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TLR-independent induction of human monocyte IL-1 by phosphoglycolipids from thermophilic bacteria

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Abstract The structures of phosphoglycolipids PGL1 and PGL2 from the thermophilic bacteria *Meiothermus taiwanensis*, *Meiothermus ruber*, *Thermus thermophilus*, and *Thermus oshimai* are determined recently (Yang *et al*. in J Lipid Res. 47:1823–1932, 2006). These bacteria belong to Gram-negative bacteria that do not contain lipopolysaccharide, but high amounts of phosphoglycolipids and glycoglycerolipids. Here we show that PGL1/PGL2 mixture (PGL1: PGL2=10:1~10:2) from *M. taiwanensis* and *T. oshimai*, but not *T. thermophilus* and *M. ruber*, up-regulate interleukin-1β (IL-1β) production in human THP-1 monocytes and blood-isolated primary monocytes. PGL2 was purified after phospholipase A2 hydrolysis of PGL1 in the PGL1/PGL2 mixture followed by column chromatography. PGL2 did not induce proIL-1 production, even, partially (35–40%) inhibited PGL1-mediated proIL-1 production, showing that PGL1 is the main inducer of proIL-1 production in PGL1/PGL2 mixture. The production of proIL-1 stimulated by phosphoglycolipids was strongly inhibited by specific PKC-α, MEK1/2, and JNK inhibitors, but not by p38-specific inhibitor. The intracellular calcium influx was involved in phosphoglycolipids-mediated proIL-1 production. Using blocking antibody and Toll-like receptor (TLR)-linked NF-κB luciferase assays, we found that the cellular receptor(s) for phosphoglycolipids on proIL-1 production was TLR-independent. Further, phosphoglycolipids isolated from *T. thermophilus* and *M. ruber* did not induce proIL-1 production, even though *T. thermophilus* possess more PGL1 than PGL2 (6:4). Specially, the fatty acid composition of phosphoglycolipids from both *T. thermophilus* and *M. ruber* consists of a low percentage of C15 (<10%) and a high percentage of C17 (>75%). It suggests, the C15 percentage of PGL may play a critical role in PGL-mediated proIL-1 induction.

Keywords Phosphoglycolipids · Thermophilic bacteria · Immunomodulators

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Introduction

Thermophilic bacteria contain unique glycolipids: glycoglycerolipids and phosphoglycolipids (PGL) [1–3] that

maintain their biological functions under extreme environmental conditions [4–8]. Glycolipids play important roles in augmenting and regulating innate and adaptive immune systems in humans [9–13], for example, some gangliosides interacting with CD1d molecules and releasing proinflammatory and immunomodulatory cytokines [12–14]. Further, monogalactosyldiacylglycerol of thermophilic blue-green alga ETS-05 had *in vivo* anti-inflammatory activity [15]. It is not known whether the glycolipids of thermophilic bacteria have immunomodulatory functions, but it has been speculated that thermophilic bacteria produce immunomodulator substances [16].

We recently determined the detailed chemical structures of PGL1 and PGL2 of thermophilic bacteria *Meiothermus taiwanensis*, *Meiothermus ruber*, *Thermus thermophilus*, and *Thermus oshimai*. These PGLs belong to two different families: PGL1, 2′-*O*-(1,2-diacyl-*sn*-glycero-3-phospho)-3′-*O*-(α-*N*-acetylglucosaminyl)-*N*-glyceroyl alkylamine; and PGL2, 2′-*O*-(2-acyl-alkyldio-1-*O*-phospho)-3′-*O*-(α-*N*-acetyl-glucosaminyl)-*N*-glyceroyl alkylamine [8]. The major fatty acids in PGL1 and PGL2 are iso- and anteiso-branched C_{15} and C_{17}. Such alkylamine-containing phospholipids are found not only in *Thermus* and *Meiothermus* species, but also in phylogenetically related species, *e.g.*, the radiation-resistant mesophilic *Deinococcus radiodurans* [17]. Our studies showed that *Thermus* and *Meiothermus ssp.* are LPS-deficient, but contain high amounts of glycolipids including glycoglycerolipids and phosphoglycolipids (PGL) in bacterial membranes.

Our studies show that the mixture of PGL1 and PGL2 of *M. taiwanensis* and *T. oshimai*, but not of *T. thermophilus* and *M. ruber*, stimulates production of the proIL-1/IL-1 in human monocytes. IL-1 is one of potent inflammatory cytokines that regulate the host defense and immune responses. The IL-1 precursor, proIL-1, is a 31–34 kDa inactive form of IL-1 in the cytosol; it is cleaved to form the 17-kDa mature functional form by the IL-1-converting enzyme [18–21]. Active IL-1 is released and carries out its diverse biological functions, such as proliferation, differentiation and activation of various cell types [22–26]. Cells respond to stimulating molecules through phosphorylation of tyrosine kinases, and then PKC or reactive oxygen species transmits the signals further. The signal transduction involved in IL-1 stimulation includes distinct subgroups, namely ERKs pathway, and the stress activated kinase pathways including JNKs and p38 mitogen-activated protein kinase [27]. The activated ERKs in turn trigger the phosphorylation of numerous cellular proteins, including transcription factors; they thus play a central role in the propagation of mitogenic signals. ERKs activation also results in cytokine induction and regulates the immune responses to bacterial products [28–30] as well as physiological stress [31–34]. JNKs is a serine/threonine protein kinase (Ser-63, Ser-73) that phosphorylates c-Jun, a component of the transcriptional factor activator protein-1 (AP-1) [35, 36], which regulates the production of various stress-induced proteins and inflammatory cytokines [34, 37, 38]. The p38 responds to stress signals such as LPS, osmotic stress, and pro-inflammatory cytokines [28, 39–41].

This work is focused on the influence of fatty acid moieties of PGL on cytokine production; and PGL-mediated signaling cascades in the regulation of cytokine production within human monocytes was also investigated.

Materials and methods

Cell cultures Human THP-1 monocytes were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Human primary monocytes were obtained from normal blood donor buffy coats (Taipei Blood Center, Taipei, Taiwan). Buffy coats cells were mixed with an equal volume of PBS, layered on Histopaque®-1077 (Sigma, St. Louis, MO, USA) and centrifuged at 400 × *g* for 30 min at 20 °C. The interface containing mononuclear cells were collected and washed twice with PBS. Thereafter, human primary monocytes (98% CD14+, analyzed by flow cytometry) were isolated from mononuclear cells by the Monocyte Isolation kit II (Miltenyi Biotech, Auburn, CA, USA). THP-1 monocytes and human blood-isolated primary monocytes were propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine (Life Technologies, Inc., MD, USA) and cultured at 37 °C under 5% CO_2 in an incubator.

