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Augmentation and Suppression of Release of Tumor Necrosis Factor from Macrophages by Negatively Charged Phospholipids

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We recently reported that some lipid species of cell membranes and lipoproteins induced the growth of peripheral macrophages. In this study, the effects of phospholipids on tumor necrosis factor (TNF)-releasing activity of macrophages were examined. Ten to 20 μg/ml of cardiolipin, which is a suboptimal concentration for macrophage growth-stimulation, augmented macrophage TNF release triggered by lipopolysaccharide (LPS) *in vitro*. This priming effect appeared with 1 day of preincubation and was still potent on day 3, whereas the priming effect of interferon-γ (IFN-γ) peaked at 3 h and then gradually decreased. In contrast, a high concentration of cardiolipin (40 μg/ml) which is optimal for the induction of macrophage growth, completely suppressed LPS-triggered TNF release from not only untreated macrophages but also IFN-γ-primed macrophages. The suppressive effect was potent even with 3 h preincubation, was still potent on day 3, and was not abolished by indomethacin. Cardiolipin had scarcely any effect on the triggering activity of LPS. Similar augmentative and suppressive activities were observed in peroxidized phosphatidylserine, which is also highly active in inducing macrophage growth, but was not found in native phosphatidylserine, which is less active, nor in phosphatidylcholine, which is an inactive species toward macrophage growth. These results suggest that lipids may be important endogenous factors in regulating both activation and growth states of peripheral macrophages.

Key words: Macrophage —Tumor necrosis factor release — Phospholipid

The function and growth of macrophages are considered to be regulated by various factors.[1, 2] We reported earlier that the growth of peripheral macrophages is induced by dead cells or denatured lipoproteins which are fated to be scavenged by macrophages in the body,[3, 4] and that the active ingredients are lipids, namely, cholesteryl esters, triglycerides,[5] and several phospholipids.[6, 7] The activities of phospholipids depend on their species; some negatively charged phospholipids such as cardiolipin (CL) and peroxidized phosphatidylserine (PS) have particularly potent macrophage growth-stimulating activity, whereas the activity of native PS is less potent and neutral phospholipids such as phosphatidylcholine (PC) have no activity.[6, 8] Since these results show that lipids are an endogenous factor regulating the quantitative aspect of peripheral macrophages, as are protein factors such as macrophage colony-stimulating factor or granulocyte/macrophage colony-stimulating factor,[9–12] it is worth investigating whether lipids also regulate the functional activity of macrophages.

In this paper, we focused on the activity of phospholipids in regulating tumor necrosis factor (TNF) release from macrophages for the following reasons. First, TNF is a cytokine located in the center of an inflammatory cytokine network and acts as a regulator throughout the stages of inflammation.[13–15] The fact that lipid-containing materials seem to be taken up by macrophages in many inflammatory processes raises the possibility that the lipids in turn affect the production of TNF. Second, TNF is extensively released by activated macrophages only after receiving two activation signals: priming and triggering stimuli.[16, 17] Accordingly, it is possible that the macrophage state of activation can be estimated from the TNF released.

Potent growth-stimulatory phospholipipids such as CL and peroxidized PS augmented (at low concentration) and suppressed (at high concentration) the release of TNF from lipopolysaccharide (LPS)-triggered macrophages. These results indicate that some lipids have the capacity to regulate the activity of macrophages qualitatively and quantitatively.

MATERIALS AND METHODS

Reagents CL from bovine heart, PS from bovine brain and PC from egg yolk were purchased from Avanti Polar Lipids (Birmingham, AL). LPS (*Escherichia coli*, 0127: B8) was from Difco Laboratory (Detroit, MI). Latex

Abbreviations used in this paper: CL, cardiolipin; PS, phosphatidylserine; PC, phosphatidylcholine; TNF, tumor necrosis factor; LPS, lipopolysaccharide; IFN-γ, interferon-γ; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; poly I:C, polyinosinic:polycytidylic acid; PGE_2, prostaglandin E_2.

beads ($\phi=1$ μm) and indomethacin were from Sigma Chemical Co. (St. Louis, MO). MTT and soluble starch were from Wako Pure Chemical Ind. Ltd. (Osaka). Recombinant murine interferon-γ (IFN-γ) was from Toray Industries Inc. (Tokyo). Heat-killed *Enterococcus faecalis* was supplied by NichiNichi Food Co. (Mie).

