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 μg ml^{-1} hydrocortisone, 10 μg ml^{-1} insulin, 0.4% (v/v) bovine pituitary extract, 0.1 ng ml^{-1} human epidermal growth factor (hEGF), 50 μg 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 μg 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 Eli-pair™ (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 μg ml^{-1}) phenylmethylsulphonylfluoride (PMSF), 30 μg 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
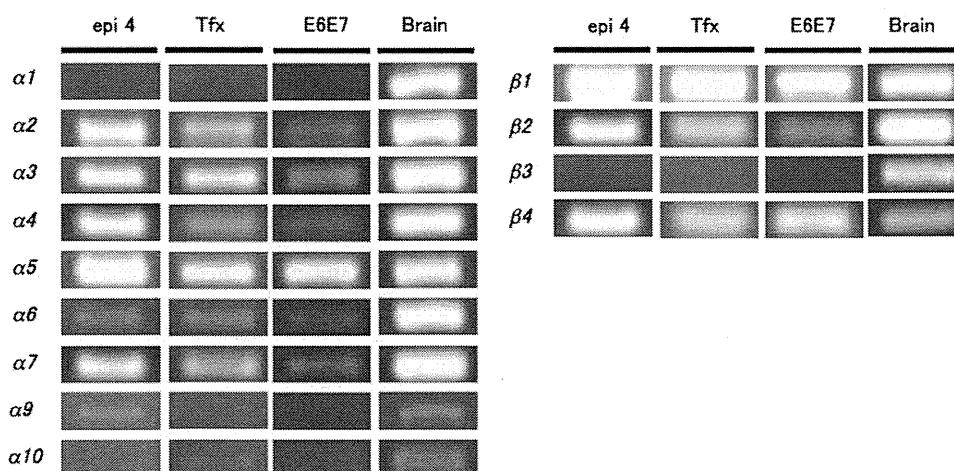
Fig. 1 – Expression profiles of nAChR mRNA in three different HGEC lines. Total RNA was extracted from subconfluent cultured cells. The expression of nAChR subunit mRNA was analysed by RT-PCR using specific primer sets. Brain RNA was used as a positive control.
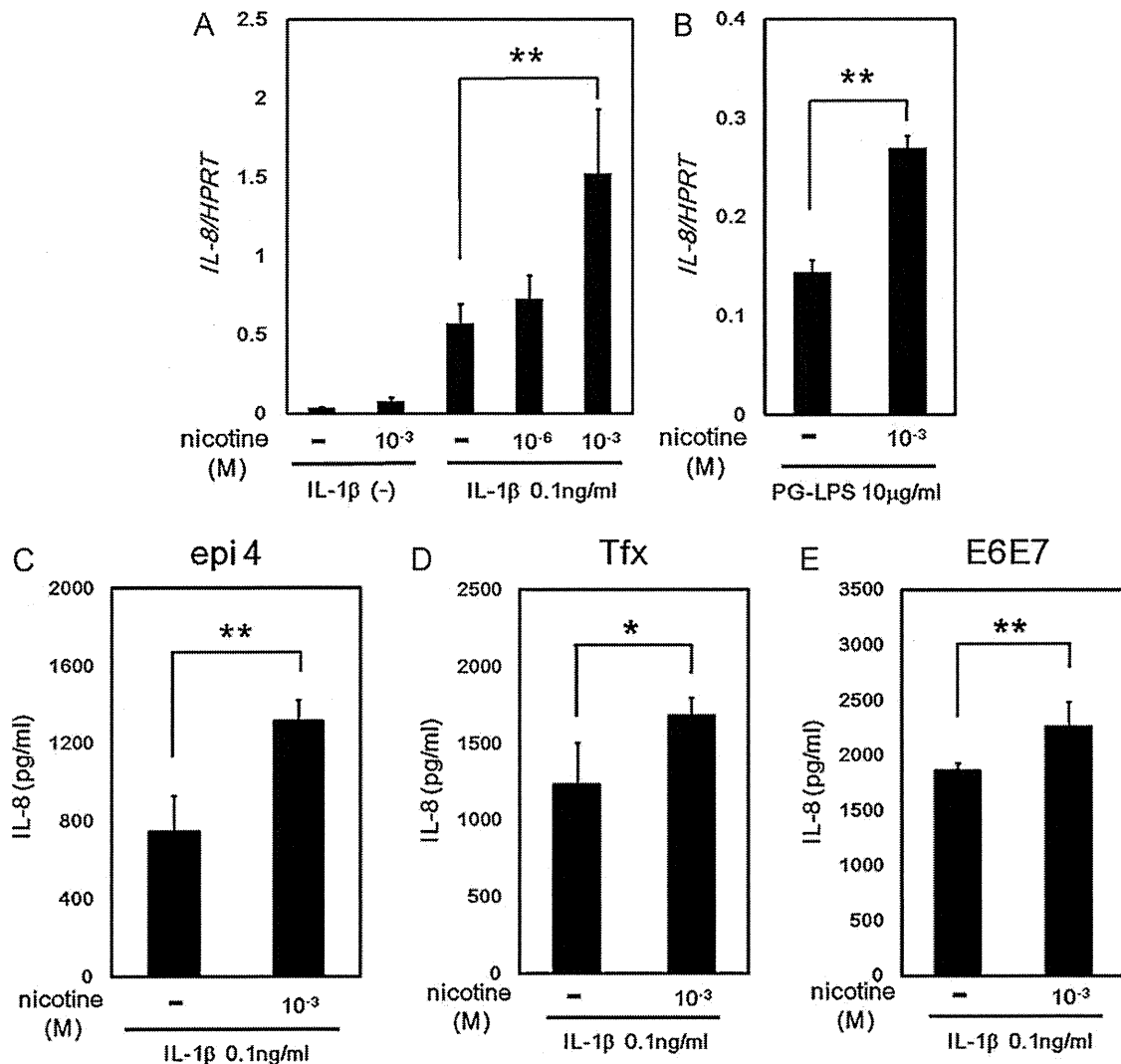
Fig. 2 – Nicotine up-regulates the expression of IL-8. IL-8 mRNA expression in epi 4 stimulated with IL-1β (A) or P. *gingivalis* LPS (B: PG-LPS) in the presence or absence of nicotine for 12 h was quantified using real-time PCR. Data are expressed as mean ± SD of three determinations. **$p < 0.01$ compared 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.
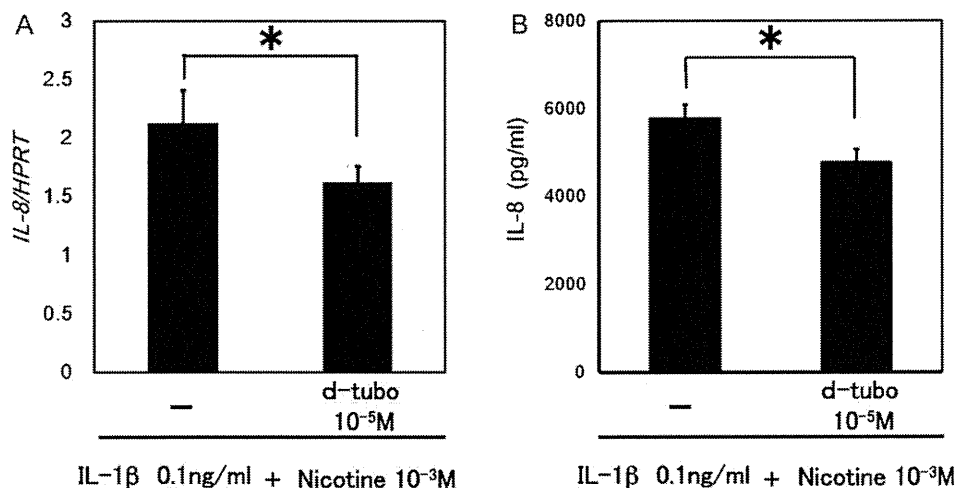