Materials Monoclonal anti-MAP kinase, activated (diphosphorylated ERK1/2) antibody, monoclonal anti-JNK kinase, activated (diphosphorylated JNK) antibody, monoclonal anti-p38 MAP kinase, activated (diphosphorylated p38) antibody, monoclonal anti-actin antibody, LPS (*Escherichia coli* 0111:B4), and polymyxin B were purchased from Sigma (St. Louis, MO, USA). Anti-IL-1β, polyclonal antibody, anti-rabbit IgG-HRP, and anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-human TLR4 (HTA125) was purchased from IMGENEX (Carlsbad, CA, USA). PD98059, SP600125, SB203580, LY294002, Gö6076, Rottlerin, U73122, and intracellular calcium chelator BAPTA/AM were purchased from Calbiochem-Novabiochem Corp (San Diego, CA, USA). Calcium chelators EGTA and TMB-8 were purchased from Biomol (PA, USA). Anti-phosphotyrosine was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-human IL-1beta ELISA kit was purchased from R & D (MN, USA). Phospholipase A2 from

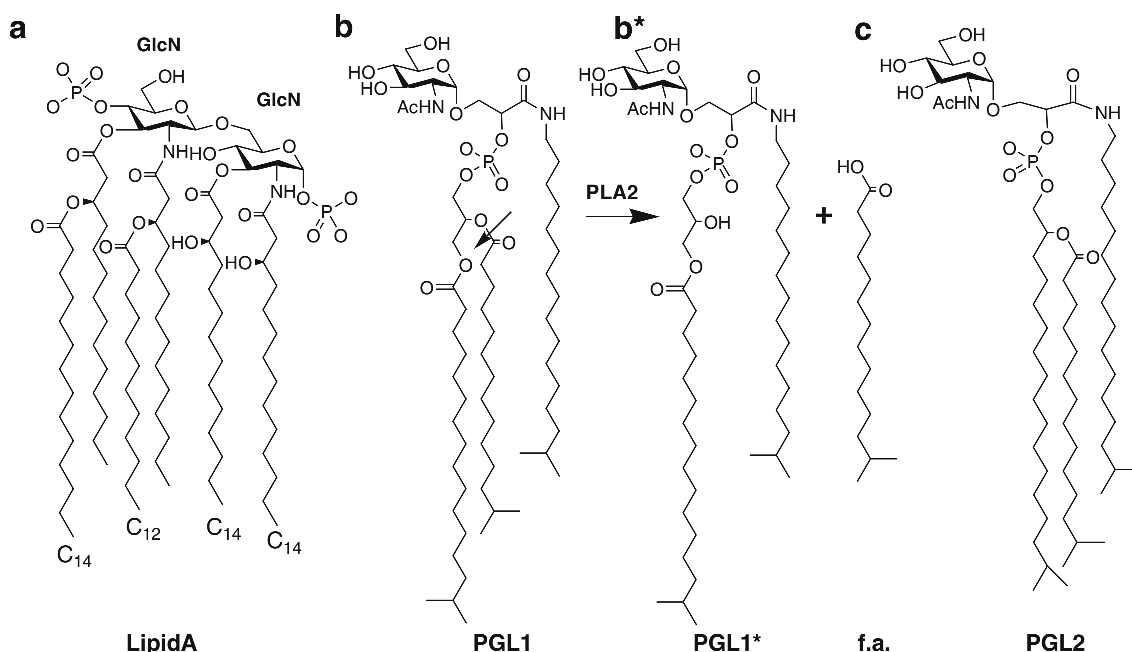

Fig. 1 The chemical structures of *M. taiwanensis* NTU220 phosphoglycolipids and *E. coli* Lipid A. **a** Chemical structure of Lipid A. **b** Chemical structure of PGL1: 2′-*O*-(1,2-diacyl-*sn*-glycero-3-phospho)-3′-*O*-(α-*N*-acetylglucosaminyl)-*N*-glyceroyl alkylamine. PGL1* (**b***) was obtained by phospholipase A2 hydrolysis of PGL1 to remove one of fatty acids. The cleavage site of the enzyme is indicated an *arrow*. **c** Chemical structure of PGL2: 2′-*O*-(2-acyl-alkyldio-1-*O*-phospho)-3′-*O*-(α-*N*-acetylglucosaminyl)-*N*-glyceroyl alkylamine

the snake venom of *Naja naja atra* was a gift from Prof. Tsai, Inn-Ho (NTU, Taiwan).

Preparation of PGL PGL1 and PGL2 (Fig. 1b and c) at a ratio of 10:2 were isolated together from the thermophilic bacteria *Meiothermus taiwanensis* ATCC BAA-400-NTU220 [8, 42] and *Thermus oshimai* NTU63 (Wu-rai hot spring, Taiwan) [43] as previously described [8]. PGL1 and PGL2 from *Thermus thermophilus* NTU77 and *Meiothermus ruber* NTU124 were isolated together following the same procedure. Briefly, a suspension of wet bacterial cells in absolute ethanol (1:10, w/v) was shaken at room temperature for 2 h. After centrifugation, the supernatant was collected, concentrated, applied to a silica gel G-60 column (Merck), and eluted with a gradient of chloroform:methanol from 20:1 to 3:1 (v/v). Carbohydrate-containing fractions were detected by TLC (stained with 0.02 M ammonium cerium sulfate dihydrate and ammonium molybdate tetrahydrate in aqueous 10% H_2SO_4) and collected. PGL1 and PGL2 were obtained together (PGL1/PGL2) in heterogeneous forms owing to the variations of fatty acid moieties (mainly straight/iso/anteiso C_{15} and C_{17}) as shown by MS analysis [8].

Western blot and ELISA analysis of PGL-stimulated THP-1 monocytes Human THP-1 monocytes in RPMI-1640 medium were treated with the PGL1/PGL2 mixture dissolved in DMSO at various concentrations at 37 °C. Ice-cold PBS containing 5 mM Na_3VO_4 was added at different time points. Cells were then immediately centrifuged at 4 °C and lysed with 300 μl of ice-cold lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton, 205 mM pyrophosphate, 1 mM glycerol phosphate, 1 mM Na_3VO_4, 2 μg/ml leupeptin, 1 mM PMSF) on ice for 10–15 min. Insoluble material was removed by centrifugation at 12,000 × *g* at 4 °C for 15 min. The protein concentrations were determined using the Bio-Rad protein assay. Samples were separated by SDS-PAGE and electrotransferred to a PVDF filter. The filters were incubated in blocking solution (5% non-fat milk in PBS with 0.1% Tween 20) at room temperature for 1 h, followed by incubation with primary anti-IL-1 antibody, monoclonal anti-phosphotyrosine kinase, monoclonal anti-MAP kinase, activated (diphosphorylated ERK1/2) antibody, monoclonal anti-JNK kinase, activated (diphosphorylated JNK) antibody, monoclonal anti-p38 MAP kinase, activated (diphosphorylated p38) antibody, or monoclonal anti-actin antibody, or other related antibodies at room temperature for 2 h. After washing three times in PBS with 0.1% Tween 20, filters were incubated with an HRP-conjugated secondary antibody directed against the primary antibody. Blots were developed using an enhanced chemiluminescence Western blotting detection system (DuPont NEN Research Product Co, Boston, MA, USA) according to the manufacturer's instructions. All other methods and procedures followed previously reported methods [44].

Preparation of pure PGL2 The PGL1/PGL2 mixture from *T. thermophilus* NTU77 cultivated at 75 °C was hydrolyzed with phospholipase A2 (*Naja naja atra*: Taiwan cobra) in diethyl ether/ 0.1 M ammonium acetate buffer, pH 7.3, 20 mM $CaCl_2$ (1:1, v/v). PGL1* (PGL1 hydrolysis product) and PGL2 were then separated by Silica G-60 chromatography (Merck) in $CHCl_3$/MeOH (3:1, v/v).

