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Nicotine up-regulates IL-8 expression in human gingival epithelial cells following stimulation with IL-1β or *P. gingivalis* lipopolysaccharide via nicotinic acetylcholine receptor signalling

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ABSTRACT

Objective: Cigarette smoking is an important risk factor for periodontal disease. The aim of this study is to evaluate the effect of nicotine, a major component of cigarette smoke, on interleukin-8 (IL-8) production and cellular signalling via nicotinic acetylcholine receptors (nAChRs) in human gingival epithelial cells (HGECs).

Design: Messenger RNA (mRNA) expression of nAChR subunits in three different HGEC lines (epi 4, Tfx and E6E7) was assessed using reverse transcription-polymerase chain reaction (RT-PCR). HGECs were stimulated by 1×10^{-3} M nicotine in the presence or absence of IL-1β or *Porphyromonas gingivalis* lipopolysaccharide (LPS). IL-8 production was then examined using real-time PCR and enzyme-linked immunosorbent assay. Nicotine-mediated signalling in the epi 4 cell line was also evaluated by Western blotting.

Results: HGECs expressed several nAChR subunits. Nicotine increased the secretion of IL-8 from HGECs that were cultured in the presence of IL-1β or *P. gingivalis* LPS and also induced the phosphorylation of extracellular signal-regulated kinase (ERK) in epi 4. Pretreatment with non-selective nAChR antagonist or intracellular calcium chelator reduced the nicotine-induced phosphorylation of ERK. Furthermore, nicotine-induced IL-8 secretion was decreased by pretreatment with non-selective nAChR antagonist, ERK1/2 inhibitor or intracellular calcium chelator.

Conclusion: These findings indicate that nicotine increases IL-8 production in gingival epithelial cells via ERK phosphorylation following Ca^{2+} signalling after nAChR activation.

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1. Introduction

Gingival epithelial cells play an important role in homeostasis of periodontal tissue. They prevent entry of periodontopathogen into periodontal tissue by forming a physical barrier and provide nonspecific, rapid host defence reaction resulting in recruitment of professional immune cells such as macrophages, dendritic cells and lymphocytes.[1–3] Several lines of evidence have indicated that bacterial stimulation induces pro-inflammatory cytokines (interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor-α) in oral and gingival epithelial cells.[4,5] In addition, we have previously revealed that human gingival epithelial cells (HGECs) and oral epithelial cell line (KB

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cells) produced IL-6, IL-8 and monocyte chemoattractant protein-1 after stimulation with *Porphyromonas gingivalis*, a causative pathogenic microorganism associated with periodontal disease.[6,7] Amongst the cytokines and chemokines produced by epithelial cells, IL-8, a neutrophil chemoattractant and activator, plays the crucial role in the first line of host defence against microorganisms.[8] Furthermore, a report demonstrating that constitutive IL-8 production was detected in non-inflamed gingival epithelium has suggested that HGECs have the ability of immunosurveillance in periodontal tissues.[9]

Cigarette smoking is an important environmental risk factor in the development of periodontal diseases.[10] Cigarette smoke consists of thousands of chemicals which can affect periodontal tissue. Amongst these, nicotine is the main constituent of cigarette smoke and a selective agonist of nicotinic acetylcholine receptors (nAChRs). Interestingly, nicotine has been documented to have an immunomodulating function, suppressing macrophage Th1-type immune responses towards Th2.[11] Although gingival epithelium is the first tissue exposed to nicotine following inhalation of cigarette smoke, little is known about the effect of nicotine on the cellular function of gingival epithelial cells, especially in terms of the innate immune response.

Chemical insults such as nicotine exposure during smoking in the presence of a bacterial plaque may affect the gingival tissue by altering the innate immune system of HGECs and may facilitate progression of periodontal diseases. In this study, we examined the effects of nicotine exposure on IL-8 production as the gingival epithelial innate immune response following stimulation with the pro-inflammatory cytokine (IL-1β) and bacterial components (*P. gingivalis* lipopolysaccharide (LPS)).

2. Materials and methods

2.1. Cell culture

All human subjects who participated in this study provided informed consent for the protocol reviewed and approved by the Institutional Review Board of the Osaka University Graduate School of Dentistry. Gingival tissue specimens were obtained from three different patients with chronic periodontitis (one male and two females; average age = 45 years) at distal wedge operation for therapeutic purposes. All patients were systemically healthy and non-smokers. Two or three gingival tissue specimens per patient were minced and treated with 0.4% dispase II (Boehringer Mannheim GmbH, Mannheim, Germany) overnight at 4 °C. The epidermal sheet was separated and trypsinised with 0.05% Trypsin-ethylene diamine tetraacetic acid (Trypsin-EDTA) (Life Technologies, Rockville, MD, USA) so that single cells would be dispersed. The cells were then seeded and subcultured in a 25-cm^2 flask (Corning Inc., Corning, NY, USA). The HGECs were grown in keratinocyte-specific growth media (HuMedia KG2, Kurabo, Osaka, Japan) containing final concentrations of 0.5 $\mu g\,ml^{-1}$ hydrocortisone, 10 $\mu g\,ml^{-1}$ insulin, 0.4% (v/v) bovine pituitary extract, 0.1 $ng\,ml^{-1}$ human epidermal growth factor (hEGF), 50 $\mu g\,ml^{-1}$ gentamycin and 50 $ng\,ml^{-1}$ 1 amphotericin B. The HGEC cell line, epi4, has previously been established.[6,12] The remaining two HGEC cultures were transformed by the SV40 T antigen using Tfx™-20 (Promega Corporation, Madison, WI, USA), and transfected with human papillomavirus 16 (HPV-16) E6 and E7 open reading frames. Transfection was performed using a retroviral system for HPV-16, named Tfx and E6E7, respectively which was kindly provided by Dr. M. Saito (Tokyo University of Science, Tokyo, Japan).[13] These cell lines survived for more than 150 culture passages. No changes in cellular characteristics were detected after culture passages.

2.2. Cell stimulation

HGEC cell lines were seeded in culture plates at a similar density for each experiment and were then grown to subconfluence. The cultured HGECs were then grown in keratinocyte-specific growth media in the absence of growth factors for 12 h. For the detection of messenger RNA (mRNA) expression of IL-8 and measurement of IL-8 production, three HGEC cell lines (epi4, Tfx, and E6E7) were treated with 0.1 $ng\,ml^{-1}$ human recombinant IL-1β (R&D System, Inc., Minneapolis, MN, USA), or 10 $\mu g\,ml^{-1}$ *P. gingivalis* LPS (Invitrogen, San Diego, CA, USA) in the presence or absence of nicotine (1×10^{-6} M, 1×10^{-3} M: Sigma–Aldrich Inc., St Louis, MO, USA). Total RNA was isolated from each well after incubation for 12 h, and the culture supernatants were harvested after incubation for 24 h. In some experiments, epi4 cells were pretreated for 1 h in the presence of a non-selective nAChR antagonist: d-tubocurarine (Sigma–Aldrich Inc.), an intracellular calcium chelator: BAPTA-AM (Dojindo, Kumamoto, Japan) or an extracellular signal-regulated kinase1/2 (ERK1/2) inhibitor: U0126 (Promega Corporation), prior to stimulation with IL-1β and nicotine. The optimal time points and the concentrations of IL-1β and *P. gingivalis* LPS were determined based on preliminary experiments for detection of IL-8 expression and IL-8 secretion.

2.3. Reverse transcription-polymerase chain reaction analysis

The total RNA of three HGEC lines was isolated from cultured cells using a prepared phenol–chloroform solution (RNABee: Tel-Test, Inc., Friendship, TX, USA), according to the manufacturer's instructions. The precipitated RNA was resolved in 0.1% diethylpyrocarbonate-treated distilled water. Complementary DNA (cDNA) synthesis and amplification via polymerase chain reaction (PCR) were performed according to previously described methods.[14,15] Primer sequences were described previously by Yanagita et al.[15] Human brain RNA (Biochain Institute Inc., Hayward, CA, USA) was used as positive control.

2.4. Real-time PCR analysis

Isolation of total RNA and cDNA synthesis were performed using the methods described above. PCR reactions were carried out using the ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master

Mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. All reactions were run in triplicate. Specific primers for human IL-8 (forward primer: 5′-ACACTGCGCCAACACAGAAATTA-3′, reverse primer: 5′-TTTGCTTGAAGTTTCACTGGCATC-3′) and hypoxanthine–guanine phosphoribosyl transferase (HPRT) (forward primer: 5′-GGCAGTATAATCCAAAGATGGTCAA-3′, reverse primer: 5′-GTCAAGGGCATATCCTACAACAAAC-3′) were purchased as pre-designed products (Takara Bio Inc., Shiga, Japan). HPRT served as a housekeeping gene.

2.5. *Measurement of IL-8 secretion*

Cytokine levels were determined by using Human IL-8 Elipair™ (R&D Systems) by following the manufacturer's protocol. All reactions were run in triplicate.

2.6. *Investigation of mitogen-activated protein kinase (MAPK) phosphorylation*

Epi 4 cells were seeded on a six-well plate and grown to confluence in keratinocyte-specific growth media supplemented with specific growth regents. After 12 h, cells were incubated in growth media without the supplemental reagents for a further 12 h. Cells were then stimulated with or without 1×10^{-3} M nicotine for 5, 10, 15, 30 and 60 min. Cells were rinsed with ice-cold PBS and lysed with radio immunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 1% NP-40, 0.5% sodium dodecyl sulphate (SDS), 1% deoxycholate) containing protease inhibitors (10 $\mu g\,ml^{-1}$) phenylmethylsulphonylfluoride (PMSF), 30 $\mu g\,ml^{-1}$ aprotinin, a phosphatase inhibitor and 1 mM sodium orthovanadate (Sigma–Aldrich Inc.). Protein was quantified using the Bradford assay. To determine phosphorylation of 21 mitogen-activated protein kinases (MAPKs), we used the Human Phospho-MAPK Array Kit (R&D Systems), according to the manufacturer's protocol. We detected immunoreactive proteins using a Western blotting detection system (ECL Plus, Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.7. *Western blotting for ERK*

Epi 4 cells were stimulated with or without 1×10^{-3} M nicotine for 5, 10, 15, 30 and 60 min. In some experiments, the inhibitor, a non-selective nAChR antagonist or an intracellular calcium chelator, was added to the cultures 1 h before stimulation with nicotine. Cells were rinsed with ice-cold phosphate buffered saline (PBS) and lysed with RIPA buffer. Equal amounts of protein (40 μg per lane) were separated using sodium dodecyl sulphate-polyacryalamide gel electrophoresis (SDS-PAGE), then electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). After blocking with PBS-Tween (PBS-T) buffer containing 5% non-fat milk, membranes were incubated with primary rabbit anti-ERK1/2 antibody (Cell Signaling Technology Inc., Danvers, MA, USA) and rabbit anti-phospho ERK1/2 antibody (Cell Signaling Technology Inc., Danvers, MA, USA) overnight at 4 °C. Membranes were then washed briefly and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) antibody (GE Healthcare JAPAN, Tokyo, Japan). We detected immunoreactive proteins using a Western blotting detection system, and densitometrically analysed bands with image analysis software (Quantity One, Bio-rad, Hercules, CA, USA).