Fig. 3 – d-Tubocurarine, a nonselective nAChR antagonist, inhibits nicotine-induced upregulation of IL-8. After pretreatment with d-tubocurarine for 1 h, epi 4 was stimulated with IL-1β and nicotine for 12 h (A: real-time PCR) or 24 h (B: ELISA). Data are expressed as mean ± SD of three determinations. *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
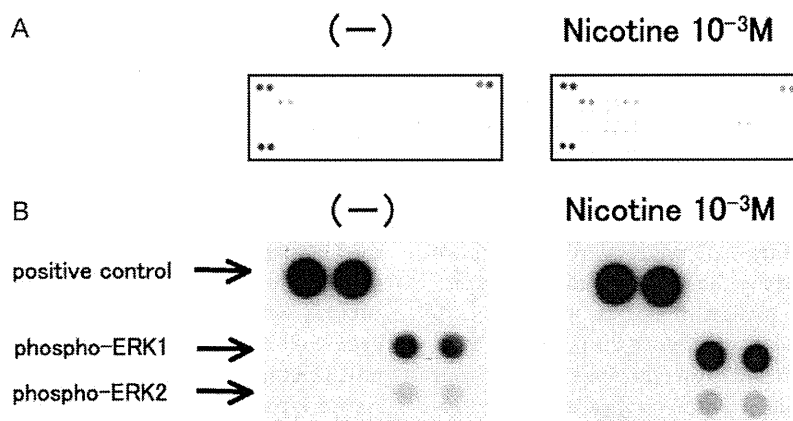
Fig. 4 – Investigation of MAPK phosphorylation in nicotine-stimulated epi4 cells. epi 4 cells were cultured in the presence or absence of nicotine for 10 min. To investigate phosphorylation of 21 MAPKs, a Human Phospho-MAPK Array Kit™ was utilized. All data (A), the enlargement of the area blotted for p-ERK1/2 and positive control (B) are shown.
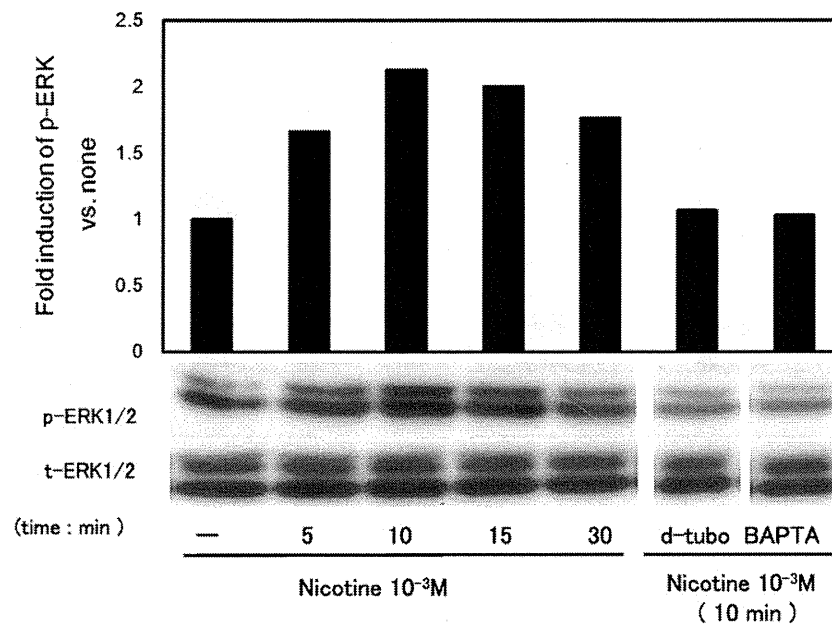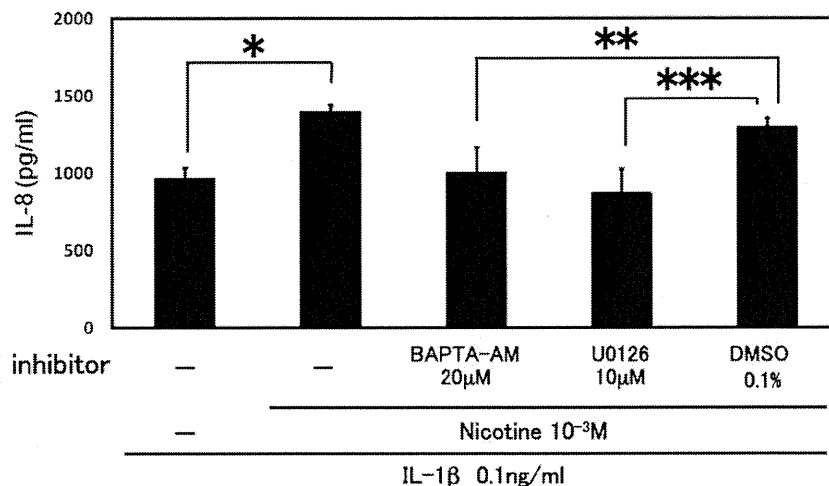
Fig. 5 – d-Tubocurarine and BAPTA-AM inhibit nicotine-induced upregulation of ERK1/2 phosphorylation. After pretreatment with d-tubocurarine or BAPTA-AM for 1 h, epi 4 cells were stimulated with nicotine for 10 min. Cell lysate was subjected to immunoblotting for p-ERK. Blotting images were analysed 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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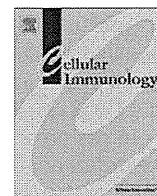
Nicotine modulates the immunological function of dendritic cells through peroxisome proliferator-activated receptor-γ upregulation

