measured at 450 nm using a microplate reader (Sunrise remote, Tecan Austria, Grödig, Austria). The concentration of MPO in each sample was calculated from the standard curve. In parallel, each sample was run additionally with 30 μ M of the specific MPO inhibitor, 4-aminobenzoic acid hydrazide [27]. The remaining peroxidase

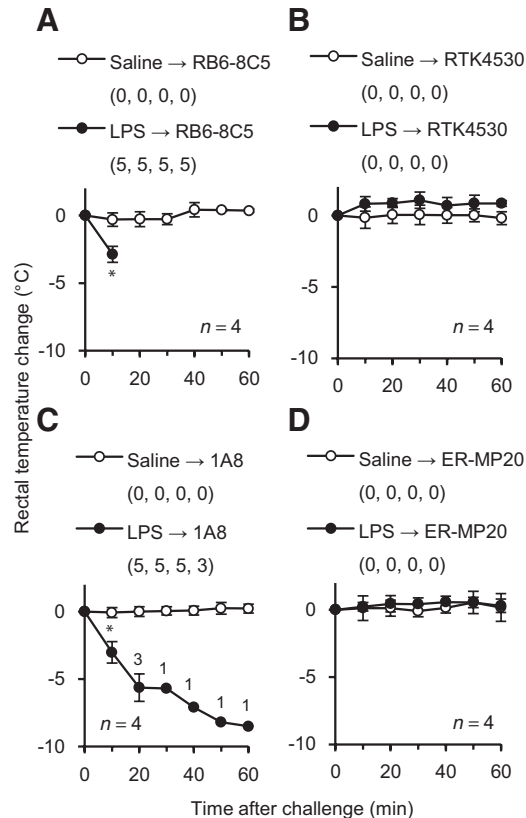


Figure 1. Anti-Ly-6G mAb induce rapid decrease in rectal temperature and death in LPS-pretreated mice. Mice were injected i.v. with saline or LPS (10 $\mu\text{g}/\text{kg}$). Two hours later, the mice were challenged with one of four mAb (500 $\mu\text{g}/\text{kg}$, i.v.): RB6-8C5 (anti-Gr-1; A), RTK4530 (isotype control; B), 1A8 (anti-Ly-6G; C), and ER-MP20 (anti-Ly6C; D). Rectal temperature was measured at the indicated time-points and expressed as temperature change versus time 0. Results are means $\pm$ SD; $n = 4$. Numbers attached to data points in C indicate the number of mice still alive at the indicated time-points. The maximal shock scores allocated to individual mice in the 1st h after the challenge are shown within parentheses. * $P < 0.01$ versus saline-primed mice at the same time-point.

activity was subtracted from the enzyme activity obtained in the absence of 4-aminobenzoic acid hydrazide.

Immunohistochemistry

Fresh lungs and livers were fixed in 4% paraformaldehyde overnight at 4°C. The lungs and livers were then embedded in paraffin and cut at 5 or 3 μm thickness, respectively. These sections were deparaffinized, washed three times with PBS, and treated with 0.1% saponin. After washing, the sections were incubated in PBS containing 3% normal goat serum for 20 min and then treated with RB6-8C5 (0.1 $\mu\text{g}/\text{ml}$ PBS) overnight at 4°C. After washing, the sections were incubated for 1 h with Simple Stain Mouse MAX PO (Rat; Nichirei Biosciences, Tokyo, Japan) as a secondary antibody. After washing, the sections were visualized with the aid of 0.025% 3,3'-diaminobenzidine and 0.01% H_2O_2 in 0.05 M Tris buffer (pH 7.6) and counterstained with Mayer's hematoxylin.

Quantitative RT-PCR

To examine the activation of the remodeling pathway of PAF synthesis in certain tissues and cells, we analyzed the mRNA expression levels of LysoPAFAT/LPCAT2 [14], as described below. Whole left lungs or sorted

cells from lungs were lysed in 1 ml Isogen (Nippon Gene, Toyama, Japan), and total RNA was extracted as described in the manufacturer's instruction manual. cDNA synthesis was carried out using a First Strand cDNA synthesis kit (Roche Diagnostics, Indianapolis, IN, USA). Real-time PCR was performed using LightCycler FastStart DNA Master SYBR Green I and the LightCycler 1.5 system (Roche Diagnostics). The primers used for PCR were as follows: LysoPAFAT/LPCAT2, forward 5'-GTCCAGCAGACTAC-GATCAGTG-3' and reverse 5'-CTTATTGGATGGGTCAGCTTTTC-3'; GAPDH, forward 5'-ATGTGTCCGTCGTGGATCTG-3' and reverse 5'-GGTGAAGAGTGGGAGTTGC-3' (Nihon Gene Research Laboratories, Sendai, Japan). The PCR conditions were 35 cycles of 95°C for 10 s, 64°C for 10 s, and 72°C for 10 s for each primer. The product size for each primer was 167 bp. The levels of LysoPAFAT/LPCAT2 mRNA were normalized to those of GAPDH mRNA.

Treatment of mice with Clo-lip

A suspension of Clo-lip was prepared according to a method described previously [28, 29]. The original suspension of Clo-lip was twofold-diluted using PBS, and the diluted suspension was injected i.v. at 0.1 ml/10 g body weight. PBS-lip were used as a control.