Mice Male C3H/He mice and C3H/HeJ mice were purchased from Shizuoka Experimental Animal Farm.

Culture of macrophages Peritoneal exudate cells were obtained from mice 3–4 days after i.p. injection of 1.5 ml of soluble starch (20 mg/ml of saline solution). These cells were suspended in RPMI-1640 medium (Nissui Seiyaku Co., Tokyo) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY), 100 units of penicillin per ml and 60 μg of kanamycin per ml (hereafter referred to as medium). The peritoneal cells were incubated in 96-well microtest plates (Corning, NY) at 1.2×10^5 cells/well. After incubation for 1.5 h at 37°C in a CO_2-incubator to allow the cells to adhere to the plates, the medium was removed and the adherent cells were vigorously washed three times with warm 0.15 *M* PBS.

More than 95% of these adherent cells were macrophages, as determined by Giemsa staining and measurement of uptake of carbon particles. These exudative macrophages were finally cultured in 200 μl of medium with or without test samples at 37°C in a humidified atmosphere of 5% CO_2 in air. The TNF concentration of macrophage supernatant was measured immediately after its collection.

Measurement of TNF activity TNF activity was measured by *in vitro* cytotoxicity assay with L-929 cells as a target.[18] Units of activity were calculated as the dilution factor of the serum allowing survival of half the L-929 cells with a human recombinant TNF preparation donated by Asahi Chemical Ind. (Tokyo) as a standard.

[^{3}H]Thymidine (^{3}H-TdR) incorporation Macrophage growth was assayed by measuring the incorporation of ^{3}H-TdR into the acid-insoluble fraction.[5] Briefly, [methyl-^{3}H]TdR (80 Ci/mmol; New England Nuclear, Boston, MA) was added at 2.5 μCi/ml and the plates were incubated for 18 h. Then the medium was removed and TCA-insoluble substances were collected on filters with Labo mash LM-101 after addition of 0.5% SDS. The filters were dried and their radioactivity was counted in a liquid scintillation spectrometer. All experiments were performed in triplicate.

Lipid samples and UV irradiation For addition to cultures, the phospholipid samples in $CHCl_3$ were dried under N_2 gas and dispersed in PBS by sonication for a few minutes in a bath-type sonicator under sterile conditions. Lipid peroxidation was induced by UV irradiation in air: 0.4–1.5 mg samples of each dry lipid in a capless glass tube were irradiated with a 15 W UV lamp for sterilization (GL-15, NEC, Tokyo) at a distance of 20 cm for the indicated period. The irradiated lipids were used within one day.

MTT assay Macrophage survival was estimated by MTT assay.[19] Twenty-five μl of MTT (5 mg/ml) per well was added to each macrophage culture and plates were incubated for 4 h. Then, the supernatant was discarded, 100 μl of acid-isopropanol solution (0.04 *N* HCl in 2-propanol) was added to each well and the optical density (A_{588}–A_{630}) was measured in an MTP-100 microplate reader (Corona Electric, Ibaraki).

Cell number determination The number of adherent cells was assessed by the method described by Nakagawara and Nathan.[20] Briefly, adherent cells were lysed by 1% (w/v) Triton X-100 and naphthol blue black-stained nuclei were counted in a hemocytometer. Assays were performed in triplicate.

Measurement of LPS content LPS content was measured by using an endotoxin-specific assay kit, Endospecy (Seikagaku Kogyo Co., Tokyo).

RESULTS

Effect of CL on TNF release from macrophages We first examined the dose-response curves of macrophage growth-stimulating activity of CL to establish the macrophage growth state and survival in a TNF release assay system. As shown in Fig. 1-a, the optimal CL concentration for induction of macrophage growth was 40 μg/ml, and 80 μg/ml was toxic to macrophages. There was a slight increase in number on day 3 when macrophages were cultured with 40 μg/ml of CL (Fig. 1-b). Furthermore, a large increase in MTT reduction was observed in macrophages cultured with 20 or 40 μg/ml of CL for 3 days (Fig. 1-c). These results indicate that $\leq$40 μg/ml of CL has no toxicity to macrophages.

