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Nicotine can skew the characterization of the macrophage type-1 (MΦ1) phenotype differentiated with granulocyte-macrophage colony-stimulating factor to the MΦ2 phenotype

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ABSTRACT

Macrophages (MΦs) exhibit functional heterogeneity and plasticity in the local microenvironment. Recently, it was reported that MΦs can be divided into proinflammatory MΦs (MΦ1) and anti-inflammatory MΦs (MΦ2) based on their polarized functional properties. Here, we report that nicotine, the major ingredient of cigarette smoke, can modulate the characteristics of MΦ1. Granulocyte-macrophage colony-stimulating factor-driven MΦ1 with nicotine (Ni-MΦ1) showed the phenotypic characteristics of MΦ2. Like MΦ2, Ni-MΦ1 exhibited antigen-uptake activities. Ni-MΦ1 suppressed IL-12, but maintained IL-10 and produced high amounts of MCP-1 upon lipopolysaccharide stimulation compared with MΦ1. Moreover, we observed strong proliferative responses of T cells to lipopolysaccharide-stimulated MΦ1, whereas Ni-MΦ1 reduced T cell proliferation and inhibited IFN-γ production by T cells. These results suggest that nicotine can change the functional characteristics of MΦ and skew the MΦ1 phenotype to MΦ2. We propose that nicotine is a potent regulator that modulates immune responses in microenvironments.

Introduction

Macrophages (MΦs) exhibit many biological functions. The functional heterogeneity and plasticity of MΦs depend on the local microenvironment [1,2]. MΦs have polarized functional properties and can be classified into at least two types, namely classical MΦ (MΦ1) and nonclassical MΦ (MΦ2) [3,4]. MΦ1 are differentiated by granulocyte-macrophage colony-stimulating factor (GM-CSF), have IL-12^{high}IL-10^{low} phenotype, participate in resistance against microorganisms and tumors, and are involved in Th1 immune responses. In contrast, MΦ2 are induced by macrophage colony-forming factor (M-CSF), produce IL-10 but not IL-12, and promote anti-inflammatory responses, tissue remodeling and angiogenesis.

Tobacco smoking is associated with increased incidences of numerous diseases such as cancers, vascular diseases, chronic obstructive pulmonary diseases and periodontal diseases [5–7]. For example, smoking-induced immunosuppression, reduction of natural killer cell cytotoxicity, and inhibition of proinflammatory cytokine production and the microbicidal activity of alveolar macrophages [8,9], have been implicated in the immunopathogenesis of these diseases, although tobacco smoke may also ameliorate inflammation [10–12]. Nicotine is one of the main components of tobacco smoke and a selective agonist of nicotinic acetylcholine receptors (nAChRs). Although the expression of nAChRs was first discovered in the central nervous system, nAChRs are also present in non-neuronal cells. A recent study suggested that acetylcholine produced after vagus nerve stimulation inhibits the release of proinflammatory cytokines from MΦs, and that nAChRα7 is essential for the attenuation of proinflammatory cytokine production [13].

In this study, we hypothesized that nicotine exposure can modulate the differentiation of MΦs. We demonstrate that nicotine promotes monocyte differentiation into IL-12^{low} MΦ1 (Ni-MΦ1) with MΦ2 features. Ni-MΦ1 are associated with reduced allogenic T cell stimulatory capacity and Th1 responses, but generate IL-10-producing T cells. Our findings suggest the possibility that nicotine exposure is involved in the heterogeneity and plasticity of the monocyte-macrophage lineage.

