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Synergistic effect of ubiquitin on lipopolysaccharide-induced TNF-α production in murine macrophage cell line RAW 264.7 cells

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Abstract

Ubiquitin synergistically augmented the production of tumor necrosis factor alpha (TNF-α) in the presence of lipopolysaccharide (LPS) in murine macrophage cell line RAW 264.7. To investigate the mechanism of this augmentation, we analyzed the effect of ubiquitin during TNF-α mRNA synthesis and degradation, and TNF-α degradation on RAW 264.7 cells stimulated by LPS. It is found that ubiquitin augmented TNF-α mRNA synthesis. Ubiquitin did not affect the degradation of TNF-α mRNA and TNF-α. In the presence of LPS, extracellular accumulation of TNF-α by ubiquitin was twice than those by LPS, but intracellular accumulation of TNF-α produced by ubiquitin with LPS or by LPS had no difference. These data indicate that ubiquitin might induce TNF-α accumulation mainly by up-regulation of the TNF-α gene transcription. Although extracellular functions of ubiquitin remain largely unknown, we postulate that ubiquitin might be involved in the modulatory mechanisms of immune response.

Keywords: Ubiquitin; Lipopolysaccharide; Tumor necrosis factor-α; Macrophage

1. Introduction

It has been reported that ubiquitin-like proteins, such as platelet activity suppressive lymphokine (PASL), ubiquitin cross-reactive protein (UCRP) and monoclonal non-specific suppressor factor (MNSF), were produced in characteristic cells, secreted to extracellular fluids, and exerted their immunoregulatory activities [1–3].

During parasitic infections, activated human platelets produce oxygen metabolites, but PASL released from activated $CD8^+$ T-cells could inhibit the oxygen metabolites production from activated platelets. Thus, PASL containing human ubiquitin moiety may be implicated in the mechanisms of cell-to-cell signaling [1,4]. Moreover, it was demonstrated that purified ubiquitin can inhibit platelet cytotoxicity [4]. UCRP, 15-kDa protein including ubiq-

Abbreviations: TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; IFN, interferon; Con-A, concanavalin-A; PASL, platelet activity suppressive lymphokine; UCRP, ubiquitin cross-reactive protein; MNSF, monoclonal non-specific suppressor factor; AGPC, acid guanidinium thiocyanate–phenol–chloroform

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uitin-like moiety, is induced by IFNs in human lymphocytes and monocytes, and is released into extracellular fluid from these cells, thereby involving in a cell-to-cell signaling pathway [2,5–7]. In our laboratory, it was reported that ubiquitin-like moiety of MNSF produced and released from murine T-cell hybridoma by Con-A, was shown to have antigen-non-specific suppressive function on the immunoresponse [8], including suppression of immunoglobulin secretion by LPS-induced murine splenocytes [3], IL-4 secretion by bone marrow-derived mast cells [9], and division in various tumor cell lines of murine origin [10].

It has been well recognized that ubiquitin, a highly conserved 8565-Da protein among the all eukaryotic cells, is involved in the degradation of short-lived or structurally abnormal intracellular proteins, and plays important roles in ubiquitin–proteasome system [11,12]. It was also reported that ubiquitin level in plasma from healthy donors was less than 50 ng/ml, although during parasitic infection, its level was significantly elevated to 150–300 ng/ml [13]. Ubiquitin was proved to stimulate differentiation in T- and B-cells derived from fractionated mouse spleen cells through β-adrenergic receptors and adenylate cyclase activation [14], and moreover ubiquitin added to the splenocytes culture significantly inhibited IgG production [5]. Therefore, not only ubiquitin-like proteins, but also ubiquitin in the extracellular space might have a possible regulatory role in the immunological and/or inflammatory processes, although its functions remain obscure.