It is well known that two stimuli are required for full induction of TNF release: a priming stimulus (typically IFN-γ) and a triggering stimulus (typically LPS).[2, 16] We first examined whether CL can prime macrophages to release TNF. As shown in Fig. 2, the priming effect of IFN-γ was observed at 3 h, but had disappeared by day 3. On the other hand, CL slowly augmented TNF release at suboptimal concentration for the induction of macrophage growth. Although the priming effect of CL was lower than IFN-γ, the CL effect seemed to be constant from day 1 to day 3. In contrast, 40 μg/ml of CL, which concentration is optimal for stimulating macrophage growth, almost completely suppressed the release of TNF. This suppressive effect was observed from 3 h to day 3. To confirm that the suppressive activity of 40 μg/ml of CL was not due to toxicity to macrophages, we examined whether these macrophages are responsive to reactivation. For this, macrophages incubated with

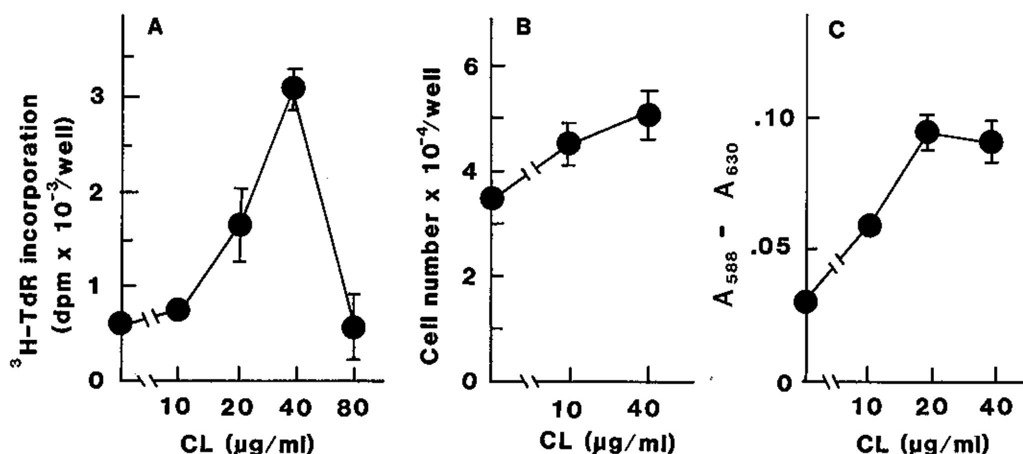

Fig. 1. Macrophage growth, cell number and macrophage survival when cultured with CL. Starch-induced macrophages were cultured with CL. A. ^{3}H-TdR incorporation was assayed on day 3. B. Cell number was determined on day 3. C. Macrophage survival was assessed by MTT assay on day 3. Each vertical bar represents SD.