2.8. *Statistical analyses*

Statistical analyses were performed using Dunnett's test for comparison. Differences with a p value <0.05 were considered significant.

3. Results

3.1. *Expressions of nAChR mRNA in three HGEC lines*

Three gingival epithelial cell lines were examined for the expressions of nAChR subunit mRNA using reverse transcription polymerase chain reaction (RT-PCR). Brain mRNA was

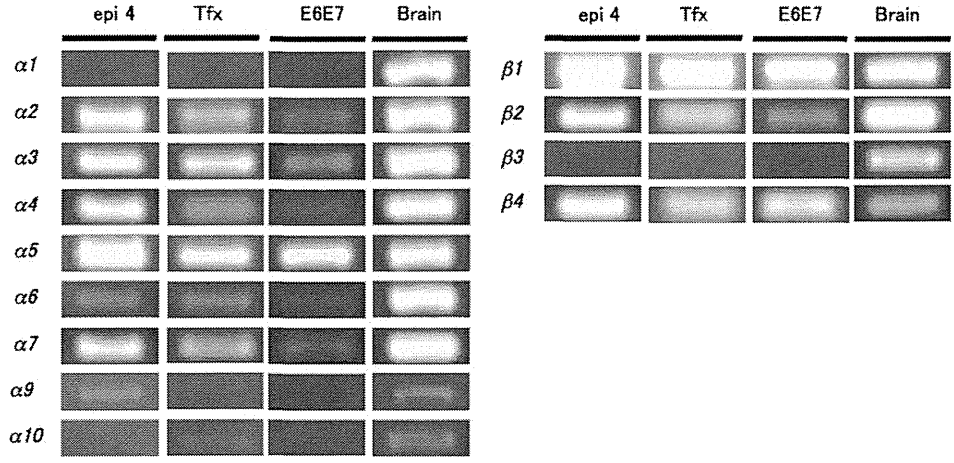

Fig. 1 – Expression profiles of nAChR mRNA in three different HGEC lines. Total RNA was extracted from subconfluent cultured cells. The expression of nAChR subunit mRNA was analysed by RT-PCR using specific primer sets. Brain RNA was used as a positive control.

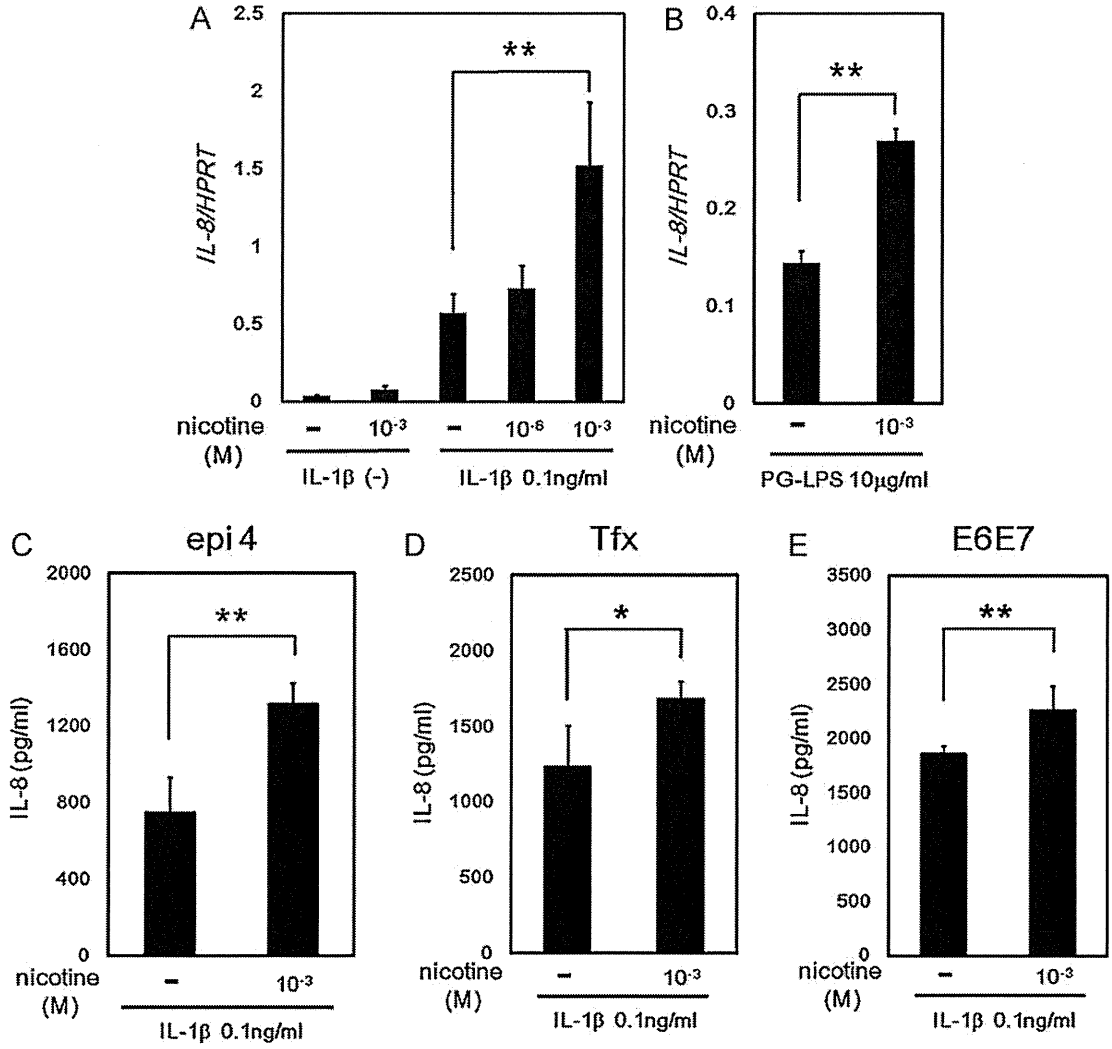

Fig. 2 – Nicotine up-regulates the expression of IL-8. IL-8 mRNA expression in epi 4 stimulated with IL-1β (A) or *P. gingivalis* LPS (B: PG-LPS) in the presence or absence of nicotine for 12 h was quantified using real-time PCR. Data are expressed as mean ± SD of three determinations. **$p < 0.01$ compared with IL-1β or *P. gingivalis* LPS alone. IL-8 production in supernatants of epi 4 (C), Tfx (D), and E6E7 (E) stimulated with IL-1β in the presence or absence of nicotine for 24 h. Concentrations of IL-8 were measured using ELISA. Data are expressed as mean ± SD of three determinations. *$p < 0.05$, **$p < 0.01$ compared with IL-1β or *P. gingivalis* LPS alone.

used as a positive control for nAChRs. Fig. 1 shows representative PCR products from three immortalised HGEC lines, epi 4, Tfx and E6E7 cells. We found that all three HGEC lines expressed mRNA for several different nAChR subunits; α2-7, α9, β1, β2 and β4 subunit mRNA were found in epi 4, whilst α2-7, α10, β1, β2 and β4 subunit mRNA were found in Tfx and E6E7.

3.2. *Effects of nicotine on IL-8 expression in HGEC lines*

We initially confirmed that nicotine (10^{-8}–10^{-3} M at 24 h) did not affect the viability of HGEC lines in our preliminary experiments. To examine the effects of nicotine on the expression of IL-8 mRNA, we extracted total RNA and performed real-time PCR for epi 4, Tfx and E6E7 cells. As shown in Fig. 2(A), 1×10^{-3} M nicotine slightly induced an increase in IL-8 mRNA expression as compared with nicotine-free conditions. Interestingly, however, in epi 4 cells that had been cultured with 0.1 ng ml^{-1} IL-1β and nicotine (1×10^{-6} M and 1×10^{-3} M), IL-8 mRNA expression was significantly enhanced as compared with IL-1β alone (Fig. 2(A)). We then examined the effect of nicotine on epi4 stimulated with *P. gingivalis* LPS. As shown in Fig. 2(B), IL-8 mRNA expression in epi 4 cells was also significantly elevated after 12 h of culture in the presence of nicotine and *P. gingivalis* LPS as compared with *P. gingivalis* LPS alone. As shown in Fig. 2(C), in the presence of IL-1β, nicotine up-regulated the secretion of IL-8 from epi 4 cells. The enhancement in IL-8 production by nicotine was also detected in Tfx, and E6E7 cells (Fig. 2(D) and (E)). Since all three HGEC lines were shown to have similar expression of nAChR subunits and IL-8 production, epi 4 cells were used for all subsequent experiments.

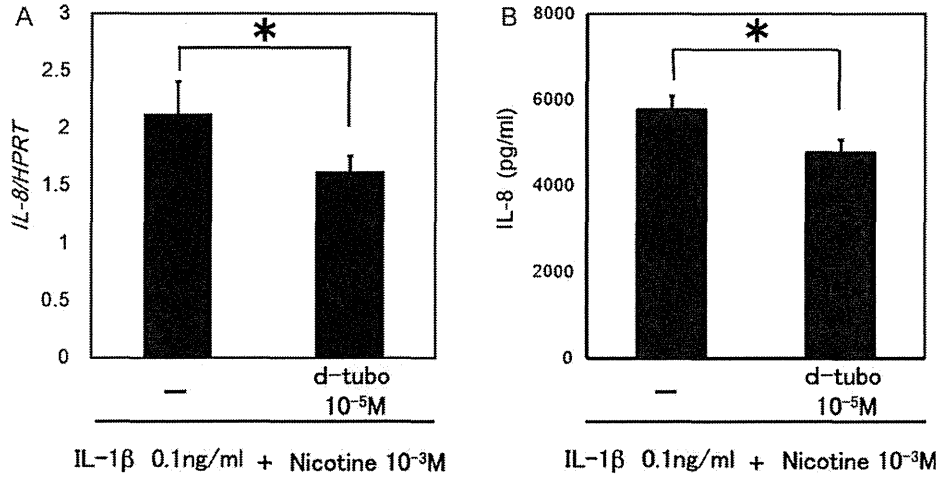

Fig. 3 – d-Tubocurarine, a nonselective nAChR antagonist, inhibits nicotine-induced upregulation of IL-8. After pretreatment with d-tubocurarine for 1 h, epi 4 was stimulated with IL-1β and nicotine for 12 h (A: real-time PCR) or 24 h (B: ELISA). Data are expressed as mean ± SD of three determinations. *$p < 0.05$ compared with IL-1β and nicotine alone.

3.3. *nAChRs are involved in IL-8 mRNA and protein secretion induced by nicotine in epi 4 cells*

We further investigated whether these effects of nicotine were mediated through nAChRs. We found that the up-regulation of IL-8 mRNA expression and protein secretion from epi 4 cells, which depended on stimulation with nicotine and IL-1β, were reduced by a non-selective nAChR antagonist (Fig. 3(A) and (B)). These results indicated that nicotine enhanced the inflammatory effect of IL-1β on epi 4 through nAChRs.