NF-kB luciferase report gene assay for TLRs HEK293T cells (2×10^4 cells /well) were seeded onto 96-well plate overnight. The cells were transfected using FuGENE6 (Roche) plus 0.003 μg of TLR-expressing plasmid, 0.03 μg of p5xNFκB-luc plasmid (Stratagene) and 0.01 μg of pcDNA3.1-βgal according to manufacturer's instruction. The cells were incubated with samples or TLR ligands for 6 h, washed twice with PBS then lysed. NFκB luciferase activities were measured using the luciferase assay system (Promega) according to the manufacturer's instruction. Levels of firefly luciferase expression were normalized against β-galactosidase activity as a control for transfection efficiency and expressed as fold stimulation over unstimulated pcDNA3.1 empty vector control [45, 46].

Results

PGL1/PGL2 up-regulates proIL-1/IL-1 production of human blood-isolated primary monocytes and human THP-1 monocytes We used Western blot to analyze and quantify the effect of PGL1/PGL2 isolated from the thermophilic bacteria on proIL-1 induction within human monocytes. Cellular actin was used as an internal control for loading equal protein amounts in each well. PGL1/PGL2 from *T. oshimai* (NTU63 PGL) and *M. taiwanensis* (NTU220 PGL) strongly stimulated proIL-1 production, whereas PGL1/PGL2 from *T. thermophilus* (NTU77 PGL) and *M. ruber* (NTU124 PGL) did not, even at higher concentrations (Fig. 2a). PGL1/PGL2 from *T. oshimai* had a relative lower activity than that of *M. taiwanensis*. As the concentration of PGL1/PGL2 from *T. oshimai* (NTU63) or *M. taiwnensis* (NTU220) increased, induction of proIL-1 also increased (Fig. 2b). When PGL1/PGL2 at various concentrations (10–100 μg/ml) was post-stimulated for 6 h, proIL-1 was detected. PGL1/PGL2 from *T. oshimai* and *M. taiwanensis* up-regulated proIL-1 production in a dosage-dependent manner (PGL: 10–100 μg/ml, equivalent to 8.2–82 μM) with an approximate fivefold and eightfold increase

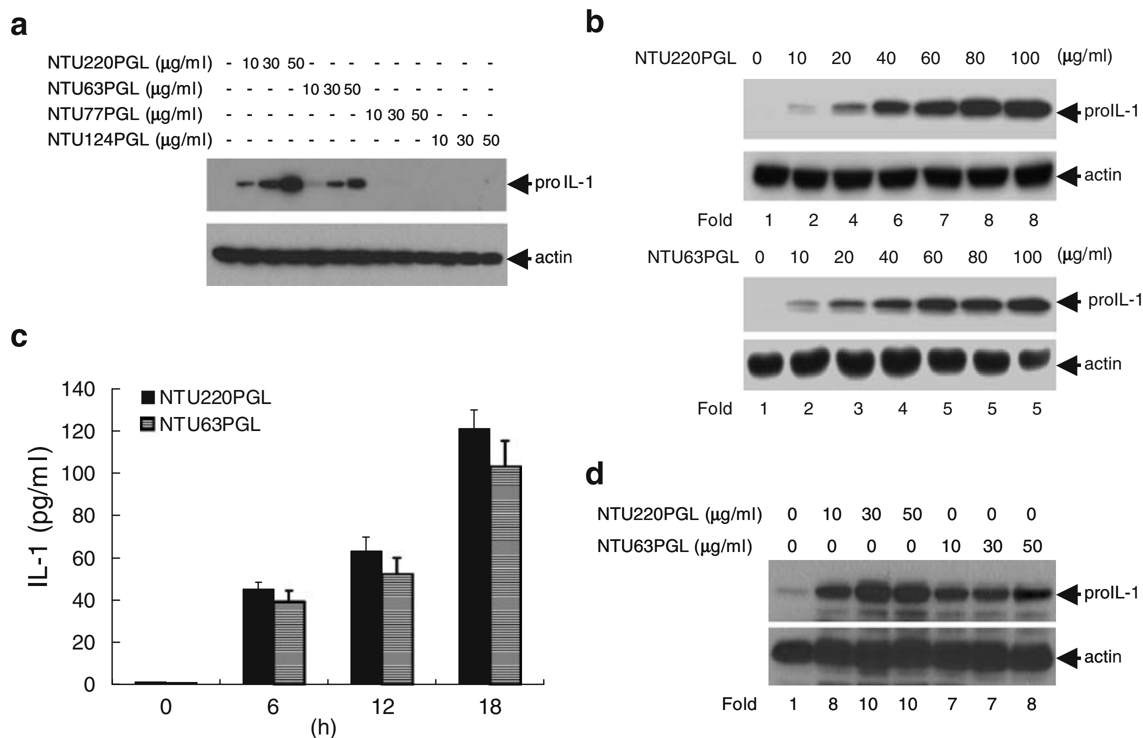

Fig. 2 Stimulation of the production of proIL-1/IL-1 in human THP-1 monocytes and human blood-isolated primary monocytes by PGL1/PGL2. **a** Cells were treated with 10 to 50 μg/ml of the PGL1/PGL2 mixture from the thermophilic bacteria (NTU220: *M. taiwanensis* ATCC BAA-400; NTU63: *T. oshimai*; NTU77: *T. thermophilus*; NTU124: *M. ruber*) at 37 °C for 6 h. **b** Cells were treated with 10 to 100 μg/ml of the PGL1/PGL2 mixture at 37 °C for 6 h. **c** Cells were treated with 50 μg/ml of the PGL1/PGL2 mixture at 37 °C for 0 ~ 18 h. **d** Human blood-isolated primary monocytes were treated with 10 to 50 μg/ml of the PGL1/PGL2 mixture at 37 °C for 6 h. ProIL-1 and IL-1 production were analyzed by Western blot analysis and ELISA, respectively, in three replicate experiments. A representative result is shown. Actin was used as a reference

in proIL-1 production, respectively. Mature IL-1 was secreted from THP-1 monocytes and increased to around 120 pg/ml after 18 h induction by PGL1/PGL2 (Fig. 2c). PGL1/PGL2 also stimulated proIL-1 production in human blood-isolated primary monocytes *in vivo* (Fig. 2d).

The effect of PGL1/PGL2 on protein tyrosine phosphorylation in THP-1 monocytes Tyrosine in total proteins was phosphorylated in the initial stage in the induction of proIL-1 production after PGL1/PGL2 bound to the specific cellular receptor (Fig. 3a). Particularly IκBα (40 kDa), identified with anti-IκBα antibody, was phosphorylated when PGL1/PGL2 from *M. taiwanensis* was used. Phosphorylation of IκBα leads to proteolytic degradation and translocation of NF-κB to the nucleus, where it induces activation of transcriptional factors [47]. ProIL-1 production induced by PGL1/PGL2 from *M. taiwanensis* was inhibited by the strong phospholipase C inhibitor U73122 (Fig. 3b). The PKC-α inhibitor Gö6976, but not the PKC-δ inhibitor Rottlerin, blocked the stimulation of proIL-1 induction by PGL1/PGL2 (Fig. 3c). PGL1/PGL2 was also involved in intracellular calcium mobilization since the Ca^{2+} chelators TMB-8 and BAPTA/AM influenced the stimulation on proIL-1 induction (Fig. 3d). However, the extracellular calcium chelator EGTA had a lesser effect than TMB-8 and BAPTA/AM on the PGL1/PGL2 stimulation of proIL-1 production (Fig. 3e), which suggested that the activity of PGL1/PGL2 is influenced more by intracellular calcium concentrations than by extracellular calcium concentrations. The inhibitors tested had a similar effect on proIL-1 induction by PGL1/PGL2 from *T. oshimai* (NTU63) and *M. taiwanensis* (NTU220) (data not shown). All the

