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Thalidomide inhibits lipopolysaccharide-induced tumor necrosis factor-α production via down-regulation of MyD88 expression

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The effect of thalidomide on lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α production was studied by using RAW 264.7 murine macrophage-like cells. Thalidomide significantly inhibited LPS-induced TNF-α production. Thalidomide prevented the activation of nuclear factor (NF)-κB by down-regulating phosphorylation of inhibitory κB factor (IκB), and IκB kinase (IKK)-α and IKK-β Moreover, thalidomide inhibited LPS-induced phosphorylation of AKT, p38 and stress-activated protein kinase (SAPK)/JNK. The expression of myeloid differentiation factor 88 (MyD88) protein and mRNA was markedly reduced in thalidomide-treated RAW 264.7 cells but there was no significant alteration in the expression of interleukin-1 receptor-associated kinase (IRAK) 1 and TNF receptor-associated factor (TRAF) 6 in the cells. Thalidomide did not affect the cell surface expression of Toll-like receptor (TLR) 4 and CD14, suggesting the impairment of intracellular LPS signalling in thalidomide-treated RAW 264.7 cells. Thalidomide significantly inhibited the TNF-α production in response to palmitoyl-Cys(*RS*)-2,3-di(palmitoyloxy) propyl)-Ala-Gly-OH (Pam_3Cys) as a MyD88-dependent TLR2 ligand. Therefore, it is suggested that thalidomide might impair LPS signalling via down-regulation of MyD88 protein and mRNA and inhibit LPS-induced TNF-α production. The putative mechanism of thalidomide-induced MyD88 down-regulation is discussed.

Keywords: Thalidomide, LPS, TNF-α, MyD88, RAW 264.7 cells

INTRODUCTION

Thalidomide (*N*-α-phthalimidoglutarimide) is known as an anti-angiogenic, anti-tumor, and anti-proliferative agent, widely used in the treatment of some immunological disorders and cancers specially in multiple myeloma.[1,2] Further, it exhibits an anti-inflammatory activity and inhibits production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6.[3,4] In particular, thalidomide has been extensively studied to modulate production of TNF-α and the TNF-α -modulating activity seems to be closely related to a series of actions of thalidomide.[5,6] Thalidomide inhibits TNF-α production via degradation of TNF-α mRNA,[7] or inactivation of nuclear factor (NF)-κB.[8] On the other hand, thalidomide is reported to enhance TNF-α production.[9] Thus, the precise action and mechanism of thalidomide on TNF-α production is still controversial.

Lipopolysaccharide (LPS) is present in the outer membranes of Gram-negative bacteria and causes systemic inflammatory response syndrome, endotoxic shock, disseminated intravascular coagulation and multiple organ failure. LPS-induced tissue injury and lethality is caused mainly by TNF-α. Therefore, the regulation of LPS-induced TNF-α production by

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thalidomide might have therapeutic potential. The LPS-induced TNF-α production is principally controlled by Toll-like receptor (TLR) 4-mediated myeloid differentiation factor 88 (MyD88) dependent signalling.[10] Further, it involves a series of signalling pathways including NF-κB,[11] mitogen-activated protein kinases (MAPKs),[12–14] such as extracellular signal regulated kinase (ERK) 1/2, p38 MAPK, stress-activated protein kinase (SAPK)/JNK, and phosphatidylinositol 3-kinase (PI3K)/AKT.[15] In the present study, we investigated if and how thalidomide modulated LPS-induced TNF-α production in RAW 264.7 murine macrophage-like cells. Here, we report that thalidomide might inhibit TNF-α production in LPS-stimulated RAW 264.7 cells via down-regulation of MyD88 expression.

MATERIALS AND METHODS

Materials

LPS from *Escherichia coli* O55 : B5 and thalidomide were purchased from Sigma Chemicals (St Louis, MO, USA) and Wako Pure Chemicals (Osaka, Japan), respectively. Palmitoyl-Cys(*RS*)-2,3-di(palmitoyloxy) propyl)-Ala-Gly-OH (Pam_3Cys) and MG 132 were obtained from Calbiochem (San Diego, CA, USA).

Cell culture

The murine macrophage-like cell line, RAW 267.4, was obtained from the Riken Cell Bank (Tsukuba, Japan) and maintained in RPMI 1640 medium containing 5% heat-inactivated fetal bovine serum (Gibco-BRL; Gaithersburg, MD, USA) and antibiotics at 37°C under 5% CO_2. Peritoneal cells were obtained by washing out the peritoneal cavity of BALB/c mice (Japan SLC; Hamamatsu, Japan) with RPMI 1640 medium and adherent cells were used as peritoneal macrophages. The experiment was carried out under the guide for care and use of laboratory animals, Aichi Medical University.