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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 proinflammatory signaling via NF-κB [28,29]. A disruption of the PPAR

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-radio-active 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′-CGG-ATGATGCAGCTCGTGA-3′: reverse, 5′-GCACGGGAGTGAAGCT-TGGTA-3′).

Apolipoprotein E: ApoE (forward, 5′-CTGCGTTGCTGGTCAC-ATTC-3′: reverse, 5′-CTCCTGCACCTGCTCAGACA-3′).

Fatty-acid-binding protein-4: FABP4 (forward, 5′-CTTCATACTGG-GCCAGGAATTTG-3′: reverse, 5′-CTCCTGCACCTGCTCAGACA-3′).

Hypoxanthine phosphoribosyl transferase: HPRT (forward, 5′-GGCAGTATAATCCAAAGATGGTCAA-3′: reverse, 5′-GTCAAGG-GCATATCCTACAACAAAC-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
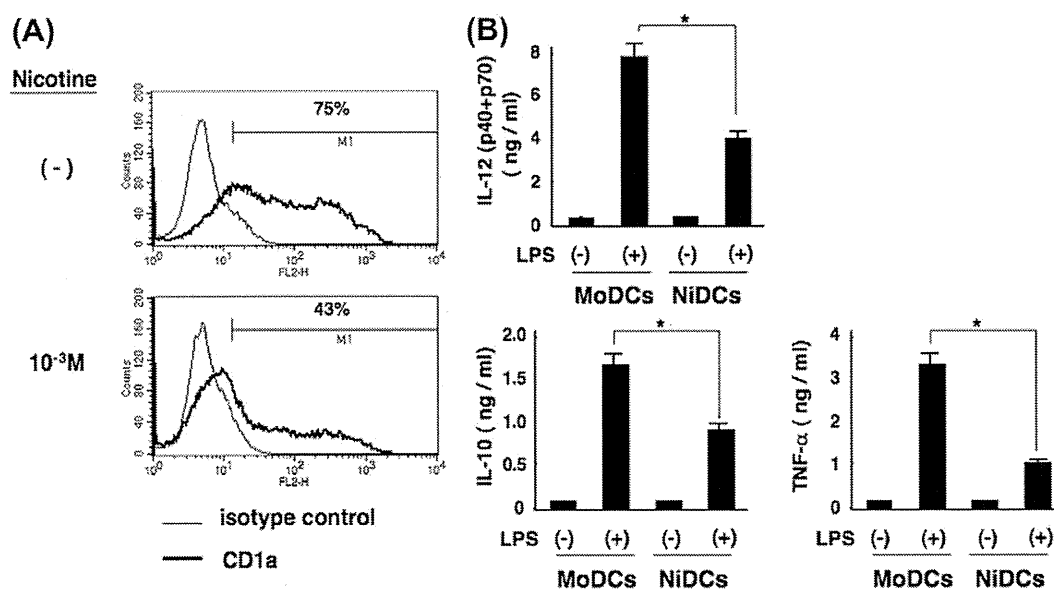
Fig. 1. (A) Differentiation of CD1a+ DC from monocytes in the presence (NiDCs) or absence (MoDCs) of 1 × 10^{-3}M nicotine. MoDCs and NiDCs were harvested at day 7 and analyzed by FACS for the expression of CD1a+. These data represent one of eight independent experiments with monocytes isolated from 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.
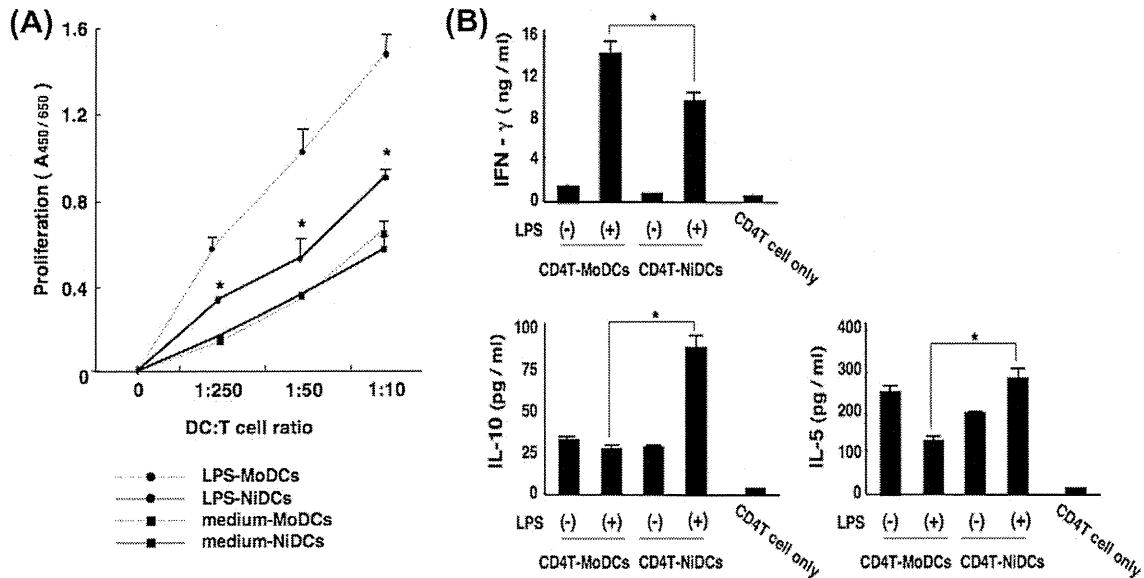
Fig. 2. Effect of nicotine on antigen-presenting properties of DC and cytokine production in MLR. (A) Comparisons of the effects of MoDCs and NiDCs on T cell proliferation. The results represent the mean OD values ± SD obtained from triplicate cultures. The data shown were obtained from one of five independent 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
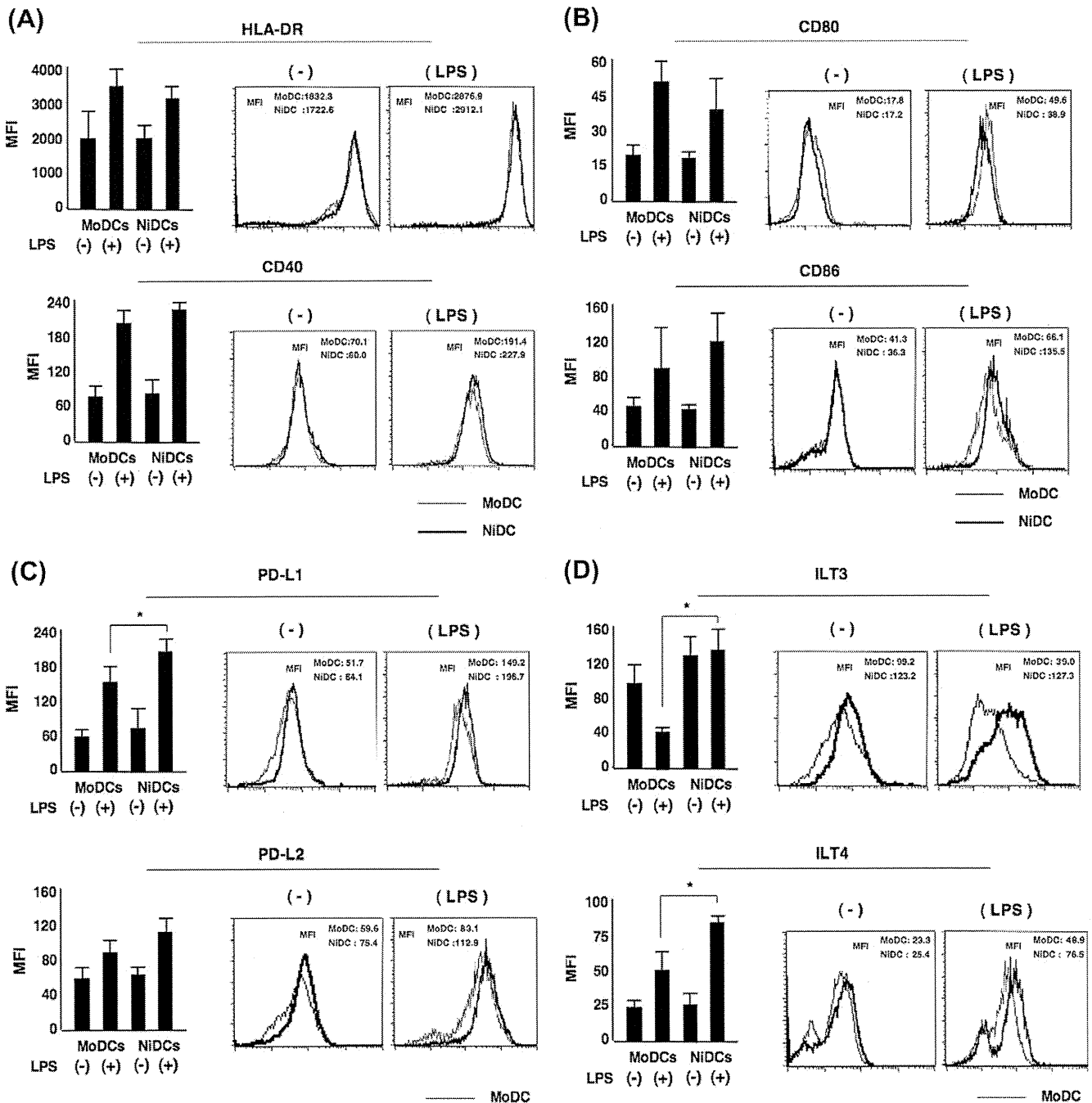
Fig. 3. Effect of nicotine on the expression of costimulatory/coinhibitory molecules on DCs. Expression of HLA-DR(A), CD40(A), CD80(B), CD86(B), PD-L1(C), PD-L2(C), ILT3(D) and ILT4 (D) on MoDCs or NiDCs stimulated with LPS (10 ng/ml) for 48 h was evaluated by FACS. Left panels: bar graphs are shown as mean values ± SD of five independent experiments. *P < 0.05 when compared with MoDCs stimulated with LPS. MFI: mean fluorescent intensity. Right panels: one representative FACS histogram of MoDCs (thin line) and NiDCs (thick line) of more than six independent experiments are shown.