On the other hand, TNF-α is a pleiotropic cytokine secreted by macrophage, mastocytes, T- and B-lymphocytes, and natural killer cells in response to various stimuli, such as LPS, viruses and parasites [15]. Multiple studies have demonstrated the important role of TNF-α in the pathogenesis of parasitic infection [16,17]. In experimental leishmaniasis, TNF-α is associated with progressive disease, and also, in malaria infection, a high level of TNF-α production has been correlated with severe complications [16].

The facts that ubiquitin, ubiquitin-like proteins and TNF-α increase in extracellular fluid during parasitic infection, and that ubiquitin or ubiquitin-like proteins also exert the immunoregulatory response in vitro were previously described [2–5,16]. Therefore, to explore the role of ubiquitin in the extracellular space, we investigated the effects of ubiquitin on TNF-α production using macrophage-like cell line, RAW 264.7. In this report, we demonstrated evidence of the synergistic effect of ubiquitin on LPS-induced TNF-α production, mainly due to the activation at the transcriptional level.

2. Materials and methods

2.1. Cells and reagents

The mouse macrophage-like cell line RAW 264.7 was obtained from RCB cell bank, and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. LPS (*Escherichia coli*, 0127: B8 Westphal type) was obtained from Difco and ubiquitin was from Sigma (St. Louis, MO). Monoclonal hamster anti-mouse TNF-α (α and β) IgG and polyclonal rabbit anti-mouse TNF-α was from Genzyme (Cambridge, MA). Peroxidase conjugated-IgG of goat anti-rabbit IgG was from Caplet. All other reagents were purchased from Miyata, Shimane, and were used without further purification.

2.2. TNF-α assay

RAW 264.7 cells (1×10^5 cells/ml) were seeded into 96-well microplates, and 5 h later, they were stimulated by the addition of LPS and/or ubiquitin. The cells were incubated for an additional 14 h, and supernatants were collected for TNF-α assay. Cultured cells in each well were sonicated with 1.0 ml of lysis buffer (25 mM HEPES, pH 7.5, 25 mM β-mercaptoethanol, 10 μM pepstatin, 10 μM leupeptin, 1% (v/v) Triton X-100) after the standing on ice bath for 30 min, and then cell lysates were assayed for cellular TNF-α. The TNF-α concentrations were determined by ELISA according to the method described previously [18].

2.3. Total RNA preparation, cDNA synthesis, and PCR amplification

Total RNA was prepared from cultured RAW

264.7 cells by AGPC method [19]. Total RNA was reverse-transcribed into cDNA by avian myeloblastosis virus-reverse transcriptase using random 9 mers as primers. PCR was carried out with Taq DNA polymerase (Takara, Japan) in an automatic DNA thermal cycler (Program Temp Control System PC-700, Astec). For TNF-α (471 bp) and the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (452 bp) amplification, the gene-specific primers were chosen as follows; 5′-CTCAGATCATCT-3′ (sense) and 5′-TCACAGAGCAAT-3′ (antisense), 5′-ACCACAGTCCATGCCATCAC-3′ (sense) and 5′-TCCACCACCCTGTTGCTGTA-3′ (antisense), respectively. PCR was performed for TNF-α and GAPDH under the following conditions; denaturation at 94°C for 1 min, and 94°C for 35 s; annealing at 42°C for 2 min, and 55°C for 35 s; extension at 72°C for 3 min, and 72°C for 2 min; 40 and 35 cycles, respectively.

2.4. Northern blot analysis

Total RNA (10 μg) was electrophoresed in 1.2% agarose-formaldehyde gels and transferred overnight to nylon filters by capillary action in 20×SSC. RNA was cross-linked to the filter by irradiation with UV light. After prehybridization, the filters were hybridized with random [α-^{32}P]dCTP (3000 Ci per nmol)-labeled probes for TNF-α. Then the filters were washed, dried and analyzed by autoradiography. The blots were also hybridized with a GAPDH cDNA probe, used as transfer control.

