

The sesquiterpene lactone parthenolide inhibits LPS- but not TNF- α -induced maturation of human monocyte-derived dendritic cells by inhibition of the p38 mitogen-activated protein kinase pathway

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Background: Dendritic cells (DCs) are the most potent antigen-presenting cells, and the manipulation of DC maturation provides a strategy for the treatment of allergic and inflammatory diseases.

Objective: In this study we examined the effect of the anti-inflammatory sesquiterpene lactone parthenolide (PTL) on DC maturation induced by LPS or TNF- α .

Methods: Human monocyte-derived DCs generated by means of culture with GM-CSF and IL-4 were pretreated with PTL and subsequently stimulated with LPS or TNF- α .

Results: PTL inhibited the upregulation of CD80, CD83, CD86, CD40, and MHC class II; the allostimulatory function; the production of TNF- α and IL-12; and the downregulation of FITC-labeled dextran uptake in human monocyte-derived DCs stimulated with LPS but not with TNF- α . The inhibitory effect of PTL on DC maturation was preceded by inhibition of the phosphorylation of p38 mitogen-activated protein kinase but not the nuclear translocation of NF- κ B.

Conclusion: These results might offer PTL not only as a promising compound for the treatment of LPS-induced disorders, including sepsis or septic shock, by inhibition of excessive DC maturation but also as a tool to further dissect the signaling pathways involved in DC maturation. (*J Allergy Clin Immunol* 2002;110:269-76.)

Key words: Dendritic cells, maturation, lipopolysaccharide, signal transduction

Dendritic cells (DCs) originate from bone marrow and function as a critical link between the innate and adaptive immune system. Immature DCs are residing in many organs, such as the skin, intestine, and respiratory tract, that are always exposed to external stimuli and function

Abbreviations used

DC:	Dendritic cell
IC ₅₀ :	Inhibitory concentration of 50%
I κ B:	Inhibitory NF- κ B
IKK:	I κ B kinase
MAPK:	Mitogen-activated protein kinase
MAPKKK:	MAPK kinase kinase
MEKK1:	MAPK/ERK kinase kinase 1
MKK:	MAPK kinase
NF:	Nuclear factor
NIK:	NF- κ B-inducing kinase
PE:	Phytoerythrin
PTL:	Parthenolide
TAK1:	Transforming growth factor β -activated kinase 1
TLR:	Toll-like receptor
TRAF:	TNF receptor-associated factor

as sentinels. Once they capture antigens, they start to migrate toward the T-cell areas of secondary lymphoid organs.^{1,2} During this migration, they lose their ability to internalize antigens and acquire the capacity to present antigens to naive T cells. This process is referred to as DC maturation or activation.^{3,4} Mature DCs are recognized by their strong surface expression of MHC class I and II, costimulatory molecules (CD40, CD80, and CD86), and the DC maturation marker CD83.^{1,5} Various stimuli, such as proinflammatory cytokines (eg, TNF- α and IL-1 β), CD40 ligation, bacterial products (eg, LPS and unmethylated DNA CpG motif), and contact sensitizers, can induce DC maturation in vivo and in vitro.⁶⁻⁹

DCs can be induced from CD34⁺ cells from human cord blood and bone marrow in the presence of GM-CSF and TNF- α or by culturing peripheral blood CD14⁺ cells with GM-CSF and IL-4.^{3,10,11} These culture systems enable us to obtain a large number of pure DCs and thus provide the opportunity to gain insight into the molecular mechanism of DC maturation. The 3 major mitogen-activated protein kinases (MAPKs), the extracellular signal-regulated kinases, the c-Jun N-terminal kinases, and p38 MAPK are activated in DCs on maturation induced by LPS or TNF- α . With the use of a specific inhibitor, SB203580, p38 MAPK was shown to play a critical role

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in DC maturation.¹²⁻¹⁴ Another intracellular component involved in DC maturation is the transcription factor nuclear factor (NF) κ B.¹⁵⁻¹⁷

Sesquiterpene lactones are found in several plant families and traditionally used as herbal remedies because of their anti-inflammatory properties. Parthenolide (PTL), the predominant sesquiterpene lactone in European feverfew (*Tanacetum parthenium*), is also used for treatment of some diseases, such as arthritis or migraine. The efficacy of this herb was originally attributed to the inhibition of platelet and polymorphonuclear leukocyte function.^{18,19} Recently, several reports showed that PTL inhibited the activation of TNF- α -induced, phorbol myristate acetate-induced, or LPS-induced NF- κ B, as well as LPS-induced MAPKs, in diverse cell lines.²⁰⁻²³

In the present study we investigated the effects of PTL on the maturation of human monocyte-derived DCs. We found that PTL inhibited molecular and functional DC maturation stimulated with LPS but not with TNF- α . This inhibition was correlated with blockade of the p38 MAPK but not the NF- κ B, pathway.