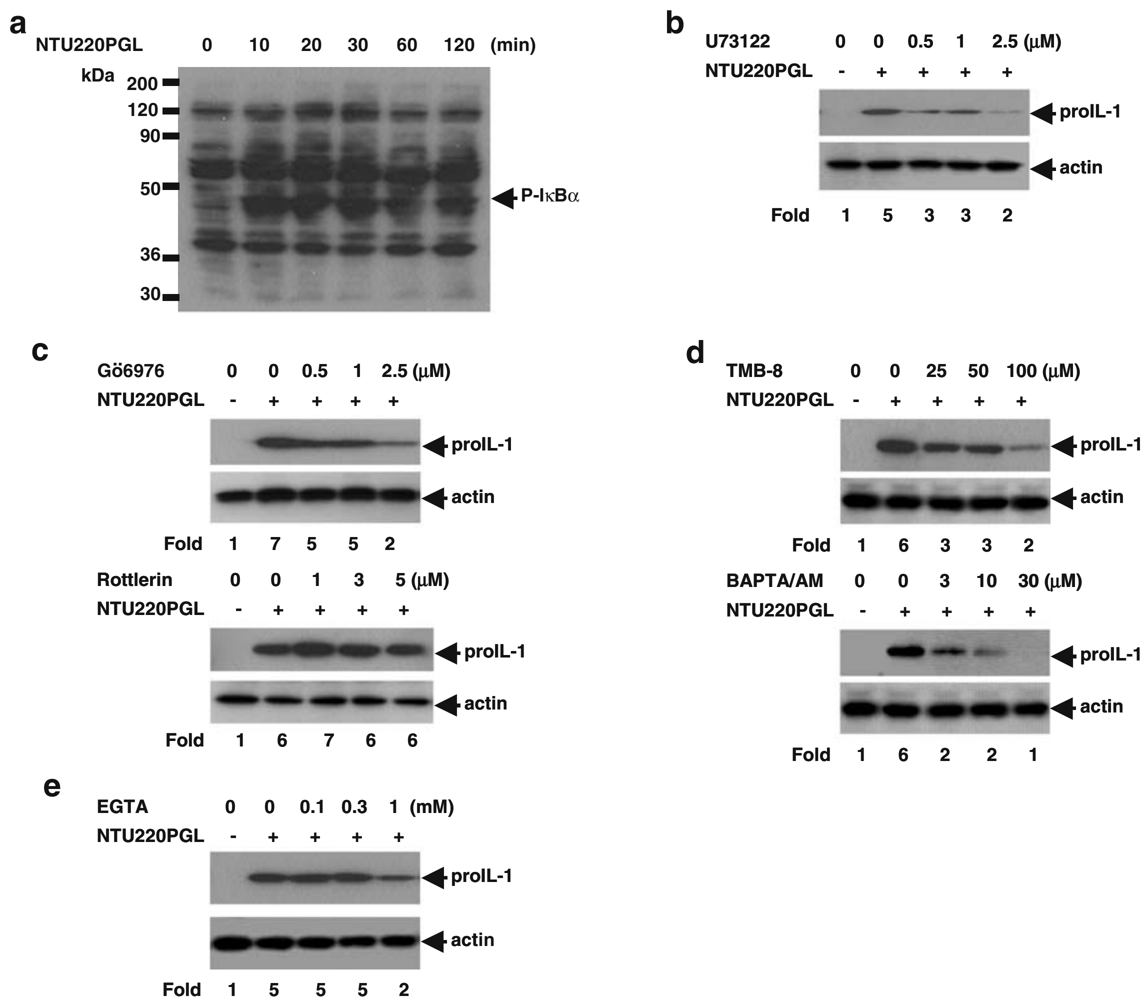

Fig. 3 Effects of various inhibitors or calcium chelators on the stimulation of proIL-1 production induced by PGL1/PGL2 from *M. taiwanensis* NTU220 in THP-1 monocytes. **a** Total protein tyrosine phosphorylation stimulated by PGL1/PGL2 from *M. taiwanensis* ATCC BAA-400 (NTU220) over time. Western blotting is described in Experimental procedures. Molecular mass (kDa) standards are indicated on the *left*; the *arrow* on the *right* indicates phosphorylated IκBα. **b** phospholipase C inhibitor: U73122; **c** protein kinase C (PKC)-α inhibitor: Gö6976 and PKC-δ inhibitor: Rottlerin; **d** intracellular calcium chelators: TMB-8, and BAPTA/AM; and **e** extracellular calcium chelator: EGTA. Actin was used as a reference

inhibitors used above did not show any cytotoxicity to the cells in the experimental condition (data not shown).

The role of mitogen-activated protein kinases on proIL-1 expression in PGL1/PGL2-stimulated THP-1 monocytes To elucidate the PGL-mediated signal transduction pathways in the regulation of proIL-1 production, cell lysates were analyzed using various monoclonal antibodies that specifically recognize phosphorylated ERK1/2, JNK1/2 and p38. The activated Thr202/Tyr204-phosphorylated form of ERK1/2 was assayed using anti-phospho-ERK1/2 antibody. Upon stimulation by *M. taiwanensis* PGL1/PGL2 (NTU220PGL), the amount of phosphorylated ERK1/2 in THP-1 monocytes slowly increased to 2.5-fold compared to untreated cells after 1 h; the amount of phosphorylated ERK1/2 was maintained even after 2 h (Fig. 4a). Upon stimulation by *T. oshimai* PGL1/PGL2 (NTU63PGL), the amount of phosphorylated ERK1/2 in THP-1 monocytes quickly reached the maximal ca. threefold increase after 10 min, and slowly decreased thereafter to return to the basal level (Fig. 4a). The differences in the stimulation of ERK1/2 phosphorylation in THP-1 monocytes by the PGL1/PGL2 mixtures from the two thermophiles might be due to their differences in fatty acid composition and distribution.

The inflammatory response of THP-1 monocytes to PGL-induced proIL-1 production prompted us to investigate the possible PGL activation of the stress-related JNK1/2 and p38 pathways. Western blot analysis using an anti-phospho-JNK1/2 antibody that recognizes the activated, Thr183/Tyr185-phosphorylated form of JNK1/2 indicated that PGL1/PGL2 from *M. taiwanensis* (NTU220) and *T. oshimai* (NTU63) stimulated JNK1/2 phosphorylation in THP-1 monocytes (Fig. 4a). The PGL1/PGL2 preparations from *M. taiwanensis* (NTU220) and *T. oshimai* (NTU63) also strongly activated the phosphorylation of p38, as detected by Western blot analyses using anti-phospho-p38, an antibody that specifically recognizes the activated, Thr180/Tyr182-phosphorylated form of p38. With both preparations, the amount of phosphorylated p38 gradually increased at a similar rate and reached the maximal ca. fivefold increase after 1 h (Fig. 4a).