tobacco toxicity that alters immune function. Considering the many components of cigarette smoke, nicotine may not be responsible for all types of tobacco toxicity. The expression of nicotinic acetylcholine receptors, however, indicates that the effects of nicotine may be transmitted and mediated by a specific cellular signaling pathway(s) coupled to immunological function. Although 10^{-3} M nicotine is greater than the pharmacological dose, the concentration of nicotine in saliva of smokers may reach mM levels in localized areas such as the oral cavity and respiratory tract [14–16]. We therefore assumed that direct and local exposure of high doses of nicotine to the inflammatory lesion with bleeding in gingival tissue initiates monocytes to differentiate into DC in inflamed gingival

microcirculation. In preliminary experiments, we used 10^{-8} to 10^{-2} M nicotine to investigate the effect of nicotine on DC phenotypic changes, and found that 10^{-3} M nicotine significantly induced DC characteristics. Additionally, we confirmed that 10^{-3} M nicotine did not impact DC viability. Therefore, we chose 10^{-3} M nicotine to induce differentiation of monocytes into DCs.

We report that in vitro differentiation of DCs from monocytes in the presence of nicotine yields a subset of DC (NiDCs) characterized by an altered phenotypic profile and modulated functions. CD1a is one of five members of the CD1 family and has been used as a DC biomarker [40]. There is however a significant heterogeneity in CD1a expression in DCs. Both CD1a (+ or −) DCs have been
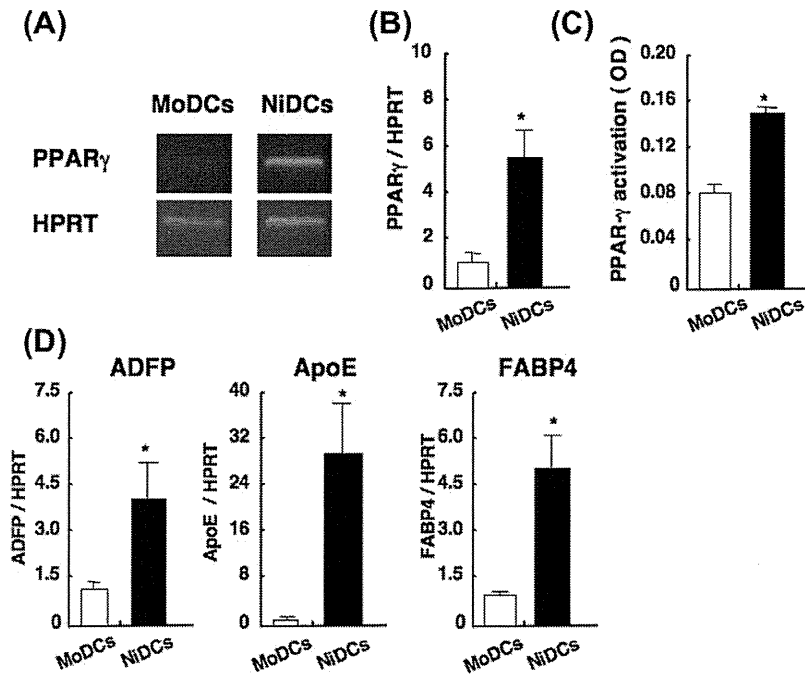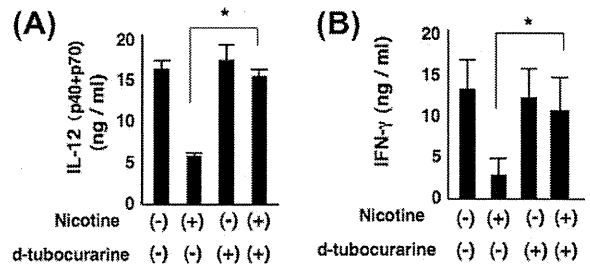
Fig. 4. Expression and activation of PPAR γ in MoDCs and NiDCs. (A) Representative RT-PCR analysis of PPAR γ mRNA expression. One representative profile of six performed. (B) Quantitative analyses of PPAR γ mRNA expression by real-time RT-PCR. mRNA levels are expressed as fold change above control mRNA (HPRT). Results are shown as mean values ± SD of five independent experiments. (C) Activation of PPAR γ in MoDCs and NiDCs. Nuclear extracts taken at a half-hour were analyzed by the TransAM PPAR γ assay. Results are shown as mean values ± SD of five independent experiments and are expressed as ratio of OD_{450}. (D) Expression of the PPAR γ target genes in MoDCs and NiDCs. Results are shown as mean values ± SD of five independent experiments. $^*P < 0.05$ when compared with MoDCs.
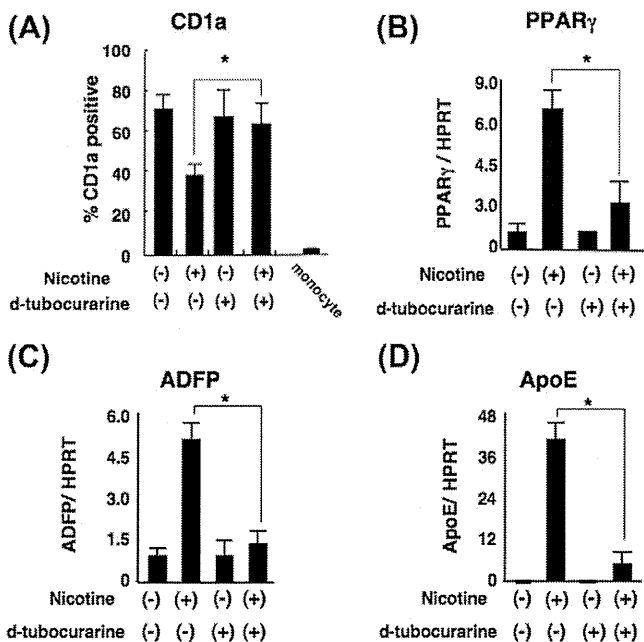
Fig. 5. Effect of d-tubocurarine, a non-selective nAChRs antagonist on mRNA expression of PPAR γ and PPAR γ target genes in MoDCs and NiDCs. (A) Expression of CD1a was examined at day 7 to evaluate the effect of nicotine and d-tubocurarine. Results are shown as mean values ± SD of five independent experiments. (B–D) Quantitative analyses of PPAR γ, ADFP, and ApoE mRNA expression by real-time RT-PCR. mRNA levels are expressed as fold change above control mRNA (HPRT). Results are shown as mean values ± SD of four independent experiments. $^*P < 0.05$ when compared with DCs differentiated in the presence of nicotine with pretreatment of d-tubocurarine.

Fig. 6. Effect of d-tubocurarine, non-selective nAChRs antagonist on Th1 responses in MoDCs and NiDCs. (A) IL-12 (p40 + p70) production by MoDCs and NiDCs. MoDCs and NiDCs pretreated with d-tubocurarine (1 μM) were cultured in the presence of 10 ng/ml LPS for 48 h. Supernatants were then tested for IL-12 (p40 + p70) production by ELISA. (B) Supernatants of restimulated T cells were harvested and measured for IFN-γ level by ELISA. Results are shown as mean values ± SD of four independent experiments. $^*P < 0.05$ when compared with NiDCs without pretreatment of d-tubocurarine.