METHODS

Culture of DCs from buffy coats

PBMCs obtained according to institutional guidelines were separated by means of Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. PBMCs (40×10^6 /mL) were incubated in 10 mL of RPMI-1640 supplemented with 5% heat-inactivated FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin (all Gibco-BRL, Paisley, United Kingdom) for 1 hour at 37°C. More than 85% of adherent cells recovered after incubation were CD14⁺ cells. CD14⁺ cells (1×10^6 /mL) were then cultured in 3 mL of RPMI-1640 supplemented with 10% heat-inactivated FCS, 10 mmol/L HEPES, 1% nonessential amino acids, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin (all Gibco-BRL), 50 μ mol/L 2-mercaptoethanol (Sigma, Buchs, Switzerland), 10^3 U/mL recombinant human (rh) GM-CSF (Stratagen, Hannover, Germany), and 500 U/mL rhIL-4 (ImmunoKontakt, Lugano, Switzerland) for 5 days. Cells were fed with 1.5 mL of fresh medium containing 100 μ mol/L 2-mercaptoethanol, 2×10^3 U/mL rhGM-CSF, and 10^3 U/mL rhIL-4 on days 2 and 4. Immature DCs (2×10^5 /mL) were then stimulated with 20 ng/mL LPS (*Escherichia coli*, strain 055:B5; Difco, Detroit, Mich) or 100 U/mL rhTNF- α (a gift of Glaxo Wellcome, Geneva, Switzerland) in 1 mL of medium containing 10^3 U/mL rhGM-CSF and 500 U/mL rhIL-4 for 48 hours to obtain mature DCs. Immature DCs were pretreated with increasing concentrations of PTL for 1 hour before stimulation, and PTL was left in culture during stimulation to investigate the effect of PTL.

Chemical reagents and antibodies

PTL (99% purity, Sigma) was dissolved in dimethyl sulfoxide (Sigma) and further diluted in HBSS (Gibco-BRL). The final concentration of dimethyl sulfoxide never exceeded 0.05%. FITC-labeled anti-CD80 mAb (mouse IgM, clone BB1), FITC-labeled CD19 mAb (mouse IgG1, clone HIB19), phycoerythrin (PE)-labeled CD86 mAb (mouse IgG2b, clone IT2.2), allophycocyanin-labeled CD40 mAb (mouse IgG1, clone 5C3), and allophycocyanin-labeled streptavidin were from PharMingen (San Diego, Calif). PE-labeled CD83 mAb (mouse IgG2b, clone HB15A) was from Immunotech (Marseille, France), biotin-conjugated HLA-

DP,DQ,DR mAb (mouse IgG2a, clone CR3/43) was from Dako (Glostrup, Denmark), FITC-labeled CD1a mAb (mouse IgG2b, clone WM-35) was from PeliCluster (Amsterdam, Netherlands), PE-labeled CD3 mAb (mouse IgG1, clone SK7) was from Becton-Dickinson (Mountain View, Calif), biotin-conjugated CD14 mAb (mouse IgG2a, clone UCHM1) was from Ancell (Bayport, Minn), and FITC-labeled dextran (40 kD) was from Molecular Probes (Eugene, Ore). FITC- and PE-labeled mouse IgG1, FITC- and PE-labeled mouse IgG2b, FITC-labeled mouse IgM, allophycocyanin-labeled mouse IgG1 (PharMingen), and biotin-conjugated mouse IgG2a (Ancell) were used as isotype-matched controls.

Flow cytometric analysis

After pretreatment with PTL for 1 hour and stimulation for 48 hours, DCs were harvested and washed twice with cold buffer (PBS containing 2% FCS and 0.1% sodium azide [Sigma]). Cells were then incubated in 50 μ L of cold buffer containing 200 μ g/mL mouse IgG (Sigma) for 15 minutes on ice. Subsequent stainings with labeled mAbs or isotype-matched controls were performed for 30 minutes on ice and then with allophycocyanin-labeled streptavidin for 15 minutes on ice in case of biotin-conjugated mAbs. Stained cells were then washed twice and resuspended in 300 μ L of cold buffer containing 10 μ g/mL 7-amino-actinomycin D (Sigma) and analyzed for 4-color immunofluorescence with a FACScalibur cell analyzer (Becton-Dickinson). More than 1×10^4 cells were analyzed for each sample, and results were processed by using Cellquest software (Becton-Dickinson).