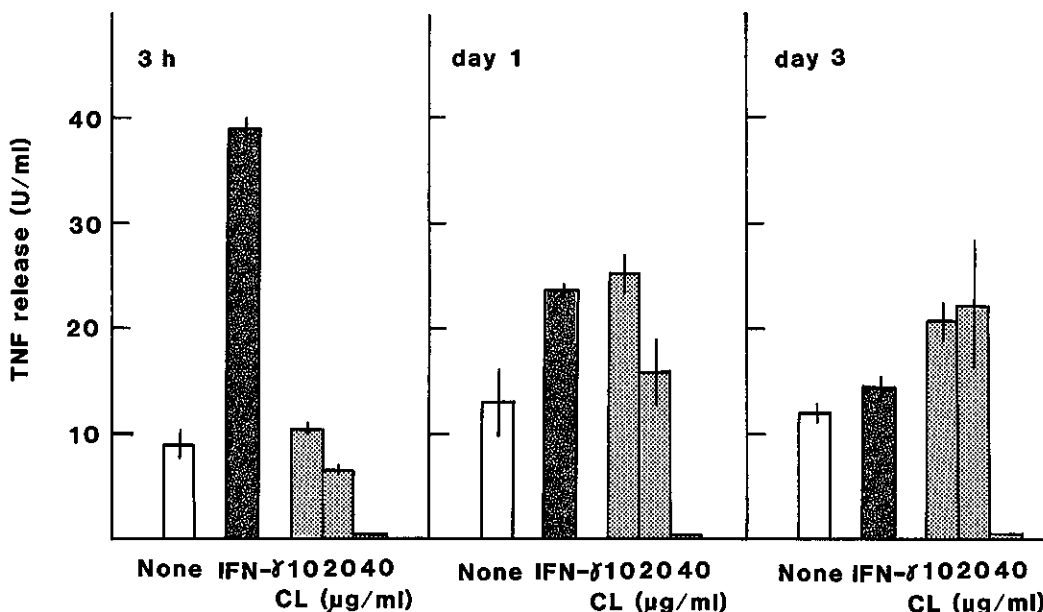

Fig. 2. Priming effect of CL on LPS-stimulated TNF release. Starch-induced macrophages were cultured with CL or IFN-γ (100 U/ml). After the indicated periods, LPS (1 μg/ml) was added to each culture. At 2 h later, TNF activity in the supernatant was measured. Bars represent the higher and lower values of duplicate estimations. Data are representative of three similar experiments.

40 μg/ml of CL for 1 day were washed, recultured with 100 U/ml of IFN-γ for 3 h, then triggered with LPS for 2 h. The TNF concentrations in supernatants were 39.1$\pm$7.7 U/ml (control culture, i.e., without CL) and 30.8$\pm$3.1 U/ml (with 40 μg/ml of CL), suggesting that the suppressive effect of a high concentration of CL did not result from toxicity of CL.

Next, to elucidate whether pretreatment of CL is necessary to modulate TNF release, we examined the effect of CL on TNF release when it was added to macrophages simultaneously with LPS. As shown in Fig. 3, in this case CL slightly enhanced TNF release at 10–40 μg/ml. However, these marginal enhancing effects were not reproduced in another experiment.

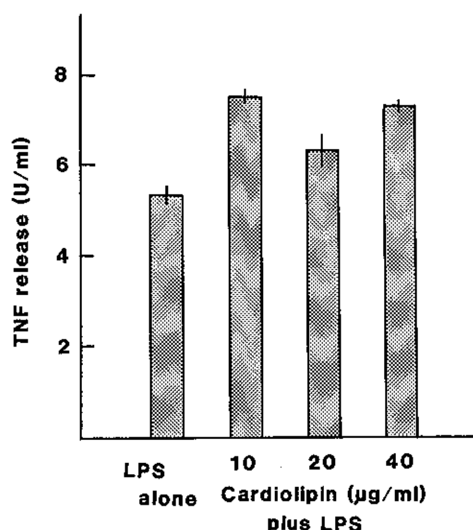

Fig. 3. Effect of CL on TNF release when added simultaneously with LPS. Starch-induced macrophages were cultured for one day without any samples, then CL and LPS (1 μg/ml) were added simultaneously. After 2-h incubation, TNF activity in the supernatant was measured. Bars represent the higher and lower values of duplicate estimations. Data are representative of two similar experiments.

Priming effect of various phospholipids on LPS-triggered TNF release We previously reported that negatively charged phospholipids such as CL and PS are active species in respect to induction of macrophage growth, whereas neutral phospholipids such as PC are not effective at all.[6,7] Moreover, the macrophage growth activity of PS is augmented by peroxidation, though PS itself is a weak mitogen.[8] To compare the activity for TNF release with the activity for macrophage growth, we examined the effects of untreated PS, PS irradiated with UV and PC on LPS-triggered TNF release.

Untreated PS did not augment TNF release but slightly suppressed it at a high concentration (80 μg/ml) (Fig. 4). In contrast, UV-irradiated PS could prime macrophages for TNF release at relatively low concentrations and almost completely suppressed it at higher concentration. MTT assay showed no reduction in macrophage survival in the presence of 80 μg/ml of UV-irradiated PS. Further, this concentration of PS or UV-irradiated PS is optimal for the induction of macrophage growth (data not shown). PC, which is incapable of inducing macrophage growth, showed no effect on TNF released from LPS-stimulated macrophages. Taken together, the data confirmed that phospholipids with higher growth-stimulating activity affect both positively and negatively the TNF release activity of LPS-triggered macrophages.