Determination of TNF-α production

The concentration of TNF-α in the culture supernatant was determined by an ELISA kit (Biosource International; Camarillo, CA, USA) according to the manufacturer's instructions. Experiments were carried out at least three times and the results are presented as the mean of triplicates ± SD.

Luciferase reporter gene assay for NF-κB activation

RAW 264.7 cells (3×10^5 cells/ml) were incubated in a 35-mm plastic dish for 24 h. The cells were transfected with 0.5 mg/ml of pNF-κB-TA-luc luciferase reporter gene (Invitrogen; Carlsbad, CA, USA) and 0.05 mg/ml of pRL-TK plasmid (Promega; Madison, WI, USA) by lipofectamine 2000 transfection reagent (Gibco-BRL). On the following day, the cells were pretreated with various concentrations of thalidomide, followed by treatment with LPS (100 ng/ml) for various times. The cells were lysed by the lysis reagent from Promega. The NF-κB luciferase activity in the cell lysates was determined with a luminometer and the β-galactosidase activity was used to normalize transfection efficiencies. Experimental results are presented as the mean ± SD for three experiments in which each transfection was performed in duplicate.

Immunoblotting

The immunoblotting method was described previously.[16] Briefly, the cell lysates were extracted by lysis buffer containing 0.5 M Tris-HCl, 4% sodium dodecyl sulfate (SDS), 2-mercaptothanol, and protease inhibitor cocktail (Sigma) and boiled at 80°C for 5 min. The protein concentration of each sample was determined by bicinchoninic acid protein assay reagent (Pierce; Rockford, IL, USA). Equal amounts of protein (20 μg) were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred to a membrane filter. A series of antibodies were used as follows: rabbit polyclonal antibodies to p65 NF-κB, the phosphorylated form, p38 and AKT, and mouse monoclonal antibodies to phosphorylated forms of IκB-α, p38 (1 : 2000) and AKT from Cell Signaling Technology (Beverly, MA, USA); rabbit polyclonal antibodies to TNF receptor-associated factor (TRAF) 6, MyD88 (1 : 3000), CD14 (1 : 2000) and β-actin (1 : 500), goat polyclonal antibody to TLR4, and mouse monoclonal antibody to interleukin-1 receptor-associated kinase (IRAK)1 (1 : 2000) from Santa-Cruz Biotechnology (Santa-Cruz, CA, USA); rabbit polyclonal antibodies to IκB kinase (IKK)-α and IKK-β and the phosphorylated forms from Abcam (Science Park, Cambridge, UK). Unless otherwise stated, antibodies were used at 1 : 1000 dilution. The immune complexes were detected with horseradish peroxidase-conjugated protein G (eBioscience; San Diego, CA, USA) at 1:5000 for 1 h and the bands were visualized with a chemiluminescent reagent (Pierce). The chemiluminescence was detected by a light capture system analyzer AE6955 (Atto; Tokyo, Japan).

Laser flow cytometric analysis

RAW 264.7 cells were seeded at 1×10^6 cells/ml in a 35-mm dish for 18 h and then treated with thalidomide (100 μg/ml) for 1 h. The cells were incubated with

PE-conjugated antibody to TLR4 (eBioscience) or CD14 (PharMingen; San Diego, CA, USA) on ice for 30 min, washed and resuspended in saline. TLR4 or CD14-positive cells were analyzed by a fluorescence-activated cells sorter (BD FACS Calibur; San Jose, CA, USA). Phycoeythrin-conjugated anti-rat isotype IgG (PharMingen) was used as a negative control.

Quantitative reverse transcription–polymerase chain reaction (PCR) analysis

Quantitative reverse transcription-PCR was performed essentially as described elsewhere.[17] Ribonucleic acid was extracted from cells using the RNeasy mini kit (Qiagen; Chatsworth, CA, USA). Ribonucleic acid was reverse-transcribed in RT buffer (Toyobo; Osaka, Japan) with a three-step incubation according to the manufacturer's instructions, and quantitative PCR was carried out using SYBR green real-time PCR master mix (Toyobo) as recommended by the manufacturer. Mouse primers were designed as follows: MyD88 (sense, 5′-CTCCTCCACATCCCTTCC-3′; antisense, 5′-CCGCACGTTCAAGAACAGAGA-3′); TNF-α (sense, 5′-TGTTGCCTCCTCTTTTGCTT-3′; antisense, 5′-TGGTCACCAAAATCAGCGTTA-3′); GAPDH (sense, 5′-TGAAGCAGGCATCTGAGGG-3′; antisense, 5′-CGAAGGTGGAAGAGTGGGAG-3′). All three primer sets were obtained from Invitrogen. Polymerase chain reaction was performed with ABI PRISM 7700 sequence detection system (Applied Biosystems; Hamilton, New Zealand) and the PCR conditions were as follows: 95°C for 10 min and 40 cycles at 95°C for 30 s, 60°C for 1 min. The relative quantitative values of MyD88 and TNF-α expression in each case were normalized by the expression levels of reference gene GAPDH. The expression levels of MyD88 and TNF-α in each sample are presented as fold increase to the mean value of the control.