[42–45]. In our study, a significant reduction of CD1a+ expression is observed in NiDCs when compared with MoDCs. The effect of nicotine on decreasing CD1a expression is inhibited by pretreatment of cells with d-tubocurarine, a non-selective nAChR antagonist. These results indicate that nicotine interaction with nAChRs can mediate DC differentiation.

As seen in Fig. 1B, LPS-stimulated NiDC secreted lower amounts of pro-inflammatory cytokines such as IL-12 (p40 + p70) and TNF-α, compared with MoDCs. These data confirm results from previous studies in which nicotine and cigarette smoke extracts (CSE) suppressed DC function, impaired antigen-presentation to induce naive T cell proliferation and alter Th1 responses into those seen in Th2 cells [23,24]. These studies also described that CSE suppressed IL-12 production in activated DCs, and diminished the effects of costimulatory molecules such as CD40 and CD80. We observed poor induction of T cell proliferation by NiDCs, elevation of the coinhibitory molecules PD-L1 and ILT4, and a slight

identified in peripheral blood [41]. In addition, recent studies suggest that the ratio of +/− forms of CD1a DCs differentiated from monocytes can be altered depending on the culture conditions

upregulation of PD-L2 in LPS-stimulated NiDCs. Furthermore, ILT3 expression was maintained in NiDC after LPS-stimulation, although expression on LPS-stimulated MoDCs was reduced. Increased PD-L1 and PD-L2 expression combined with positive costimulatory molecules such as CD86 induce an immunotolerogenic function in DCs [46–48]. In addition, high expression of ILT3 and ILT4 on DCs is associated with immunotolerogenic characteristics [49,50]. The reduction in priming capacity of NiDCs may result from induction of inhibitory cell surface receptors.

PPAR γ has been reported to mediate several DC functions. PPAR γ-activated DCs altered the differentiation of naive CD4 T cells into Th2 cells [32,51]. In addition, PPAR-γ agonists inhibited TLR-mediated DC activation by interfering with the NF-κB and MAP kinase pathway [52]. In a conditional PPAR γ knockout mouse study, PPAR γ-activated DCs induced naive T cell anergy [31]. Other studies suggest that PPAR γ plays an important role in induction and maintenance of natural and induced Treg cells [53,54]. A recent report described that activation of PPAR γ strongly enhanced the expression of B7H1 (also termed PD-L1) [55]. We show that expression of PPAR-γ gene and PPAR-γ target genes are upregulated in NiDCs. ApoE plays important roles in lipoprotein clearance and homeostasis. ADRP plays a role in lipid body formation and cross-presentation of phagocytosed antigens to $CD8^+$ T cells. FABP4 plays an important role in the regulation of insulin sensitivity. In addition, both ApoE and FABP4 deficiency lead to development of atheloscle-rosis. Among those molecules, ADRP is likely to have a role in DC antigen presenting function. However, there is no information at present that these genes are involved in T cell proliferation and differentiation. With regard to PPAR-γ expression in NiDCs, further studies are needed to investigate the possible role of nicotine in induction of Treg cells as our data suggests that nicotine may differentiate monocytes into tolerogenic DCs.

The molecular mechanism of elevated PPAR γ expression in NiDCs needs to be clarified. Results from recent studies indicate that α7nAChR is crucial to the regulation of systemic inflammation, and that nicotine and acetylcholine control inflammatory cytokine production from endotoxin-stimulated macrophages by inhibiting NF-κB pathway via α7nAChRs [11,13]. We demonstrate that pretreatment of DCs with d-tubocurarine, a non-selective nAChR antagonist, reversed inhibition of CD1a expression, IL-12 production and Th1 responses in the presence of nicotine. One point that appears to be clear is that nicotine and nicotinic agonists prevent endotoxin-induced activation of the NF-κB pathway and induce expression of PPAR γ. Upregulation of PPAR γ and downreg-ulation of NF-κB may synergistically induce the NiDC phenotype described previously. Blocking nAChRs by antagonist recovered Th1 response, however, there was no significant difference in IL-5 and IL-10 production between antagonist-treated NiDCs and control-NiDCs (data not shown). Presumably, the pathway inducing Th2 immune responses may be influenced by endocytosed nicotine, and not only by nicotine acting via nAChRs signaling.

Cigarette smoking leads to many health problems worldwide by significantly increasing the risk of diseases such as lung cancer, cardiovascular disease, COPD, rheumatoid arthritis and periodontal disease [1–5,56–58]. Smoking-related suppression of immune re-sponses includes reduction of NK activity [59], and inhibition of microbicidal activity of macrophages [8,60]. Cigarette smoking and nicotine, however, may actually reduce severe inflammation in patients with ulcerative colitis (UC) [61,62]. In an animal study, conditional deletion of the PPAR γ-encoded gene in intestinal epi-thelial cells or macrophages caused an exacerbation of experimen-tal colitis [30,53]. In addition, PPAR γ expression in the colonic mucosa is impaired in UC patients [63]. Results from these studies in addition to results from our study, indicate that nicotine signal-ing may induce expression and activation of PPAR γ.

In conclusion, our study provides new data indicating that nic-otine reduces inflammatory cytokine production, and suppresses T cell priming capacity of DC via nAChRs. The suggested mechanism is nicotinic upregulation of coinhibitory molecules. The effect of nicotine may be mediated by PPAR γ expression. These studies suggest a link between the effects of nicotine on DC function and smoking-related diseases. Understanding the immuno-modulatory effects of nicotine will provide new and useful information for the prevention of smoking-related diseases.

Acknowledgments

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Adiponectin regulates functions of gingival fibroblasts and periodontal ligament cells

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Iwayama T, Yanagita M, Mori K, Sawada K, Ozasa M, Kubota M, Miki K, Kojima Y, Takedachi M, Kitamura M, Shimabukuro Y, Hashikawa T, Murakami S. Adiponectin regulates functions of gingival fibroblasts and periodontal ligament cells. J Periodont Res 2012; doi: 10.1111/j.1600-0765.2012.01467.x. © 2012 John Wiley & Sons A/S

Background and Objective: Adiponectin is a cytokine constitutively produced by adipocytes and exhibits multiple biological functions by targeting various cell types. However, the effects of adiponectin on primary gingival fibroblasts and periodontal ligament cells are still unexplored. Therefore, we investigated the effects of adiponectin on gingival fibroblasts and periodontal ligament cells.

Material and Methods: The expression of adiponectin receptors (AdipoR1 and AdipoR2) on human gingival fibroblasts (HGFs), mouse gingival fibroblasts (MGFs) and human periodontal ligament (HPDL) cells was examined using RT-PCR and western blotting. HGFs and MGFs were stimulated with interleukin (IL)-1β in the presence or absence of adiponectin, and the expression of IL-6 and IL-8 at both mRNA and protein levels was measured by real-time PCR and ELISA, respectively. Furthermore, small interfering RNAs (siRNAs) in MGFs were used to knock down the expression of mouse AdipoR1 and AdipoR2. The effects of adiponectin on the expression of alkaline phosphatase (*ALP*) and runt-related transcription factor 2 (*Runx2*) genes were evaluated by real-time PCR. Mineralized nodule formation of adiponectin-treated HPDL cells was revealed by Alizarin Red staining.

Results: AdipoR1 and AdipoR2 were expressed constitutively in HGFs, MGFs and HPDL cells. Adiponectin decreased the expression of IL-6 and IL-8 in IL-1β-stimulated HGFs and MGFs. AdipoR1 siRNA in MGFs revealed that the effect of adiponectin on reduction of IL-6 expression was potentially mediated via AdipoR1. Adiponectin-treated HPDL cells promoted the expression of *ALP* and *Runx2* mRNAs and up-regulated ALP activity. Furthermore, adiponectin enhanced mineralized nodule formation of HPDL cells.