3.4. *Cell signalling molecules, related to activation by nicotine, in epi4 cells*

MAPK phosphorylation in nicotine-stimulated HGECs was examined to evaluate the functional significance of nAChR in HGECs. A strong ERK1/2 signal was detected using a Human Phospho-MAPK Array Kit™, as shown in Fig. 4. To examine whether the activation of ERK was involved in nicotine-induced IL-8 release, protein extracts prepared from epi 4 cells, which had been incubated with 1×10^{-3} M nicotine, were immunoblotted with antibodies against p-ERK1/2 and total ERK1/2. p-ERK1/2 levels increased after 10 min of incubation with nicotine (Fig. 5). This nicotine-induced ERK phosphorylation was suppressed in the presence of either d-tubocurarine or BAPTA-AM (Fig. 5). These results confirmed that nicotine-induced activation of ERK was associated with Ca^{2+} signalling via nAChRs. Interestingly, as shown in Fig. 6, an increase in nicotine-induced IL-8 production from epi 4 cells was significantly reduced in the presence of either an intracellular calcium chelator or a selective inhibitor of MAPK/ERK kinase (MEK) as compared with that of IL-1β alone.

4. Discussion

Recent works have shown that keratinocytes or epithelial cells express nAChR. Several studies reported that α1, α3-7, α9 and

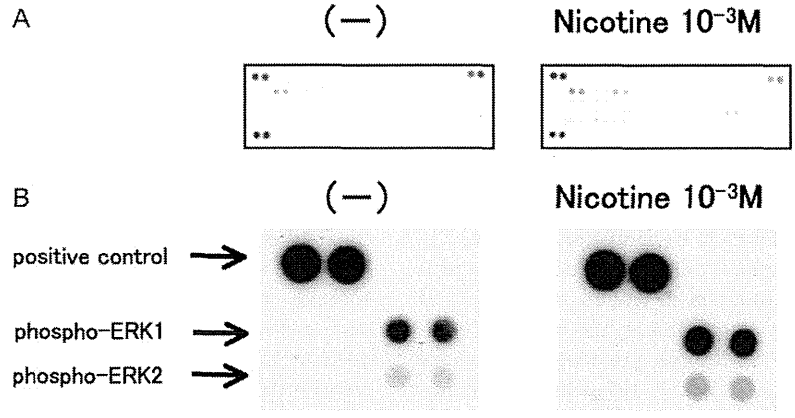

Fig. 4 – Investigation of MAPK phosphorylation in nicotine-stimulated epi4 cells. epi 4 cells were cultured in the presence or absence of nicotine for 10 min. To investigate phosphorylation of 21 MAPKs, a Human Phospho-MAPK Array Kit™ was utilized. All data (A), the enlargement of the area blotted for p-ERK1/2 and positive control (B) are shown.

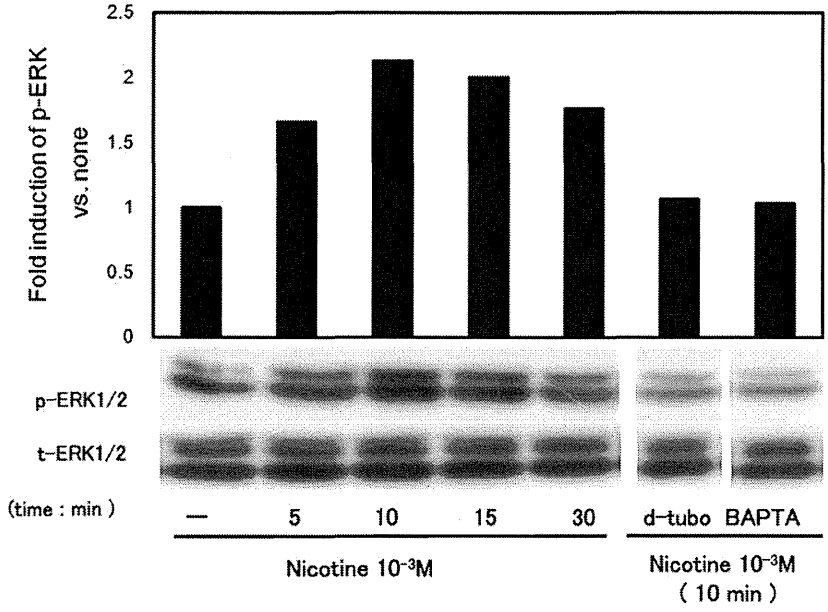

Fig. 5 – d-Tubocurarine and BAPTA-AM inhibit nicotine-induced upregulation of ERK1/2 phosphorylation. After pretreatment with d-tubocurarine or BAPTA-AM for 1 h, epi 4 cells were stimulated with nicotine for 10 min. Cell lysate was subjected to immunoblotting for p-ERK. Blotting images were analysed densitometrically.

10 and β1, 2 and 4 nAChR subunits were present in human airway epithelial cells.[16–18] Nguyen et al.[19] characterised nAChR on gingival keratinocytes and oesophageal epithelia and found the expression of α3, α5, α7 and β2 nAChR subunits. In this study, we established three HGEC lines from three different patients to detect nAChR expression and IL-8 production in these cells. These studies confirmed that these three HGECs showed the same nAChR expression and IL-8 production phenotype. RT-PCR experiments revealed that α2-7, β1, 2 and 4 subunit mRNAs are expressed in three established HGEC lines. Two additional subunits, α9 and α10, were also expressed in epi 4, and in Tfx and E6E7, respectively. The subtle difference in nAChR subunit expression may be caused by the difference in differentiation stage of the HGECs examined. To our knowledge, this is the most extensive analysis of nAChR subunit expression in HGECs. Furthermore, we demonstrated that the non-selective nAChR antagonist, d-tubocurarine, suppressed nicotine-induced IL-8 production and enhanced phosphorylation of ERK. This suggests that the effect of nicotine on HGECs can be transmitted through nAChRs on the cell surface.

It is well known that gingival or oral epithelial cells can secrete IL-8 in response to several periodontal pathogens or pro-inflammatory cytokines.[20,21] Our previous study reported that *P. gingivalis* LPS can induce IL-8 production in HGECs via Toll-like receptor-2.[6] To explore the effect of nicotine on pro-inflammatory cytokine production in HGECs, we used IL-1β and *P. gingivalis* LPS as stimulants to induce the synthesis of IL-8 in this study. Consistent with the previous studies, our results showed that both IL-1β and *P. gingivalis* LPS can up-regulate IL-8 production in three established HGECs. In addition, IL-8 expression was enhanced in the presence of nicotine in a dose-dependent manner. Nicotine has been shown to attenuate IL-8 production following LPS stimulation in activated monocytic cells.[22] Furthermore, nicotine inhibited the production of pro-inflammatory cytokines via nicotine signalling.[23] Conversely, nicotine has been reported to stimulate neutrophils and gingival fibroblasts to produce IL-8.[24,25] In addition, Mahanonda et al.[26] reported that nicotine and cigarette smoke extract stimulated IL-8 expression in HGEC cultures, which is consistent with this report. This discrepancy in the effect of nicotine may be dependent on cell type and the difference in expression levels of nuclear factor (NF)-κB, which controls inflammatory cytokine gene transcription.[27] Nicotine prevented activation of the NF-κB pathway in professional antigen-presenting cells, such as macrophages,[23,24] whereas it stimulated NF-κB activation in neutrophils, the innate immune sentinels. Like neutrophils, HGECs, which are the primary interface between gingival tissue and the oral cavity, can sense pathogens and chemical insults. Because these cells play an important role in providing the first line of host defence, they may sense nicotine as a foreign stress and induce a pro-inflammatory response to maintain homeostasis. Further studies are required to elucidate the mechanism responsible for the diversity in effects of nicotine amongst different cell types.

Nicotine has been reported to activate ERK1/2 in oral keratinocytes.[28] In addition, Ca^{2+} influx can be induced after the binding of nicotine to nAChR.[29] Amongst previous studies of signal transduction via nAChR subunits, the α7 nAChR subunit, which can form homopentameric α7 nAChR, has been well documented. For example, the interaction with α7 nAChR stimulates JAK-2-signal transducer and activator of transcription 3-suppressor of cytokine signalling 3 (JAK-2-STAT-3-SOCS3) pathway in macrophage.[23,30] In oral keratinocytes, α7 nAChR can use Ras/Raf-1/MEK1/ERK and JAK-2/STAT-3 signalling pathways.[28] α7 nAChR has also been reported to activate phosphatidylinositol-3 kinase (PI3K), a

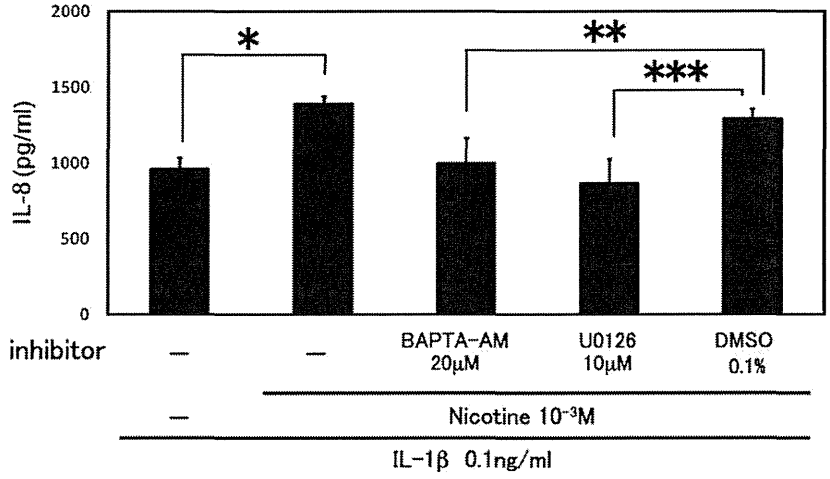

Fig. 6 – BAPTA-AM and U0126 inhibit nicotine-induced up-regulation of IL-8 production. After pretreatment with BAPTA-AM and U0126 for 1 h, epi 4 cells were stimulated with IL-1β in the presence or absence of nicotine for 24 h. The concentration of IL-8 was evaluated using ELISA. Data are expressed as mean ± SD of three determinations. *$p < 0.05$ compared with IL-1β alone, **$p < 0.01$ and ***$p < 0.05$ compared with DMSO.

Ca^{2+}-dependent kinase in neuronal cells.[31] In the present study, nicotine rapidly induced the activation of ERK1/2 phosphorylation in the HGEC line, epi 4. Furthermore, nicotine-induced phosphorylation in epi 4 was suppressed by pretreatment with a non-selective nAChR antagonist or an intracellular calcium chelator. Previous study has documented that nicotine induces an elevation in Ca^{2+} levels via nAChRs, which is dependent on the activation of a voltage-operated Ca^{2+} channel, and also involves Ca^{2+} release from intracellular stores.[32] Our study showed that both signalling via nAChR and Ca^{2+} release from intracellular stores were at least involved in nicotine-induced IL-8 production and ERK1/2 phosphorylation in epi4 cells. This result suggests that nicotine-induced IL-8 production and ERK1/2 activation in HGECs is dependent on Ca^{2+} signalling, possibly via nAChRs.