2.5. Determination of mRNA stability

RAW 264.7 cells were stimulated in the presence of 1 μg/ml LPS with or without 1 μM ubiquitin for 2 h, and then 5 μg/ml actinomycin D was added to culture medium. Total RNA was prepared and used for Northern blot analysis as described above. Densitometry of the gel photography was used to determine and quantify the levels of TNF-α mRNA. Estimates of the relative TNF-α mRNA amounts were obtained by dividing the peak densitometry area of TNF-α mRNA band by the area of GAPDH band.

2.6. Assay for protein degradation

RAW 264.7 cells were stimulated in the presence of 1 μg/ml LPS with or without 1 μM ubiquitin for 14 h, and then 10 μg/ml cycloheximide was added to culture medium. At 30 and 60 min after addition of cycloheximide, the cells were collected and sonicated with 1.0 ml of lysis buffer after the standing on ice bath and used for TNF-α assay.

3. Results

3.1. Effect of ubiquitin on LPS-induced TNF-α production

RAW 264.7 cells (1×10^5 cells/ml) were cultured with 10 ng/ml to 5 μg/ml LPS to confirm the effect of LPS for TNF-α production. The maximum accumulation of TNF-α in the culture medium was observed at 14 h and 1 μg/ml LPS concentration with about 7.4-fold increase compared with that in the absence of LPS (data not shown).

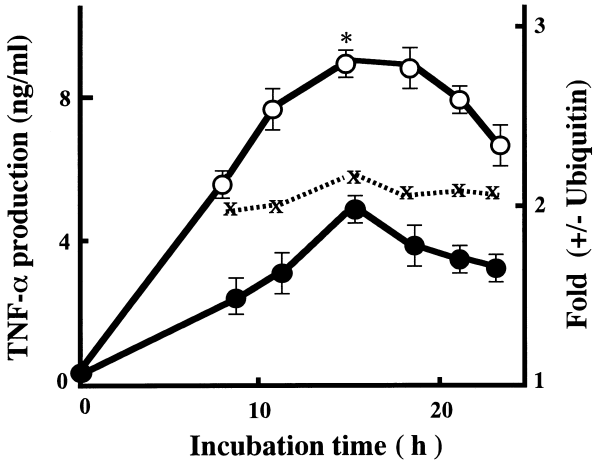

Fig. 1. Time course of LPS or LPS plus ubiquitin-induced TNF-α production. RAW 264.7 cells (1×10^5 cells/ml) were cultured with (○) or without (●) 1 μM ubiquitin in the presence of 1 μg/ml LPS. The ratio of the TNF-α production by 1 μg/ml LPS plus 1 μM ubiquitin to that by 1 μg/ml LPS alone is shown (×). The amounts of TNF-α in the culture supernatants were determined by ELISA as described in the Section 2. Data are the mean ± S.D. of six different experiments conducted in triplicate. *$P < 0.002$, compared with LPS alone at 14 h.

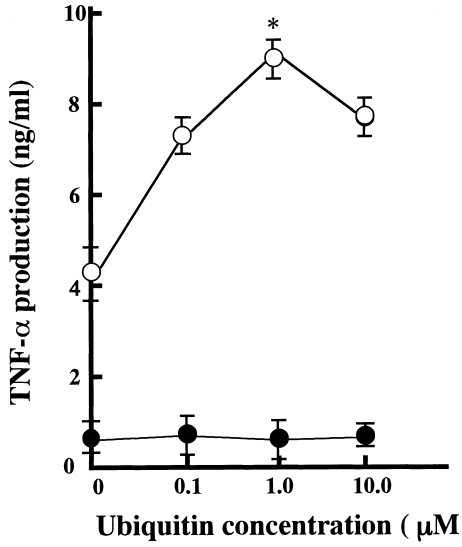

Fig. 2. Effect of increasing concentration of ubiquitin on LPS-induced TNF-α production. RAW 264.7 cells (1×10^5 cells/ml) were cultured with the indicated doses of ubiquitin (0.1, 1 and 10 μM, respectively) for 14 h in the presence (○) or absence (●) of 1 μg/ml LPS. The amounts of TNF-α in the culture supernatants were determined by ELISA as described in the Section 2. Data are the mean ± S.D. of six independent experiments conducted in triplicate. $*P<0.002$, at the concentration of 1 μM ubiquitin compared with LPS alone.