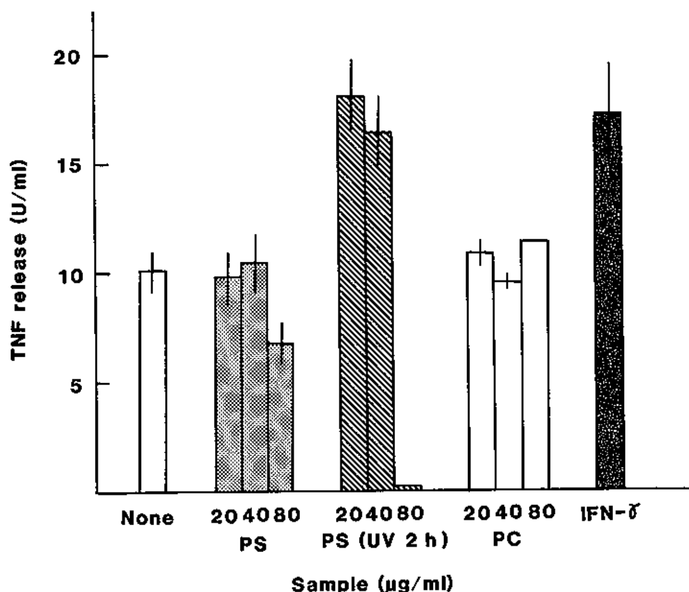

Fig. 4. Priming effects of various phospolipids on TNF release from macrophages. Starch-induced macrophages were cultured with each phospholipid. One day later, LPS (1 μg/ml) was added to each culture. After 2-h incubation, TNF activity in each supernatant was measured. Bars represent the higher and lower values of duplicate estimations. Data are representative of three similar experiments.

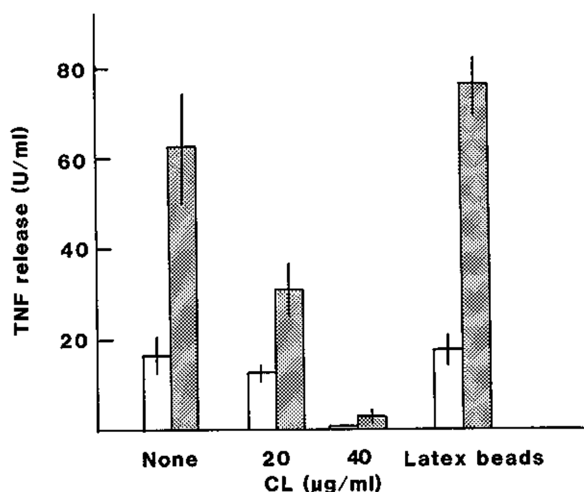

Fig. 5. Suppressive effect of CL on IFN-γ-primed TNF release. Starch-induced macrophages were cultured with CL or latex beads (0.01% solid) simultaneously with or without IFN-γ for 3 h. Then, LPS (1 μg/ml) was added to each culture. After 2 h of incubation, TNF activity in each supernatant was measured. ▭: without IFN-γ, ▩: with IFN-γ (100 U/ml). Bars represent the higher and lower values of duplicate estimations. Data are representative of four similar experiments.

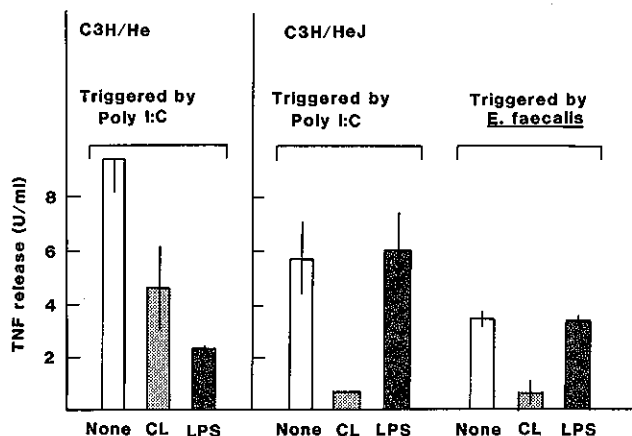

Fig. 6. Suppressive effect of CL on TNF release from macrophages of C3H/HeJ mice. Starch-induced macrophages from C3H/He or C3H/HeJ mice were cultured with CL (40 μg/ml) or LPS (1 ng/ml) for 1 day. Then, as a triggering stimulus, poly I:C (10 μg/ml) or *E. faecalis* (1 μg/ml) was added. After 2 h of incubation, TNF activity in each supernatant was measured. Bars represent the higher and lower values of duplicate estimations.