Statistical analysis

Experimental data are presented as the mean ± SD from at least three independent experiments. The significance of differences between experimental and control groups was determined with Student's *t*-test. A value of $P < 0.01$ was considered statistically significant.

RESULTS

Thalidomide inhibits the production of TNF-α in LPS-stimulated RAW 264.7 cells

The effect of thalidomide on LPS-induced TNF-α production was examined by using RAW 264.7 cells (Fig. 1).

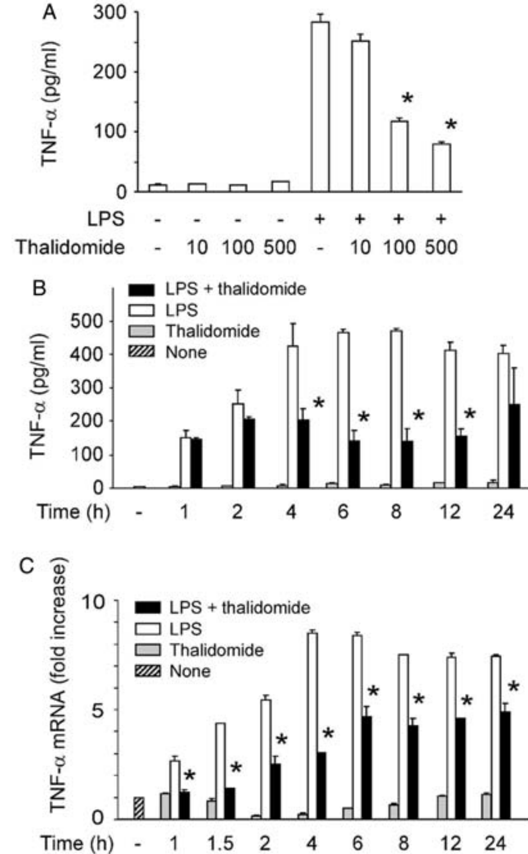

Fig. 1. Effect of thalidomide on LPS-induced TNF-α production. (A) RAW 264.7 cells were pretreated with thalidomide at 10, 100 or 500 μg/ml for 1 h and stimulated with LPS (100 ng/ml) for 6 h. The concentration of TNF-α in the supernatant was measured with ELISA. (B) RAW 264.7 cells were pretreated with thalidomide at 100 μg/ml for 1 h and stimulated with LPS (100 ng/ml) for various time periods. The level of TNF-α protein was determined by ELISA. $*P < 0.01$ versus LPS alone. (C) RAW 264.7 cells were pretreated with thalidomide at 100 μg/ml for 1 h and stimulated with LPS (100 ng/ml) for various time periods. The level of TNF-α mRNA was determined by real-time PCR. $*P < 0.01$ versus LPS alone.

RAW 264.7 cells produced a large amount of TNF-α in response to LPS whereas the cells pretreated with thalidomide at 100 μg/ml and 500 μg/ml produced much less (Fig. 1A). Thalidomide inhibited LPS-induced TNF-α production roughly in a concentration-dependent manner. Thalidomide did not significantly inhibit LPS-induced IL-6 production at 6 h (data not shown). The inhibition of LPS-induced TNF-α production by thalidomide was consistent with previous studies.[6,7] Thalidomide at 500 μg/ml exhibited no cytotoxic action against RAW 264.7 cells with the MTT assay (Chemicon; Temecula, CA, USA) and the cell viability of thalidomide-pretreated RAW 264.7 cells did not decrease in response to LPS (data not shown).

The time course of LPS-induced TNF-α production in thalidomide-pretreated RAW 264.7 cells was examined (Fig. 1B). Thalidomide-pretreated cells produced

a decreased amount of TNF-α 4 h after LPS stimulation than untreated cells, and the reduction continued up to 12 h. The expression of TNF-α mRNA in thalidomide-pretreated RAW 264.7 cells was also examined (Fig. 1C). Thalidomide pretreatment inhibited the TNF-α mRNA expression 1 h after LPS stimulation and the inhibition was seen at 24 h.