To see whether ubiquitin could affect on the TNF-α accumulation induced by LPS, RAW 264.7 cells (1×10^5 cells/ml) were cultured with or without 1 μM ubiquitin in the presence of 1 μg/ml LPS for 0–24 h. One μM ubiquitin and 1 μg/ml LPS added to RAW 264.7 cell cultures increased TNF-α production by about 2-fold compared with LPS alone at 14 h (Fig. 1). Although maximum accumulation was achieved at 14 h after addition of 1 μM ubiquitin in the presence of 1 μg/ml LPS, TNF-α accumulation gradually declined with further incubation. As included in Fig. 1, the ratio of ubiquitin plus LPS-stimulated TNF-α accumulation to LPS-induced one was almost constant at the level of about 2-fold elevation. During the incubation of RAW 264.7 cells with ubiquitin in the presence of LPS, the number of RAW 264.7 cells was almost constant, therefore this elevation of TNF-α was not dependent on cell proliferation.

Next, we tested the effect of ubiquitin concentration on LPS-induced TNF-α production. As shown in Fig. 2, ubiquitin increased TNF-α production in dose-dependent manner in the presence of LPS. Maximal accumulation with about 2-fold elevation was observed at the concentration of 1 μM ubiquitin compared with 1 μg/ml LPS alone. The addition of 10 μM ubiquitin to RAW 264.7 cell cultures decreased TNF-α production compared with 1 μM ubiquitin. When ubiquitin (0.1, 1 and 10 μM, respectively) alone was added to RAW 264.7 cells culture, no significant increase of TNF-α was detected (Fig. 2).

To eliminate the possibility that some cytokines, such as IFN-γ, contaminated during the ubiquitin preparation, might confuse the effect of ubiquitin since ubiquitin used in these experiments was purified from bovine red cells, we treated the ubiquitin preparation at 100°C, for 5 min. Ubiquitin is stable on heat-treatment at 100°C for 5 min [1], but not cytokines [20–23]. One μM heat-treated ubiquitin caused appreciable LPS-induced TNF-α accumulation; the magnitude of increase by heat-treated ubiquitin was comparable to that of non-heat-treated ubiquitin (9.02 ± 0.61 ng/ml by 1 μM heat-treated ubiquitin

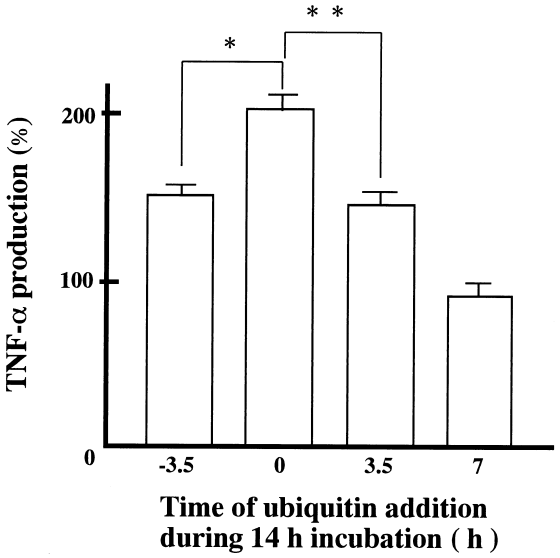

Fig. 3. Effect of the time point of ubiquitin addition on LPS-induced TNF-α production. RAW 264.7 cells (1×10^5 cells/ml) were stimulated with 1 μg/ml LPS for 14 h. Ubiquitin was added at the 3.5 h before and 0, 3.5, 7 h after 1 μg/ml LPS addition. The amounts of TNF-α in the culture supernatants were determined by ELISA as described in the Section 2. The values are the percentages mean ± S.D. of six different experiments conducted in triplicate. Percent value was set as 100% when TNF-α was induced by 1 μg/ml LPS alone for 14 h (4.13 ± 0.47 ng/ml). $*P<0.01$, at −3.5 h compared with 0 h. $*P<0.01$, at 3.5 h compared with 0 h.