Conclusion: Our observations demonstrate that adiponectin exerts anti-inflammatory effects on HGFs and MGFs, and promotes the activities of osteoblastogenesis of HPDL cells. We conclude that adiponectin has potent beneficial functions to maintain the homeostasis of periodontal health, improve periodontal lesions, and contribute to wound healing and tissue regeneration.

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Periodontal disease is a chronic inflammatory disease initiated by the biofilm of periodontopathic bacteria, leading to the destruction of periodontal tissues. Periodontal disease can be exacerbated by many factors;

for example, systemic diseases, such as diabetes, osteoporosis and immunodeficiency diseases, have been shown to result in an increased risk for periodontal disease (1). Interestingly, recent epidemiologic studies have suggested that obesity is also a risk factor for periodontitis (2,3). Obesity, which induces insulin resistance following systemic chronic inflammation (4), is one of the leading causes of type 2 diabetes, which is closely associated with periodontal diseases. Thus, it is possible that obesity and metabolic syndrome could be risk factors for the progression of periodontal diseases; however, the precise mechanism of how obesity results in the destruction of periodontal tissue remains unclear.

Adipokines, secreted by adipose tissue, can influence insulin resistance, inflammation and the cardiovascular system (5). Adiponectin – an adipokine – circulates in high concentrations in plasma (6). Two adiponectin receptors (AdipoRs) have been reported to be expressed on various tissues and cells (7). Importantly, hypo-adiponectinemia has been observed in patients with type 2 diabetes mellitus, obesity and coronary artery disease (8,9). Physiological concentrations of adiponectin suppressed tumor necrosis factor-α-induced inflammatory responses in human endothelial cells and macrophages (10). Recent studies have revealed that the concentrations of adiponectin in serum of patients with severe periodontitis are lower than those in serum from healthy subjects (11,12). Periodontal treatment has also been shown to increase the levels of adiponectin in chronic periodontitis (13). Interestingly, Yamaguchi *et al.* (14) reported that the levels of expression of AdipoRs were decreased in sites of severe periodontitis. These data suggest that adiponectin is involved in the homeostasis of periodontal tissues and may modulate inflammatory responses at periodontal lesions.

In recent years, adiponectin and AdipoRs have been reported to be expressed in osteoblasts (15,16), suggesting that adiponectin may be involved not only in anti-inflammatory functions but also in bone metabolism. Among periodontal tissues, periodon-

tal ligament (PDL) cells have the potential to regulate neogenesis of alveolar bone and cementum and play important roles in events of wound healing and regeneration following periodontal tissue breakdown caused by progression of periodontal diseases. Considering the multifunctional role of adiponectin, adiponectin may affect the functional characteristics of PDL cells, which can differentiate into mineralized tissue-forming cells such as osteoblasts and cementoblasts (17).

In this study we investigated the anti-inflammatory effect of adiponectin on human gingival fibroblasts (HGFs) and mouse gingival fibroblasts (MGFs). In addition, we examined the physiological effect of adiponectin on cytodifferentiation of human PDL (HPDL) cells. The results showed that adiponectin suppressed proinflammatory cytokines induced by interleukin (IL)-1β stimulation, possibly via AdipoR1. Furthermore, adiponectin promoted the differentiation and mineralization of HPDL cells.

Material and methods

Reagents

Recombinant human and mouse IL-1β, adiponectin and normal rabbit IgG were obtained from R&D Systems (Minneapolis, MN, USA). Anti-adipoR1 IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Alpha Diagnostic Intl. Inc. (San Antonio, TX, USA).

Cells

Before participating in this study, all human subjects provided informed consent according to a protocol that was reviewed and approved by the Institutional Review Board of the Osaka University Graduate School of Dentistry. HGFs were obtained from biopsies of healthy gingiva taken from healthy volunteers, as previously described (18). HGFs were used for experiments at passages 4–10. MGFs were isolated from healthy gingival tissue of the first premolar teeth of BALB/c mice. When the cells that grew out from the explants reached

confluence, they were separated by treatment with 0.53 mM EDTA containing 0.05% trypsin, collected by centrifugation and cultured on plastic culture dishes containing standard medium (standard medium is α-minimal essential medium containing 10% fetal calf serum) until they reached confluence. After 12 passages, the clonal MGF cell line was established using the limiting-dilution method. HPDL cells were isolated and maintained as described previously (19,20). For the induction of cytodifferentiation, HPDL cells were cultured in α-minimal essential medium (α-MEM) containing 10% fetal calf serum, 10 mM β-glycerophosphate and 50 μg/mL of ascorbic acid [(calcification-inducing medium (C-Med)]. C-Med was replaced every 3 d.

RT-PCR

Total RNA was isolated from HGFs, HPDL cells and MGFs using an RNA-Bee kit (TEL-TEST, Inc., Friendswood, TX, USA) according to the manufacturer's instructions. cDNA was synthesized and amplified using PCR, as described previously (18). Oligonucleotide PCR primers specific for adiponectin and AdipoRs were synthesized by Clontech (Palo Alto, CA, USA). The sequences of the primers are shown in Table 1. Hypoxanthine phosphoribosyltransferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as housekeeping genes.

Western blot analysis

HGFs, HPDL cells and MGFs were lysed in RIPA buffer [25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1% sodium deoxycholate, 0.1% SDS, 10 mM Na_3VO_4 and 10 μg/mL each of aprotinin and leupeptin]. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with 10% bovine serum albumin for 1 h and subsequently with rabbit polyclonal anti-adiponectin Ig (Alpha Diagnostic International inc.) or goat polyclonal anti-AdipoR Ig (Santa Cruz Biotechnology) for 1 h at

Table 1. Primers used for RT-PCR

Gene		Sequence	
Human	Forward	5'-CGA GAT GTG ATG AAG GAG ATG GG-3'	304 bp
HPRT1	Reverse	5'-GCC TGA CCA AGG AAA GCA AAG TC-3'	
Human	Forward	5'-CAA ACA GCC CCA AAG TCA AT-3'	288 bp
Adiponectin	Reverse	5'-TCT CAG GTG AGG TGG GAA AC-3'	
Human	Forward	5'-AAA CTG GCA ACA TCT GGA CC-3'	300 bp
AdipoRl	Reverse	5'-GCT GTG GGG AGC AGT AGA AG-3'	
Human	Forward	5'-ACA GGC AAC ATT TGG ACA CA-3'	267 bp
AdipoR2	Reverse	5'-CCA AGG AAC AAA ACT TCC CA-3'	
Mouse	Forward	5'-AGG TTG TCT CCT GCG ACT TC-3'	211 bp
GAPDH	Reverse	5'-CTT GCT CAG TGT CCT TGC TG-3'	
Mouse	Forward	5'-ATC TGA CGA CAC CAA AAG GG-3'	226 bp
Adiponectin	Reverse	5'-TCT CCA GGA GTG CCA TCT CT-3'	
Mouse	Forward	5'-TGC CCT CCT TTC GGG CTT GC-3'	529 bp
AdipoRl	Reverse	5'-GCC TTG ACA AAG CCC TCA GCG ATA G-3'	
Mouse	Forward	5'-TCT TCC TGT GCC TGG GGA TCT T-3'	254 bp
AdipoR2	Reverse	5'-CCC GAT ACT GAG GGG TGG CAA A-3'	

AdipoRl, adiponectin receptor 1; *AdipoR2*, adiponectin receptor 2; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HPRT1*, hypoxanthine phosphoribosyltransferase-1.