Thalidomide inhibits the activation of NF-κB in LPS-stimulated RAW 264.7 cells

Thalidomide is reported to inhibit NF-κB activation,[18] and subsequent TNF-α production.[8] Therefore, the effect of thalidomide on LPS-induced NF-κB activation was examined in RAW 264.7 cells (Fig. 2). First, we studied the NF-κB activation with a luciferase reporter gene assay. As shown in Figure 2A, LPS markedly enhanced the reporter gene activity in RAW 264.7 cells. On the other hand, thalidomide at 100 μg/ml definitely inhibited LPS-induced augmentation of the NF-κB-dependent reporter gene activity. Subsequently, we examined the time course of LPS-induced NF-κB activation in thalidomide-pretreated cells (Fig. 2B). The NF-κB activation assessed by the reporter gene activity was significantly inhibited in thalidomide-pretreated cells 2 h after LPS stimulation. The inhibition continued up to 24 h.

Further, we examined the phosphorylation of p65 NF-κB by immunoblotting using anti-phosphorylated p65 antibody to confirm the impaired NF-κB activation. Thalidomide remarkably inhibited LPS-induced phosphorylation of p65 (Fig. 2C).

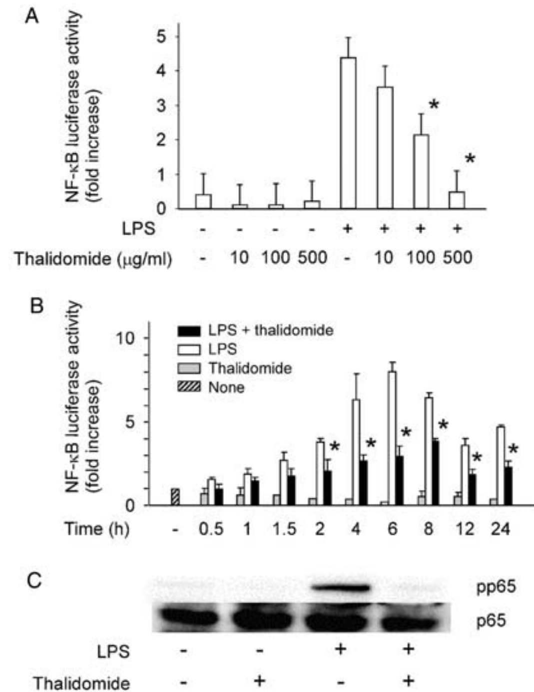

Fig. 2. Effect of thalidomide on LPS-induced NF-κB activation. (A) RAW 264.7 cells transfected with the reporter gene were pretreated with thalidomide at 10, 100 or 500 μg/ml for 1 h and stimulated with LPS (100 ng/ml) for 8 h. *$P<0.01$ versus LPS alone. (B) RAW 264.7 cells transfected with the reporter gene were pretreated with thalidomide (100 μg/ml) for 1 h and stimulated with LPS (100 ng/ml) for various time periods. NF-κB activity is expressed as a relative luciferase activity. *$P<0.01$ versus LPS alone. (C) RAW 264.7 cells were pretreated with thalidomide (100 μg/ml) for 1 h and stimulated with LPS (100 ng/ml) for 30 min. The phosphorylated form of p65 was detected by immunoblotting with an anti-phosphorylated p65 (pp65) antibody.

Thalidomide inhibits the phosphorylation of IκB, IKK and AKT in LPS-stimulated RAW 264.7 cells

In the preceding section, we demonstrated the inhibition of NF-κB activation by thalidomide. Therefore, the effect of thalidomide on the phosphorylation of IκB as the upstream molecule of NF-κB was examined by immunoblotting with anti-phosphorylated IκB-α antibody (Fig. 3A). Inhibitory κB-α was phosphorylated within 15 min following LPS stimulation and the phosphorylation was detectable 2 h after LPS stimulation. On the other hand, thalidomide significantly inhibited LPS-induced IκB-α phosphorylation.

IKK-α and IKK-β are the upstream kinases of IκB in the NF-κB signalling pathway and phosphorylate IκB-α and IκB-β, followed by NF-κB activation.[19] Based on the inhibition of LPS-induced IκB-α phosphorylation by thalidomide, the effect of thalidomide on LPS-induced IKK-α and IKK-β activation was examined by immunoblotting using antibodies to phosphorylated IKK-α and IKK-β (Fig. 3B). Thalidomide inhibited LPS-induced phosphorylation of IKK-α and IKK-β, although LPS definitely induced phosphorylation of IKK-α and IKK-β 30 min after the treatment.

LPS-induced NF-κB activation is regulated by several signal molecules, such as NIK,[20] and AKT.[21] The AKT signal molecule is known to regulate NF-κB activation via IKK activation.[22] Therefore, the effect of thalidomide on LPS-induced AKT activation was examined (Fig. 3C). LPS clearly induced the phosphorylation of AKT whereas thalidomide definitely inhibited LPS-induced AKT phosphorylation.