and 8.85 ± 0.02 ng/ml by 1 μM non-heat-treated ubiquitin, respectively). These observations ruled out that ubiquitin-stimulated TNF-α accumulation in the presence of LPS was caused by cytokines contaminated in the prepared ubiquitin. Hence, these results suggest that ubiquitin itself acts as a synergistic effector for LPS on TNF-α production.

To investigate the time dependence for the synergistic effect of ubiquitin and LPS on TNF-α production, RAW 264.7 cells (1×10^5 cells/ml) were stimulated with LPS before or after treatment of ubiquitin. As shown in Fig. 3, the pretreatment with ubiquitin for 3.5 h before LPS treatment showed about 152% of TNF-α accumulation compared with the addition of LPS and ubiquitin simultaneously (approximately 200% at 0 h). The addition of ubiquitin at 3.5 h after the treatment of RAW 264.7 cells with LPS showed about 147% of TNF-α accumulation, and when ubiquitin was added at 7 h after LPS-treatment, no significant stimulation was observed. These results suggest that the stimulatory effect of ubiquitin on the LPS-induced TNF-α production required the presence of ubiquitin at the early period of the TNF-α production.

3.2. Effect of ubiquitin on TNF-α secretion

TNF-α is composed of membrane-bound precursor and mature secretable form [24–30]. To investigate which form was increased by ubiquitin and whether ubiquitin accelerates the release of LPS-induced TNF-α to extracellular culture medium, both cellular and extracellular TNF-α concentrations were measured. Following 14 h incubation with or without 1 μM ubiquitin in the presence of 1 μg/ml LPS, TNF-α in the culture medium and cell lysates were measured, and determined as the extracellular and cellular TNF-α, respectively. As shown in Table 1, the accumulation of extracellular TNF-α exhibited about two-fold elevation by the addition of 1 μM ubiquitin in the presence of LPS (see Fig. 1). However, no elevation of the cellular TNF-α amounts was observed (compared with that of LPS alone). These observations suggest that the extracellular TNF-α accumulation is major action of ubiquitin, but not cellular accumulation.

Table 1
Cellular and extracellular TNF-α concentrations after LPS or LPS plus ubiquitin treatment

Substances added to culture	TNF-α (ng/ml)	
	Cellular	Extracellular
None	0.68 ± 0.20	0.65 ± 0.18
LPS (1 μg/ml)	0.91 ± 0.23	4.80 ± 0.83
LPS (1 μg/ml)+ubiquitin (1 μM)	0.89 ± 0.11	8.82 ± 0.03[a]

RAW 264.7 cells (1×10^5 cells/ml) were cultured with or without 1 μM ubiquitin in the presence of 1 μg/ml LPS for 14 h. The amounts of TNF-α in the culture supernatants and cell lysates are indicated as extracellular and cellular TNF-α, respectively. The TNF-α concentrations were determined by ELISA is as described in the Section 2. The values are the mean ± S.E. of six experiments conducted in triplicate.

[a] $P < 0.006$, compared with extracellular TNF-α stimulated by LPS alone.