Statistical analysis

Experimental values are expressed as means $\pm$ SD. The statistical significance of differences was assessed using a two-tailed unpaired t test with a Welch correction. Data were analyzed by means of a two-way ANOVA, followed by a Bonferroni post hoc test for multiple comparisons (see Fig. 6D). Values of $P < 0.01$ were considered to indicate significance.

RESULTS

Anti-Ly-6G mAb induce anaphylaxis-like shock in LPS-pretreated mice

Injection of RB6-8C5 induced rapid hypothermia and death in mice pretreated with LPS (Fig. 1A). In these mice, apparent signs of shock (such as staggering) started at 3–4 min, and death occurred within 20 min. No obvious signs of shock were observed

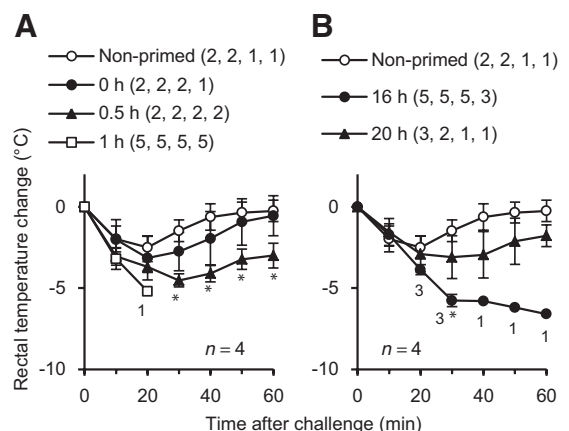
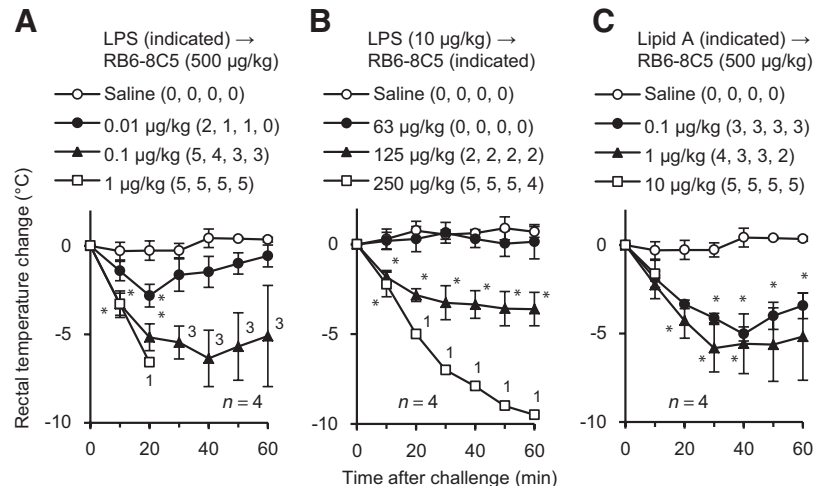


Figure 2. Effect of time interval between LPS and RB6-8C5 injections on subsequent shock. Mice were challenged with RB6-8C5 (1 mg/kg, i.v.) in one of three ways: (1) the RB6-8C5 given alone (nonprimed; same data shown in A and B), (2) simultaneously with LPS (10 $\mu\text{g}/\text{kg}$, i.v.; interval=0 h; data shown in A), or (3) after LPS (interval=0.5–20 h; data split between A and B). In other respects, the explanation of the figure is the same as for Fig. 1. * $P < 0.01$ versus nonprimed mice at the same time-point.

Figure 3. Dose dependency of the shock-inducing effects of LPS, RB6-8C5, and lipid A. (A) Mice were injected i.v. with saline or LPS (0.01–1 $\mu\text{g}/\text{kg}$), followed 2 h later by RB6-8C5 (500 $\mu\text{g}/\text{kg}$, i.v.). (B) Mice were injected with LPS (10 $\mu\text{g}/\text{kg}$, i.v.), followed 2 h later by an i.v. injection of saline or RB6-8C5 (63–250 $\mu\text{g}/\text{kg}$). (C) Mice were injected i.v. with saline or synthetic lipid A (0.1–10 $\mu\text{g}/\text{kg}$), followed 2 h later by RB6-8C5 (500 $\mu\text{g}/\text{kg}$, i.v.). The explanation of the figure is the same as for Fig. 1. * $P < 0.01$ versus saline-injected mice at the same time-point.



in mice not pretreated with LPS (Fig. 1A). An isotype control mAb, RTK4530, did not induce any shock signs in LPS-pretreated mice (Fig. 1B). As RB6-8C5 reportedly binds to Ly-6G and Ly-6C [6], we next tested 1A8 (anti-Ly-6G mAb) and ER-MP20 (anti-Ly-6C mAb) as substitutes for RB6-8C5. 1A8 yielded essentially the same results as RB6-8C5 (Fig. 1C), but ER-MP20 had no effect (Fig. 1D). These results suggest that antibody binding to Ly-6G antigen, a specific surface marker of neutrophils [6], induces the above anaphylaxis-like shock in LPS-pretreated mice.

