Toll-like receptor 2-mediated dendritic cell activation by a *Porphyromonas gingivalis* synthetic lipopeptide

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A PG1828 gene-encoded triacylated lipoprotein was previously isolated from a *Porphyromonas gingivalis* lipopolysaccharide preparation as a Toll-like receptor (TLR) 2 agonist and its lipopeptide derivatives were synthesized based on the chemical structure. In the present study, granulocyte–macrophage colony stimulating factor-differentiated bone marrow-derived dendritic cells (BMDDCs) were stimulated separately with the *P. gingivalis* synthetic lipopeptide *N*-palmitoyl-S-[2-pentadecanoyloxy, 3-palmitoyloxy-(2*R*)-propyl]-L-Cys-Asn-Ser-Gln-Ala-Lys (PGTP2-RL) and its glyceryl stereoisomer (PGTP2-SL). Only PGTP2-RL activated BMDDCs from wild-type mice to secrete tumour necrosis factor- α , interleukin (IL)-6, IL-10 and IL-12p40, whilst PGTP2-RL-induced cytokine production was eliminated in TLR2 knockout ($^{-/-}$) BMDDCs. BMDDCs from wild-type mice but not TLR2 $^{-/-}$ mice responded to PGTP2-RL as well as Pam₃CSK₄ by increasing the expression of maturation markers, including CD80 (B7-1), CD86 (B7-2), CD40, CD275 (B7RP-1/inducible T-cell co-stimulatory ligand) and major histocompatibility complex class II. Taken together, these results indicate that the fatty acid residue at the glycerol position in the *P. gingivalis* lipopeptide plays a pivotal role in TLR2-mediated dendritic cell activation.

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INTRODUCTION

Porphyromonas gingivalis, a black-pigmented Gram-negative anaerobic rod, is considered to be associated with chronic periodontal disease (Slots *et al.*, 1986). The bacterium has been shown to possess various bioactive components including a cytoplasmic membrane, peptidoglycan, outer-membrane proteins, LPS and fimbriae on its cell surface (Offenbacher, 1996). These components have been demonstrated to induce multiple cytokines in periodontal tissues (Holt *et al.*, 1999). We previously separated a 16 kDa PG1828-encoded triacylated lipoprotein composed of two palmitoyl groups and one pentadecanoyl group at the N-terminal glycerocysteine from *P. gingivalis* strain 381 and found that it showed definite biological activities (Hashimoto *et al.*, 2004). More recently, we synthesized the derivatives of that lipopeptide on the basis of its chemical structure (Makimura *et al.*, 2006).

Dendritic cells (DCs) play an important role as professional antigen-presenting cells, which induce naïve T cells to

differentiate into T-helper type (Th) 1, Th2 and cytotoxic T cells (Banchereau & Steinman, 1998). Normally, DCs take up foreign antigens in the periphery, process the antigens and migrate to the T-cell area of lymph nodes, where they present the antigens. During this process of migration, DCs markedly change their characteristics; this is termed maturation. DC maturation is associated with enhanced expression of major histocompatibility complex (MHC) class II, B7 family co-stimulatory [CD80 (B7-1), CD86 (B7-2) and CD275 (B7RP-1/inducible T-cell co-stimulator ligand; ICOSL)] and signalling (CD40) molecules and with the secretion of inflammatory and immunoregulatory cytokines (Drakes *et al.*, 2004; Mellman & Steinman, 2001; Palucka & Banchereau, 2002). The process is reported to be induced by bacterial components such as LPS, lipoprotein/lipopeptide, polysaccharide, porin and CpG DNA (De Smedt *et al.*, 1996; Hertz *et al.*, 2001; Lin *et al.*, 2005; Nishiguchi *et al.*, 2001; Singleton *et al.*, 2005; Sparwasser *et al.*, 1998).

Toll-like receptors (TLRs), a family of mammalian homologues of *Drosophila* Toll, have been identified as pattern-recognition receptors that are expressed on cells of the innate immune system (Akira & Takeda, 2004). The recognition of microbial pathogens by TLRs leads to activation of various intracellular signalling cascades that regulate transcriptional nuclear factor- κ B, which subsequently produces cytokines

Abbreviations: BMC, bone marrow cell; BMDDC, bone marrow-derived dendritic cell; DC, dendritic cell; ICOSL, inducible T-cell co-stimulatory ligand; IL, interleukin; MHC, major histocompatibility complex; PE, phycoerythrin; rmGM-CSF, recombinant mouse granulocyte–macrophage colony stimulating factor; Th, T-helper type; TLR, Toll-like receptor; TNF, tumour necrosis factor.