Real-time PCR

HGFs and MGFs were seeded in a six-well plate at a density of 3×10^5 cells and 1.2×10^6 cells/well, respectively. Cells were grown to confluence in standard medium. Following 18 h of preincubation in the presence or absence of adiponectin, cells were treated with or without 0.1 ng/mL of IL-1β, then total RNA was isolated and precipitated. cDNA was synthesized and mixed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and gene-specific primers (Takara Bio, Shiga, Japan). Real-time PCR was performed using a 7300 Fast Real-Time PCR System (Applied Biosystems). The sequences of the primers are shown in Table 2. HPRT and GAPDH served as housekeeping genes.

Measurement of inflammatory cytokines in culture supernatants

HGFs and MGFs were seeded in a 12-well plate at a density of 1.8×10^5 and 7.2×10^5 cells, respectively, and grown to confluence in standard medium. Following 18 h of preincubation

with or without adiponectin, cells were treated with or without 0.5 ng/mL of IL-1β. In some experiments, cells were pretreated for 1 h with anti-adiponectin Ig. At the end of the incubation periods, the supernatants were collected and the levels of IL-6 and IL-8 (HGFs only) protein were measured using ELISA kits (R&D Systems) according to the manufacturer's instructions.

RNA interference

Small interfering RNA (siRNA) was used to knock down the expression of mouse AdipoR1 and AdipoR2. The AdipoR1 and AdipoR2 siRNAs, and a

negative-control siRNA (Silencer Select Negative Control #1 siRNA), were synthesized by Applied Biosystems. Silencer 1 Negative Control #1 siRNA was designed to have no significant sequence similarity to mouse, rat or human transcript sequences. MGFs were placed on a six-well culture dish. Twenty-four hours after incubation, MGFs, at 40–50% confluence, were transfected with siRNA AdipoR1, siRNA AdipoR2 or negative-control siRNA. The cells were transfected with 200 pmol of siRNA and negative-control siRNA using Lipofectamine 2000 (Invitrogen Corp, Carlsbad, CA, USA) according to the

Table 2. Primers used for real-time PCR

Gene		Sequence
Human	Forward	5'-GGC AGT ATA ATC CAA AGA TGG TCA A-3'
HPRT1	Reverse	5'-GTC AAG GGC ATA TCC TAC AAC AAA C-3'
Human	Forward	5'-AAG CCA GAG CTG TGC AGA TGA GTA-3'
IL6	Reverse	5'-TGT CCT GCA GCC ACT GGT TC-3'
Human	Forward	5'-ACA CTG CGC CAA CAC AGA AAT TA-3'
IL8	Reverse	5'-TTT GCT TGA AGT TTC ACT GGC ATC-3'
Human	Forward	5'-GGA CCA TTC CCA CGT CTT CAC-3'
ALP	Reverse	5'-CCT TGT AGC CAG GCC CAT TG-3'
Human	Forward	5'-CAC TGG CGC TGC AAC AAG A-3'
RUNX2	Reverse	5'-CAT TCC GGA GCT CAG CAG AAT AA-3'
Mouse	Forward	5'-TGT GTC CGT CGT GGA TCT GA-3'
GAPDH	Reverse	5'-TTG CTG TTG AAG TCG CAG GAG-3'
Mouse	Forward	5'-CCA CTT CAC AAG TCG GAG GCT TA-3'
IL6	Reverse	5'-GCA AGT GCA TCA TCG TTG TTC ATA C-3'

ALP, alkaline phosphatase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HPRT1*, hypoxanthine phosphoribosyltransferase-1; *IL6*, interleukin-6; *IL8*, interleukin-8; *RUNX2*, runt-related transcription factor 2.

manufacturer's instructions. The cells were then analyzed using real-time PCR.

Determination of alkaline phosphatase activity, and staining with Alizarin Red

Alkaline phosphatase (ALP) activity was assessed according to the procedure described previously (20,21). Histochemical analysis of calcified nodules was performed using the Alizarin Red staining method (20,22). The density of calcified nodules in each well was calculated using the WinRoof software program (Mitani Corporation, Fukui, Japan).

Statistical analysis

The results were analyzed for statistical significance using the Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results

Expression of AdipoR1 and AdipoR2, but not adiponectin, was detected in HGFs, HPDL cells and MGFs

To examine the expression of adiponectin and its receptors (AdipoR1 and AdipoR2) and mRNA and protein in HGFs, HPDL cells and MGFs, we performed RT-PCR amplification and western blotting. As shown in Fig. 1A and 1B, mRNA transcripts and protein for AdipoR1 and AdipoR2, but not for adiponectin, were detected in all cell types investigated.

Adiponectin reduced the expression of proinflammatory cytokines in IL-1β-stimulated HGFs

To the effect of adiponectin on the expression of proinflammatory cytokines in IL-1β-stimulated HGFs, HGFs were pretreated with adiponectin for 18 h before 2.5 h of stimulation with IL-1β, whereupon real-time PCR was performed. As shown in Fig. 2A, adiponectin significantly reduced the expression of *IL6* and *IL8* mRNAs. For assessment of IL-6 and IL-8 pro-
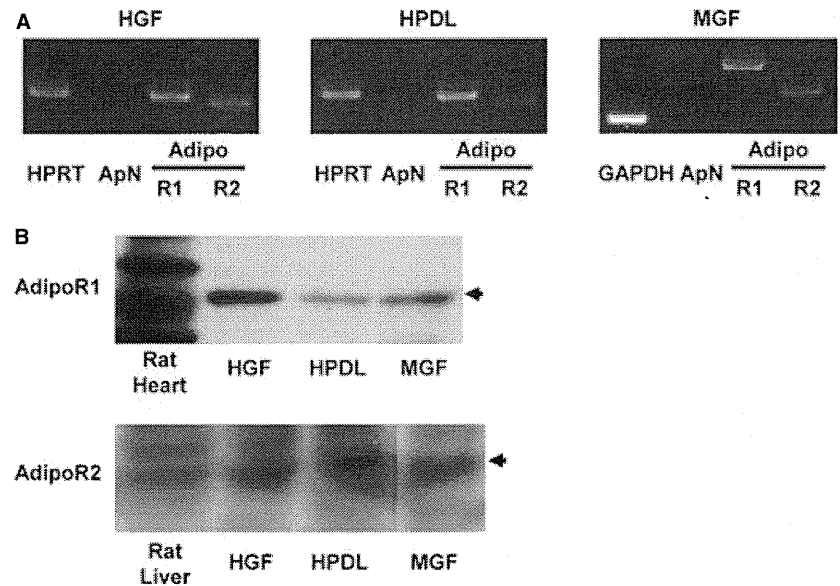

Fig. 1. Expression of adiponectin (ApN), adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2). (A) Expression of *ApN*, *AdipoR1* and *AdipoR2* genes in human gingival fibroblasts (HGFs), human periodontal ligament (HPDL) cells and mouse gingival fibroblasts (MGFs) was examined by RT-PCR. (B) Expression of AdipoR1 and AdipoR2 proteins in HGFs, HPDL cells and MGFs was detected using western blotting. The data represent one of three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase.

duction, adiponectin-pretreated HGFs were stimulated with 0.5 ng/mL of IL-1β, harvested after 12 h and then assayed using ELISA. Adiponectin also reduced the production of IL-6 protein and IL-8 protein (Fig. 2B).