The present findings demonstrate that HGECs express an array of nAChR subunits that can temporarily transmit nicotine signalling to synergistically induce the secretion of IL-8 in the presence of IL-1β or *P. gingivalis* LPS. However, further studies regarding the effect of smoking, and therefore nicotine on cellular characteristics in HGECs, are required. In particular, the effect of the long-term exposure of nicotine, or the other cigarette smoke constituents (e.g., carbon monoxide, acetaldehyde, acrolein and so on) on HGECs needs to be investigated. These further studies may clarify the mechanism for initiation and progression of periodontal diseases.

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Competing interests

None declared.

Ethical approval

Not required.

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Immunomodulation of dendritic cells differentiated in the presence of nicotine with lipopolysaccharide from *Porphyromonas gingivalis*

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Tobacco smoking is a significant risk factor for periodontal diseases. Nicotine, one of the most studied constituents in cigarette smoke, is thought to modify immune responses. Dendritic cells (DCs), which are key mediators between innate and adaptive immunity, stimulate naive T cells to differentiate to effector T-cell subsets that may be actively involved in the immunopathogenesis of periodontal diseases. In this study, we evaluated the effects of nicotine and lipopolysaccharide (LPS) from *Porphyromonas gingivalis*, alone and in combination, on the functions of human monocyte-derived DCs to elucidate the mechanism of tissue destruction of smoking-associated periodontal diseases. *P. gingivalis* LPS-stimulated DCs differentiated with nicotine (NiDCs) induced lower T-cell proliferation and human leukocyte antigen (HLA)-DR expression, but elevated expression of programmed cell death ligand 1. Additionally, NiDCs impaired interferon-γ production but maintained interleukin (IL)-5 and IL-10 production in co-cultured T cells. Furthermore, NiDCs produced lower levels of proinflammatory cytokines compared with DCs differentiated in the absence of nicotine. Interestingly, NiDCs preferentially produced the T helper 2 (Th2)-type chemokines macrophage chemotactic protein-1 and macrophage-derived chemokine. These results suggest that the presence of nicotine during differentiation of DCs modulates the immunoregulatory functions of *P. gingivalis* LPS-stimulated DCs.

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Among lifestyle-related risk factors of periodontal diseases, tobacco use is considered as one of the most important. It alters immune responses, including the production of cytokines and proinflammatory mediators, which are important for the immunopathogenesis of periodontal diseases (1, 2). Nicotine is one of the main components of tobacco and the most investigated constituent among thousands in tobacco smoke. It has been reported to affect the secretion of cytokines and inflammatory mediators from immune cells, such as neutrophils and mononuclear cells, and from several cell types derived from periodontal tissues, including gingival fibroblasts, gingival epithelial cells, and periodontal ligament cells (1–4).

The functional roles of T cells in periodontitis lesions remain to be elucidated. In human studies, T cells infiltrating gingival lesions expressed mRNA for T-helper (Th)1/Th2 and for regulatory cytokines (5, 6). Furthermore, previous studies, using mice, revealed the important role of the $CD4^+$ T-cell immune response in the mechanisms of tissue destruction associated with periodontal diseases (7, 8). These findings suggest that T cells may be actively involved in the immunopathogenesis of periodontal diseases. Naive T cells are stimulated to differentiate into effector T cells by dendritic cells (DCs), which are the most efficient antigen-presenting cells and play an important role in coupling innate and adaptive immune responses (9). Priming of DCs with bacterial components, such as lipopolysaccharide (LPS), up-regulates the production of proinflammatory cytokines and the expression of costimulatory molecules, which drives T-cell differentiation to Th1, Th2, regulatory T cells (Treg), or Th17 cells (10, 11).

Porphyromonas gingivalis, a causative microorganism in the development of chronic inflammation in periodontal tissues, contains various virulence factors, such as LPS, fimbrial proteins, and proteases (12, 13). *P. gingivalis* LPS can elicit various types of immune and inflammatory responses. Incubation with *Escherichia coli* LPS induces high levels of production of

proinflammatory cytokines from DCs and strong Th1 responses (14). In contrast, previous studies showed that DCs stimulated with *P. gingivalis* LPS produce low levels of inflammatory cytokines and a skewed Th2 immune response (15–17). In addition, *P. gingivalis* LPS stimulated the expression of costimulatory molecules (CD80 and CD86) and tolerogenic molecules [B7-H1 (also called programmed cell death ligand 1, PD-L1) and ILT-3 (also called immunoglobulin-like transcripts 3)] on DCs (18). However, the type of immune responses that are caused by DCs differentiated with nicotine in the presence of *P. gingivalis* LPS remain to be clarified.

The aim of this study was to determine the effects of nicotine on the differentiation of DCs and to analyse the characteristics and ability of DCs to prime T cells upon stimulation with *P. gingivalis* LPS. We observed that *P. gingivalis* LPS-stimulated DCs that had been differentiated in the presence of nicotine promoted Th2 immune responses more prominently than did DCs differentiated in the absence of nicotine stimulated with *P. gingivalis* LPS. This was accompanied by the regulation of chemokine expression, the suppression of human leukocyte antigen (HLA)-DR expression, and the up-regulated expression of PD-L1. These results suggest that one of the potent mechanisms of periodontal destruction induced by smoking is nicotine-induced alteration of DC functions.

Material and methods

Cell culture

All human subjects who participated in this study provided informed consent. The protocol was reviewed and approved by the Institutional Review Board of the Osaka University Graduate School of Dentistry. Human monocytes were obtained from peripheral blood mononuclear cells (PBMCs) isolated from healthy volunteers by standard density-gradient centrifugation using Histo-Paque 1077 (Sigma-Aldrich, St Louis, MO, USA), followed by anti-CD14 microbead magnetic-cell sorting, according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). The purity of $CD14^+$ monocytes was >95%. Monocyte-derived DCs without nicotine (MDDCs) or monocyte-derived DCs with nicotine (NiDCs) were generated in complete medium (RPMI-1640 containing a final concentration of 10% heat-inactivated fetal calf serum, 20 mM Hepes, and 50 $\mu g\ ml^{-1}$ of gentamicin) supplemented with 25 $ng\ ml^{-1}$ of interleukin (IL)-4 (R&D Systems, Minneapolis, MN, USA) and 50 $ng\ ml^{-1}$ of granulocyte–macrophage colony-stimulating factor (GM-CSF) (R&D Systems) for 6 d in the presence or absence of nicotine (0, 10^{-6}, 10^{-4}, or 10^{-3} M; Sigma-Aldrich) prepared in PBS and adjusted to pH.7.2. Non-adherent cells were harvested as DCs on day 6.

Flow cytometric analysis

The expression of cell-surface molecules was evaluated by flow cytometry. Immature DCs, with or without nicotine, were cultured with 100 $ng\ ml^{-1}$ of *P. gingivalis* LPS (InvivoGen, San Diego, CA, USA). After 48 h, the cells were harvested and incubated at 4°C in the dark for 30 min with either specific monoclonal antibodies at 5 $\mu g\ ml^{-1}$ or isotype-matched control antibodies. Fluorescein isothiocyanate (FITC)-conjugated antibodies (BD Biosciences, San Jose, CA, USA), used for the experiments, were as follows: anti-HLA-DR, anti-CD40, anti-CD80, anti-CD86, and anti-PD-L1. The cells were washed twice and data were acquired using a FACSCalibur (BD Biosciences). Data from viable cells were analysed using CELLQUEST software (BD Biosciences).

Allogeneic T-cell proliferation

$CD4^+$ $CD45RA^+$ naive T cells were obtained from CD4 T cells isolated from PBMCs using a Naive $CD4^+$ T cell Isolation Kit and anti-CD45RO microbeads, according to the manufacturer's instructions (Miltenyi Biotec). The purity of the $CD4^+$ $CD45RA^+$ T cells was >90%. Dendritic cells were seeded at various cell densities, of 4×10^2 cells to 1×10^4 cells, in 96-well plates and were exposed to 100 $ng\ ml^{-1}$ of *P. gingivalis* LPS for 48 h at 37°C in an incubator with a 5% CO_2 atmosphere, treated for 1 h with 50 $\mu g\ ml^{-1}$ of mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan) to inhibit DC proliferation, and were then co-cultured with 1×10^5 $CD4^+$ $CD45RA^+$ naive T cells for 6 d. Allogeneic T-cell proliferation was measured using the non-radioactive colorimetric assay WST-1 system (Roche Diagnostics, Penzberg, Germany), according to the manufacturer's instructions, and the absorbance (A)450/A650 was measured after 2 h on a microplate reader (Bio-Rad, Hercules, CA, USA).

Cytokine and chemokine detection

To measure cytokine and chemokine secretion by MDDCs and NiDCs, these DCs were stimulated for 48 h with or without 100 $ng\ ml^{-1}$ of *P. gingivalis* LPS, and the supernatants were collected and frozen at −80°C until used for measurement of cytokine secretion. To measure cytokine production by T cells, 1×10^5 naive $CD4^+$ $CD45RA^+$ T cells were co-cultured for 6 d with 1×10^4 MDDCs or NiDCs treated with mitomycin C. After 6 d of culture, the cells were reseeded at 2×10^5 cells/well and restimulated for 48 h with plate-bound anti-CD3 (10 $\mu g\ ml^{-1}$; eBiosciences, San Diego, CA, USA) and soluble anti-CD28 (1 $\mu g\ ml^{-1}$; eBiosciences). The supernatants were collected and frozen at −80°C until use. The levels of the cytokines interferon-γ (IFN-γ), IL-5, IL-8, IL-10, IL-12p40 + p70, and tumour necrosis factor-α (TNF-α), and of the chemokines monocyte chemotactic protein-1 (MCP-1), regulated upon activation, normal, T-cell expressed, and secreted (RANTES), and macrophage-derived chemokine (MDC), in the culture supernatants were measured by ELISA (R&D Systems), according to the manufacturer's protocol. The plates were read in a microplate reader (Bio-Rad) and the A450/A540 ratio was measured.

Statistical analyses

Data were expressed as mean ± SD. Statistical analysis of the results was performed using the Student's *t*-test or ANOVA followed by Dunnett's multiple comparison tests. Differences were considered statistically significant when the *P*-value was <0.05.

Results

Effects of nicotine on T-cell priming on *P. gingivalis* LPS-stimulated MDDCs and NiDCs

To determine the capacity of MDDCs and NiDCs to stimulate allogeneic $CD4^+$ $CD45RA^+$ naive T cells, DCs were cultured with medium alone or with medium containing 100 ng/ml of *P. gingivalis* LPS, harvested 48 h later, and then co-cultured with naive T cells for 6 d. As shown in Fig. 1, *P. gingivalis* LPS-stimulated MDDCs induced elevated proliferation levels with increasing DC numbers. However, DCs that had been cultured in the presence of nicotine (10^{-4} and 10^{-3} M) significantly impaired T-cell proliferation. These results suggest that *P. gingivalis* LPS-stimulated NiDCs had a reduced antigen-presenting ability to stimulate allogeneic T cells.