Materials and methods

Isolation of monocytes, and generation of MΦ1 and MΦ2. The protocol for this study was reviewed and approved by the Institutional Review Board of the Osaka University Graduate School of Dentistry. All the subjects participated in the study after providing informed consent. Human monocytes were purified from peripheral blood mononuclear cells (PBMCs) isolated from healthy volunteers by

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standard density gradient centrifugation using Histo-Paque 1077 (Sigma–Aldrich, St. Louis, MO). The cells were further purified by magnetic cell sorting with anti-CD14 microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of the $CD14^+$ monocytes was more than 98%. MΦ1 and MΦ2 were generated in complete RPMI-10 (RPMI-1640 containing 10% heat-inactivated fetal calf serum, 20 mM Hepes, 50 μg/ml gentamicin, 100 U/ml penicillin and 100 μg/ml streptomycin) supplemented with GM-CSF (5 ng/ml) or M-CSF (25 ng/ml) for 5 or 6 days, respectively. In some experiments, MΦ1 and MΦ2 were generated in the presence of nicotine (10^{-3} M). Nicotine was prepared in PBS and neutralized to pH 7.2. $CD4^+$ naive T cells were obtained from PBMC-isolated CD4 T cells using a Human $CD4^+$ T cell isolation kit (Miltenyi Biotec). $CD45RA^+$ cells were isolated from $CD4^+$ T cells using CD45RO Microbeads (Miltenyi Biotec).

Analysis of MΦ1 and MΦ2 surface molecules by flow cytometry. The expressions of surface molecules were evaluated by flow cytometry. Briefly, the cells were incubated with fluorescently labeled monoclonal antibodies at 10 μg/ml or isotype-matched control antibodies for 30 min at 4 °C in the dark. The FITC-conjugated antibodies (anti-CD14 and anti-CD163) and PE-conjugated antibodies (anti-CD1a, anti-CD11b, anti-CD16, and anti-CD206) used were obtained from BD Biosciences (San Jose, CA). The cells were washed twice and data were acquired using a FACSCalibur (BD Biosciences). Analyses of viable cells were performed using the CELLQuest™ software (BD Biosciences).

Assays for antigen-uptake activity. To examine the endocytic activity of MΦ1 and MΦ2, the cells were incubated with 0.1 mg/ml FITC-dextran (Sigma–Aldrich) or Lucifer yellow (Sigma-Aldrich) for 1 h at 4 °C or 37 °C. Cells were washed with PBS and analyzed by flow cytometry.

T cell proliferation assay. MΦs were cultured under the above-described conditions for 24 h, treated with mitomycin (50 μg/ml) for 1 h and cocultured with 1×10^5 allogeneic naive CD4 T^+ cells for 6 days. The cells were pulsed with 0.5 μCi/well of ^{3}H-labeled thymidine (Amersham Pharmacia, Buckinghamshire, UK) for the last 8 h of the 6-day culture period, followed by scintillation counting. The results were calculated as the mean cpm values ± SD obtained from triplicate cultures.

Assays for cytokine and chemokine production. To measure cytokine secretion, monocytes, MΦ1 and MΦ2 were stimulated with 10 ng/ml lipopolysaccharide (LPS; *Salmonella minnesota*; List Biological Laboratories Inc., Campbell, CA) for 24 h, and the supernatants were harvested. The cytokine levels in the supernatants were measured using IL-8, IL-10, IL-12 and MCP-1 ELISA kits (Pierce Endogen, Rockford, IL). Each sample was assayed in triplicate. The supernatants harvested from the above-described allogeneic T cell proliferation assays were measured for their IFN-γ and IL-10 levels. In some experiments, T cells were restimulated with a plate-bound anti-human CD3 antibody (2 μg/ml; BD Biosciences) for 24 h.

Results and discussion

Characteristics of surface markers of MΦ1 and MΦ2 in the presence or absence of nicotine

Monocytes were differentiated into MΦs in the presence of GM-CSF or M-CSF for MΦ1 or MΦ2, and GM-CSF or M-CSF plus nicotine