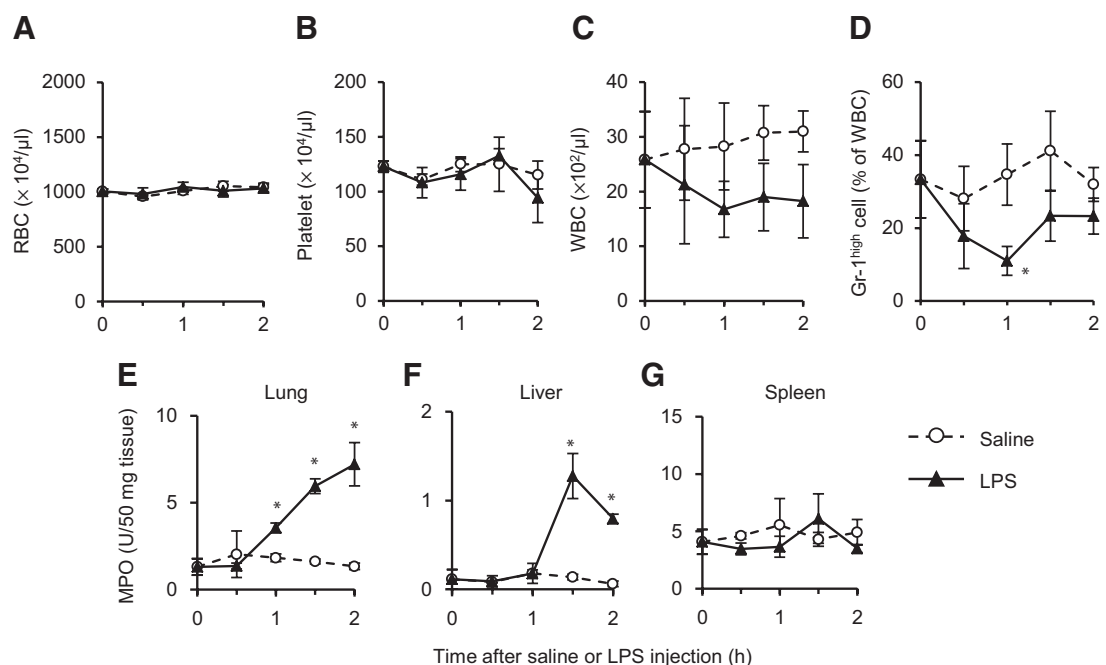
The following experiments were conducted mainly using RB6-8C5, as 1A8 hybridoma was not available when we started this study. First, we examined the effects of varying the time interval between the LPS and RB6-8C5 injections on the severity of the shock, as well as the effects of varying the doses of LPS and RB6-8C5. Lethal shock was observed when the time interval was 1, 4, 8, 12, or 16 h (Fig. 2; data for 4, 8, and 12 h are not shown). It

should be noted that in nonprimed mice, injection of RB6-8C5 at this dose (1 mg/kg) caused weak shock signs (Fig. 2), together with a slight decrease in rectal temperature, whereas no shock signs were induced by a lower dose (500 $\mu\text{g}/\text{kg}$; Fig. 1A). In four mice injected i.v. with RB6-8C5 (1 mg/kg), followed 1 h later by LPS (10 $\mu\text{g}/\text{kg}$; the reverse of the above order), no shock signs were observed (data not shown). The shock was dependent on the dose of LPS and RB6-8C5 (Fig. 3A and B, respectively). In addition, synthetic lipid A—the active principle of LPS [30]—exhibited essentially the same priming ability as LPS (Fig. 3C).

Recruitment of neutrophils from peripheral blood to lung and liver in the LPS-induced priming phase

In the following experiments, we elected to use 10 $\mu\text{g}/\text{kg}$ LPS for priming, 500 $\mu\text{g}/\text{kg}$ RB6-8C5 for challenge, and a 2-h time interval between LPS and RB6-8C5, as at these doses and at this time

Figure 4. Neutrophil kinetics in blood and organs during the LPS-induced priming phase. Mice were injected i.v. with saline or LPS (10 $\mu\text{g}/\text{kg}$), and then peripheral blood was collected at the indicated time-points for the measurement of RBCs (A), platelets (B), WBCs (C), and the percentage of Gr-1^{high} cells among WBCs (D). Lung (E), liver (F), and spleen (G) were also collected for the measurement of MPO activity. Results are means $\pm$ SD; $n = 4$ –6. * $P < 0.01$ versus saline-injected mice at the same time-point.



interval, maximal shock was observed (see previous section). An influx of inflammatory cells, such as neutrophils, into susceptible organs (including lung, liver, and spleen), is thought to be an important feature of LPS-induced tissue injury [31]. We therefore investigated neutrophil kinetics in peripheral blood and these organs during the LPS-induced priming phase. The RBC and platelet counts remained almost constant during the priming phase (Fig. 4A and B), whereas the WBC count tended to decrease from 1 h after the LPS injection (Fig. 4C). The percentage of Gr-1^{high} cells (neutrophils) among WBCs was significantly decreased at 1 h after LPS (Fig. 4D). Concomitantly, lung MPO activity exhibited a significant increase (Fig. 4E). Lagging slightly behind the pulmonary MPO increase, liver MPO activity was significantly increased at 1.5 h after LPS (Fig. 4F). In contrast, spleen MPO activity remained fairly constant throughout the experimental period (Fig. 4G). These results indicate that circulating neutrophils are recruited to lung and liver at about 1 and 1.5 h, respectively, after a LPS injection.