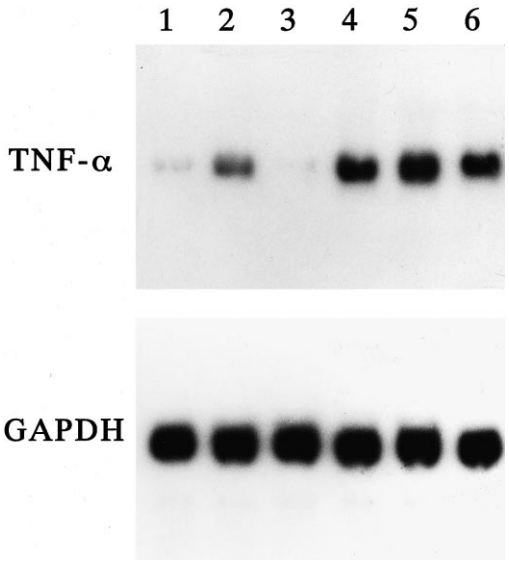

Fig. 4. Effect of ubiquitin, LPS and both on TNF-α mRNA expression. RAW 264.7 cells (1×10^5 cells/ml) were stimulated without (lane 1) or with 1 μg/ml LPS (lane 2), 1 μM ubiquitin (lane 3), and with 0.1 μM (lane 4), 1.0 μM (lane 5), or 10 μM ubiquitin (lane 6) plus 1 μg/ml LPS for 2 h. Cells were then analyzed for the TNF-α mRNA expression by Northern blot analysis. Total RNA (10 μg) was hybridized either to radiolabeled TNF-α cDNA probe or to radiolabeled GAPDH cDNA probe and exposed to X-ray film for 18 h. The data are representative of four independent experiments.

3.3. Effect of ubiquitin on TNF-α mRNA synthesis and degradation

Next, we considered whether ubiquitin treatment with LPS could affect the transcriptional level of TNF-α production. To approach this issue, we inves-

tigated the effect of ubiquitin on TNF-α mRNA production in RAW 264.7 cells. When 1×10^5 cells were cultured with 1 μg/ml LPS for 2 h, the increase of TNF-α mRNA production was observed (Fig. 4, lane 2), although the addition of 1 μM ubiquitin alone did not enhance TNF-α mRNA expression (lane 3). The coexistence of 1 μM ubiquitin with 1 μg/ml LPS exhibited apparent augmentation (two-fold) compared with 1 μg/ml LPS alone (lanes 5 and 2). The treatment of 0.1 or 10 μM ubiquitin plus 1 μg/ml LPS slightly increased TNF-α mRNA production compared with that of 1 μg/ml LPS alone (lanes 4, 6 and 2). It is worth noting that these results present unambiguous evidence supporting the dose-dependency of ubiquitin-induced TNF-α production observed in Fig. 2.

Next, we performed TNF-α mRNA stability experiments to assess whether ubiquitin would affect the half-life of TNF-α mRNA. After RAW 264.7 cells were cultured in the presence of 1 μg/ml LPS with or without 1 μM ubiquitin for 2 h, subsequently 5 μg/ml actinomycin D was added into the culture medium. The cells were cultured additional 1, 2 and 4 h, and the TNF-α mRNA levels were determined. As shown in Fig. 5, the half-life of TNF-α mRNA stimulated with LPS alone and that with ubiquitin in the presence of LPS were determined to be approximately 2.0 and 2.1 h, respectively. These results suggest that the effect of ubiquitin on the up-regulation of TNF-α mRNA was dependent on the transcription rate and scarcely on mRNA stability.