AdipoR1 knockdown abrogated the adiponectin-induced reduction of IL-6 expression in MGFs

To examine the effect of adiponectin on the expression of IL-6 in IL-1β-stimulated MGFs, MGFs were pretreated with adiponectin for 18 h before stimulation with IL-1β for 2.5 h, whereupon real-time PCR was performed. As shown in Fig. 3A, adiponectin significantly reduced the expression of *IL6* mRNA. To assess the production of IL-6 protein, adiponectin-pretreated MGF were stimulated with 0.5 ng/mL of IL-1β, harvested after 12 h and then assayed using ELISA. Adiponectin also reduced the production of IL-6 protein (Fig. 3B). To elucidate the effect of adiponectin via AdipoRs, siRNA was used to block the expression of *AdipoR1* and *AdipoR2* mRNAs. As shown

in Fig. 4A and 4B, real-time PCR revealed that treatment with siRNA-AdipoR1 and siRNA-AdipoR2 significantly reduced the expression of AdipoR1 and AdipoR2 in MGFs compared with negative-control siRNA (Fig. 4A and 4B). Whereas IL-1β-induced expression of IL-6 in MGF treated with negative-control siRNA was significantly suppressed by adiponectin treatment, these suppressive effects were attenuated in MGFs treated with siRNA for AdipoR1 (Fig. 4C). By contrast, treatment with siRNA for AdipoR2 did not suppress IL-1β-induced expression of IL-6 by adiponectin (Fig. 4D). These results suggest that adiponectin reduces IL-6 expression in MGFs possibly via AdipoR1.

Anti-AdipoR1 Ig attenuated the adiponectin-induced reduction of IL-6 expression in HGF

To elucidate the effect of adiponectin via AdipoR1, HGFs were pretreated with control antibodies or with two types of anti-AdipoR1 polyclonal Igs and then stimulated with IL-1β in the presence or absence of adiponectin.
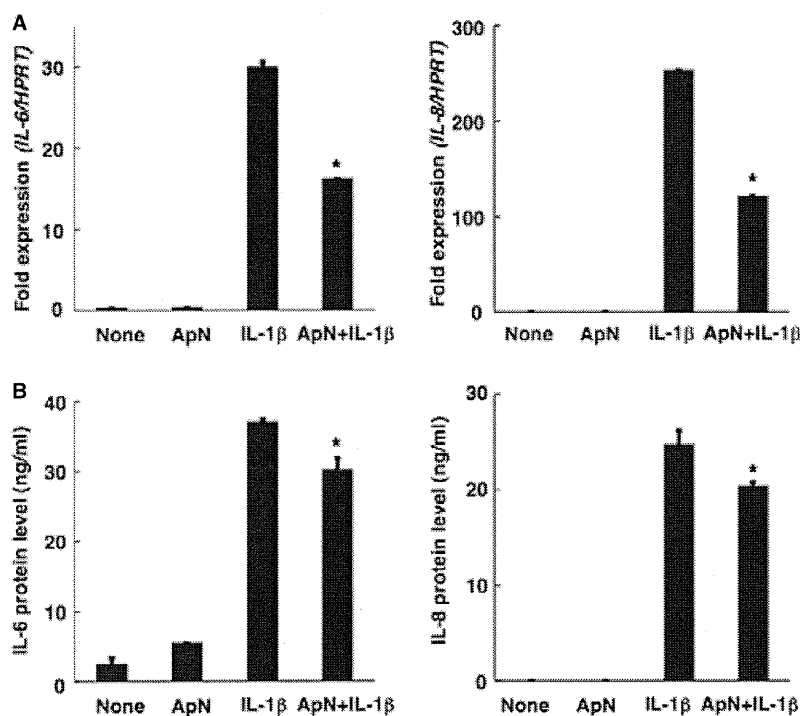
Fig. 2. Adiponectin reduced interleukin (IL)-1β-induced production of IL-6 and IL-8 in human gingival fibroblasts (HGFs). (A) Expression of *IL6* and *IL8* genes in HGFs was examined using real-time PCR. HGFs were pretreated with adiponectin (ApN; 5 μg/mL) for 18 h, stimulated with IL-1β (0.1 ng/mL) for 2.5 h and then total RNA was isolated. (B) The levels of IL-6 and IL-8 protein in HGF cultured condition medium were measured using ELISA. HGFs were pretreated with adiponectin (ApN; 10 μg/mL) for 18 h and stimulated for 12 h with IL-1β (0.5 ng/mL). Data are the mean ± standard deviation of triplicate determinations. *p < 0.05 compared with IL-1β-stimulated HGF without ApN pretreatment. HPRT, hypoxanthine phosphoribosyltransferase.

Fig. 3. Adiponectin reduced interleukin (IL)-1β-induced expression of IL-6 in mouse gingival fibroblasts (MGFs). (A) Expression of the *IL6* gene in MGFs was examined by real-time PCR. MGFs were pretreated with adiponectin (ApN; 20 μg/mL) for 18 h, stimulated with IL-1β (0.5 ng/mL) for 2.5 h and then total RNA was isolated. (B) The level of IL-6 protein in MGF cultured condition medium was measured by ELISA. MGFs were pretreated with adiponectin (ApN; 20 μg/mL) for 18 h and stimulated with IL-1β (0.5 ng/mL) for 12 h. Data are the mean ± standard deviation of triplicate determinations. *p < 0.05 compared with IL-1β-stimulated MGFs without pretreatment of adiponectin. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

After 12 h, IL-6 production in the culture supernatants was assayed using ELISA. As shown in Fig. 5, anti-AdipoR1 Igs significantly attenuated the suppression of IL-1β-induced IL-6 expression by adiponectin. These results suggest that adiponectin reduces IL-6 expression in the IL-1β-stimulated HGFs possibly via AdipoR1.

Adiponectin promoted the differentiation and mineralization of HPDL cells

Next, we examined whether or not adiponectin would promote the differentiation and mineralization of HPDL cells. As shown in Fig. 6A, ALP activity in HPDL cells was significantly enhanced in the presence of adiponectin. Real-time PCR revealed that adiponectin significantly enhanced the expression of *ALP* and runt-related transcription factor 2 (*Runx2*; an important transcription factor involved in osteoblastic differentiation and mineralization) (23) genes in HPDL cells cultured with C-Med in the presence of adiponectin (Fig. 6B and 6C) compared with C-Med only. Subsequently, mineralized nodule formation by HPDL cells on day 18 was investigated. As shown in Fig. 7A and 7B, adiponectin significantly increased the intensity of Alizarin Red staining. These results suggest that adiponectin promotes the differentiation and mineralization of HPDL cells.

Discussion

Adiponectin is an abundant serum protein, with concentrations in the order of 3–30 μg/mL (10). In this study, we demonstrated, for the first time, that physiological concentrations of adiponectin suppress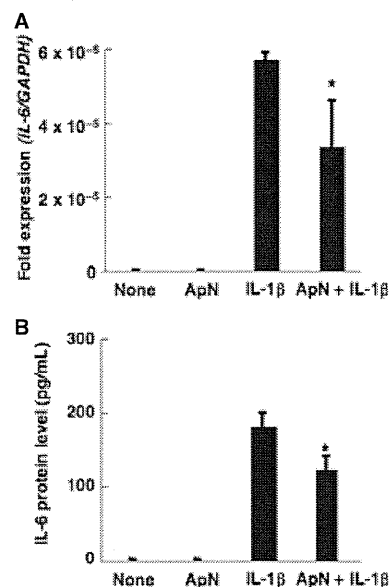 IL-1β-induced IL-6 and IL-8 expression in HGFs, and IL-6 in MGFs, at mRNA and protein levels, possibly via AdipoR1 signaling. We also showed that adiponectin enhances the differentiation and mineralization of HPDL cells. Unfortunately, however, mouse IL-8 has not yet been identified. As mouse CXCL1 is known to be the functional homolog of human IL-8, we examined the expression CXCL1 in preliminary studies (data not shown). In these studies, we found that adiponectin significantly reduced the expression of *CXCL1* mRNA. However, the expression of CXCL1 protein was not reduced by adiponectin (data not shown).

Several previous studies have reported that adiponectin is the immunomodulatory cytokine for the function of monocytes, macrophages and