Stimulation of neutrophils within pulmonary and hepatic blood vessels after RB6-8C5 challenge in LPS-pretreated mice

We next investigated blood cell kinetics during the RB6-8C5-induced challenge phase (Fig. 5A–D). The RBC count of mice given LPS plus RB6-8C5 increased rapidly and markedly after the

RB6-8C5 injection (Fig. 5A). This hemoconcentration may be explained as occurring as a result of the vascular leakage of plasma that accompanies severe anaphylaxis-like shock [32]. In contrast, the platelet count of mice given LPS plus RB6-8C5 displayed a rapid and marked decrease after the RB6-8C5 (Fig. 5B). With or without LPS pretreatment, the percentage of Gr-1^{high} cells among WBCs decreased rapidly after RB6-8C5 (Fig. 5D).

In line with the results of the MPO assay (Fig. 4, E and F), a marked accumulation of Gr-1^{high} cells was observed in the lung and liver of mice given LPS plus saline or LPS plus RB6-8C5 (versus the control groups; Fig. 5E). These Gr-1^{high} cells were localized within the pulmonary and hepatic blood vessels. Moreover, Gr-1^{high} cell aggregates were observed within the pulmonary and hepatic blood vessels of mice given LPS plus RB6-8C5 (rightmost panels in Fig. 5E).

Involvement of PAF in the shock induced by LPS plus RB6-8C5

To clarify the molecular mechanism underlying LPS + RB6-8C5-induced shock, we looked for differences in sensitivity among various mice (Table 1), and we also evaluated the modulating effects of various drugs on the severity of the shock (Table 2).

LPS stimulates innate immunity via TLR4 [33]. Unlike control C3H/HeN mice, C3H/HeJ mice (with a mutated, dysfunctional TLR4) were resistant to LPS + RB6-8C5-induced shock