and increases the expression of cell-surface molecules, including DC maturation markers (Akira, 2003; Imler & Hoffmann, 2003). To date, 10 and 13 TLRs have been reported in humans and mice, respectively (Kawai & Akira, 2005; Tabeta *et al.*, 2004). Among the TLR family proteins, TLR2 plays a central role in recognizing a broad range of microbial products and is crucial for recognizing microbial lipopeptides (Akira & Takeda, 2004). Furthermore, TLR2 has been shown to form heteromers with either TLR1 or TLR6 to recognize the lipopeptides (Takeuchi *et al.*, 2001, 2002). Interestingly, we recently showed that *P. gingivalis* synthetic lipopeptides activate immune cells through TLR2 but not TLR1/TLR6 (Makimura *et al.*, 2006). The present study was designed to investigate the biological activities of *P. gingivalis* synthetic lipopeptides with bone marrow-derived DCs (BMDDCs) and determine whether TLR2 mediates this process.

METHODS

Reagents. Synthetic lipopeptide derivatives derived from *P. gingivalis* lipoprotein, *N*-palmitoyl-*S*-[2-pentadecanoyloxy, 3-palmitoyloxy-(2*R*)-propyl]-*L*-Cys-Asn-Ser-Gln-Ala-Lys (PGTP2-RL) and its *S* stereoisomer (PGTP2-SL), were prepared according to a method described previously (Makimura *et al.*, 2006) and the structures are shown in Fig. 1. The lipopeptides were dissolved at a concentration of 10 mg ml⁻¹ in DMSO and used as stock solutions. The synthetic bacterial lipopeptide Pam₃CSK₄ was obtained from EMC Microcollections (Tuebingen, Germany) and dissolved at 1 mg ml⁻¹ in pyrogen-free double-distilled water (Otsuka Pharmaceutical). *Escherichia coli*-type lipid A (compound 506) was synthesized chemically as described previously (Imoto *et al.*, 1984) and dissolved at 2 mg ml⁻¹ in 0.1% (v/v) triethylamine aqueous solution. These stock solutions were diluted with culture medium immediately before use.

Mice. C57BL/6 mice were obtained from Japan SLC and TLR2 knockout (TLR2^{-/-}) mice (C57BL/6 background) were kindly provided by Dr S. Akira (Department of Host Defence, Research Institute for Microbial Diseases, Osaka University, Japan). The animals received humane care in accordance with our institutional guidelines and the legal requirements of Japan.

Isolation and differentiation of BMDDCs. BMDDCs were differentiated as described by Lutz *et al.* (1999), with minor modifications. Briefly, bone marrow cells (BMCs) were obtained by flushing

the femora and tibiae with a 26-gauge needle. To generate DCs, 10 ml cell suspension containing 2 × 10⁶ BMCs in RPMI 1640 (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma), 50 U penicillin ml⁻¹, 50 µg streptomycin ml⁻¹ and 20 ng recombinant mouse granulocyte-macrophage colony stimulating factor (rmGM-CSF; R&D Systems) ml⁻¹ was seeded in a 100 mm cell culture dish (day 0) and incubated for 10 days at 37 °C in a humidified 5% (v/v) CO₂ atmosphere. During the incubation period, the culture medium was changed to fresh medium containing 20 ng rmGM-CSF ml⁻¹ on days 3, 6 and 8. On day 10, non-adherent cells were collected and resuspended in culture medium without rmGM-CSF and incubated for 24 h before stimulation.

Stimulation of BMDDCs. The collected BMDDCs were centrifuged and resuspended in antibiotic-free RPMI 1640 containing 10% (v/v) FBS. The cells were cultured in a Falcon 2058 tube (Becton Dickinson Labware) at 4 × 10⁵ cells per 200 µl at 37 °C in a 5% (v/v) CO₂ atmosphere for 24 h, after which 200 µl culture medium containing the indicated doses of the test specimens was added to the DC culture tubes. Following 24 h incubation at 37 °C, the tubes were centrifuged and supernatants were collected to measure cytokine secretion. The collected cells were analysed for the induction of cell-surface markers. Pam₃CSK₄ and compound 506 were used as control TLR2 and TLR4 agonists, respectively (Aliprantis *et al.*, 1999; Hoshino *et al.*, 1999).

Cytokine quantification in culture supernatants. Interleukin (IL)-6 and tumour necrosis factor (TNF)-α levels were determined using commercially available ELISA kits (eBioscience), and IL-10 and IL-12p40 were analysed using Duo ELISA Development Sets (R&D Systems).