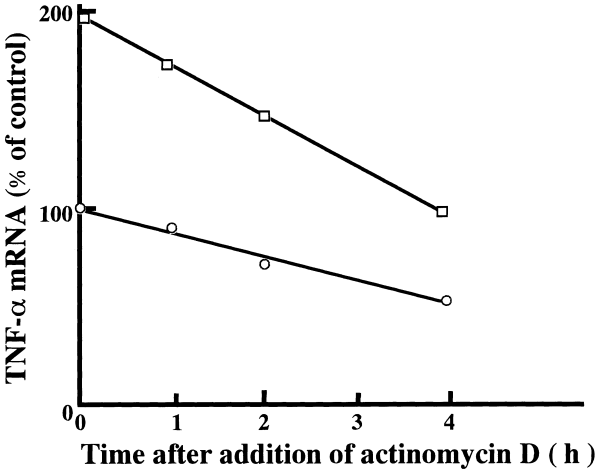

Fig. 5. Effect of ubiquitin on TNF-α mRNA stability. RAW 264.7 cells (1×10^5 cells/ml) were stimulated with 1 μg/ml LPS alone (○) or 1 μg/ml LPS plus 1 μM ubiquitin (□) for 2 h. After addition of 5 μg/ml actinomycin D, total RNA was prepared at the indicated time points. Cells were then analyzed for the TNF-α mRNA expression by Northern blotting. Total RNA (10 μg) was hybridized either to radiolabeled TNF-α cDNA probe or to radiolabeled GAPDH cDNA probe and exposed to X-ray film for 18 h. TNF-α mRNA obtained before the addition of actinomycin D by LPS alone was set as 100%. The data are representative of four independent experiments.

3.4. Effect of ubiquitin on TNF-α degradation

The possibility that ubiquitin treatment may cause TNF-α accumulation by the suppression of TNF-α degradation remains to be ruled out. To see TNF-α degradation, RAW 264.7 cells were treated for 14 h with or without 1 μM ubiquitin in the presence of 1 μg/ml LPS, and then 10 μg/ml cycloheximide was added to the culture medium. The cells were collected and sonicated with the lysis buffer at 30 and 60 min, and each lysate was then assayed for TNF-α [18]. The TNF-α concentration in the cells stimulated with or without ubiquitin in the presence of LPS both proportionally decreased (Table 2). No obvious difference on TNF-α degradation between the cells stimulated by LPS and LPS plus ubiquitin was detected. These results indicate that ubiquitin treatment never affects the TNF-α degradation in the RAW 264.7 cells.

Table 2
Effect of ubiquitin on TNF-α degradation

Substances added to culture	Incubation time (min)		
	0	30	60
LPS (1 μg/ml)	0.99 ± 0.03	0.86 ± 0.06	0.71 ± 0.03
LPS (1 μg/ml)+ubiquitin (1 μM)	1.01 ± 0.05	0.81 ± 0.08	0.66 ± 0.02

RAW 264.7 cells (1×10^5 cells/ml) were cultured with or without 1 μM ubiquitin in the presence of 1 μg/ml LPS for 14 h. After 10 μg/ml cycloheximide was added to culture medium, the cells were sonicated with the lysis buffer at the indicated time, and each lysate was used for the TNF-α assay. The data are the mean ± S.D. of six experiments conducted in triplicate.

4. Discussion

Ubiquitin, a highly conserved protein composed of 76 amino acids, is present in all eukaryotic cells, and involved in the degradation of short-lived or structurally abnormal proteins [31–34]. Though an extracellular localization of ubiquitin was described in human seminal fluid or in plasma where the ubiquitin levels is less than 50 ng/ml, its function in this fluid remains unclear [13]. The production of cytotoxic metabolites, which is produced by IgE antibodies-activated platelets and exhibit killing properties against larvae of *Schistosoma mansoni* [1,35–37] is remarkably inhibited by PASL, released from Con-A or antigen-stimulated $CD8^+$ T-cells. It was identified that its encoded nucleic acid and predicted amino acid sequences were partially identical to human ubiquitin, moreover purified ubiquitin was able to inhibit platelet cytotoxicity [4]. Thus, ubiquitin itself may possess unrecognized immunoregulatory activities in addition to its role in a variety of cellular processes. TNF-α and PASL concentrations would increase in patients with parasitic disease, such as shistosomiasis, *Hymenoptera* venom hypersensitivity (HVH), bacterial infection and neoplastic disease [1,4,38]. Therefore, we began to interest ourselves in the research whether ubiquitin had some influence on TNF-α production.