Suppressive effect of CL on priming activity of IFN-γ We next examined whether a high concentration of CL inhibits TNF release from macrophages primed with IFN-γ, or whether IFN-γ reverses the suppressive effect of CL. For this, macrophages were primed with IFN-γ with or without CL for 3 h, then LPS-triggered TNF release was measured. The results showed that 40 μg/ml of CL strongly suppressed TNF release in spite of the presence of IFN-γ (Fig. 5). On the other hand, latex beads did not suppress TNF release from macrophages cultured either with or without IFN-γ, suggesting that the suppressive effect of CL is not due merely to the phagocytic reaction of CL micelles by macrophages.

Suppressive effect of CL is not due to contaminating LPS Very recently, pretreatment with a low concentration of LPS was shown to inhibit TNF release from macrophages triggered by a second stimulation with LPS.[21] We therefore measured LPS content in a CL sample by modified *Limulus* assay and found the LPS concentration in 40 μg/ml CL to be below 10 pg/ml. Further, to confirm the absence of the effect of LPS, we checked whether a suppressive effect was observed in macrophages from C3H/He J mice, an LPS-low-responding strain.[22] In this study, we used poly I:C instead of LPS as the triggering stimulus since the former is a potent activator of macrophages of C3H/HeJ.[23] As shown in Fig. 6, 1 ng/ml of LPS inhibited poly I:C-triggered TNF release by macrophages from C3H/He mice, but this inhibition was not observed in C3H/HeJ mice. CL (40 μg/ml), on the other hand, inhibited TNF release from macrophages of not only C3H/He but also C3H/HeJ strain. This confirms that the suppressive effect of a high concentration of CL is not due to contamination by LPS.

The suppressive effect of CL does not seem to be specific to macrophages triggered by LPS or poly I:C, since similar results were obtained in macrophages triggered by the whole body of *Enterococcus faecalis*. Although 1 ng/ml of LPS suppressed TNF release in macrophages from C3H/He mice when triggered with *E. faecalis* (3.3±0.5 U/ml; without LPS, 1.8±0.3 U/ml; with 1 ng/ml of LPS), pretreatment with LPS did not inhibit TNF release from C3H/HeJ macrophages (Fig. 6).

Table I. Effect of Indomethacin on Suppressive Activity of Cardiolipin[a]

Treatment	Indomethacin (μM)		
	None	1	10
None	4.6±0.5[b]	13.7±3.6	5.9±0.8
CL (40 μg/ml)	0.0±0.0	0.1±0.0	0.0±0.0
LPS (1 ng/ml)	1.0±0.1	7.4±2.1	4.2±0.6

a) Starch-induced macrophages were incubated with CL (40 μg/ml) or LPS (1 ng/ml) for 1 day, then 1 μg/ml of LPS was added. The plates were incubated for a further 2 h, and TNF activity in the supernatant was measured. Indomethacin was added 30 min before the addition of CL or LPS and remained present in the cultures. Data are representative of two similar experiments.
b) TNF release (U/ml).

No effect of indomethacin on suppressive ability of CL at high concentration Since PGE_2, which is secreted from macrophages, is known to exert a suppressive effect on many macrophage functions, including TNF release activity,[24-26] we examined the possibility that the suppressive effect of a high concentration of CL could be mediated by PGE_2, using the cycloxygenase inhibitor indomethacin. As shown in Table I, indomethacin partially compensated for the suppression at a low concentration of LPS; however, it did not cancel the suppressive effect of a high concentration of CL.