Flow cytometric analysis of NF- κ B translocation in DC nuclei preparations

The detection by means of flow cytometry of NF- κ B nuclear translocation in stimulated immature DCs was performed as previously described.²⁴ Immature DCs cultured on flat-bottom 24-well plates at a concentration of 2×10^5 cells per well were pretreated with 10 μ mol/L PTL for 1 hour at 37°C and subsequently stimulated with 20 ng/mL LPS or 100 U/mL TNF- α for 30 minutes. Cells were then transferred to V-bottom 96-well plates and washed twice with PBS. Nuclei preparations were obtained by incubating cells with 200 μ L of Pipes-Triton buffer (10 mmol/L Pipes, 0.1 mol/L NaCl, 2 mmol/L MgCl₂, and 0.1% Triton X-100 in H₂O) for 30 minutes at 4°C. After 2 washes in cold buffer, nuclei were stained with anti-human NF- κ B p65 mAb (Santa Cruz Biotechnology, Santa Cruz, Calif) at 5 μ g/mL or with appropriate isotype-matched control for 30 minutes at 4°C. After 2 additional washes, nuclei were incubated for 30 minutes at 4°C with FITC-conjugated F(ab')₂ goat anti-mouse IgG (1:300 dilution, Dako) and then washed twice in cold buffer. Nuclei were then analyzed by means of flow cytometry on a FACSVantage (Becton-Dickinson) equipped with a pulse processor module, allowing doublet discrimination. Nuclei were counterstained with 1 μ g/mL propidium iodide (Molecular Probes) and gated on the basis of propidium iodide staining (FL-3 measured at 630 nm) after doublet elimination by FL-3 area versus FL-3 width measurement. Two to 3×10^4 events were recorded for each sample.

Allogeneic mixed leukocyte reaction

PBMCs were obtained as described above, and after incubation for 1 hour at 37°C in a Petri dish, nonadherent cells were passed through a nylon wool column. Allogeneic CD3⁺ T cells thus obtained were distributed at 5×10^4 cells per well and incubated for 5 days in the presence of graded numbers of irradiated DCs (3000 rad, ¹³⁷Cs source). Tritiated thymidine (1 μ Ci/well; New England Nuclear, Boston, Mass) incorporation over 12 hours was determined with a liquid scintillation counter (Wallac, Regensdorf, Switzerland).

TABLE I. Effect of PTL on expression of surface molecules on DCs

	CD86	CD83	CD80	CD40	MHC II	CD1a
Controls						
G4	45.0 ± 4.1	21.6 ± 1.9	23.6 ± 2.0	25.0 ± 3.7	334.1 ± 70.1	363.5 ± 89.8
PTL (10 μmol/L)	18.8 ± 2.4*	13.0 ± 1.9	17.8 ± 1.5	20.1 ± 4.4	221.0 ± 45.3	253.8 ± 68.9
LPS						
PTL (0 μmol/L)	245.1 ± 32.2	134.0 ± 17.2	53.2 ± 5.6	54.4 ± 9.8	745.5 ± 62.8	331.3 ± 79.5
PTL (1 μmol/L)	218.8 ± 22.1	129.9 ± 13.5	45.1 ± 4.6	49.8 ± 9.1	517.8 ± 32.0	367.0 ± 70.7
PTL (5 μmol/L)	54.6 ± 12.4†	26.7 ± 6.1†	25.4 ± 3.9†	23.5 ± 4.9*	414.1 ± 61.9*	250.7 ± 63.0
PTL (10 μmol/L)	28.9 ± 7.2†	13.8 ± 2.5†	18.3 ± 2.7†	23.4 ± 5.5*	220.2 ± 37.1*	259.9 ± 71.3
TNF-α						
PTL (0 μmol/L)	86.7 ± 16.4	71.6 ± 12.3	27.2 ± 3.2	35.4 ± 7.2	338.3 ± 56.5	291.6 ± 64.0
PTL (10 μmol/L)	91.8 ± 24.0	65.2 ± 11.2	22.8 ± 1.7	32.3 ± 5.2	316.6 ± 50.6	255.2 ± 51.5

Immature DCs were pretreated with PTL and stimulated with LPS or TNF-α for 48 hours. The expression of each surface molecule was then analyzed by means of flow cytometry. These results are means of 7 independent experiments and shown as units of mean fluorescence intensity ± SEM.

G4, Immature DCs cultured for 48 hours in the presence of GM-CSF and IL-4.

**P* < .05 and †*P* < .005 compared with respective stimulants.

FITC-labeled dextran uptake

Cultured DCs were washed twice and resuspended in 1 mL of RPMI-1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 25 mmol/L HEPES. Cells were then incubated with FITC-labeled dextran (0.2 mg/mL) either at 4°C or 37°C for 1 hour. Cells were washed twice with cold buffer and analyzed with a FACScalibur cell analyzer, as described above.

Production of TNF-α, IL-12 p40, and p70 from DCs

After pretreatment with increasing concentrations of PTL (0.01–10 μmol/L) for 1 hour, immature DCs were stimulated with LPS (20 ng/mL) for 24 hours (TNF-α) or 48 hours (IL-12 p40 and p70), and the amount of TNF-α, IL-12 p40, and p70 in each supernatant was measured with TNF-α (BioSource International, Camarillo, Calif), IL-12 p40 (BioSource), and IL-12 p70 (Endogen, Woburn, Mass) ELISA kits, respectively.