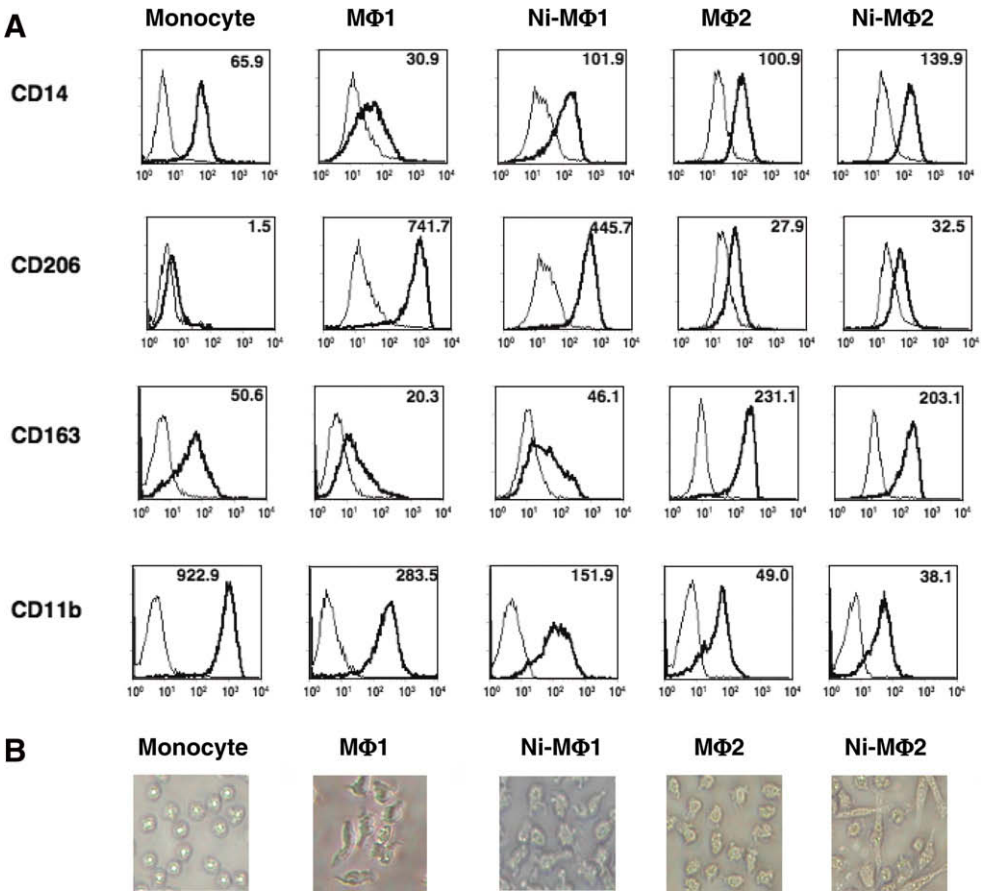

Fig. 1. Characterization of monocytes, MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2. Monocytes were isolated from PBMCs, and MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2 were generated in parallel from the same donor by culture for 6 days. (A) The surface molecule expressions of CD14, CD206, CD163, and CD11b on the cells were determined by flow cytometry (thick lines). The thin lines represent the isotype-matched control antibodies. Data are representative of 3–6 independent experiments. Monocytes were analyzed at day 0. (B) Morphologies of monocytes, MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2. Data are representative of three independent experiments.

for Ni-MΦ1 or Ni-MΦ2, respectively. Following a previously described method for differentiation [14], we reproduced similar characteristics of MΦ1 and MΦ2. MΦ1 and MΦ2 shared the typical macrophage phenotype of $CD1a^-$ (data not shown), $CD11b^+$ and $CD14^+$. MΦ1 expressed consistently lower levels of CD14 and CD163 than MΦ2, but higher levels of CD206 and CD11b (Fig. 1A). Although some review papers have documented that the expression level of CD206 is higher in MΦ2 than in MΦ1 [1,2], it seems to depend on the environmental conditions for differentiation. After GM-CSF-mediated differentiation, MΦ1 express high levels of CD206 [14,15]. In contrast, M-CSF-derived MΦ2 express very low levels of CD206 [14]. CD163, a hemoglobin scavenger receptor, is associated with the nonclassical MΦ2 phenotype [16]. In the presence of nicotine, the phenotype of the surface molecules of MΦ1 (Ni-MΦ1) was somewhat similar to that of MΦ2. Ni-MΦ1 expressed higher levels of CD14 and CD163 than MΦ1, but expressed lower levels of CD206 and CD11b (Fig. 1A). The morphological findings for monocytes, MΦ1, Ni-MΦ1, MΦ2 and Ni-MΦ2 are shown in Fig. 1B. MΦ2 were less adherent, while Ni-MΦ2 exhibited a stretched spindle-like morphology. On the contrary, Ni-MΦ1 were adherent, but had rounder and more irregular shapes than MΦ1.