Effects of nicotine and *P. gingivalis* LPS on T-cell differentiation

To further characterize the effects of nicotine and *P. gingivalis* LPS on the priming ability of DCs, expanded T cells were restimulated with anti-CD3 and anti-CD28 for 48 h, the supernatants were collected, and the levels of IFN-γ, IL-5, and IL-10 were measured. As shown in Fig. 2, $CD4^+$ $CD45RA^+$ T cells cultured with *P. gingivalis* LPS-stimulated MDDCs produced elevated levels of IFN-γ, IL-5, and IL-10. Although $CD4^+$ $CD45RA^+$ T cells cultured with *P. gingivalis* LPS-stimulated NiDCs produced significantly lower levels of IFN-γ, there was no difference in the production of IL-5 and IL-10 relative to $CD4^+$ $CD45RA^+$ T cells primed with MDDCs.

Effects of nicotine on surface molecule expression on *P. gingivalis* LPS-stimulated MDDCs and NiDCs

We hypothesized that one of the mechanisms by which *P. gingivalis* LPS-stimulated NiDCs reduced T-cell proliferation and polarized the cytokine profile of Th1/Th2 was the different expression of antigen-presentation-related surface molecules between MDDCs and NiDCs.

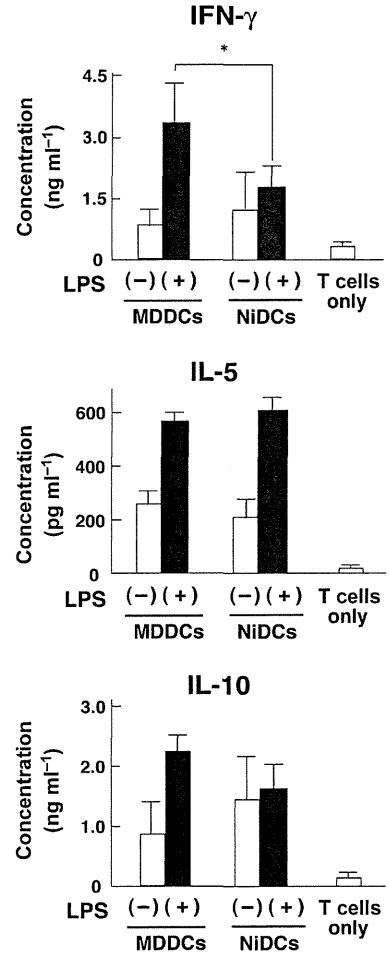

Fig. 2. Effect of nicotine on cytokine production in T cells co-incubated with monocyte-derived DCs without nicotine (MDDCs) or with monocyte-derived dendritic cells with nicotine (NiDCs). Supernatants of restimulated T cells were harvested and the concentrations of interferon-γ (IFN-γ), interleukin (IL)-5, and IL-10 were measured by ELISA. The results represent the mean values + SD obtained from triplicate cultures. These data represent one of five independent experiments. $*P < 0.05$ compared with *Porphyromonas gingivalis* LPS-stimulated MDDCs.

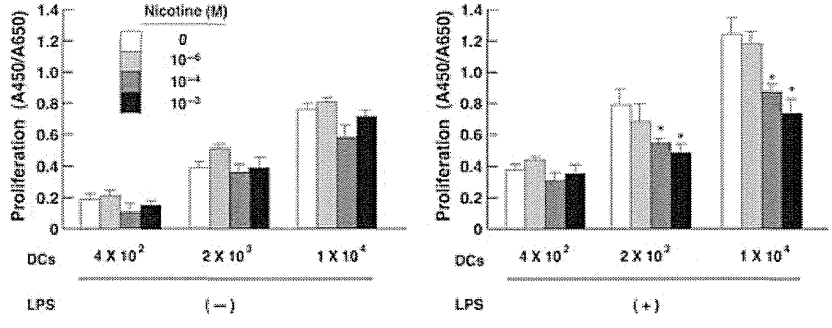

Fig. 1. Effect of nicotine on the antigen-presenting properties of dendritic cells (DCs). Comparison of the effects of monocyte-derived DCs without nicotine (MDDCs) and monocyte-derived DCs with nicotine (NiDCs) on T-cell proliferation. Various cell numbers of DCs were co-cultured for 6 d with 1×10^5 naive $CD4^+$ $CD45RA^+$ T cells in the presence or absence of *Porphyromonas gingivalis* lipopolysaccharide (LPS) and then T-cell proliferation was measured using a non-radioactive colorimetric assay WST-1 system at an absorbance of 450/650 (A450/A650). The results represent the mean absorbance (A450/A650) values + SD obtained from triplicate cultures. The data represent one of six independent experiments. $*P < 0.05$ compared with *P. gingivalis* LPS-stimulated MDDCs.

Thus, we subsequently analysed the levels of expression of molecules involved in antigen presentation, such as HLA-DR, CD40, CD80, CD86, and PD-L1, on MDDCs and NiDCs. As shown in Fig. 3, *P. gingivalis* LPS up-regulated the expression of all five molecules on DCs, and the expression levels of CD40, CD80, and CD86 on NiDCs were similar to those on MDDCs. However, HLA-DR expression on *P. gingivalis* LPS-stimulated NiDCs was lower than on MDDCs. In contrast, expression of the co-inhibitory molecule, PD-L1, was slightly elevated in *P. gingivalis* LPS-stimulated NiDCs compared with MDDCs.

Differential cytokine and chemokine expression patterns of MDDCs and NiDCs in response to *P. gingivalis* LPS

The difference in cytokine/chemokine production induced by DCs also affects subsequent DC maturation and T-cell responses (9). To investigate the effects of nicotine on the production of cytokines by DCs, the production of different types of cytokines and chemokines (TNF-α, a proinflammatory cytokine; IL-8, a proinflammatory chemokine; IL-12, a Th1-biasing cytokine; RANTES, a Th1-biasing chemokine; IL-10, a Th2-biasing cytokine; and MCP-1 and MDC, Th2-biasing chemokines) by *P. gingivalis* LPS-stimulated MDDCs and NiDCs was examined. *P. gingivalis* LPS-stimulated MDDCs produced significantly higher amounts of IL-12, IL-10, and TNF-α compared with *P. gingivalis* LPS-stimulated NiDCs and non-stimulated MDDCs (Fig. 4A). Furthermore, *P. gingivalis* LPS-stimulated NiDCs produced significantly lower amounts of all cytokines examined (Fig. 4A). However, both MDDCs and NiDCs stimulated with *P. gingivalis* LPS produced high amounts of IL-8 compared with non-stimulated DCs (Fig. 4A). We next assessed the levels of chemokine production from MDDCs and NiDCs in the absence or presence of *P. gingivalis* LPS. As shown in Fig. 4B, RANTES was produced by MDDCs but not by NiDCs stimulated with *P. gingivalis* LPS. In contrast, non-stimulated NiDCs spontaneously produced MCP-1 at significantly higher levels than did unstimulated MDDCs. Stimulation of NiDCs with *P. gingivalis* LPS resulted in a slight elevation in the production of MCP-1 compared with non-stimulated NiDCs, and MCP-1 was significantly increased compared with *P. gingivalis* LPS-stimulated MDDCs (Fig. 4B). Similarly to MCP-1, non-stimulated NiDCs produced significantly increased levels of MDC compared with MDDCs without *P. gingivalis* LPS stimulation. However, stimulation with *P. gingivalis* LPS did not alter the production of MDC from either MDDCs or NiDCs. These results suggest that nicotine modulates DC function by suppressing the production of

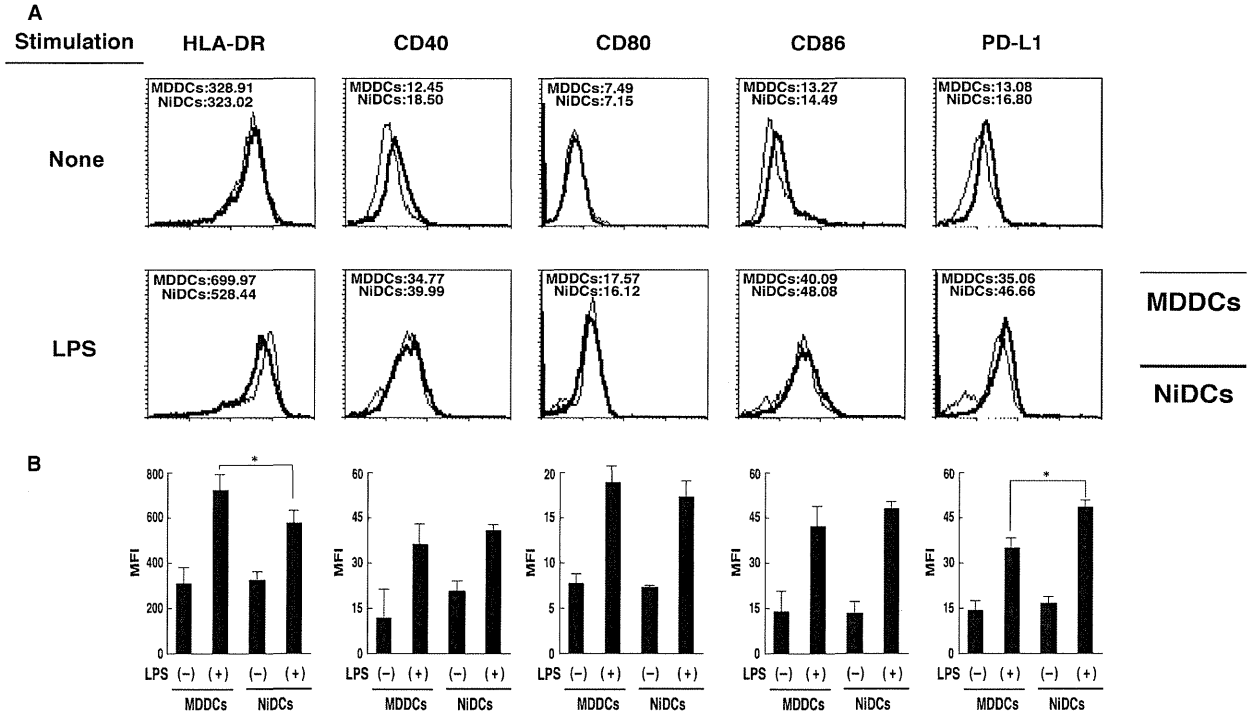

Fig. 3. Effect of nicotine on the expression of surface molecules on dendritic cells (DCs). Expression of human leucocyte antigen (HLA)-DR, CD40, CD80, CD86, and programmed cell death ligand 1 (PD-L1) on monocyte-derived DCs without nicotine (MDDCs) or on monocyte-derived DCs with nicotine (NiDCs), stimulated for 48 h with *Porphyromonas gingivalis* lipopolysaccharide (LPS) (100 ng ml^{-1}), was evaluated by fluorescence-activated cell sorting (FACS). MFI, mean fluorescence intensity. (A) One representative FACS histogram of MDDCs (thin line) and NiDCs (thick line) of more than three independent experiments are shown. (B) Bar graphs represent the mean values + SD of three independent experiments. $*P < 0.05$ compared with P.gingivalis LPS-stimulated MDDCs.