Immunocyto staining and flow cytometry. BMDDCs were stained with fluorochrome-conjugated antibodies at 10 µg ml⁻¹ and incubated for 30 min. Thereafter, the cells were washed with PBS containing 0.1% (w/v) NaN₃ and fixed with 1% (w/v) paraformaldehyde. Stained cells were analysed using a FACSCalibur with CellQuest software (BD Biosciences). The following antibodies were obtained from eBioscience: fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD11c, clone N418 (hamster IgG); FITC-conjugated anti-mouse MHC class II (I-A/I-E), clone M5/114.15.2 (rat IgG2b); FITC-conjugated anti-mouse CD80 (B7-1), clone 16-10A1 (hamster IgG); phycoerythrin (PE)-conjugated anti-mouse CD86 (B7-2), clone GL1 (rat IgG2a); PE-conjugated anti-mouse CD40, clone 1C10 (rat IgG2a); PE-conjugated anti-mouse CD275 (B7RP-1/ICOSL), clone HK5.3 (rat IgG2a); and isotype controls for FITC-conjugated hamster IgG, FITC-conjugated rat IgG2b and PE-conjugated rat IgG2a.

Statistical analysis. Statistical significance between groups was evaluated by analysis of variance and a Tukey multiple-comparison test using the Microsoft EXCEL 2004 and STATCEL2 (OMS Publishing) software packages. Differences between groups were considered significant at the level of *P* < 0.05.

RESULTS AND DISCUSSION

Expression of DC marker CD11c

DCs play a pivotal role in the relationship of innate immunity with adaptive immunity. A number of studies have indicated that epidermal immature DCs, termed Langerhans cells, exist in human gingiva (Cutler *et al.*, 1999; Gemmell *et al.*, 2002; Jotwani & Cutler, 2003; Jotwani *et al.*, 2001; Segulier *et al.*, 2000). DCs apparently mature upon contact with a variety of bacteria in the gingiva and

Fig. 1. Schematic representation of synthetic PGTP2 compounds used in this study. *, Glyceryl stereoisomer (*R* or *S*).

then contribute to T-cell modulation in periodontal lesions. To address the interaction of *P. gingivalis* lipopeptide derivatives with DCs, we prepared BMDDCs derived from BMCs of wild-type and TLR2^{-/-} mice. BMCs were cultured in the presence of rmGM-CSF for 10 days and the cells were investigated for surface expression of CD11c, a well-known marker antigen of DCs (Wallet *et al.*, 2005), on days 0 and 10. As shown in Fig. 2, both wild-type and TLR2^{-/-} cells showed CD11c expression on day 10, indicating that these cells had differentiated successfully into DCs.

Cytokine-producing activity of PGTP synthetic compounds

Cytokine production during the activation process of DCs is also an important parameter in deciding the outcome of B- and T-cell responses. Following bacterial stimulation, DCs first induce TNF- α , which is able to activate vicinal non-infected DCs, and then produce IL-6 and IL-12 (Rescigno *et al.*, 2000). IL-6 promotes terminal differentiation of B cells into plasma cells and polarization of naïve T cells to effector Th2 cells (Rincon *et al.*, 1997), whilst IL-12 is known to be a critical factor in the development of Th1 cells (Hsieh *et al.*,

Fig. 2. Cell-surface expression of CD11c. BMCs derived from wild-type and TLR2^{-/-} mice were cultured with rmGM-CSF for 10 days. Cells were stained on day 0 and again on day 10 with FITC-conjugated anti-CD11c antibody (bold line) or an isotype-matched control antibody (thin line).

Fig. 3. Cytokine secretion by BMDDCs in response to PGTP2 compound. Wild-type BMDDCs were stimulated with the indicated doses of PGTP2-RL (●), PGTP2-SL (○), Pam₃CSK₄ (▲) or compound 506 (□) for 24 h and the levels of secreted cytokines (IL-6, TNF- α , IL-10 and IL-12p40) in culture supernatants were determined by ELISA. All samples were assayed in triplicate and the results are expressed as means $\pm$ SD. The mean values were significantly different from non-stimulated cultures. **, $P < 0.01$; *, $P < 0.05$.

Fig. 4. TLR2 dependency of cytokine secretions by BMDDCs in response to PGTP2-RL. BMDDCs from wild-type (open bars) and TLR2^{-/-} (filled bars) mice were stimulated or not with PGTP2-RL, Pam₃CSK₄ or compound 506 at 1 µg ml⁻¹ for 24 h and the levels of secreted cytokines (IL-6, TNF-α, IL-10 and IL-12p40) in culture supernatants were determined by ELISA. All samples were assayed in triplicate and the results are expressed as means ± SD. The mean values were significantly different. **, $P < 0.01$; *, $P < 0.05$.