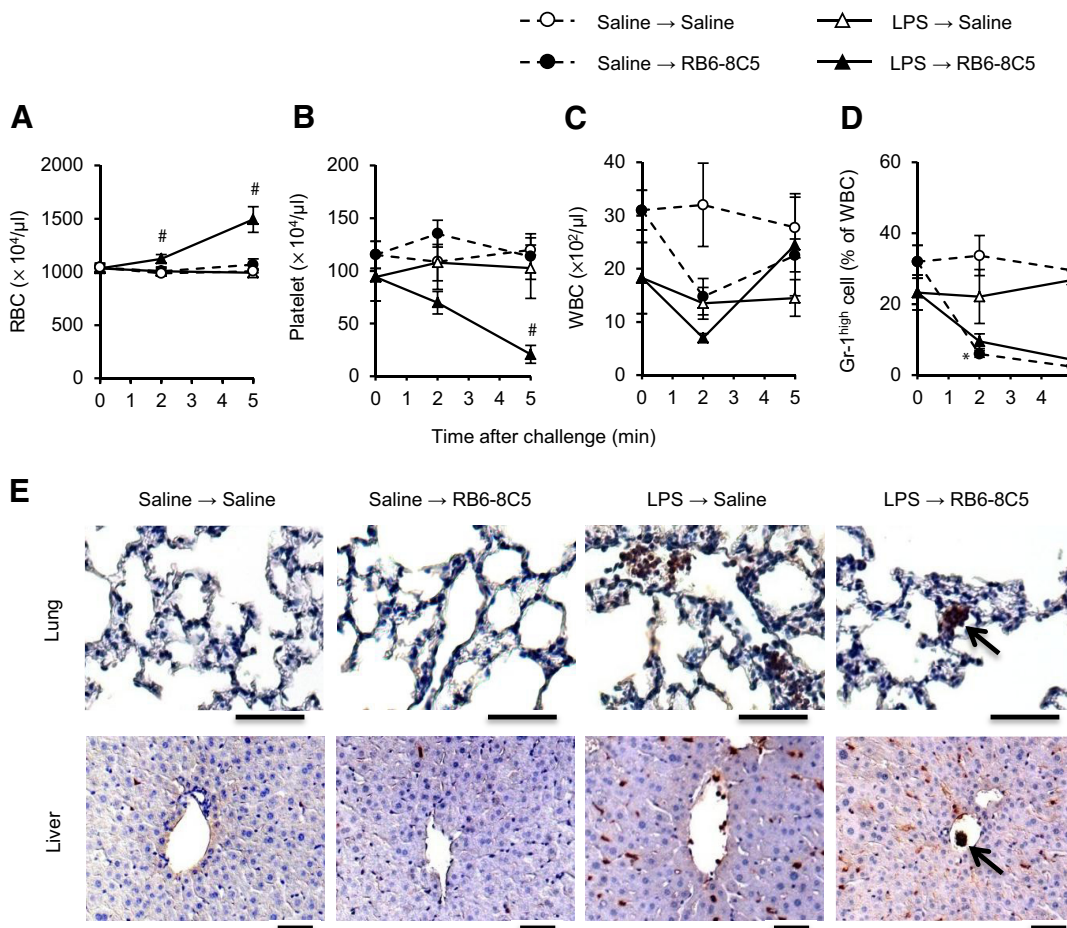


Figure 5. Neutrophil kinetics in blood and organs during the RB6-8C5-induced challenge phase. Mice were injected i.v. with saline or LPS (10 $\mu\text{g}/\text{kg}$), followed 2 h later by an i.v. injection of saline or RB6-8C5 (500 $\mu\text{g}/\text{kg}$). Then, peripheral blood was collected at the indicated time-points for the measurement of RBCs (A), platelets (B), WBCs (C), and the percentage of Gr-1^{high} cells among WBCs (D). Results are means $\pm$ SD; $n = 4-8$. * $P < 0.01$ versus mice given saline followed by saline; # $P < 0.01$ versus mice given LPS followed by saline. (E) Lung and liver specimens were sampled at 5 min after the saline or RB6-8C5 challenge, and tissue sections were stained with RB6-8C5 and secondary antibody (brown). Arrows show Gr-1^{high} cell aggregates. Data are representative of three independent experiments. Original bars represent 50 μm .

TABLE 1. Sensitivities to the Shock Induced by LPS Plus RB6-8C5 in Various Mice

Genetic background	Shock score	Mortality
C3H/HeN	5 ^a	4/4
C3H/HeJ	0 ^a	0/4
C57BL/6	5 ^a	6/6
C57BL/6 TLR2 KO	5 ^a	5/5
C57BL/6 HDC KO	5 ^a	4/4
C57BL/6 H1R KO	5 ^a	7/7
C57BL/6 PAFR KO	3, 3, 3, 2	0/4 ^b
BALB/c	5 ^a	4/4
BALB/c IL-1 KO	5 ^a	5/5
BALB/c TNF- α KO	5 ^a	5/5
BALB/c IL-1 and TNF- α KO	5 ^a	5/5
WBB6F1-W/W ^u	5 ^a	4/4

Male mice (8–12 weeks old) were injected with LPS (10 μ g/kg, i.v.), followed 2 h later by RB6-8C5 (500 μ g/kg, i.v.). ^aAll mice tested within a given group were allocated the same shock score. ^bThe rectal temperature changes in the same experiment are shown (see Fig. 6B).

(Table 1). Although TLR2 has also been implicated in the signaling induced by some types of LPS [34], TLR2 KO mice were sensitive to shock induction by the LPS used in this study plus RB6-8C5 (Table 1). IL-1 and TNF- α are important inflammatory mediators produced by immunocompetent cells, such as macrophages, in response to LPS. However, IL-1 KO mice, TNF- α KO mice, and IL-1 plus TNF- α KO mice were all sensitive to LPS + RB6-8C5-induced shock (Table 1).

Recent studies have suggested that histamine and PAF are two major mediators of murine anaphylaxis [35, 36]. However, HDC KO mice, which show histamine deficiency, and H1R KO mice were sensitive to LPS + RB6-8C5-induced shock (Table 1). On the other hand, WEB-2086, a PAFR antagonist that prevents death being caused by PAF injection in mice [37], prevented the lethal effects of LPS plus RB6-8C5 when injected before the RB6-8C5 injection but not when injected before the LPS injection (Fig. 6A and Table 2). In addition, PAFR KO mice showed resistance to LPS + RB6-8C5-induced shock (Fig. 6B and Table 1), as well as to LPS + 1A8-induced shock (Fig.

6C). To look for further evidence of PAF production in response to LPS plus RB6-8C5, we examined the mRNA level of a PAF biosynthetic enzyme, LysoPAFAT/LPCAT2, in lungs taken from mice at 5 min after an RB6-8C5 challenge (Fig. 6D). This mRNA level tended to be increased by LPS and was increased greatly by subsequent RB6-8C5. In contrast, an RB6-8C5 alone did not increase it. Moreover, dexamethasone, which attenuates the LPS-induced up-regulation of LysoPAFAT/LPCAT2 in vitro [14], prevented the lethal effects of LPS plus RB6-8C5 when applied before the LPS (Fig. 6E and Table 2). These results indicate that LPS + RB6-8C5/1A8-induced shock is mediated by PAF, not by histamine.

Tissue injury during endotoxemia is often attributed to neutrophil-derived ROS [38], and some biological activities of PAF may be mediated by NO [39]. However, antioxidant pretreatment with NAC or edaravone did not prevent the lethal effects of LPS plus RB6-8C5, nor did NO synthase inhibition by L-NAME (Table 2).

Neutrophils and macrophages are involved in shock induction by LPS plus RB6-8C5

Next, we tried to identify the cells responsible for LPS + RB6-8C5-induced shock by in vivo depletion of individual cell types (Table 3).

Prior neutrophil depletion by 1A8 completely prevented LPS + RB6-8C5-induced shock (Table 3), indicating that neutrophils play a central role in this shock and that RB6-8C5 binding to Ly-6C antigen on non-neutrophil Ly-6C⁺ cells contributes little or nothing to the development of this shock.

Rapid thrombocytopenia shown in Fig. 5B is one of the characteristic events in platelet-dependent systemic anaphylaxis [40]. Platelet depletion by Pm-1 prevents anaphylaxis-like shock being induced in mice by a certain type of LPS [23, 41, 42]. However, such platelet depletion failed to prevent the lethal effects of LPS plus RB6-8C5 (Table 3), suggesting that platelets are not a major mediator of this type of shock.