The findings shown in Fig. 1 suggested that Ni-MΦ1 retained the MΦ1 phenotype but were partially skewed to obtain the characteristics of the MΦ2 phenotype. Therefore, we speculated that nicotine could promote MΦs to obtain MΦ2 properties.

Effects of nicotine on antigen-uptake by Ni-MΦ1

To evaluate the antigen-uptake ability of MΦs, lectin-mediated endocytosis and macropinocytosis were examined using FITC-dextran and Lucifer yellow, respectively. Although Ni-MΦ1 expressed a lower level of CD206 than MΦ1, their uptake of FITC-dextran was more efficient than that of MΦ1 (Fig. 2A). A recent study showed that MΦ1 expressed a higher level of CD206 than MΦ2, but their uptake of FITC-dextran was comparable to that of MΦ2 owing to the involvement of lectin-independent mechanisms such as macropinocytosis [14]. The uptake of Lucifer yellow by Ni-MΦ1 was also more efficient than that of MΦ1 (Fig. 2A). Although the reason why Ni-MΦ1 were able to uptake FITC-dextran is unclear, nicotine may induce presently unidentified molecules involved in receptor-mediated endocytosis. MΦ2 have a higher capacity for Lucifer yellow uptake than MΦ1 [14]. Our data confirm that MΦ2 are active in macropinocytosis-mediated uptake of Lucifer yellow, and that Ni-MΦ1 share a strong phagocytic function with MΦ2. Unlike MΦ1, which have a low capacity for antigen-uptake, Ni-MΦ1 not only had a similar capacity to MΦ2 for macropinocytosis but also had strong characteristics for endocytosis.

The cytokine profile of Ni-MΦ1 differs from that of MΦ1

To investigate the effects of nicotine on cytokine production by MΦs, the cytokine production capacities of LPS-stimulated MΦs were examined. MΦ1 have been reported to produce large amounts of IL-12, whereas IL-10 and MCP-1 are hardly produced [2,3]. Ni-MΦ1 produced significantly lower amounts of IL-12 than MΦ1, but maintained the production of comparable levels of IL-10. Furthermore, Ni-MΦ1 showed a high MCP-1-producing capacity, which is typical of MΦ2. IL-8 was produced at constitutively high levels by all cell types. These data suggest that Ni-MΦ1 may not be as completely polarized as MΦ2, since Ni-MΦ1 were unable to produce IL-10 to the same extent as MΦ2. However, Ni-MΦ1 pro-