Thalidomide inhibits the phosphorylation of a series of MAPKs in LPS-stimulated RAW 264.7 cells

LPS is known to activate a series of MAPKs, such as p38 and SAPK/JNK, in macrophages and trigger TNF-α production via the signalling pathways.[12–14] Therefore, the effect of thalidomide on the activation of these MAPK pathways was examined by immunoblotting using antibodies to their phosphorylated forms (Fig. 4). LPS rapidly induced the phosphorylation of p38 and

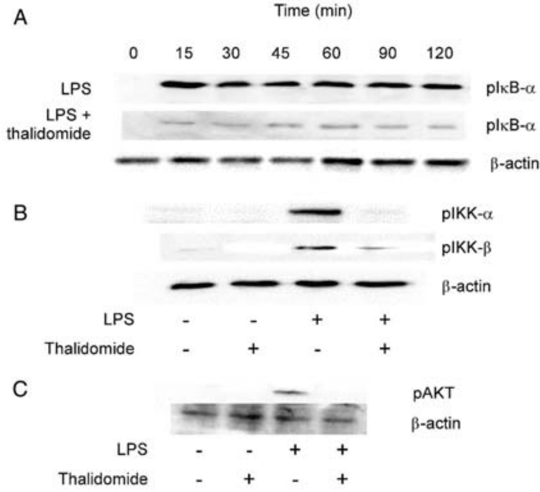

Fig. 3. Effect of thalidomide on LPS-induced phosphorylation of IκB-α, IKK-α, IKK-β and AKT. (A) RAW 264.7 cells were pretreated with thalidomide (100 μg/ml) for 1 h and stimulated with LPS (100 ng/ml) for various time periods. The phosphorylation of IκB-α was detected by immunoblotting with an anti-phosphorylated IκB-α (pIκB-α) antibody. (B,C) RAW 264.7 cells were pretreated with thalidomide (100 μg/ml) for 1 h and stimulated with LPS (100 ng/ml) for 30 min. The phosphorylated forms of IKK-α and IKK-β (B), and AKT (C) were detected by immunoblotting with antibodies to IKK-α, IKK-β, AKT, and their phosphorylated forms (pIKK and pAKT). β-Actin was used as the control.

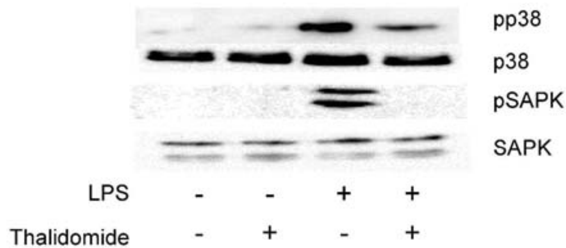

Fig. 4. Effect of thalidomide on LPS-induced activation of p38 and SAPK/JNK. RAW 264.7 cells were pretreated with thalidomide (100 μg/ml) for 1 h and stimulated with LPS (100 ng/ml) for 30 min. The phosphorylation of p38 and SAPK/JNK were detected by immunoblotting with antibodies to p38, SAPK/JNK, and their phosphorylated forms (pp38 and pSAPK).

SAPK/JNK 30 min after the stimulation. Thalidomide significantly inhibited the phosphorylation of SAPK and p38. The inhibition of p38 by thalidomide was less marked than that of SAPK.

Thalidomide inhibits MyD88-dependent signal pathway via reduced MyD88 expression

It became clear that thalidomide inhibited the activation of NF-κB, p38 and SAPK/JNK. Those molecules belong to the MyD88-dependent pathway in LPS signalling. Therefore, we examined the effect of thalidomide on