As previous studies have implicated mast cells, basophils, and/or macrophages in systemic anaphylaxis [35, 36, 43], we next focused on these cell types. Mast cell-deficient WBB6F1-W/W^u mice [44] were sensitive to LPS + RB6-8C5-induced

TABLE 2. Effects of Various Drugs on the Severity of the Shock Induced by LPS Plus RB6-8C5

Treatment	Timing of drug injection	Shock score	Mortality
None		5 ^a	4/4
WEB-2086 (1 mg/kg, i.v.)	10 min before LPS	5 ^a	4/4
	10 min before RB6-8C5	3, 2, 2, 2	0/4 ^b
Dexamethasone (5 mg/kg, i.p.)	120 min before LPS	2, 2, 2, 0	0/4 ^b
NAC (100 mg/kg, i.p.)	60 min before LPS	5 ^a	4/4
	60 min before RB6-8C5	5 ^a	4/4
Edaravone (8 mg/kg, i.v.)	60 min before LPS	5 ^a	4/4
	60 min before RB6-8C5	5 ^a	4/4
L-NAME (10 mg/kg, i.p.)	30 min before LPS	5 ^a	4/4
	30 min before RB6-8C5	5 ^a	4/4

Mice were injected with LPS (10 μ g/kg, i.v.), followed 2 h later by RB6-8C5 (500 μ g/kg, i.v.). Drug treatment was given as indicated in Column 1. ^aAll mice tested within a given group were allocated the same shock score. ^bThe rectal temperature changes in the same experiment are shown (see Fig. 6A and E).

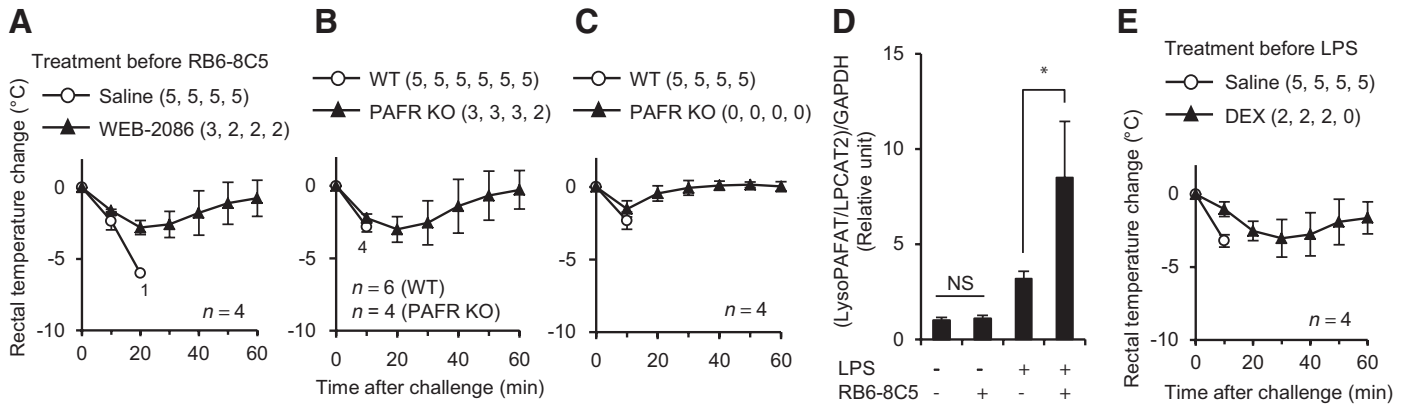


Figure 6. PAF is critical for induction of severe anaphylaxis-like shock by LPS plus a given anti-Ly-6G mAb. Mice were injected i.v. with LPS (10 $\mu\text{g}/\text{kg}$), followed 2 h later by an i.v. injection of RB6-8C5 (A, B, and E) or 1A8 (C; 500 $\mu\text{g}/\text{kg}$). Saline or WEB-2086 (1 mg/kg) was injected i.v. at 10 min before RB6-8C5 (A). PAFR KO mice and WT mice were examined (B and C). Saline or dexamethasone (DEX; 5 mg/kg) was injected i.p. at 120 min before LPS (E). The explanation of the figure is the same as for Fig. 1. (D) Mice were injected i.v. with saline or LPS (10 $\mu\text{g}/\text{kg}$), followed 2 h later by an i.v. injection of saline or RB6-8C5 (500 $\mu\text{g}/\text{kg}$). Five minutes later, lungs were collected, and the expression levels of LysoPAFAT/LPCAT2 mRNA and GAPDH mRNA were analyzed by quantitative RT-PCR. Results are means $\pm$ SD; $n = 3$. * $P < 0.01$.

shock (Table 1). Basophil depletion by Ba103 did not prevent the lethal effects of LPS plus RB6-8C5 (Table 3). In contrast, macrophage depletion by Clo-lip greatly suppressed LPS + RB6-8C5-induced shock, as well as LPS + 1A8-induced shock (Fig. 7A and Table 3). These results indicate that macrophages, but not mast cells or basophils, are involved in LPS + RB6-8C5/1A8-induced shock.