The protein-kinase-mediated signaling pathways in the regulation of proIL-1 production were also tested using three specific antagonists: PD98059, SP600125 and SB203580. These antagonists inhibit the phosphorylation of MEK1, JNK1/2 and p38, respectively. The MEK1 inhibitor, PD98059, significantly down-regulated the PGL1/PGL2-induced proIL-1 production at a concentration of 10 μM (Fig. 4b and c). At 100 μM, PD98059 almost completely inhibited proIL-1 production induced in the MEK1/ERK1/2 pathway, which indicated that the activation of the MEK1/ERK1/2 pathway is important in the stimulation of proIL-1 mediated by PGL1/PGL2. The JNK1/2 pathway inhibitor, SP600125, at concentrations above 10 μM, also inhibited induction of proIL-1 production by PGL1/PGL2 from *M. taiwanensis* and *T. oshimai*, complete inhibition was observed at concentrations >10–30 μM (Fig. 4b and c). *M. taiwanensis* PGL1/PGL2 activated JNK1/2 phosphorylation (Fig. 4a), but the JNK1/2 inhibitor, SP600125, inhibited proIL-1 production. The specific p38 inhibitor, SB203580, is known to be very effective even at low concentration, but shows no significantly inhibitory effect on PGL1/PGL2-induced proIL-1 production at 0.1–10 μM (Fig. 4b and c). This observation suggested that p38 is not significant in PGL1/PGL2-mediated proIL-1 production. Although the p38 phosphorylations were activated by PGL, the failure of SB203580 to block PGL1/PGL2-induced proIL-1 production indicated that the PGL1/PGL2-induced p38 pathway is probably not involved in the regulation of proIL-1 production. Instead, the activation of MEK1/ERK1/2 by PGL1/PGL2 is mainly responsible for proIL-1 production by THP-1 monocytes. The kinase activity of ERK1/2, JNK1/2 and p38 were inhibited completely by the PD98059 (100 μM), SP600125 (30 μM) and SB203580 (10 μM), respectively, as demonstrated by *in vitro* kinase assay (by measuring the phosphorylation of Elk, c-jun and ATF-2, respectively). PD98059, SP600125 and SB203580 did not show any cytotoxicity to the cells in the experimental condition, yet high concentration of PD98059 reduced cell proliferation (data not shown).

TLRs are not the PGL receptors in the induction of proIL-1 production TLR4 is usually the main receptor for bacterial polysaccharides, *e.g.* lipopolysaccharide (LPS), in influencing cellular responses. The previous studies showed that the outer membranes of *Thermus* and *Meiothermus* species are not composed of LPS, instead of peculiar glycolipids (GL) including glycoglycerolipids and phosphoglycolipids (PGL) [3, 8, 48, 49]. Further, all our experimental materials, there were in the absence of 2-keto-3-deoxyoctonate (KDO) which belongs to the component of typical LPS. Moreover, in order to rule out the possibility of PGL-induced proIL-1 expression being due to any LPS-like molecules during sample preparation, we used polymyxin B (PMB) to neutralize the activity LPS, on PGL- and LPS-induced proIL-1 expression within THP-1 monocytes. We found that PMB could be used to completely inhibit LPS-induced proIL-1 expression (Fig. 5a, compare lanes 6–7 to lanes 8–9); yet by contrast, PMB was not able to inhibit PGL-induced proIL-1 expression (Fig. 5a, compare lanes 2–3 to lanes 4–5). In addition, we determined the LPS content of PGL samples by means of a Limulus amoebocyte lysate (LAL) assay, and we further observed an insignificant level of endotoxin (<1EUml^{-1}) in the tested PGL samples (data

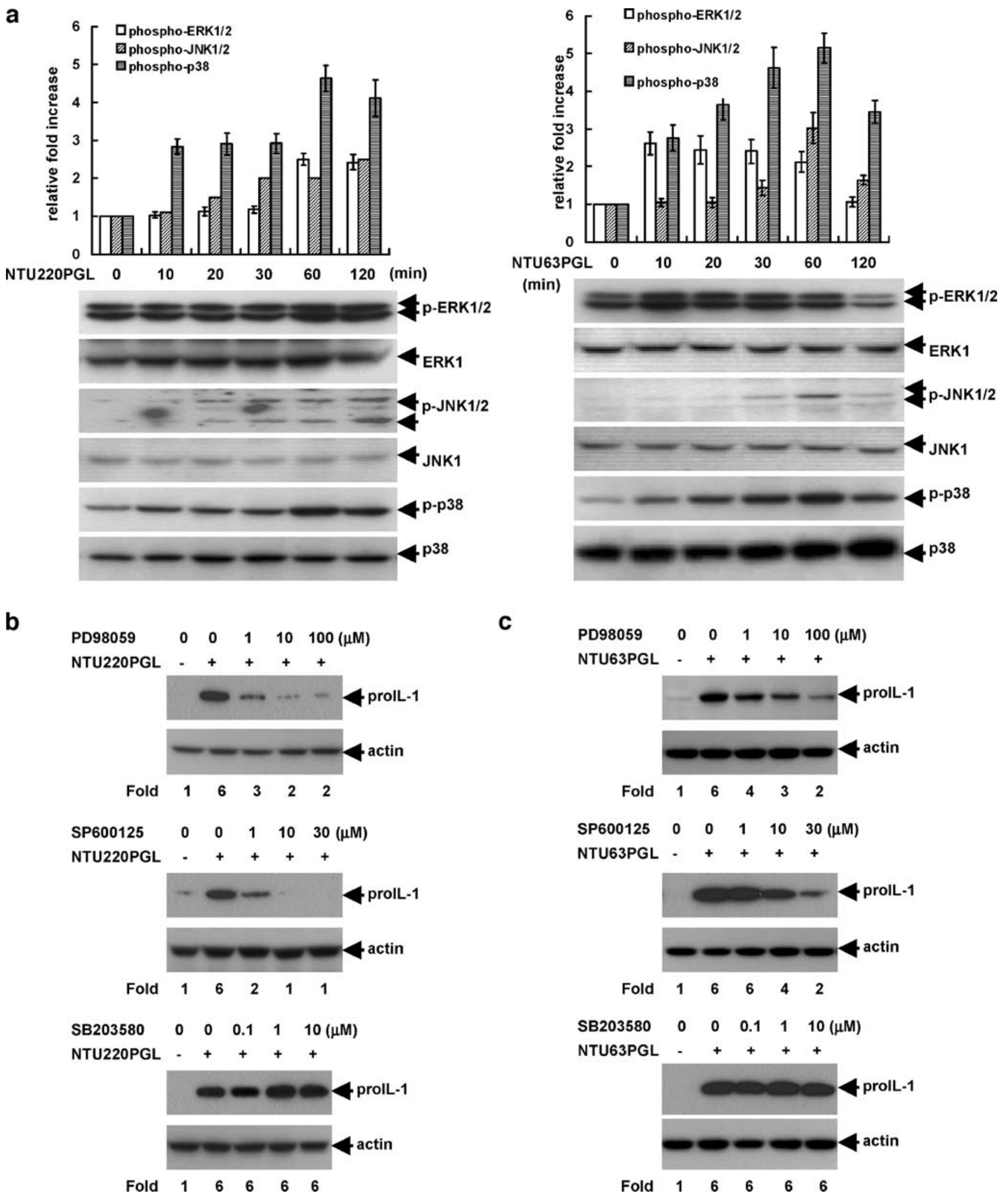

Fig. 4 Role of ERK1/2, JNK1/2, and p38 on proIL-1 expression in PGL1/PGL2-stimulated human THP-1 monocytes. **a** Cells were cultured in medium containing 50 μg/ml of PGL1/PGL2. Cells were harvested at the indicated times, and cell lysates were analyzed by Western blotting using specific monoclonal antibodies. The data are the means±SD of three experiments. Proteins were quantified using PhosphorImager® and ImageQuaNT® software Molecular Dynamics. **b** *M. taiwanensis* NTU220; **c** *T. oshimai* NTU63. Cells were incubated with the inhibitor at various concentrations at 37 °C for 1 h. PGL1/PGL2 (50 μg/ml) was then added and incubation continued for another 6 h. proIL-1 production was analyzed by Western blotting with actin as reference. The experiments were carried out in three replicates. A representative blot is shown