Western blot analysis

After PTL treatment, immature DCs were stimulated with 20 ng/mL LPS for 30 minutes or 100 U/mL TNF-α for 5 minutes, according to preliminary kinetics experiments. Cells (1 × 10⁶ per condition) were then washed twice with cold PBS and lysed with 100 μL of lysis buffer (50 mmol/L Tris/HCl [pH 7.4], 1% Triton X-100, 150 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, 2 mmol/L ethyleneglycol-bis-[β-aminoethylether]-N,N,N',N'-tetraacetic acid, 40 mmol/L β-glycerophosphate, 50 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 200 μmol/L sodium orthovanadate, 0.3 mmol/L leupeptin, 1 mmol/L pepstatin A, 1 mmol/L phenylmethylsulfonyl fluoride, and 100 nmol/L okadaic acid). The homogenates were centrifuged at 14,000 rpm for 15 minutes at 4°C. Ten to 20 μg of cell lysates were electrophoresed on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking with 5% fat-free dried milk, membranes were incubated with either anti-phosphorylated p38 MAPK antibody or anti-phosphorylated MAPK kinase (MKK) 3/6 antibody (Cell Signaling Technology, Beverly, Mass) for 12 hours at 4°C and then with horseradish peroxidase-labeled goat anti-rabbit IgG (Bio-Rad, Hercules, Calif) for 1.5 hours. Immunoreactive bands were visualized with ECL detection reagent (Amersham, Zurich, Switzerland) and quantified with a scanning densitometer (Molecular Dynamics, Sunnyvale, Calif).

After stripping, the membranes were reprobed with either anti-total p38 MAPK antibody or anti-total MKK3 antibody (Cell Signaling) as respective loading controls. Immature DCs (1 × 10⁶ per condition) were also treated with PTL (10 μmol/L) and subsequently stimulated with 20 ng/mL LPS for 10 to 60 minutes or 100 U/mL TNF-α for 5 minutes and then lysed with lysis buffer. Lysates were electrophoresed on gels and transferred to nitrocellulose membranes. Membranes were then incubated with anti-phosphorylated inhibitory NF-κBα (IκBα) antibody (Cell Signaling) for 12 hours at 4°C. After visualization, membranes were stripped and reprobed with anti-total IκBα antibody (Cell Signaling).

Statistical analysis

The Student *t* test was used to analyze the results, and a *P* value of less than .05 was considered to be statistically significant.

RESULTS

PTL inhibits upregulation of costimulatory molecules on DCs induced by LPS but not by TNF-α

Immature DCs were pretreated with PTL at concentrations between 0.01 and 50 μmol/L for 1 hour and subsequently stimulated with 20 ng/mL LPS or 100 U/mL TNF-α for 48 hours in the presence of PTL to investigate the effect of PTL on DC maturation. The expression of CD86, CD83, CD80, CD40, MHC class II, and CD1a on DCs was analyzed by means of flow cytometry. First, the toxicity of PTL was examined by use of 7-amino-actinomycin D, which discriminates between viable, apoptotic, and necrotic cells.²⁵ Up to 10 μmol/L PTL did not induce apoptosis or necrosis in DCs with mortality of less than 20%, whereas more than 90% of DCs underwent apoptosis at a concentration of 50 μmol/L after 48 hours of incubation (data not shown). LPS strongly upregulated the expression of CD86, CD83, CD80, CD40, and MHC class II on DCs, and this upregulation was abrogated by PTL in a concentration-dependent manner, with an inhibitory concentration of 50% (IC₅₀) of approximately 2.5 μmol/L, whereas the expression of CD1a was not significantly affected by PTL. On the other hand, TNF-

α -induced upregulation of these molecules was only marginally affected by 10 μ mol/L PTL (Table 1). Although PTL is also known to cause allergic contact dermatitis,²⁶ it did not show any stimulatory effect on immature DCs and further inhibited the expression of surface molecules, especially CD86, on immature DCs (Table 1, upper panel). PTL was thought to inhibit potential activation of immature DCs that had occurred during the experimental process.

PTL inhibits the upregulation of allostimulatory capacity and the downregulation of endocytosis in LPS-stimulated DCs

Mature DCs have the capacity to induce proliferation in allogeneic T cells at a much higher level than immature DCs.³ We therefore performed mixed leukocyte reaction to determine the effect of PTL on the allostimulatory capacity of DCs. PTL inhibited the allostimulatory capacity of LPS-stimulated DCs in a concentration-dependent manner. DCs treated with 10 μ mol/L PTL showed an allostimulatory capacity equal to that of immature DCs (Fig 1, A), whereas the effect of PTL on TNF- α -stimulated DCs was marginal up to 10 μ mol/L (Fig 1, B). Immature DCs lose their high level of endocytotic activity during the maturation process.⁴ Thus we tested whether PTL affected the uptake of FITC-labeled dextran by DCs. PTL completely inhibited the downregulation of FITC-labeled dextran uptake induced by LPS, whereas PTL did not significantly prevent TNF- α -induced downregulation (Fig 1, C). These results show that PTL can prevent the acquisition by LPS-stimulated DCs of functional features normally present in mature DCs.