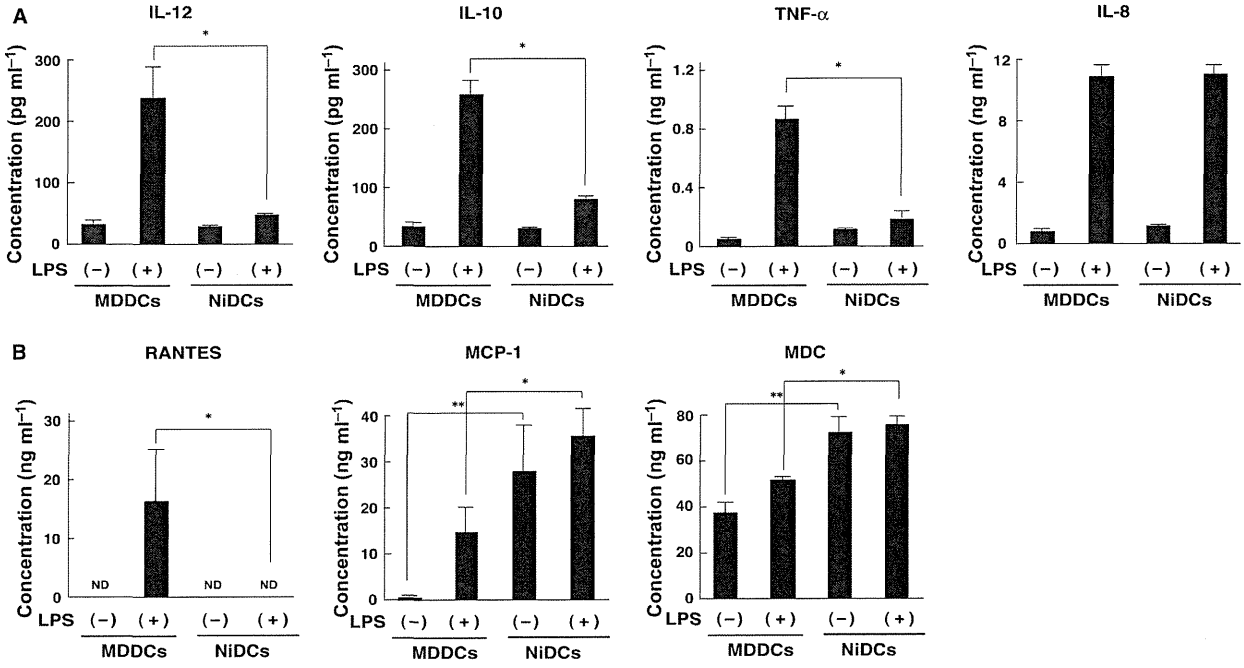

Fig. 4. Cytokine (A) and chemokine (B) production by monocyte-derived dendritic cells without nicotine (MDDCs) and by monocyte-derived dendritic cells with nicotine (NiDCs). The MDDCs and the NiDCs were cultured for 48 h in the absence or presence of 100 ng ml^{-1} of *Porphyromonas gingivalis* lipopolysaccharide (LPS). Supernatants were tested for interleukin (IL)-12, IL-10, tumour necrosis factor-α (TNF-α), IL-8, regulated upon activation, normal, T-cell expressed, and secreted (RANTES), monocyte chemotactic protein-1 (MCP-1), and macrophage-derived chemokine (MDC) secretion by ELISA. The results are shown as mean values + SD obtained from triplicate cultures. These data represent one of five independent experiments. $^{*}P < 0.05$ compared with *P. gingivalis* LPS-stimulated MDDCs. $^{**}P < 0.05$ compared with non-stimulated MDDCs.

Th1-biasing chemokines and by enhancing the secretion of Th2-biasing chemokines from DCs.

Discussion

In this study, we investigated the effects of nicotine and *P. gingivalis* LPS on the immunomodulation of DCs. In previous studies, nicotine concentrations ranging between 10^{-7} and 10^{-2} M were used to investigate its effects on various cell types (3, 4, 19–26). Hence, intermediate doses of nicotine (from 10^{-6} to 10^{-3} M) were used in the present study. We hypothesized that local exposure to high doses of nicotine in inflammatory periodontal lesions with bleeding may prime monocytes to differentiate into DCs in the inflamed microcirculation. In a previous study, we used several different concentrations of nicotine to examine its effect on DC characteristics, and found that 10^{-3} M nicotine induced significant alterations in the DC phenotype (27). Furthermore, we confirmed that 10^{-3} M nicotine did not affect DC viability. Therefore, we investigated the effects of 10^{-3} M nicotine on the expression of surface molecules on DCs and on DC-stimulated T-cell polarization.

Dendritic cells stimulated with *P. gingivalis* LPS skew naive $CD4^{+}$ T cells to a Th2 phenotype, whereas *E. coli* LPS induce Th1 responses (15). The current study showed that the combination of nicotine and *P. gingivalis* LPS accelerated Th2 responses more potently than did *P. gingivalis* LPS alone, by inhibiting the production of IFN-γ (Fig. 2); however, IL-10 production by *P. gingivalis* LPS-stimulated NiDCs was reduced (Fig. 4A). We also observed a difference in chemokine production between NiDCs and MDDCs (Fig. 4B). Dendritic cells differentiated in the presence of nicotine could produce MCP-1 and MDC constitutively without *P. gingivalis* LPS stimulation. Previously, we demonstrated that macrophages differentiated in the presence of nicotine produced high amounts of MCP-1 in the absence of LPS stimulation (28). Interestingly, MCP-1 is thought to be important for inducing Th2 immune responses (29, 30). Macrophage-derived chemokine is a potent chemoattractant for Th2 cells and, together with its receptor, CCR4, is an important determinant of Th2 responses (31). In marked contrast, RANTES was not detected in the supernatants of NiDCs. RANTES is thought to be important in T-cell responses by promoting the differentiation and proliferation of Th1 cells and by cooperating with IFN-γ to activate macrophages, natural killer cells, and T cells (32–34). Furthermore, Kawai *et al.* (35) demonstrated that RANTES produced by endothelial cells enhanced Th1-type selective migration. These results suggest the mechanism by which NiDCs induce Th2 cells is not mediated by IL-10, but rather than the polarization of the T-cell response may be a result of the difference in expression of chemokines induced by nicotine.

JOTWANI *et al.* (15) reported that DCs stimulated with *P. gingivalis* LPS were less effective at stimulating allogeneic T-cell proliferation than were DCs stimulated with *E. coli* LPS; however, both types of LPS induced equivalent T-cell maturation and costimulatory molecule expression. A previous study demonstrated that *P. gingivalis* LPS induced elevated expression of the costimulatory molecules CD80 and CD86 and of the tolerogenic markers ILT3 and B7-H1 (also known as PD-L1) on DCs (18). In this study, we demonstrated that *P. gingivalis* LPS-stimulated NiDCs induced lower levels of allogeneic T-cell proliferation compared with MDDCs (Fig. 1). A possible explanation for the suppression of T-cell proliferation by *P. gingivalis* LPS-stimulated NiDCs may be the reduced expression of HLA-DR and the elevated expression of PD-L1 compared with *P. gingivalis* LPS-stimulated MDDCs (Fig. 3).

In periodontal tissues, DCs and Langerhans cells (LCs) play crucial roles to connect innate and adaptive immunity against infectious microbes (36). Previous studies demonstrated that DCs and LCs were present in the epithelium and the lamina propria of healthy gingiva, and during gingivitis and periodontitis (37–40). However, little is known about the status of DCs and LCs in the gingival tissue of smokers. Souto *et al.*(41) reported that the numbers of $CD1a^+$ LCs and $CD83^+$ DCs were decreased in the gingival tissue of smokers who were diagnosed with gingivitis. Both the distribution and the phenotypic changes of DCs may regulate immune responses in periodontal tissues and lead to the periodontal destruction typically observed in smokers.

This study provides new information on immune responses induced by *P. gingivalis* LPS-stimulated DCs differentiated in the presence of nicotine. These results may help to elucidate the mechanism of tissue destruction in smoking-associated periodontal disease.

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Nicotine modulates the immunological function of dendritic cells through peroxisome proliferator-activated receptor-γ upregulation

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ABSTRACT

We examined the effects of nicotine on differentiation and function of monocyte-derived human dendritic cells (DCs). NiDCs, which were the DCs differentiated in the presence of nicotine, showed lower levels of CD1a. Secretion of IL-12 and TNF-α by lipopolysaccharide (LPS)-stimulated NiDCs was significantly suppressed compared to monocyte-derived DCs grown without nicotine. NiDCs displayed a diminished capacity to induce allogeneic T cell proliferation with a reduced production of IFN-γ, and maintained/enhanced LPS-mediated expression of coinhibitory molecules. Interestingly, NiDCs enhanced the expression of nuclear receptor peroxisome proliferator-activated receptors γ (PPAR γ), which has immunomodulatory properties. Expression of PPAR γ and PPAR γ-target genes was significantly inhibited by pretreatment with d-tubocurarine, antagonist of non-selective nicotinic acetylcholine receptors (nAChR). In addition, reduction of Th1 responses was inhibited after blocking nAChR-mediated signal. These data suggest the effect of nicotine on altering DC immunogenicity by impeding Th1 immunity is partially mediated by upregulation of PPAR γ.

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1. Introduction

Cigarette smoking significantly increases the risk of developing numerous diseases such as cancer, vascular disease, periodontal disease, and chronic obstructive pulmonary disease (COPD) [1–5]. It has been suggested that the increased incidence of these diseases in smokers may be due to chronic inhalation of chemicals in cigarette smoke that eventually leads to altered immune responses [6]. Among thousands of chemical components in cigarette smoke, nicotine is a main component and is known to induce T-cell anergy and immunosuppression [7]. Nicotine is a selective agonist of the nicotinic acetylcholine receptors (nAChRs). Human nAChRs are pentamers that are also agonist-regulated ion channels. nAChRs are expressed by neuronal as well non-neuronal cells, including epithelial cells [8], lymphocytes [9], alveolar macrophages [10,11], and eosinophils [12]. Recent reports suggest that nicotine inhibits systemic inflammation via an anti-inflammatory, cholinergic pathway coupled to α7nAChRs [11,13]. During inhalation of cigarette smoke, the epithelial surface of the oral cavity, bronchi and lungs are exposed to localized, high doses of nicotine (>10^{-3} M). In particular, nicotine concentrations in the saliva of long-term snuff users can reach mM levels [14–16].