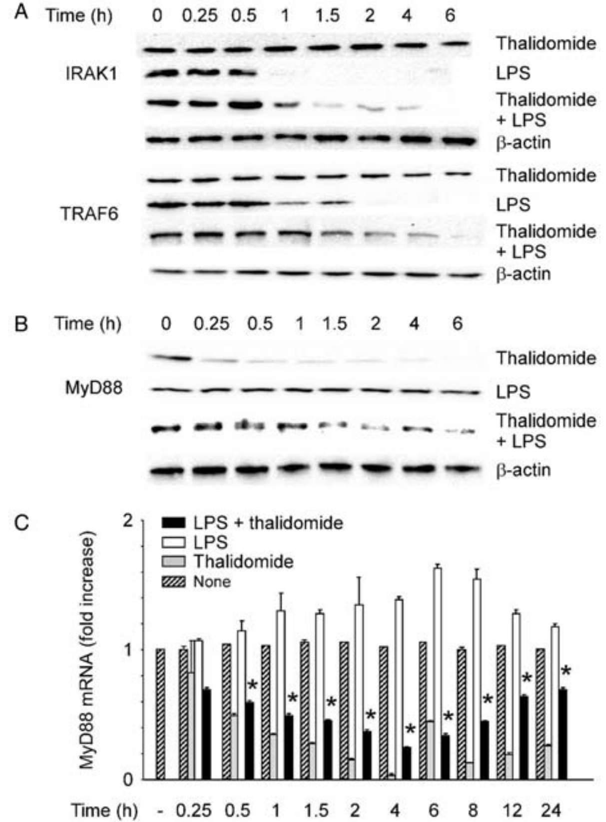

Fig. 5. Effect of thalidomide on IRAK1, TRAF6, and MyD88 expression. RAW 264.7 cells were pretreated with thalidomide (100 μg/ml) for 1 h and stimulated with LPS (100 ng/ml) for various time periods. (A) The expression of IRAK1 and TRAF6 was detected by immunoblotting with antibodies to IRAK1 and TRAF6. (B) The expression of MyD88 was detected by immunoblotting with an antibody to MyD88. (C) MyD88 mRNA expression was measured by real-time PCR. $*P < 0.01$ versus LPS alone.

a series of signal molecules acting in the MyD88-dependent pathway. First, the effect of thalidomide on the expression of IRAK1 and TRAF6 in RAW 264.7 cells was examined. The cell lysates were analyzed by immunoblotting with antibodies against IRAK1 and TRAF6 (Fig. 5A). Thalidomide did not affect the expression of IRAK1 or TRAF6 protein. IRAK1 and TRAF6 disappeared in response to LPS which was prevented by thalidomide from 1–4 h.

Next, the effect of thalidomide on the expression of MyD88 protein in RAW 264.7 cells was examined. Cell lysates were analyzed by immunoblotting using an antibody against MyD88. The expression of MyD88 protein decreased 15 min after thalidomide treatment and the reduction continued up to 6 h. LPS did not markedly affect MyD88 expression but thalidomide down-regulated MyD88 expression in LPS-stimulated RAW 264.7 cells (Fig. 5B) at 1.5 h. We also examined the effect of thalidomide on the expression of MyD88 mRNA. Thalidomide significantly reduced the

expression of MyD88 mRNA after 30 min in either untreated or LPS-stimulated cells and the reduction was seen at 24 h (Fig. 5C). Furthermore, we examined the effect of thalidomide on MyD88 mRNA expression in mouse peritoneal macrophages. Peritoneal macrophages were pretreated with thalidomide (100 μg/ml) and then stimulated with LPS (100 ng/ml) for 1 h. Thalidomide alone significantly decreased MyD88 mRNA expression in non-treated cells (38% reduction). Although LPS enhanced expression in non-treated cells (162% increase), thalidomide reduced this in LPS-stimulated cells (42% reduction).

Thalidomide does not alter the expression of cell surface CD14 and TLR4 in RAW 264.7 cells

The effect of thalidomide on the cell surface expression of CD14 and TLR4 in RAW 264.7 cells was examined. Laser flow cytometric analysis demonstrated that there was no significant difference in the cell surface expression of CD14 and TLR4 between untreated or thalidomide-pretreated RAW 264.7 cells (Fig. 6A). The result was also confirmed by immunoblotting with an anti-CD14 or TLR4 antibody (Fig. 6B). In addition, CD14 and TLR expression in LPS-treated cells was not altered by thalidomide pretreatment (data not shown). The possibility that thalidomide might inhibit LPS-induced TNF-α production via altered cell surface expression of CD14 and TLR4 was, therefore, excluded.

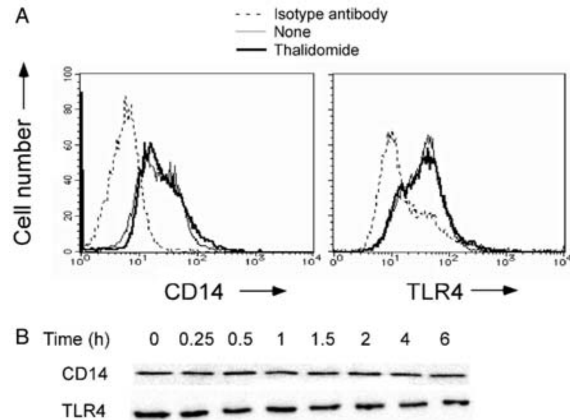

Fig. 6. Effect of thalidomide on CD14 and TLR4 expression. (A) RAW 264.7 cells were treated with thalidomide (100 μg/ml) for 1 h and stained with PE-conjugated isotype matched IgG, and PE-conjugated anti-CD14 or TLR4 antibody. The fluorescent intensity is expressed on a log scale. (B) RAW 264.7 cells were pretreated with thalidomide (100 μg/ml) for 1 h and stimulated with LPS (100 ng/ml) for various time periods. Cell lysates were analyzed by immunoblotting with an antibody to CD14 or TLR4.