PTL prevents TNF- α and IL-12 secretion by LPS-stimulated DCs

DCs produce many cytokines, including TNF- α and IL-12, during maturation.¹ We wondered whether LPS-induced secretion of TNF- α , IL-12 p40, and IL-12 p70 was affected by PTL. Immature DCs were pretreated with increasing concentrations of PTL and subsequently stimulated with LPS. The production of TNF- α , IL-12 p40, and IL-12 p70 were quantified by means of ELISA. LPS induced significant production of TNF- α , IL-12 p40, and IL-12 p70, and these cytokine productions were abrogated by PTL in a concentration-dependent manner, with an IC₅₀ of approximately 1 μ mol/L (Fig 2, A-C).

PTL inhibits p38 MAPK and MKK3/6 phosphorylation induced by LPS but not by TNF- α

PTL inhibited DC maturation induced by LPS but not by TNF- α . We demonstrated previously that inhibition of p38 MAPK by SB203580, unlike PTL, inhibited DC maturation induced by both LPS and TNF- α .¹² We therefore hypothesized that PTL might differentially affect the p38 MAPK pathway activated by LPS or TNF- α . To test this, we performed Western blot analysis to detect the phosphorylated p38 MAPK. Immature DCs were pre-

treated with PTL and subsequently stimulated with 20 ng/mL LPS for 30 minutes or 100 U/mL TNF- α for 5 minutes. Both LPS and TNF- α induced phosphorylation of p38 MAPK. PTL inhibited LPS-induced phosphorylation of p38 MAPK in a concentration-dependent manner (IC₅₀, 7.5 μ mol/L), whereas PTL enhanced, rather than prevented, TNF- α -induced phosphorylation (Fig 3, A).

p38 MAPK is activated by the dual phosphorylation of both threonine and tyrosine residues in the Thr-Gly-Tyr motif, which is induced by 2 specific MKKs, MKK3 and MKK6.^{27,28} We next asked whether MKK3 and MKK6 were affected by PTL. PTL also inhibited the phosphorylation of MKK3/6 induced by LPS (IC₅₀, 5.5 μ mol/L) but not by TNF- α (Fig 3, B). These results indicate that PTL prevented the activation of the p38 MAPK pathway induced by LPS.

PTL prevents NF- κ B nuclear translocation and I κ B degradation induced by both LPS and TNF- α

NF- κ B is another molecular family whose activation is associated with DC maturation.¹⁵ NF- κ B normally binds I κ B, which impedes NF- κ B nuclear translocation from the cytoplasm to the nucleus. Once cells are exposed to inflammatory stimuli, including LPS and TNF- α , I κ B is phosphorylated, leading to I κ B degradation and nuclear translocation of NF- κ B.²⁹ We thus examined whether PTL influenced nuclear translocation of NF- κ B induced by LPS or TNF- α . After PTL treatment, nuclei were isolated from stimulated DCs, and the effect of PTL on fluorescence intensity of nuclear p65 was analyzed by means of flow cytometry. Although LPS and TNF- α increased p65 fluorescence intensity in isolated nuclei, preincubation of DCs with PTL partially inhibited both LPS- and TNF- α -induced increase of nuclear p65 fluorescence intensity (Fig 4, A). The effect of PTL on I κ B α phosphorylation and degradation was next examined by means of Western blot analysis. Both LPS and TNF- α induced the phosphorylation of I κ B α and reduced the amount of total I κ B α , indicating its degradation. PTL preincubation before stimulation with LPS or TNF- α prevented the phosphorylation of I κ B α , whereas the total I κ B α signal did not diminish, as seen with LPS or TNF- α alone (Fig 4, B). This indicates that PTL prevented the phosphorylation and subsequent degradation of I κ B α .

DISCUSSION

We found that PTL inhibited the upregulation of CD80, CD83, CD86, CD40, and MHC class II; the allostimulatory function; the production of TNF- α and IL-12; and the downregulation of FITC-labeled dextran uptake in human monocyte-derived DCs stimulated with LPS but not with TNF- α . The inhibitory effect of PTL on DC maturation was preceded by inhibition of the phosphorylation of p38 MAPK and MKK3/6 induced by LPS but not by TNF- α . We have previously shown that the p38 MAPK