Dendritic cells (DCs) are the most efficient antigen-presenting cells for coupling the innate to the adaptive immune responses [17]. In the presence of bacterial components such as LPS, DC maturation can be induced by stimulation of TLRs expressed on DCs. Matured DCs produce proinflammatory cytokines and up-regulate the expression of costimulatory molecules [18]. In addition, they detect, capture and process foreign antigens and evoke a variety of immunological responses by presenting foreign antigens to naive CD4 T cells, resulting in differentiation into Th1, Th2, regulatory T cells (Treg) and Th17 cells [19]. It is well established that DCs can display unique functional characteristics depending on the different tissue microenvironments to which they are exposed *in vivo* and on different tissue culture conditions *in vitro* [20–22]. Recent studies indicate that cigarette smoke and nicotine suppressed DC-mediated immune responses in human *in vitro* [23,24]. In contrast, another study showed that nicotine strongly activated DC-mediated adaptive immune responses [25]. The difference in effects may be due to the concentration of nicotine used in the experiments. The effects of nicotine on *in vitro*-differentiated DC function remains controversial, however, since those reports suggest the possibility that nicotine modulates DC function regardless of actual nicotine-induced DC activation.

One of the nuclear transcription factors that influence the DC immune function is the peroxisome proliferator-activated receptor-γ (PPAR γ). PPAR γ was originally identified as a promoter of adipose differentiation and regulator of insulin and glucose metabolism [26,27]. Recently, PPAR γ has also been shown to mediate anti-inflammatory effects via negative interference with pro-inflammatory signaling via NF-κB [28,29]. A disruption of the PPAR

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γ gene in macrophages caused an upregulation of inflammatory cytokine production [30]. In addition, PPAR γ regulated the maturation and function of DC [31–34]. These findings suggest that PPAR γ plays an important role in inflammation and immunity.

In the present study, we investigated the effects of nicotine on the differentiation of human monocytes into DCs. Our results demonstrate that DCs differentiated in the presence of nicotine (NiDCs) reduce inflammatory cytokine production and induce the expression of coinhibitory molecules compared to those in the absence of nicotine (MoDCs). Furthermore, LPS-stimulated NiDCs induce differentiation of naive CD4 T cells into Th2 cells, whereas LPS-stimulated MoDCs induce Th1 immune responses. NiDCs are also associated with increased expression of PPAR γ and PPAR γ-target genes. Finally, our study suggests that nicotine modulates the DC phenotype by upregulation of PPAR γ gene expression.

2. Materials and methods

2.1. Isolation of monocytes, and generation of monocyte-derived dendritic cells

All human subjects participating in this study after provided informed consent to a protocol that was reviewed and approved by the Osaka University Graduate School of Dentistry Institutional Review Board. Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers, and monocytes were isolated by standard density gradient centrifugation using Histo-Paque 1077 (Sigma–Aldrich, St. Louis, MO), followed by anti-CD14 microbeads magnetic cell sorting, and processed according to the manufacturer's instruction (Miltenyi Biotec, Auburn, CA). The purity of the CD14 positive monocytes was >95%. Control DC (-nicotine) or test DC ($+10^{-8}$ to 10^{-2} M nicotine) were generated in complete RPMI-10 (RPMI-1640 with a final concentration of 10% heat-inactivated FCS, 20 mM Hepes, 50 μg/ml gentamicin) supplemented with 25 ng/ml, IL-4 (R&D Systems, Minneapolis, MN) and 50 ng/ml GM-CSF (R&D Systems). Non-adherent cells were harvested on day 6 or 7. Nicotine (Sigma–Aldrich) was prepared in PBS and neutralized to pH 7.2. In some experiments, monocytes were pretreated for 30 min in the presence of the non-selective and competitive nAChR antagonist, d-tubocurarine (Sigma–Aldrich) prior to supplementation with IL-4 and GM-CSF. $CD45RA^+$ and $CD4^+$ naive T cells were obtained from PBMC isolation of CD4 T cells using a Naive $CD4^+$ T cells Isolation kit (Miltenyi Biotec).

2.2. Analysis of DC surface molecules by flow cytometry

Expression of cell surface molecules was evaluated by flow cytometry. Immature DCs with or without nicotine (NiDCs and MoDCs, respectively) were cultured with 10 ng/ml lipopolysaccharide (LPS; *Salmonella minnesota*; List Biological Laboratories, INC, Campbell, CA) to induce cytokine and chemokine production. After 48 h, cells were harvested and incubated at 4 °C in the dark for 30 min with mAbs at 5 μg/ml or isotype-matched control Abs. FITC-conjugated Abs (BD Biosciences, San Jose, CA, unless noted) used for the experiments were anti-CD14, anti-HLA-DR, anti-CD40, anti-CD80, and anti-CD86. PE-conjugated Abs used for the experiments were anti-CD1a, anti-PD-L1, anti-PD-L2, anti-ILT3 (Beckman Coulter, Marseille, France) and anti-ILT4 (Beckman Coulter). Cells were washed twice and data were acquired on a FACSCalibur (BD Biosciences). Data from viable cells were analyzed with CELLQuest™ software (BD Biosciences).

2.3. Antigen uptake by DCs

MoDCs or NiDCs were washed with PBS and suspended in complete RPMI-10 containing FITC-dextran (200 μg/ml: Molecular Probes, Eugene, OR). After 60 min-incubation at 37 °C or 4 °C (as negative control), cells were washed three times, resuspended with PBS, and analyzed by flow cytometry.

2.4. Allogeneic T cell proliferation

MoDCs and NiDCs were stimulated with 10 ng/ml LPS for 48 h, treated with mitomycin C (50 μg/ml for 1 h) to inhibit DC proliferation, and then co-cultured with 10^5 naive $CD4^+$ T cells for 6 days. Allogeneic T cell proliferation was measured using the non-radioactive colorimetric assay WST-1 system (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions and the OD450/650 measured after 2 h on a micro plate reader (Bio-Rad, Hercules, CA).

2.5. Measurement of cytokine secretion

MoDCs and NiDCs were stimulated with 10 ng/ml LPS for 48 h, and supernatants were frozen at −80 °C until used for measurement of cytokine secretion. Supernatants cytokine levels were determined using IL-12 (p40 + p70), IL-10 and TNF-α ELISA kits (Pierce Endogen, Rockford, IL). To measure cytokine production by T cells, 10^5 naive $CD4^+$ T cells were co-cultured for 6 days with 10^4 unstimulated DCs or LPS-stimulated DCs treated with mitomycin C. After 6 days culture, cells were restimulated at 2×10^5 cells/well with plate-bound anti-CD3 (eBioscience) and soluble anti-CD28 (eBioscience) for 24 h. Supernatants were frozen at −80 °C until use. Cytokine levels were determined in supernatants using IFN-γ, IL-5, and IL-10 ELISA kits (Pierce Endogen).

2.6. RT-PCR assay and real-time quantitative RT-PCR assay

Total RNA was extracted from cells using the RNAbee kit (TEL-TEST, Friendswood, TX) according to the manufacture's instructions. cDNA synthesis and amplification via PCR were performed as previously described. HPRT (hypoxanthine phosphoribosyl transferase) was used as a positive control for RNA integrity. After denaturation at 95 °C for 5 min, each cycle consisted of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Amplified products were analyzed by electrophoresis at 100 V for 30 min on 1.5% TAE agarose gels containing 0.5 mg/ml ethidium bromide. Band density was quantified with Quantity One software (Bio-Rad, Hercules, CA). Quantitative real-time PCR was performed with an ABI7700 system (Applied Biosystems, Tokyo, Japan) using the following primers;

PPAR γ (forward, 5′-TGGAATTAGATGACAGCGACTTGG-3′: reverse, 5′-CTGGAGCAGCTTGGCAAACA-3′).
CD1a (forward, 5′-TCGGGTGAAGCACAGCAGTC-3′: reverse, 5′-GGCACTATCACCGCCAAGATG-3′).
Adipose differentiation-related protein: ADFP (forward, 5′-CGGATGATGCAGCTCGTGA-3′: reverse, 5′-GCACGGGAGTGAAGCTTGGTA-3′).
Apolipoprotein E: ApoE (forward, 5′-CTGCGTTGCTGGTCACATTC-3′: reverse, 5′-CTCCTGCACCTGCTCAGACA-3′).
Fatty-acid-binding protein-4: FABP4 (forward, 5′-CTTCATACTGGGCCAGGAATTTG-3′: reverse, 5′-CTCCTGCACCTGCTCAGACA-3′).
Hypoxanthine phosphoribosyl transferase: HPRT (forward, 5′-GGCAGTATAATCCAAAGATGGTCAA-3′: reverse, 5′-GTCAAGGGCATATCCTACAACAAAC-3′). HPRT served as a housekeeping gene.

2.7. Measurement of PPAR γ activation

PPAR γ activation in nuclear extracts was determined by TransAM PPAR γ kit (Active Motif Inc., Carlsbad, CA) according to

the manufacture's protocol. In brief, 5 μg of nuclear extract was incubated for 1 h in a 96-well plate immobilized with an oligonucleotide containing PPAR γ binding site. Antibody was added and incubated for 1 h. Anti-IgG horseradish peroxidase was added and incubated for an additional 1 h. Plates were washed and developing solution added, followed by stop solution, and the OD450/650 measured on a micro plate reader (Bio-Rad).

2.8. Statistical analysis

Data were expressed as the mean ± SD. Statistical analysis of the results was performed with Student's *t* test or ANOVA followed Dunnett multiple comparison test. Differences were considered statistically significant when *p* value were less than 0.05.

3. Results

3.1. Effect of nicotine on differentiation of monocytes into DCs

Monocyte can differentiate into DCs in the presence of IL-4 and GM-CSF. The addition of IL-4 and GM-CSF to cells when they are initially cultured will lead to upregulation of CD1a expression and downregulation of CD14 expression. Different concentrations of nicotine were added with IL-4 and GM-CSF to determine the possible effect of nicotine on differentiation. FACS acquisition of cell surface expression data was obtained on day 7. As shown in Fig. 1A, MoDCs without nicotine presented the typical phenotype of monocyte-derived DCs characterized by high CD1a expression and low level CD14 expression (data not shown). Of the different nicotine concentrations tested, 10^{-3} M reduced CD1a expression. In preliminary experiments, we confirmed that nicotine (10^{-8} to 10^{-3} M) did not affect DC viability as indicated by trypan blue exclusion and WST-1 assays. The cell viabilities with or without 10^{-3} M nicotine were 29.1% and 30.7% by trypan blue exclusion, and 34.0% and 35.3% by WST-1 assay, respectively.

3.2. The pattern of cytokine production is altered by nicotine

DCs produce several cytokines and chemokines depending on the extracellular environment and stimuli. Recent studies have shown that CD1a is a marker for DC production of IL-12 and Th1 polarization [35,36]. As shown in Fig. 1A, nicotine reduced the expression of CD1a in DCs, whereas the effect of nicotine on Th1/2 polarization remained unclear [23–25]. We were interested in whether DCs differentiated in the presence of nicotine would produce Th1/Th2 cytokines. We therefore examined the production of IL-12 (p40 + p70), IL-10, and TNF-α. In this experiment, DCs were activated in the presence of LPS, which augments the Th1 response. Supernatants of non-stimulated and LPS-stimulated MoDCs and NiDCs were assayed for IL-12 (p40 + p70), IL-10 and TNF-α. IL-12 (p40 + p70) and TNF-α production by NiDCs after LPS stimulation was significantly reduced (Fig. 1B). Since IL-10 production of LPS-stimulated NiDCs was also reduced, it is unlikely that inhibition of IL-12 and TNF-α secretion in the presence of nicotine was mediated through IL-10, an anti-inflammatory cytokine. These results suggest that nicotine may impair Th1 polarization.