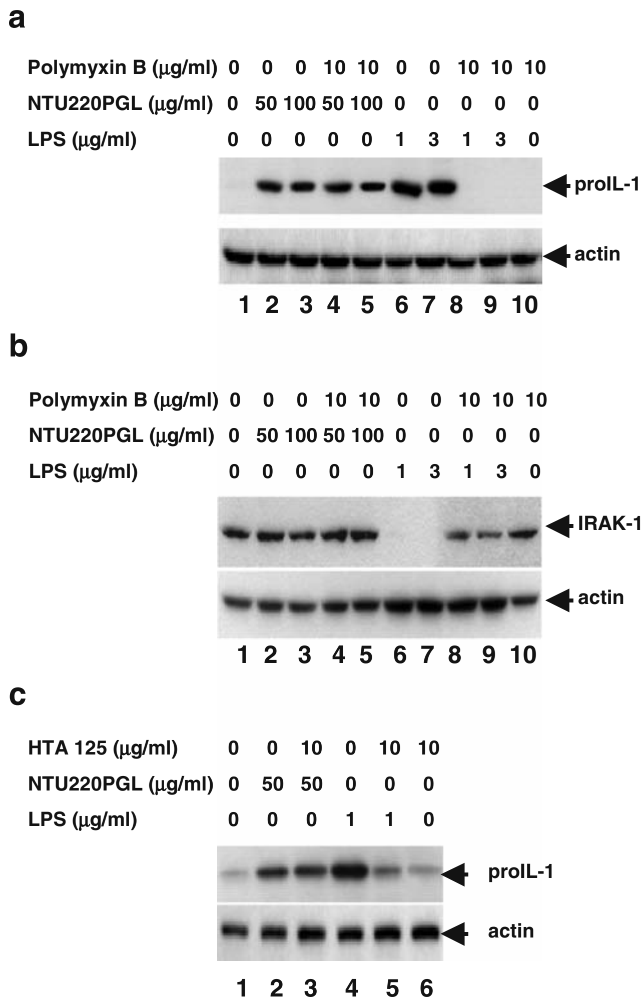

Fig. 5 TLR4 is not the receptor for PGL in induction of proIL-1 production. **a** Pre-incubation of THP-1 monocytes with polymyxin B (10 μg/ml) or medium for 30 min, followed by treatment with *M. taiwanensis* PGL1/PGL2 (50 and 100 μg/ml) or *E. coli* 0111:B4 LPS (1 and 3 μg/ml) for 6 h. Whole cell lysates were analyzed by Western blot with anti-IL-1 antibody. **b** The same procedures as in (**a**), but analyzed with anti-IRAK-1 antibody. **c** Pre-incubation of THP-1 monocytes with specific mouse anti-human TLR4 monoclonal antibody HTA125 (10 μg/ml) or control IgG (10 μg/ml) for 30 min, followed by treatment with *M. taiwanensis* PGL1/PGL2 (50 μg/ml) or *E. coli* LPS (1 μg/ml) for 6 h. Whole cell lysates were analyzed by Western blotting with anti-IL-1 antibody. Each experiment was carried out in three replicates. A representative blot is shown

not shown). Taking these results together, we believe that the effect of PGL upon IL-1 gene expression was not due to LPS contamination of the PGL, but to the presence of the PGL itself. Next, we investigated whether TLR4 participates in PGL-mediated signal transduction. The LPS stimulation of a TLR-4-associated molecule, the IL-1 receptor-associated kinase-1 (IRAK-1), located further downstream in the signal pathway, was evaluated. As shown in Fig. 5b, LPS treatment resulted in IRAK-1 degradation, and this effect was prevented by PMB (Fig. 5b, compare lanes 8–9 to lanes 6–7). Interestingly, PGL1/PGL2 from *M. taiwanensis* (NTU220 PGL) did not lead to IRAK-1 degradation (Fig. 5b, compare lanes 4–5 to lanes 2–3), with or without PMB. Further, the specific mouse anti-human TLR4 monoclonal antibody HTA125 inhibits LPS-induced signals through TLR4. As expected, HTA125 inhibited LPS-induced TLR4-mediated induction of proIL-1 production (Fig. 5c, compare lane 4 to lane 5); however, PGL-mediated proIL-1 production was not affected (Fig. 5c, compare lane 2 to lane 3). These results indicated that TLR4 is not as a receptor for PGL1/PGL2. The effect of PMB and TLR4 monoclonal antibody in the stimulation by PGL1/PGL2 from *T. oshima* (NTU63) in THP-1 monocytes was similar to that from *M. taiwanensis* (NTU220) described above (data not shown). Other TLRs, 2, 3, 5, 7, 8, 9 were assayed for TLR-linked NF-kB luciferase activity [45, 46]; all results were negative (data not shown). Therefore, not TLRs, but rather other cellular PGL receptors are involved in the induction of proIL-1 production.

PGL2 partially inhibits PGL1 in proIL-1 production We reported previously [8] and here that the PGL isolated from the thermophilic bacterial strains studied contained a mixture of PGL1 and PGL2, and these could not be separated by general chromatographic methods. Based on their structures, PGL1 should be hydrolyzed at the sn-2 position by phospholipase A2, whereas PGL2 would not be a substrate of the enzyme. This approach could help us to get pure PGL2, therefore, to distinguish the real role between PGL1 and PGL2. The PGL mixture isolated from *T. thermophilus* NTU77 grown at 75 °C contained had PGL2 as major and PGL1 as minor components. Other bacteria had more PGL1 than PGL2. Phospholipase could not hydrolyze PGL1 in the latter PGL1/PGL2 mixtures, completely. PGL1 was not the best substrate for phospholipase A2. The products PGL1* and one fatty acid in reaction caused feedback inhibition for phospholipase A2 to make optimal enzyme reaction. PGL2, the dominant PGL in the mixture of *T. thermophilus* NTU77 was separated from the PGL1 hydrolysis product (PGL1*) by silica G-60 chromatography. The fatty acid composition of pure PGL2 of *T. thermophilus* NTU77 was similar to that of the PGL1/PGL2 mixture, iso-C17 as major fatty acid and iso-C15 as minor (Table 1). Purified PGL2 alone failed to stimulate proIL-1 production, but partially inhibited (35–40%) PGL1 in the induction of proIL-1 production instead: as is evident from calculation between PGL1/PGL2 quantitative ratio and their combined activities (Fig. 6). These results indicate that PGL1 induces proIL-1 production. Further, PGL1*, the hydrolysis product from PGL1 by phospholipase A2, did not stimulate induction of proIL-1 production (Fig. 6). The chemical difference between PGL1 and PGL1* was only one fatty acid chain. The complete fatty