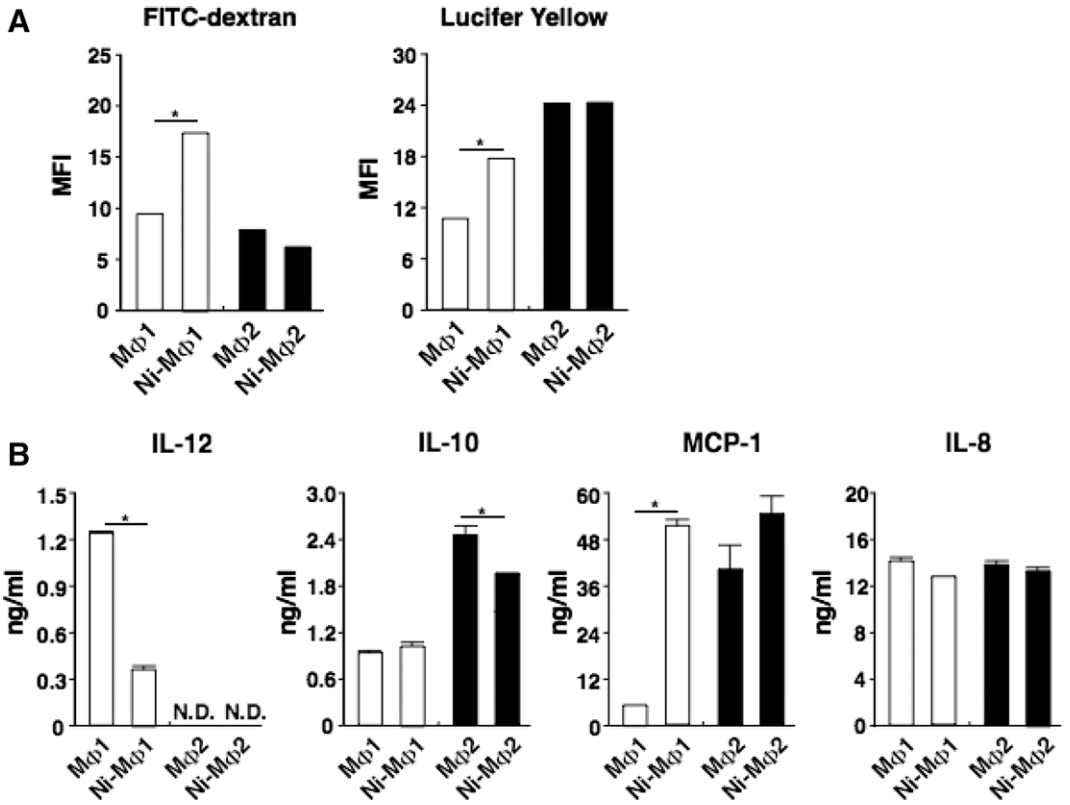

Fig. 2. Effects of nicotine on antigen-uptake and cytokine production by MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2. (A) Uptakes of FITC-dextran (0.1 mg/ml) and Lucifer yellow (0.1 mg/ml) by MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2 after 1 h. The results are shown as the mean fluorescence intensity (MFI) values ± SD obtained from three independent experiments. The MFI values were calculated as the MFI value at 37 °C minus the MFI value at 4 °C. $^*P < 0.05$ compared with MΦs without nicotine. (B) Cytokine productions by MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2 in the presence or absence of LPS. The data are represent the means ± SD from triplicate cultures. The data shown were obtained in one of three or four independent experiments. $^*P < 0.05$ compared with MΦs without nicotine.

duced low amounts of IL-12 and large amounts of MCP-1, suggesting that Ni-MΦ1 share anti-inflammatory properties with MΦ2.

Ni-MΦ1 show hampered T cell stimulatory activities

Next, we examined the induction of T cell proliferation by MΦs. LPS-stimulated MΦ1 induced strong allogeneic T cell proliferation, compared with MΦ2. However, LPS-stimulated Ni-MΦ1 resulted in significantly reduced T cell proliferation (Fig. 3A).

IFN-γ production by activated T cells cocultured with LPS-stimulated Ni-MΦ1 was reduced to almost half the level produced by MΦ1 (Fig. 3B). In contrast, Ni-MΦ1 exhibited reduced IL-10 production in the presence or absence of LPS stimulation, while MΦ1 failed to produce IL-10 (Fig. 3B). MΦ2 and Ni-MΦ2 were unable to induce IFN-γ production, but produced the same levels of IL-10 after LPS stimulation. Taken together, Ni-MΦ1 and MΦ2 had similar characteristics with respect to the reduction of T cell proliferation and induction of IL-10 production.