Thalidomide inhibits TNF-α production mediated by TLR2 as well as TLR4

LPS (a TLR4 ligand) and Pam_3Cys (a TLR2 ligand) induce TNF-α production via MyD88-dependent NF-κB activation.[23,24] To confirm that thalidomide inhibits TNF-α production in a MyD88-dependent fashion, we determined the level of TNF-α in thalidomide-pretreated or untreated RAW 264.7 cells stimulated with Pam_3Cys. Thalidomide significantly inhibited TNF-α production in response to Pam_3Cys as well as LPS (Fig. 7), suggesting that thalidomide might inhibit MyD88-dependent TNF-α production in TLR2 and TLR4 signalling.

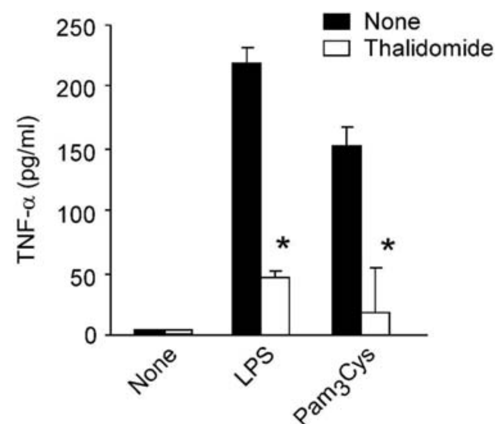

Fig. 7. Effect of thalidomide on TNF-α production induced by LPS, Pam_3Cys and polyI:C. RAW 264.7 cells were pretreated with thalidomide (100 μg/ml) for 1 h and stimulated with LPS (100 ng/ml), Pam_3Cys (10 μg/ml), or polyI:C (10 μg/ml). The concentration of TNF-α in the culture supernatant was measured with ELISA. *$P < 0.01$ versus no thalidomide treatment.

Thalidomide-induced reduction of MyD88 protein is prevented by a proteasome inhibitor

Thalidomide reduced the expression of MyD88 protein rapidly (15 min) in RAW 264.7 cells, although it also decreased the level of MyD88 mRNA. Therefore, we studied the participation of degradation in the reduction of MyD88 protein by thalidomide. The ubiquitin–proteasome pathway is known to regulate the stability of many proteins,[25] and plays an important role in a number of processes critical to immune function.[26] The effect of MG 132 as a proteasome inhibitor on the reduction of MyD88 in thalidomide-pretreated RAW 264.7 cells was examined (Fig. 8A). The expression of MyD88 was analyzed by immunoblotting with anti-MyD88 antibody. MG 132, significantly prevented the reduction of MyD88 protein by thalidomide, suggesting the involvement of proteasome-dependent degradation in the MyD88 reduction. In addition, MG 132 at 2.5 μM

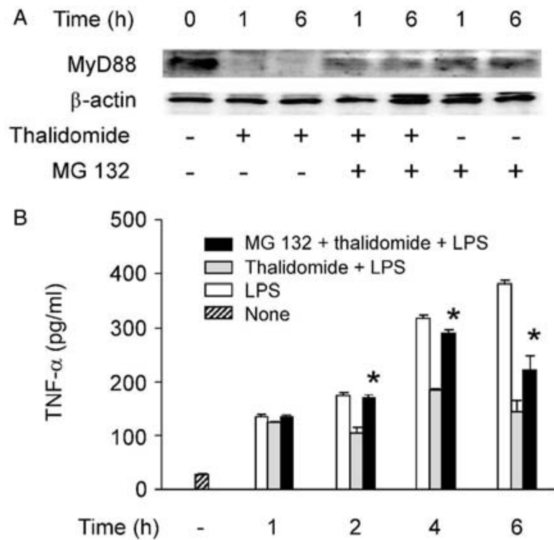

Fig. 8. Effect of MG 132 as a proteasome inhibitor on thalidomide-induced MyD88 reduction and thalidomide-induced TNF-α inhibition. (A) RAW 264.7 cells were pretreated with MG 132 at 2.5 μM for 1 h and treated with thalidomide (100 μg/ml) for 1 h or 6 h. The expression of MyD88 was determined by immunoblotting with an anti-MyD88 antibody. (B) RAW 264.7 cells were pretreated with MG 132 at 2.5 μM for 1 h and again treated with thalidomide (100 μg/ml) for 1 h following LPS (100 ng/ml) at various time periods. The level of TNF-α in the culture supernatant was determined by ELISA. $^{*}P < 0.01$ versus no MG 132 treatment.

did not exhibit a cytotoxic action against RAW 264.7 cells. Further, we examined the effect of MG 132 on the inhibition of LPS-induced TNF-α production by thalidomide (Fig. 8B). MG 132 significantly restored the reduction of LPS-induced TNF-α production by thalidomide. MG 132 also reduced LPS-induced TNF-α production to approximately half at 6 h.