Macrophages have been reported to play a role in neutrophil recruitment into inflamed tissues [45, 46]. Therefore, one possibility was that the shock-preventing effect of macrophage depletion might be a result of reduced neutrophil recruitment into lung and/or liver in response to LPS. However, prior macrophage depletion by Clo-lip did not affect LPS-induced neutrophil accumulation in lung and liver (Fig. 7C).

TABLE 3. Effects of Neutrophil, Platelet, Basophil, or Macrophage Depletion on the Severity of the Shock Induced by LPS Plus RB6-8C5

Pretreatment	Shock score	Mortality
Control IgG (8 mg/kg, i.p.)	5 ^a	4/4
1A8 (8 mg/kg, i.p.)	0 ^a	0/4
Pm-1 (10 mg/kg, s.c.)	5 ^a	4/4
Ba103 (80 $\mu\text{g}/\text{mouse}$, i.v.)	5 ^a	4/4
PBS-lip (0.1 ml/10 g, i.v.)	5 ^a	4/4 ^b
Clo-lip (0.1 ml/10 g, i.v.)	2, 2, 2, 1	0/4 ^b

RB6-8C5 (500 $\mu\text{g}/\text{kg}$, i.v.) was injected 2 h after injection of LPS (10 $\mu\text{g}/\text{kg}$, i.v.) into mice, which had been injected with the indicated antibodies or liposomes 1 day before the injection of RB6-8C5. We confirmed that at the doses indicated, 1A8, Pm-1, Ba103, and Clo-lip depleted mice of most of their neutrophils, platelets, basophils, and macrophages, respectively (data not shown). ^aAll mice tested within a given group were allocated the same shock score. ^bThe rectal temperature changes in the same experiment are shown (see Fig. 7A).

Neutrophils are the source of PAF in response to LPS plus 1A8

Finally, we tried to identify the cells responsible for PAF production by sorting of pulmonary cells obtained from mice given LPS plus 1A8. The mRNA level of LysoPAFAT/LPCAT2 was increased significantly in neutrophils (Ly-6G^{high}) but not in macrophages (Ly-6G^{low} F4/80⁺) or in other cells (Ly-6G^{low} F4/80⁺; Fig. 8). These results indicate that neutrophils are the source of PAF in this type of shock.

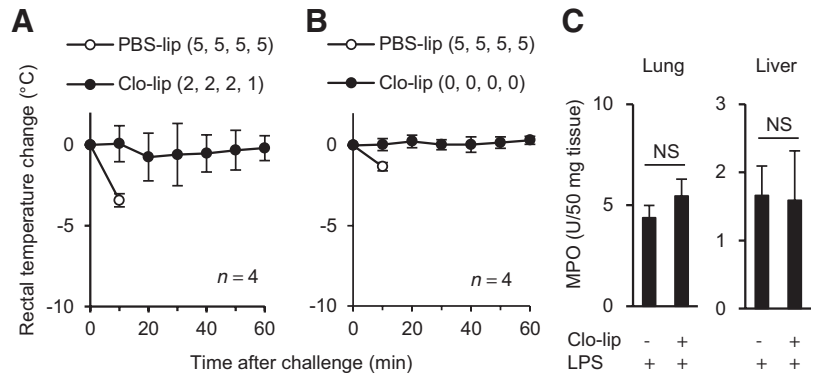
DISCUSSION

It has been suggested that the triggering of neutrophil activation requires an initial “priming” stage [47]. Neutrophils are primed by exposure to inflammatory agents (e.g., LPS) and/or by adherence to activated endothelium or to the surfaces of foreign materials. Once neutrophils have been primed within the circulation, they become trapped, mainly in the pulmonary microcirculation. Priming not only enhances neutrophil responses to subsequent stimuli but also fixes neutrophils at the inflamed site and increases their functional longevity [31, 47]. Given that for the optimal effect of LPS pretreatment, it needed to be administered at least 1 h before RB6-8C5, we suppose that neutrophils may become primed during and/or after their accumulation in the lung and liver via exposure to LPS and/or adherence to LPS-activated endothelium, and the subsequent stimulation of Ly-6G on the primed neutrophils may lead to their excessive activation, resulting in shock.

Worthen et al. [48] demonstrated that priming human neutrophils with LPS results in enhanced production of PAF upon stimulation with fMLP. Moreover, Shindou et al. [14, 49] reported that long-term (16 h) LPS treatment of murine macrophages up-regulates the expression of LysoPAFAT/LPCAT2 and that subsequent short-term stimulation with PAF markedly augments the activation of this enzyme. The present study sug-

Figure 7. In vivo depletion of macrophages ameliorates the shock induced by LPS plus a given anti-Ly-6G mAb.

Mice were injected i.v. with PBS-lip or Clo-lip at 1 day before the following experiments. Mice were injected i.v. with LPS (10 $\mu\text{g}/\text{kg}$), followed 2 h later by RB6-8C5 (A) or 1A8 (B; 500 $\mu\text{g}/\text{kg}$). The explanation of the figure is the same as for Fig. 1. (C) Mice were injected i.v. with LPS (10 $\mu\text{g}/\text{kg}$). Lung and liver were collected at 2 h after the LPS injection for the measurement of MPO activity. Results are means $\pm$ SD; $n = 4$.



gests that LysoPAFAT/LPCAT2 is moderately induced in neutrophils by LPS in the priming phase and that the subsequent stimulation of the Ly-6G on their cell surface rapidly and substantially induces LysoPAFAT/LPCAT2, resulting in production of a large amount of PAF.