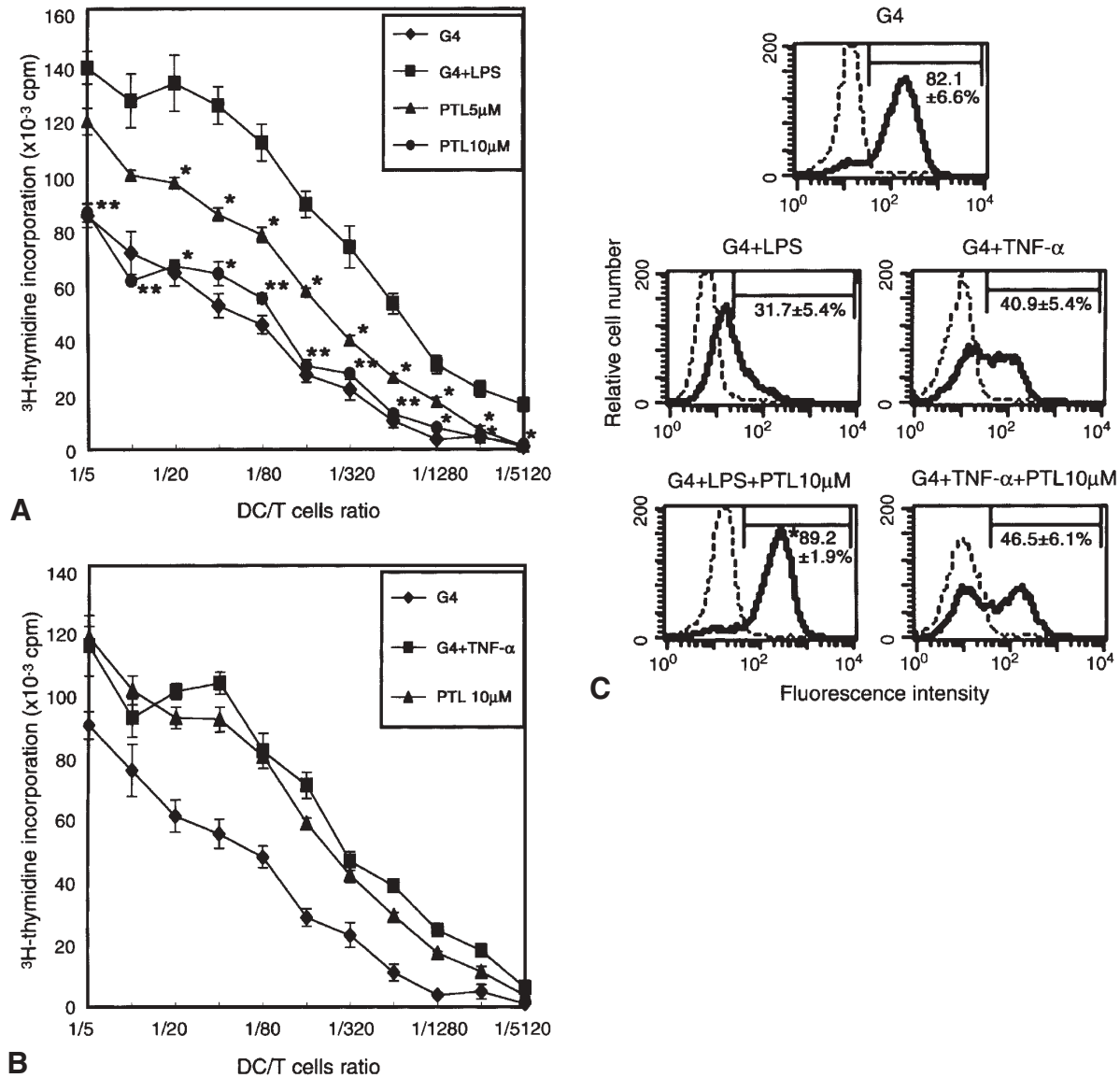


FIG 1. The effect of PTL on the allostimulatory and endocytotic capacities of DCs. **A** and **B**, After PTL treatment, immature DCs were stimulated with LPS or TNF- α for 48 hours. Allogeneic T-cell proliferation was measured after 5 days of coculture with DCs. These data are means $\pm$ SEM of quadruplicates and representative of 4 independent experiments. **C**, Immature DCs were prepared as described above, and cells were then incubated with FITC-labeled dextran for 1 hour at 4°C (dotted lines) or 37°C (solid lines). Representative results and mean percentages $\pm$ SEM of FITC-positive cells of 4 independent experiments are shown. * P < .05 and ** P < .005 compared with respective stimulants. G4, Immature DCs cultured for 48 hours in the presence of GM-CSF and IL-4.

pathway was involved in DC maturation using a p38 MAPK specific inhibitor.¹² The results obtained in this report with PTL are consistent with this view.

LPS is the major surface membrane component of the gram-negative bacteria and can strongly stimulate the innate immune system. LPS is recognized by toll-like receptor 4 (TLR4), and that signal is transmitted through pathways remarkably similar to IL-1 and IL-18 receptors, which share some components, ultimately leading

to the activation of NF- κ B and MAPKs.³⁰ Immature DCs express several TLRs, including TLR4, and increase their expression transiently after LPS stimulation.^{31,32} The signal from TLR4 is transmitted to IL-1 receptor-associated kinases through adapter proteins.^{30,33,34} After autophosphorylation, IL-1 receptor-associated kinase interacts with TNF receptor-associated factor 6 (TRAF6), which mediates the activation of 2 distinct MAPK kinase kinases (MAPKKKs), transforming