Discussion

In the present study, we have demonstrated that thalidomide inhibits LPS-induced TNF-α production via reduced MyD88 expression. The inhibition of LPS-induced TNF-α production by thalidomide is due to intracellular events because thalidomide does not affect the cell surface expression of LPS-related molecules, such as CD14 and TLR4. Several lines of evidence suggest that the inhibition by thalidomide of LPS-induced TNF-α production may be responsible for reduced MyD88 expression: (i) thalidomide down-regulated the expression of MyD88 protein and mRNA in RAW 264.7 cells and mouse peritoneal cells; (ii) thalidomide inhibited the activation of NF-κB, p38 and SAPK/JNK present downstream of the MyD88 signal molecule; and (iii) thalidomide inhibited TLR4- and TLR2-mediated, MyD88-dependent TNF-α production. LPS-induced TNF-α production is reported to diminish in MyD88-deficient mice.[10] Once again, thalidomide is strongly suggested to inhibit LPS-induced TNF-α production via down-regulation of MyD88 expression.

Thalidomide is reported to regulate TNF-α production via NF-κB-dependent mechanisms.[8] It has been reported that thalidomide blocks NF-κB activation through suppression of IKK activity,[18] and subsequently by the inhibition of IκB degradation.[27] The present study also confirmed that thalidomide inhibited NF-κB activation in LPS-stimulated RAW 264.7 cells and that the inactivation of NF-κB was due to the inhibition of phosphorylation of IKK-α, IKK-β and IκB-α by thalidomide. Moreover, we provide new evidence that thalidomide inhibits LPS-induced phosphorylation of AKT, which is an upstream molecule of IKK and causes the activation of IKK. Thalidomide-induced AKT inactivation indicates the involvement of further upstream molecules. Therefore, the impaired activation of all such signal molecules can be explained by reduction of MyD88 expression as this upstream molecule.

Thalidomide reduced the expression of MyD88 protein and mRNA in RAW 264.7 cells and mouse peritoneal cells. Therefore, the reduced expression of MyD88 protein may be mediated by down-regulation of MyD88 mRNA synthesis. However, the reduction of MyD88 protein occurs immediately (15 min) after thalidomide treatment. Moreover, a proteasome inhibitor, MG 132, prevented the reduction of MyD88 protein by thalidomide. Therefore, there is the possibility that thalidomide may induce reduced expression of MyD88 protein via enhanced proteasomal degradation. Thus, a series of actions of thalidomide might be responsible for degradation of target molecules. In fact, preclinical *in vitro* and *in vivo* studies show the remarkable anti-multiple myeloma activity of bortezomib/PS-341, a proteasome inhibitor as an analog of thalidomide, refractory to prior therapies of thalidomide.[28] Other investigators also showed that bortezomib/PS-341 inhibits NF-κB activation.[29,30] Regarding the degradation of MyD88, TGF-β is reported to reduce MyD88 protein with no alteration of the mRNA probably via proteasomal degradation of MyD88.[17] It is of particular interest to clarify whether thalidomide causes the degradation of MyD88 protein.

TLR4-mediated signalling occurs following sequential recruitment of the adaptor molecule MyD88 and the IRAK family, TRAF6, and NIK.[31,32] The down-regulation of MyD88 protein is certain to result in the functional failure of IRAK and TRAF6 as the down-stream molecules. Further, TRAF6 triggers NF-κB activation via TAK1- or AKT-mediated IKK activation, and also triggers p38 and SAPK/JNK activation.

Considering that the activation of NF-κB and MAPKs are involved in LPS-induced TNF-α production, it is reasonable that thalidomide-induced MyD88 reduction inhibits LPS-induced TNF-α production via impaired activation of the downstream molecules.

Conclusions

Thalidomide seems to inhibit LPS-induced inflammatory responses via reduced MyD88 expression and might be useful for prevention of LPS-mediated tissue injury and lethality. In fact, thalidomide is reported to protect LPS-induced lung and hepatic injury and LPS-induced shock.[33,34] On the other hand, thalidomide is reported to enhance LPS-mediated lethality via up-regulated TNF-α production.[9] Therefore, the therapeutic use of thalidomide in LPS-related tissue injury and shock is still a matter for speculation.

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