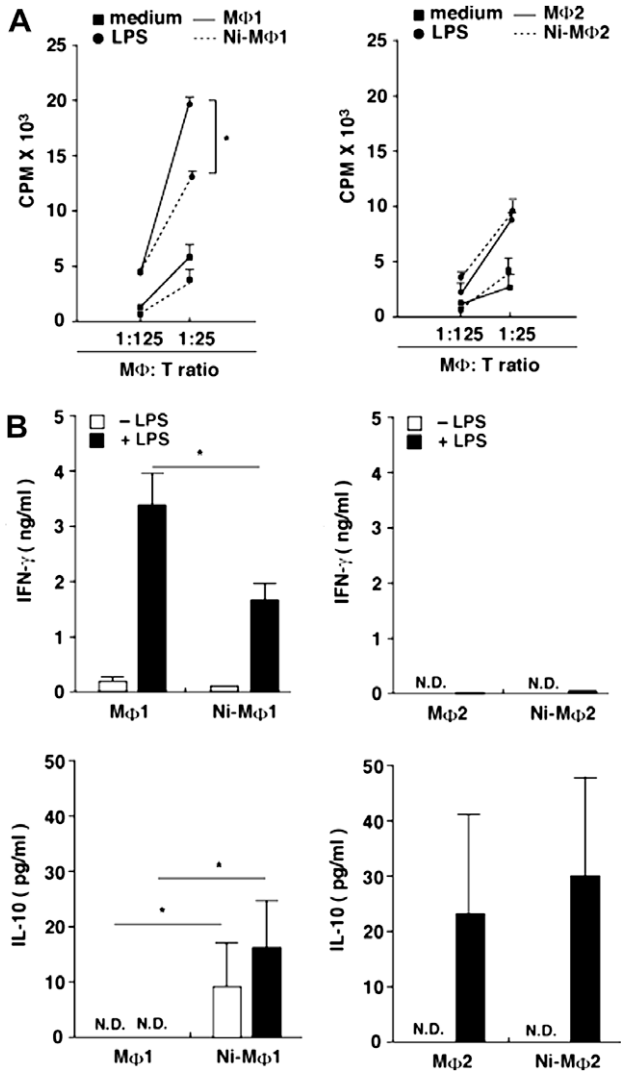

Fig. 3. T cell stimulation capacities of MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2. (A) Comparisons of the effects of MΦ1, Ni-MΦ1, MΦ2, and Ni-MΦ2 on T cell proliferation. The results represent the mean cpm values ± SD obtained from triplicate cultures. The data shown were obtained in one of three independent experiments. $^{*}P < 0.05$ compared with MΦ1 without nicotine. (B) Supernatants obtained from T cell proliferation assays were measured for their IFN-γ and IL-10 levels by ELISA. The results represent the mean values ± SD obtained from triplicate cultures. The data shown were obtained in one of three independent experiments. $^{*}P < 0.05$ compared with MΦ1 without nicotine.

M-CSF-derived MΦs have been reported to induce poor T cell proliferation and T cell anergy [17,18], suggesting that MΦ2 have anti-inflammatory effects and function in the maintenance of peripheral tolerance. We confirmed that Ni-MΦ1 suppressed T cell proliferation, similar to the case for MΦ2, and induced IL-10 production. In the steady-state condition in peripheral blood, GM-CSF, a proinflammatory cytokine, is hardly detected [19] whereas M-CSF is detectable [20]. As an inflammatory condition, ulcerative colitis (UC) is characterized by epithelial barrier disruption and abnormal immune responses, which induce the formation of ulcer-like lesions [21]. In UC patients, nicotine in cigarette smoke may be involved in ameliorating the disease severity, although the mechanisms remain unclear [10]. In the case of periodontal diseases, smokers tend to demonstrate reduced clinical inflammatory signs (bleeding on probing, tissue redness and edema) [11,12]. These findings for both UC and periodontal diseases suggest that cigarette smoke including nicotine can conceal the actual signs of disease severity, although nicotine may also contribute to UC remission. Our present data suggest that nicotine induces MΦs possessing anti-inflammatory and immunosuppressive properties in GM-CSF-dominant inflammatory regions. In this study, we have shown that nicotine modulates MΦ functions. However, the findings do not completely explain the effects of nicotine on human health because several kinds of cells express nAChRs and can respond to nicotine. Further studies are necessary to clarify the effects of nicotine.

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