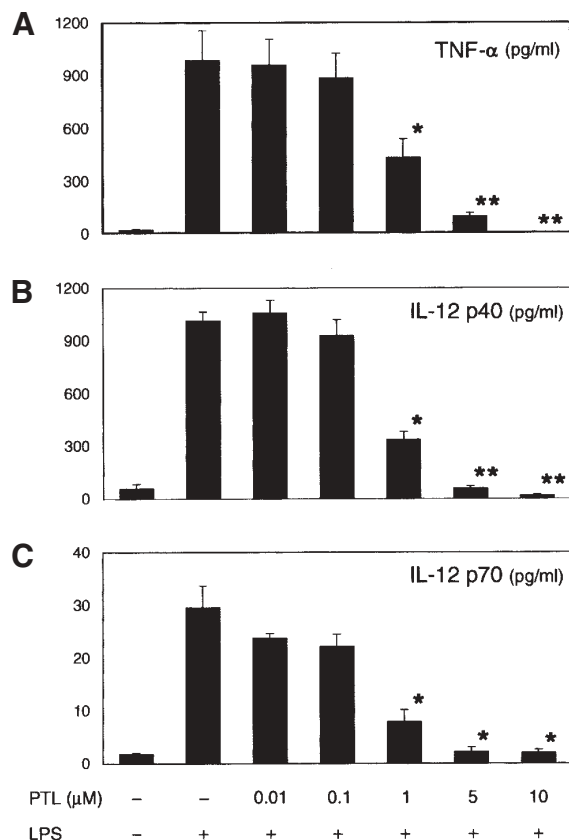


FIG 2. The effect of PTL on LPS-induced secretion of TNF- α , IL-12 p40, and IL-12 p70 by DCs. Immature DCs were pretreated with PTL and stimulated with LPS. TNF- α (A), IL-12 p40 (B), and p70 (C) in the supernatant were measured by means of ELISA. These data are means $\pm$ SEM of 4 (TNF- α and p40) and 3 (p70) independent experiments. * P < .05 and ** P < .005 compared with LPS.

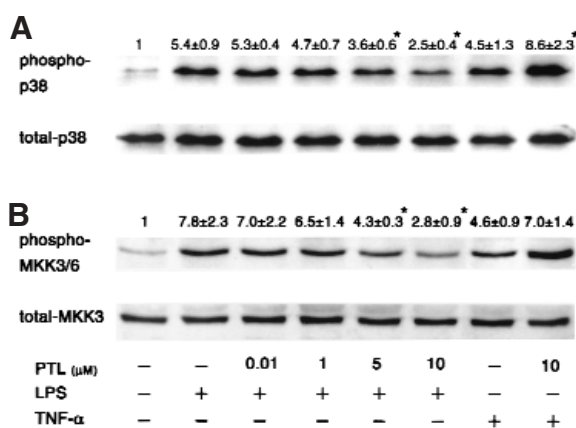


FIG 3. The effect of PTL on LPS- or TNF- α -induced phosphorylation of p38 MAPK and MKK3/6. Immature DCs were pretreated with PTL for 1 hour and then stimulated with LPS for 30 minutes or TNF- α for 5 minutes. Cellular DC lysates were then subjected to Western blot analysis for p38 MAPK (A) or MKK3/6 (B). The areas of the immunoreactive bands representing phosphorylated p38 MAPK and MKK3/6 were quantified by means of scanning densitometry and normalized by the areas of respective loading controls. Fold inductions compared with the area of control were calculated. Representative results and mean fold inductions $\pm$ SEM of 4 (p38 MAPK) and 3 (MKK3/6) independent experiments are shown. * P < .05 compared with respective stimulants.

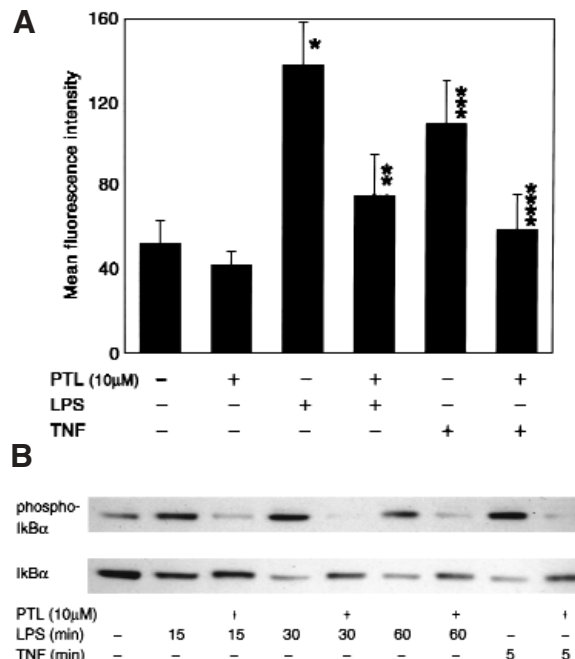


FIG 4. The effect of PTL on LPS- or TNF- α -induced NF- κ B p65 nuclear translocation and I κ B α degradation. **A**, After pretreatment with PTL for 1 hour, immature DCs were stimulated with LPS or TNF- α for 30 minutes. DC nuclei were then stained with anti-human NF- κ B p65 mAb or isotype-matched control and then subjected to flow cytometric analysis. Means $\pm$ SEM of 4 independent experiments are shown for each condition. * P < .012, ** P < .004, *** P < .009, and **** P < .005, as calculated with the 2-tailed Student t test. **B**, Immature DCs were stimulated with LPS or TNF- α for indicated periods of time after pretreatment with PTL. Cellular DC lysates were then subjected to Western blot analysis for phosphorylated and total I κ B α . These results are representative of 3 independent experiments.