In RAW 264.7 cell cultures, we observed that the addition of 1 μM ubiquitin to 1 μg/ml LPS resulted in about two-fold increase in TNF-α production compared with 1 μg/ml LPS alone, however ubiquitin alone did not affect on TNF-α production. Therefore, we aimed at determining the mechanism by which ubiquitin augmented TNF-α production in LPS-induced RAW 264.7 cells. When 1 μM ubiquitin and 1 μg/ml LPS were simultaneously added to RAW 264.7 cells culture, maximal production of TNF-α was observed. These results clearly demonstrate the synergistic effect of ubiquitin on LPS induced TNF-α production in RAW 264.7 cells. We found that 1 μM ubiquitin with LPS increased the TNF-α mRNA levels about two-fold compared with LPS alone. These data are almost paralleled with the data obtained in Fig. 2, that TNF-α production was increased about two-fold by the addition of 1 μM ubiquitin in the presence of LPS compared with LPS alone. Ubiquitin did not influence on the half-life of LPS-induced TNF-α mRNA, and also on the degradation of TNF-α. The effect of ubiquitin on the translational level was not undertaken, although the increase in the both TNF-α mRNA and protein levels by the ubiquitin with LPS was almost two-fold, probably suggesting few implication of ubiquitin on the translational level of TNF-α. Extracellular accumulations of TNF-α by LPS plus ubiquitin were twice than those by LPS alone, but cellular accumulations of TNF-α were no difference. These data indicate that TNF-α induced by ubiquitin and LPS would be immediately released into the extracellular space, and never remain as the pro TNF-α molecule. Many studies showed that processing of 26-kDa pro TNF-α to mature 17-kDa TNF-α was blocked by inhibitors of metalloproteinases, and pro TNF-α was processed to mature TNF-α by several matrix metalloproteinases, such as matrilysin and collagenase [39–41]. Recently, the processing protease of TNF-α, called TNF-α-converting enzyme (TACE), was purified and cloned [42]. We did not research whether ubiquitin promoted TNF-α processing or not, although the processing enzyme might possess enough activities to cleave the pro TNF-α, even if the pro TNF-α would increase twice by the treatment of ubiquitin with LPS. It is widely accepted that the expressions of the TNF-α gene by LPS are regulated both at the transcriptional and post-transcriptional levels [15]. Thus, we concluded that the synergistic effect of ubiquitin with LPS on TNF-α accumulation was mainly caused by the up-regulation of TNF-α gene transcription. This is the first evidence as far as we know.

Recently, we observed that treatment with ubiquitin alone did not augment the activity of the inducible nitric oxide synthase (i-NOS), and stimulation of RAW 264.7 cells with LPS plus ubiquitin also did not affect on the i-NOS production compared with LPS alone (T. Nabika and I. Momose, unpublished data). LPS binds to its own receptor and induces i-NOS expression as well as TNF-α expression via the activation of NF-κB [15,43]. Since ubiquitin influenced on the TNF-α production in RAW 264.7 cells stimulated by LPS, but not on the i-NOS production, ubiquitin may interact with transcriptional factor(s) other than NF-κB. Cytokines produced by activated macrophages, such as interleukin-1 (IL-1), IL-6 and IL-8, also have important effects on immu-

nological and inflammatory responses [43–45]; therefore, it would be interesting to examine the effect of ubiquitin on these cytokines to clarify the specificity for the action or the signal transduction pathways of ubiquitin.

Liovera et al. [46] reported that injections of polyclonal goat anti-murine TNF IgG preparation into tumor-bearing rats abolished the increase in muscle ubiquitin gene expression, showing that TNF could have an important role in the activation of the ubiquitin-dependent proteolytic system during tumor growth. This experiment was conducted in isolated rat soleus muscles and human recombinant TNF-α resulted in a remarkable increase in ubiquitin gene expression [47]. Taken together with our own data, it can be speculated that ubiquitin and TNF-α may interact with each other in a multiple network in vivo. Moreover, it remains unknown whether ubiquitin acts on RAW 264.7 macrophage via its own receptor or another mode, but Pancré et al. [4] described that PASL probably acted via a receptor present on the platelet membrane. Therefore, several studies in regard to the above described network or action mechanisms of ubiquitin are currently underway to confirm the precise function of ubiquitin in macrophage activation.

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