Table 1 Distribution of PGL1 and PGL2 and their major fatty acid profiles

Strain (abbreviation)	Growth temperature	PGL1:PGL2	Ratio of major fatty acids $C_{15:0}$: $C_{16:0}$: $C_{17:0}$	ProIL-1 induction
T. oshimai (NTU63)	65 °C	10:1~10:2	47.5: 9.7: 40.2	+
T. thermophilus (NTU77)	75 °C	4:6	5.2: 13.7: 81.1	−
M. taiwanensis (NTU220)	55 °C	10:1~10:2	33.0: 11.2: 48.9	+
M. ruber (NTU124)	65 °C	6:4	2.5: 9.7: 80.4	−
T. thermophilus (NTU77)	75 °C	PGL2 only	4.7: 16: 78.4	−

chains probably constructed an effective spatial conformation for PGL1 to induce cytokine expression. Deficiency of fatty acid chains caused to reduce the biological activities.

Discussion

Cell surface receptors can transduce information from the extracellular environment into the cell by recruiting a variety of cytoplasmic proteins involving in regulating cellular pathways. IL-1 can activate a plethora of intracellular signaling pathways [50–54]. We studied the PGL-mediated signal transduction pathways, that up-regulate the production of IL-1 in THP-1 monocytes and found that thermophilic bacteria produce immunomodulatory molecules. It has not been reported before. Some studies have shown that even monosugar α-galactosylceramide glycolipid (α-GalCer) could cause a strong immune response, potently induce antitumor immunity through increasing IL-12 production by dendritic cells [55] and can activate iNKT cells and protect mice against a variety of diseases, including cancer, infection and several autoimmune and inflammatory conditions in preclinical studies [10]. Phosphoglycolipids of the cyanobacterium *Scytonema julianum* antagonize the actions of platelet-activating factor [56].

Among the four thermophilic bacteria tested, PGL1/PGL2 from *T. oshimai* NTU63 and *M. taiwanensis* NTU220, not *T. thermophilus* NTU77 and *M. ruber* NTU124, did induce proIL-1 production (Fig. 2a). We were unable to separate PGL1 and PGL2 using the traditionally standard chromatographic methods. The quantitative ratio of PGL1:PGL2 could be estimated only from the intensities of their oxymethine groups in NMR analyses. The ratio of PGL1:PGL2 in some of the thermophilic bacteria is affected by growth temperature. In *T. oshimai* NTU63, the ratio of PGL1:PGL2 keeps about 10:1 to 10:2 and does not change in the different growth temperature ranging from 55 to 75 °C. In *M. taiwanensis* NTU220 and *M. ruber* NTU124, the amount of PGL2 increases at higher growth temperatures; the ratio of PGL1:PGL2 in *M. ruber* varies from 10:1 to 6:4 depending on the growth temperature [8]. In *T. thermophilus* NTU77, PGL2 is predominant at 75 °C and varies from 10:1 (55 °C) to 4:6 (PGL1:PGL2) depending on the growth temperature. The PGL1:PGL2 ratios at the optimal growth temperature and the ratios of major fatty acids of PGL1 and PGL2 in the four thermophilic bacteria are summarized in Table 1. The chemical characteristics of PGL1/PGL2 in *T. oshimai* and *M. taiwanensis* are PGL1 major (PGL1/PGL2=10/1–10/2) and higher percentages of C15 and C17 in fatty acids. However, in *T. thermophilus*, even though it contains more PGL1 than PGL2 (PGL1:PGL2, 6:4), the release of proIL-1 stimulated by PGL was inactive probably due to the lower C_{15} content (<10%) and higher C_{17} content (>75%) of fatty acids in PGL1/PGL2. Therefore, it implies that the chain-lengths of the fatty acid constituents in PGL may play a significant role in the biological activity since PGLs from *T. oshimai* and *M. taiwanensis* with higher C15 (more than 30%) percentages possess biological activity to stimulate the release of proIL-1. Furthermore, PGL1*, the hydrolytic product of PGL1 by PLA2 with the removal of one fatty acid chain, showed no activity whereas PGL2 displayed partial inhibition for PGL1 activity. It may reveal that three complete fatty acid chains in PGL1 (including one amide-bound acyl group and the two ester-bound acyl groups) may form a spatial conformation for the induction of signaling through putative receptors.

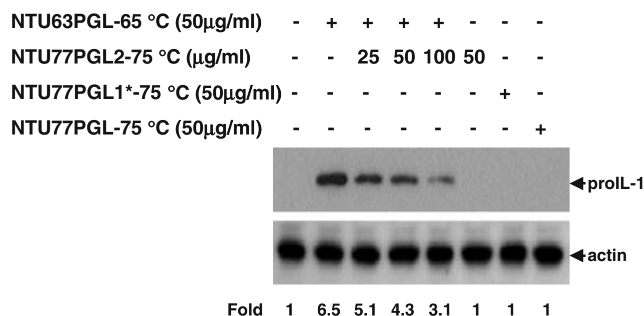

Fig. 6 PGL2 inhibits PGL-induced proIL-1 production in human THP-1 monocytes. Cells were incubated with PGL in the absence or presence of PGL2 for 6 h. The proIL-1 expression was analyzed by Western blot. PGL1* was the product of PGL1 hydrolyzed with phospholipase 2. (NTU63: *T. oshimai*; NTU77: *T. thermophilus*). Actin was used as a reference

Alkyl chain length is known to be the factor controlling the loading of PGL into the hydrophobic cavities of lipid-binding receptors [57]. According to the previous reports, lipopeptides interact with TLR2 might give one example about the effect of length (or carbon number) of fatty acid in biological activity [58, 59]. In this lipopeptide, two ester-bound palmitic acids mediate high activity, while one amide-bound and another ester-bound palmitic acid in the molecule or absence of ester-bound fatty acids would be inactive. These results are quite similar to those of active PGL1, inactive PGL1* and PGL2. On the other hand, lipoprotein analogues with two ester-bound fatty acids having short chain length of eight carbons or less were incapable of activating cells through TLR2, whereas a chain length of 16 carbon atoms was optimal in activity. Fatty acid lengths were also reported to influence the tripalmitoylated macrophage-activating lipopeptide from *Mycoplasma fermentans* (MALP2) analog through TLR2 in a TLR6-independent manner [60].

Glycosphingolipids from non-pathogenic Gram-negative bacteria *Sphingomonas*, have α-linked sugars similar to α-GalCer [61]. Both α-GalCer and glycosphingolipids can stimulate iNKT, especially for Vα14i and Vα24i NKT cells [12]. From stereochemical point of view, α-configuration is important for their biological activities. In this study, PGL1 also possesses α-configuration and shows IL-1 stimulation activity. One kind of synthetic monogalactosyl diacylglycerol BbGL-IIc (from *Borrelia burgdorferi*), the species of fatty acids seem to play an important role in biological activity since $C_{18:1}$ in the sn-1 position and $C_{16:0}$ in the sn-2 position of glycerol, was the most potent antigen in most mouse and human NKT cells [13]. The overall conformation of PGL1 of thermophilic bacteria (*T. oshimai, M. taiwanensis*) could influence the activity through interaction with cellular receptors. Our future work will clarify how the fatty acids in PGL1 play a role in the activity through chemical synthesis of PGL1 with homogeneous fatty acids.