DISCUSSION

In previous work, we reported on the effects of lipids on growth of peripheral macrophages.[3-8] In this study of the effects of phospholipids on the functional activity of macrophages, we focused on TNF release activity since this cytokine plays a significant part in initiating and regulating inflammatory processes,[13-15] and TNF release may be an indicator of macrophage activation.

A correlation was found between the activity of TNF release induced by phospholipids and that of the induction of macrophage growth; active phospholipids for inducing macrophage growth (that is CL and UV-irradiated PS) are potent regulators of TNF release, while inactive PC or weakly active lipid (untreated PS) has scarcely any effect on TNF release (Fig. 4). PC had no effect even when it had been UV-irradiated (data not shown); this is consistent with the finding that oxidized PC did not induce macrophage growth.[8)]

The effects of CL or UV-irradiated PS are biphasic. At suboptimal concentrations for induction of macrophage growth, they augmented LPS-stimulated TNF release. On the other hand, their optimal concentration almost completely suppressed TNF release (Figs. 2 and 3). The latter suppressive effect was also observed when they coexisted with IFN-γ (Fig. 5). This is in striking contrast with the effects of glucocorticoids or transforming growth factor-β 2. They were recently reported to suppress TNF release from LPS-triggered macrophages, but IFN-γ overcame the suppression.[27, 28)]

The suppressive effect of CL does not appear to be a result of cellular damage, since macrophages incubated with the suppressive concentration of CL were synthesizing DNA and showed no decrease of cell number or loss of the ability to reduce MTT (Fig. 1). However, there is a possibility that a small amount of LPS which may be contaminating CL is the actual suppressive substance, since LPS reportedly can suppress TNF release from macrophages.[21)] We discount this possibility for several reasons. First, the actual concentration of LPS in the CL sample was extremely low (see "Results"). Second, CL fully suppressed TNF release from macrophages of C3H/HeJ mice. Third, a potent suppressive effect was observed by UV-irradiated PS, but not by untreated PS. Fourth, the suppressive effect of CL was not affected by indomethacin, whereas that of LPS was partially cancelled by this inhibitor (Table I). The suppressive effect of LPS may be, at least in part, mediated by PGE_2. This seems to be in accordance with the report that suppression of macrophage H_2O_2 release by a low concentration of LPS was partly mediated by PGE_2.[29)]

There are two other possibilities: that CL inhibits TNF release by masking the next triggering agent, LPS, or that CL blocks the binding of LPS to appropriate macrophage sites. However, these explanations do not seem reasonable. First, the suppressive effect of CL was still observed when macrophages were pretreated with CL, washed and triggered by LPS, or when these CL-pretreated macrophages were triggered by a 100-fold excess of LPS (data not shown). Second, CL had no inhibitory effect on TNF release when added simultaneously with LPS (Fig. 3). Moreover, the inhibitory activity of CL was observed when gram-positive bacteria, *E. faecalis*, were used as the triggering agent. Thus, CL may actually deactivate macrophages and reduce their response to the next triggering stimulus.

The mechanism by which CL augments and suppresses TNF release is unknown and is the subject of ongoing research. In this respect, the recent report by Hamilton *et al.* may be relevant to our study. They reported that oxidized low-density lipoprotein (LDL) or lipids extracted from oxidized LDL (and not from native LDL) suppressed the expression of TNF-α mRNA in maleylalbumin-stimulated macrophages.[30)]

Our results may be physiologically significant in many respects. When a relatively small amount of damaged cells is present in a local environment, peripheral macrophages may respond and be activated to scavenge these lipid-including materials. Under such circumstances, no proliferating response of macrophages may be necessary. In contrast, when a large number of dead cells are present in local sites, for example, in the later phase of inflammation, the activation of local macrophages no longer occurs and macrophages may start to proliferate and increase their activity to scavenge a large quantity of lipid-including materials. Moreover, considering the result that at a high concentration of active phospholipids, the TNF release activity of macrophages was almost completely abolished, there might be a negative regulatory mechanism in the macrophage activation state in the regions where a large number of cells are destroyed. Peroxidized phospholipids contained in damaged cells or denatured lipoproteins may be important regulatory substances affecting both positively and negatively the growth and activation states of peripheral macrophages.

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