1993). In contrast to these cytokines, IL-10 has been shown to inhibit IL-12, leading to Th2 responses (Brightbill *et al.*, 2000). To evaluate the cell-activating capacities of the PGTP2 compounds, we examined cytokine production by BMDDCs stimulated with the indicated doses of PGTP2-RL, PGTP2-SL, Pam₃CSK₄ or compound 506 for 24 h (Fig. 3). PGTP2-RL induced IL-6, TNF-α, IL-10 and IL-12p40 production by BMDDCs in a dose-dependent manner, whereas PGTP2-SL induced little or no production at the highest concentration tested (10 µg ml⁻¹). Both Pam₃CSK₄ and compound 506 showed stronger cytokine production compared with PGTP2-RL. These results are in agreement with those of our previous study that examined IL-6 and IL-8 production by a murine macrophage cell line and human peripheral blood mononuclear cells (Makimura *et al.*, 2006) and suggest that the fatty acid residue at the glycerol position of the PGTP2 compounds plays an important role in cell activation.

Involvement of TLR2 in PGTP-induced cytokine production

We investigated the contribution of a cell-surface receptor on BMDDCs to the signalling of PGTP2-RL. BMDDCs from wild-type and TLR2^{-/-} mice were stimulated with 1 µg PGTP2-RL ml⁻¹ for 24 h and levels of secreted cytokines were determined by ELISA (Fig. 4). Stimulation of TLR2^{-/-} BMDDCs with PGTP2-RL as well as with Pam₃CSK₄, a TLR2 agonist, resulted in almost no induction of IL-6, TNF-α, IL-10 or IL-12p40. On the other hand, compound 506, a TLR4 agonist, induced approximately the same amount of cytokine production in both wild-type BMDDCs and TLR2^{-/-} BMDDCs. These results clearly indicate that PGTP2-RL activates BMDDCs to induce these cytokines in a TLR2-dependent fashion. It has been reported that the TLR4 agonist LPS predominantly activates Th1 responses, whilst the TLR2 agonist Pam₃CSK₄ induces Th2 responses at higher levels (Dillon *et al.*, 2004). In agreement with those

Fig. 5. TLR2 dependency of upregulation of cell-surface maturation markers on BMDDCs in response to PGTP2-RL. BMDDCs derived from wild-type and TLR2^{-/-} mice were stimulated with (bold line) or without (thin line) PGTP2-RL (a), PGTP2-SL (b), Pam₃CSK₄ (c) or compound 506 (d) at 1 µg ml⁻¹ for 24 h, after which the cells were stained with FITC- or PE-conjugated antibodies, as described in Methods.

results, wild-type BMDDCs in the present study significantly induced the Th2-restricted cytokine IL-10 in response to PGTP2-RL at the lowest concentration ($0.1 \mu\text{g ml}^{-1}$) among PGTP2-RL-induced cytokines (Fig. 3). Furthermore, the lowest concentration of Pam₃CSK₄, but not of compound 506, induced definite IL-10 production by wild-type BMDDCs (Fig. 3).

Involvement of TLR2 in PGTP2-induced upregulation of maturation markers

Increased surface expression of MHC, co-stimulatory and signalling molecules on DCs was found to be closely related to antigen presentation to T cells and was followed by activation of the acquired immune system. Therefore, we next examined the involvement of TLR2 in PGTP2-induced upregulation of DC surface maturation markers. Slight upregulation of CD80, CD86, CD40, CD275 and MHC class II was seen in wild-type BMDDCs stimulated with PGTP2-RL for 24 h, whilst upregulation did not occur in TLR2^{-/-} BMDDCs (Fig. 5a). In comparison with the cells treated with PGTP2-RL, BMDDCs treated with $1 \mu\text{g}$ PGTP2-SL ml⁻¹ showed no increased expression of these markers (Fig. 5b). Similar to PGTP2-RL, Pam₃CSK₄-induced upregulation of maturation markers was eliminated in TLR2^{-/-} BMDDCs (Fig. 5c). In contrast, compound 506 increased the expression of maturation markers on the surface of both wild-type and TLR2^{-/-} BMDDCs (Fig. 5d). A synthetic 19 kDa lipopeptide derived from *Treponema pallidum* 47 kDa lipoprotein has been demonstrated previously to enhance the expression of CD80 and CD86 on human DCs in a TLR2-dependent manner (Hertz *et al.*, 2001). It has also been shown that a synthetic diacylated lipopeptide from *Mycoplasma salivarium* (FSL-1) augments the expression of CD80, CD86 and MHC class II on BMDDCs (Kiura *et al.*, 2006). In addition, it has been demonstrated that human monocyte-derived DCs mature upon contact with *P. gingivalis* cells (Jotwani *et al.*, 2001). Thus PGTP2-RL induced upregulation of maturation markers on the surface of BMDDCs through TLR2 in a manner similar to these synthetic lipopeptides. We concluded that the lipopeptide may be a crucial component in *P. gingivalis*-induced DC activation.

Taken together, our results demonstrated that a *P. gingivalis* synthetic lipopeptide is capable of activating BMDDCs to induce cytokine production and increase maturation surface markers through TLR2. These findings may have important implications for host defence responses in periodontal lesions.

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