growth factor β -activated kinase 1 (TAK1) and MAPK/ERK kinase kinase 1 (MEKK1).^{35,36} Then TAK1 activates p38 MAPK through MKK3/6 activation, as well as NF- κ B through NF- κ B-inducing kinase (NIK).³⁷ TNF- α , on the other hand, triggers a receptor complex formation, including the type I TNF receptor, and TRAF2 is further recruited into the complex.³⁸ TRAF2 mediates the activation of NIK and MEKK1, leading to NF- κ B activation, as well as the MAPKKK apoptosis signal-regulating kinase 1, which can activate MKK3/6.³⁹⁻⁴² Therefore both MKK3 and MKK6 are activated by at least 2 distinct MAPKKKs (ie, TAK1 through the IL-1 receptor/TLR pathway and apoptosis signal-regulating kinase 1 through the type I TNF receptor pathway). Although further investigations are needed to identify the molecular target or targets of PTL inhibition, our observations suggest that PTL prevents p38 MAPK activation by inhibiting the IL-1 receptor/TLR pathway at the level of MAPKKK or farther upstream.

Another signaling pathway that seems to play a major role in DC maturation is the NF- κ B pathway.¹⁵⁻¹⁷ Furthermore, PTL was reported to inhibit NF- κ B activation induced by LPS or TNF- α in diverse cell lines.²⁰⁻²² Hehner et al²¹ reported that PTL inhibited a component of I κ B kinases (IKKs) in TNF- α -stimulated HeLa cells.

The activation of IKKs induces I κ B degradation and the nuclear translocation of NF- κ B.²⁹ IKKs are activated by LPS or TNF- α through 2 direct activators, NIK or MEKK1.^{43,44} We thus investigated the effect of PTL on NF- κ B in the context of DC maturation. Our results show that PTL inhibited the nuclear translocation of p65 induced by both LPS and TNF- α . In addition, PTL prevented the phosphorylation and subsequent degradation of I κ B α induced by LPS or TNF- α . This is unlike the effect of PTL on p38 MAPK phosphorylation in stimulated DCs. The phosphorylation of p38 MAPK induced by LPS was inhibited by PTL, whereas that induced by TNF- α was not. Furthermore, in contrast to LPS, PTL could not prevent phenotypical and functional features of DC maturation in response to TNF- α stimulation. Thus the correlation between p38 MAPK phosphorylation and DC maturation was better than that between NF- κ B activation and DC maturation in our experiments (Fig 5). Collectively, these results reinforce the crucial role played by p38 MAPK but not by NF- κ B, in the initiation of DC maturation. Our results further suggest that the TNF signaling pathway toward NF- κ B might have a PTL-sensitive element that the TNF pathway toward p38 MAPK might not. Both pathways, however, appear to possess the PTL-sensitive elements when activated with LPS. The molecular identification of any PTL-sensitive element or elements in these pathways remains to be established.

In conclusion, PTL appears to be a useful tool to dissect the intracellular signaling pathways involved in DC maturation and might provide the basis for development of compounds for therapeutic use in situations with excessive activation of DCs.

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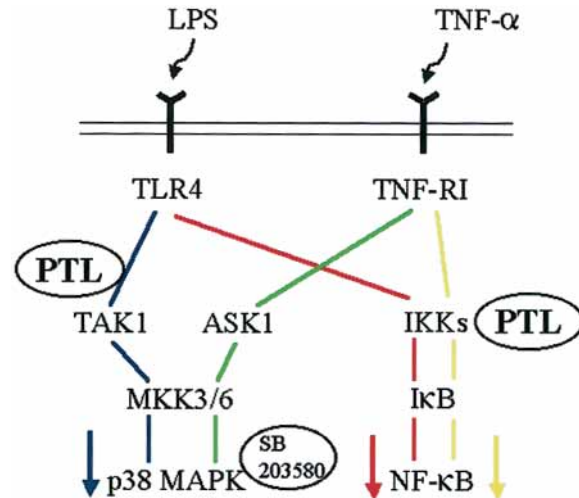


FIG 5. Schema for the effect of PTL on DC maturation. PTL inhibited LPS-induced but not TNF- α -induced, phosphorylation of p38 MAPK. On the other hand, both LPS- and TNF- α -induced nuclear translocation of p65 were inhibited by PTL. Because only LPS-induced DC maturation was inhibited by PTL, PTL might preferentially affect the molecule or molecules on the p38 MAPK pathway from the TLR4 involved in DC maturation. *TNF-RI*, Type I TNF receptor; *ASK1*, Apoptosis signal-regulating kinase 1.

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