Our results indicate the involvement of macrophages in the LPS + RB6-8C5/1A8-induced shock. Macrophages produce various inflammatory substances. For example, LPS primes macrophages for enhanced production of ROS [50] and arachidonic acid metabolites [51] and for an augmented increase in LysoPAFAT activity [49]. We speculate that in the challenge phase, macrophages may produce arachidonic acid metabolites in response to neutrophil-derived PAF. It is also possible that macrophages may play a role in the priming phase, although the mechanism remains to be clarified.

In vitro studies suggest a role for Ly-6 molecules in cellular signaling. Indeed, antibody binding to Ly-6A/E antigen can trigger T cell activation [52], and GPI anchoring seems to be critical for Ly-6A/E-mediated T cell activation [53]. In general,

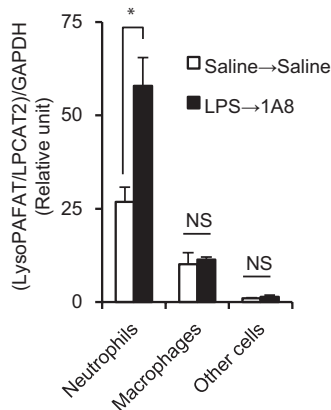


Figure 8. Identification of PAF-producing cells in lungs of mice given LPS plus 1A8.

Mice were injected i.v. with saline or LPS (10 $\mu\text{g}/\text{kg}$), followed 2 h later by an i.v. injection of saline or 1A8 (500 $\mu\text{g}/\text{kg}$), respectively. Five minutes later, lungs were collected, and pulmonary cells were sorted into three populations: neutrophils (Ly-6G^{high}), macrophages (Ly-6G^{low} F4/80⁺), and other cells (Ly-6G^{low} F4/80⁻). The expression levels of LysoPAFAT/LPCAT2 mRNA and GAPDH mRNA in these populations were analyzed by quantitative RT-PCR. Results are means $\pm$ SD; $n = 3$. * $P < 0.01$.

most of the GPI-anchored proteins are localized and packed in the lipid raft, and their cross-linking by suitable antibodies results in signal transduction, including an increase in the intracellular Ca^{2+} concentration and activation of lipid raft-associated signaling molecules such as tyrosine kinases [54]. For example, Morgan et al. [55] showed that antibody cross-linking of CD59, a Ly-6-related molecule [7, 8], on human neutrophils causes a rapid increase in the intracellular Ca^{2+} concentration and also causes ROS production. The remodeling pathway of PAF biosynthesis consists of two enzymes—cytosolic PLA₂ α and LysoPAFAT—each of which exhibits Ca^{2+} -dependent activity [14, 56, 57]. In addition, calcium ionophore stimulation causes maximal PAF production by murine neutrophils within 5 min [13]. Thus, we speculate that antibody binding to Ly-6G on LPS-primed neutrophils may induce intracellular Ca^{2+} mobilization (via an unidentified mechanism), which in turn, leads to PAF synthesis.

Interestingly, two recent studies reported that cross-linking of Ly-6G on neutrophils causes neutropenia and death in mice pretreated with TNF- α [58, 59]. The fatal reaction induced by TNF- α plus RB6-8C5 resembles the shock reported here, although TNF- α seems not to be involved in LPS + RB6-8C5-induced shock. The molecular mechanism underlying the fatal reaction induced by TNF- α plus RB6-8C5 has not been fully clarified, including whether or not PAF is involved [58, 59]. We speculate that cross-linking of Ly-6G on TNF- α -primed neutrophils may also induce PAF production. Indeed, not only LPS but also TNF- α has been reported to prime the lethal effects of PAF in vivo [60]. Thus, LPS and TNF- α may prime neutrophils via different pathways.

In an isolated, perfused rabbit lung, LPS primes the lung for enhanced injury in response to PAF [61], suggesting a possible association with the pathogenesis of acute lung injury and adult respiratory distress syndrome. Other interesting reports include the following: LPS primes PAF-induced gastrointestinal damage and mortality [61]. Norman et al. [59] associated the fetal reaction induced by TNF- α plus RB6-8C5 with disseminated intravascular coagulation. Simultaneous injection of LPS and PAF into rats induces ischemic bowel necrosis [62]. The PAF-mediated anaphylaxis-like shock induced by mannan in a certain strain of mouse is augmented by coadministration of LPS or pretreatment with muramyl dipeptide [41]. These findings and the present study suggest that LPS-

associated PAF production is involved in a number of disease states, including infections and/or anaphylaxis.

In conclusion, the present study suggests that the PAF production by neutrophils in response to LPS plus Ly-6G stimulation may be involved in various pathologic conditions, such as acute lung injury and adult respiratory distress syndrome, although it remains to be clarified which molecule is the natural ligand for Ly-6G.

AUTHORSHIP

Y.T. performed experiments and wrote the paper. Y.N. designed and performed experiments. T.K. advised on experimental design. Y.E. and S.S. provided overall direction and edited the paper.

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