3.3. NiDCs show impaired T cell proliferation

The ability of MoDCs and NiDCs to cause proliferation of allogeneic naive T cells was compared. MoDCs and NiDCs were cultured with or without LPS, harvested after 48 h, and co-cultured with naive T cells for 6 days. As shown in Fig. 2A, MoDCs and NiDCs in the presence of LPS resulted in significant T cell proliferation compared to MoDCs and NiDCs without LPS. Interestingly, however, there was significant reduction of T cell proliferation when cultured with LPS-stimulated NiDCs compared to LPS-stimulated MoDCs. These results suggest that the ability of antigen-presentation by DC to stimulate allogeneic T cells is diminished following nicotine treatment.

3.4. Cytokine-secretion profile of $CD4^+$ T cells primed with DCs developed with or without nicotine

To further characterize the effect of nicotine on the priming capacity of DCs, the expanded T cells were restimulated with anti-CD3 and anti-CD28. Supernatants were collected and levels of IFN-γ, IL-5, and IL-10 were measured. The results summarized in Fig. 2B showed that $CD4^+$ T cells cultured with MoDCs produced elevated IFN-γ and decreased levels of IL-10. $CD4^+$ T cells cultured

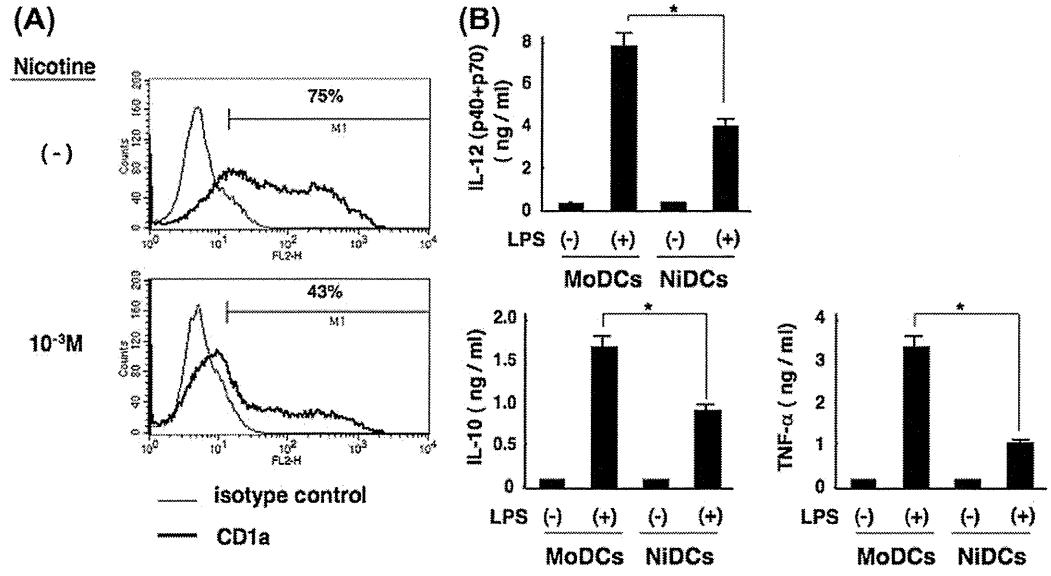

Fig. 1. (A) Differentiation of $CD1a^+$ DC from monocytes in the presence (NiDCs) or absence (MoDCs) of 1×10^{-3}M nicotine. MoDCs and NiDCs were harvested at day 7 and analyzed by FACS for the expression of $CD1a^+$. These data represent one of eight independent experiments with monocytes isolated from different donors. (B) Cytokine production by MoDCs and NiDCs. MoDCs and NiDCs were cultured in the absence or presence of 10 ng/ml LPS for 24 h. Supernatants were tested for cytokine secretion by ELISA. Results are shown as mean values ± SD of at least five independent experiments. *$P < 0.05$ compared with LPS-stimulated MoDCs.

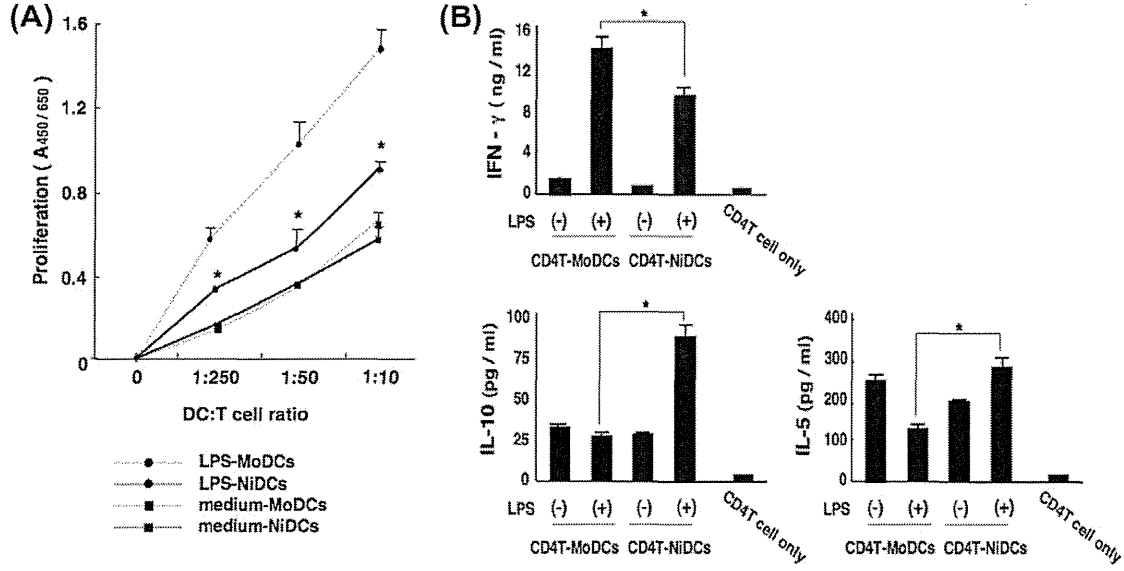

Fig. 2. Effect of nicotine on antigen-presenting properties of DC and cytokine production in MLR. (A) Comparisons of the effects of MoDCs and NiDCs on T cell proliferation. The results represent the mean OD values ± SD obtained from triplicate cultures. The data shown were obtained from one of five independent experiments. *$P < 0.05$ compared with LPS-stimulated MoDCs. (B) Supernatants of restimulated T cells were harvested and measured for IFN-γ, IL-5 and IL-10 levels by ELISA. The results represent the mean values ± SD obtained from triplicate cultures. These data represent one of five independent experiments. *$P < 0.05$ compared with LPS-stimulated MoDCs.

with NiDCs produced elevated levels of IL-5 and IL-10 relative to control $CD4^+$ T cells (MoDCs group). These results suggest that nicotine has the ability to differentiate naive T cells into Th2 $CD4^+$ T cells. We tested for additional Th2 cytokine (IL-4 and IL-13) by ELISA, but neither was detected.

3.5. Nicotine altered coinhibitory/costimulatory molecule expression

As shown in Fig. 2A, T cell proliferation was reduced in the presence of NiDCs. Although we assessed antigen uptake by MoDCs and NiDCs, no difference was observed between the two cell types (data not shown). To investigate the differences in T cell stimulation mediated by MoDCs and NiDCs, we analyzed expression levels of HLA-DR, CD40, B7 costimulatory molecules (CD80, CD86), coinhibitory molecules [the programmed cell death ligand (PD-L)1 and PD-L2] and inhibitory receptors [the immunoglobulin-like transcripts (ILT)3 and ILT4] on MoDCs and NiDCs after activation with LPS. In the absence of LPS, expression levels did not differ between MoDCs and NiDCs. Following LPS stimulation, NiDCs showed significantly elevated levels of PD-L1 and ILT4 when compared with MoDCs (Fig. 3C and D). The expression of CD86 and PD-L2 on LPS-stimulated NiDCs was slightly elevated in average but not significantly compared to LPS-stimulated MoDCs. Interestingly, ILT3 expression on NiDCs was not changed after LPS stimulation whereas MoDCs showed a reduction (Fig. 3D). The expression of HLA-DR, CD40, and CD80 on NiDCs was not significantly different from those on MoDCs following LPS stimulation (Fig. 3A and B).

3.6. PPAR γ expression in NiDCs

Recent studies have shown that PPAR γ is a potential regulator of antigen-presenting cells and T cells. NiDCs showed a reduction in inflammatory cytokine production, and had a lower capacity to induce T cell proliferation, and Th2 polarization. Characteristics of NiDCs were similar to those of PPAR γ agonist-treated MoDCs. Therefore, we examined PPAR γ expression in MoDCs and NiDCs. We observed increased expression of PPAR γ mRNA in NiDCs (Fig. 4A and B). We then measured PPAR γ activity in nuclear extracts from MoDCs and NiDCs using a TransAM PPAR γ ELISA kit. Results indicate that PPAR γ activity in NiDCs was significantly higher than in MoDCs (Fig. 4C). Since PPAR γ was induced in NiDCs, we further investigated expression of adipose differentiation-related protein (ADRP), apolipoprotein E (ApoE), and fatty-acid-binding protein-4 (FABP4), which are known target genes of PPAR γ. As shown in Fig. 4D, upregulation of these three genes occurred only in NiDCs and correlated directly to PPAR γ expression.

3.7. Effect of non-selective nAChR antagonist on DC differentiation in the presence of nicotine

To examine whether effect of nicotine on DC development is mediated by nicotinic acetylcholine receptors (nAChRs), monocytes were preincubated with the non-selective and competitive nAChR antagonist, d-tubocurarine (1 μM) 30 min before culture. As shown in Fig. 5A, CD1a expression was recovered by pretreatment of cells with d-tubocurarine. These data indicates that the effect of nicotine on CD1a expression depends mainly on specific interaction with nAChRs. In addition, the effect of nicotine on the induction of PPAR-γ, ADFP, and ApoE gene expressions was clearly inhibited by pretreatment with d-tubocurarine (Fig. 5B–D).

3.8. Blocking nAChRs recovered Th1 response

As shown in Fig. 6, inhibition of nicotine signal by pretreatment with d-tubocurarine (1 μM), caused inhibition of PPAR γ expression (Fig. 6). Thus, we investigated whether d-tubocurarine-treated NiDCs recovered Th1 responses. As shown in Fig. 6, pretreatment of d-tubocurarine reversed reduction of IL-12 (p40 + p70) secretion by NiDCs (Fig. 6A) and IFN-γ secretion by T cells cocultured with LPS-activated NiDCs (Fig. 6B).

4. Discussion

Nicotine and cigarette smoke extracts containing nicotine are reported to have immuno-modulating effects in human and mouse [23–25,37–39]. Nicotine is a major chemical component of cigarette smoke that contains 3000–4000 chemical compounds. We tested our hypothesis that nicotine is one of the main causes of