TLR4 is usually the main cellular receptor for LPS or polysaccharides. In our studies, PGL1/PGL2 from the thermophilic bacteria, unlike *E. coli* LPS, did not stimulate proIL-1 production through TLR4 or through TLRs 2, 3 and 5, 7, 8, 9 since mouse anti-human TLR4 monoclonal antibody did not block the stimulation of proIL-1 production and TLR-linked NF-κB luciferase assays including TLRs 2, 3, 5, 7–9 showed no positive responses. We suggest that this stimulation is TLR-independent. Like glycosphingolipids

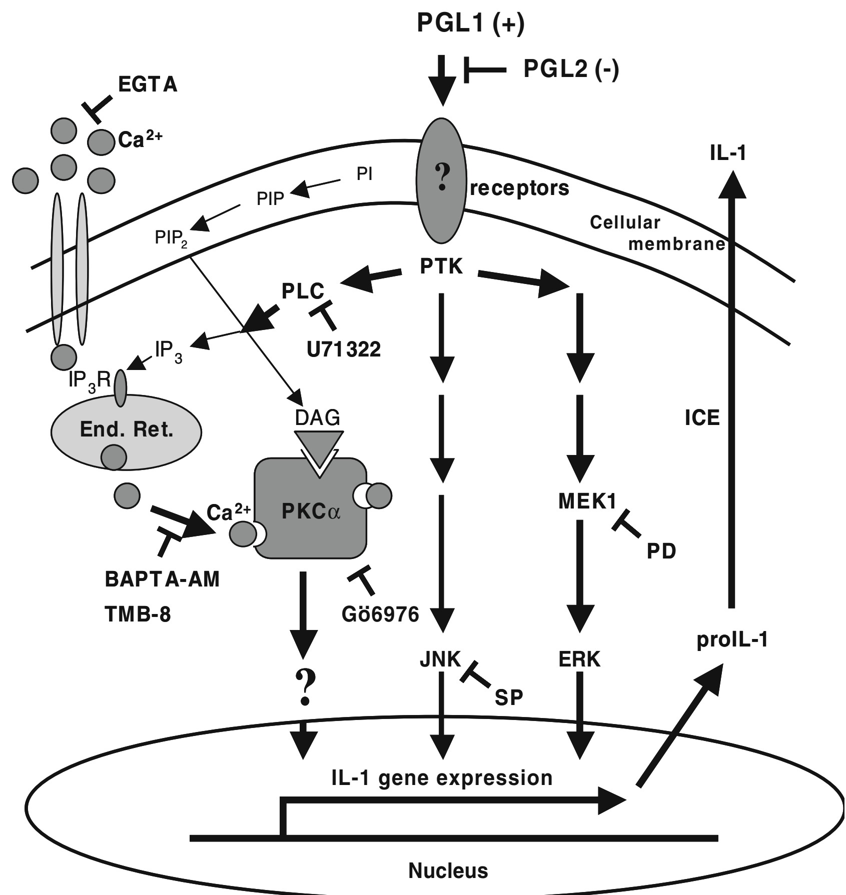

Fig. 7 The proposed PGL-mediated signal transduction pathways in the regulation of proIL-1 protein production and IL-1 secretion

from *Sphingomonas*, it is recognized by most mouse and human NKT cells that provide an innate-type immune response to certain microorganisms through recognition by their antigen receptor, and that they might be useful in providing protection from bacteria that can not be detected by pattern recognition receptors such as Toll-like receptor 4 [12, 61].

LPS stimulates macrophages to release inflammatory cytokines, such as IL-1 and TNF-α. The molecular mechanism for IL-1 production includes the activation of ERK, JNK and p38, and the release of ROS, which play key roles in the LPS-mediated signal transductions between extracellular membrane stimulation, cytoplasmic response, and gene activation [44, 62, 63]. LPS-mediated PI3K/Rac/p38 pathways play a dominant role in the regulation of proIL-1/IL-1; other pathways such as PKC/MEK/ERK and PI3K/Rac/JNK are less important [44]. From structural comparison between lipid A of *E. coli* LPS and PGL of thermophilic bacteria in this study, there are some similarities in structure. However, they have some different signal pathways. The MEK1 inhibitor PD98059 prevented ERK1/2 phosphorylation and significantly down-regulated proIL-1 production within monocytes stimulated by PGL from the thermophilic bacteria *Thermus* and *Meiothermus*, the PKC-α/MEK1/ERK1/2 pathway is important in the regulation of the activity. ROS did not have a positive effect on PGL1/PGL2 from *Thermus* or *Meiothermus* spp (data not shown). Moreover, G protein of the Gi/o family was not the receptor since its inhibitor, pertussis toxin, did not block the proIL-1 expression in PGL1/PGL2 stimulated cells; Src kinases were also not involved (data not shown). The intracellular calcium chelators BAPTA/AM [64] and TMB-8 influenced stimulation of proIL-1 production by PGL, which suggests that PGL could be involved in intracellular metabolism and efflux.

The structures of PGL1 and PGL2 are partially similar to that of lipid A (Fig. 1b and c to Fig. 1a). Lipid A is a bacterial cell wall phospholipid composed of a diglucosamine with several ester-linked and amide-linked long-chain fatty acids. It is the principal component of Gram-negative bacteria that activates the innate immune system [65]. This endotoxin is able to trigger clinical sepsis, which is a major issue among the critically ill [66]. Some studies have shown that lipid A-like compounds, such as lipid X, lipid IVa, form inactive complexes with TLR4 or its accessory proteins and result in the inhibition of acute lethal toxicity induced by LPS [67, 68]. They act as agonists of pro-inflammatory responses in the mouse and as antagonist in humans [22, 67, 68] probably due to the similar structures of lipid A and PGL. In medical therapy, the pro- and anti-inflammatory responses following infection are complex and involve many mediators with unique actions. These mediators, however, have multiple inter-relationships. TNF and IL-1 are considered to be central in the sepsis responses; therefore, blocking their actions appears to be an attractive strategy in combating the infection. Studies to date have focused mainly on the IL-1 receptor antagonist, which is synthesized by macrophages and inhibits IL-1 by binding to its cellular receptor [69]. That is the reason why we studied the *Thermus* and *Meiothermus* PGL in proIL-1 expression and their signal pathways. Further, we found that PGL can cause also endotoxin tolerance in experiments and this feature is currently under investigation in our laboratory.

In conclusion, we have demonstrated that PGL from thermophilic bacteria is able to up-regulate the production of proIL/IL-1 in THP-1 monocytes mainly through the PLC/PKC-α and MEK1/ERK1/2 pathways and partially through the JNK1/2 pathway (Fig. 7). PGLs from *M. taiwanensis* and *T. oshimai* have similar biological functions. Some of the differences in the responses probably arise from the different length, branching, and percentage of fatty acids in the PGLs. PGL2 has inhibitory effects on PGL1 in proIL-1 induction. Unlike the receptor of LPS induction, TLR4 and some other TLRs are not the receptors for proIL-1 production induced by PGL of thermophilic bacteria.

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