

Nicotine Protects Dendritic Cells from Apoptosis and Support DCs-dependent CD4⁺ T-cell Priming *in vitro*

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Tao *et al.*: Antiapoptotic Effects of Nicotinic Environment Support DCs-dependent CD4⁺ T-cells Priming

Dendritic cells are antigen-presenting cells that act as messengers between the innate and adaptive immune systems. Their major function is to process foreign material and present it to CD4⁺ T-cells. Previous studies have demonstrated that nicotine suppresses the immune function of dendritic cells. However, the role of nicotine on CD4⁺ T-cells differentiation remains elusive. In this study, the effect of nicotine was examined on the phenotype, activity and apoptosis of murine bone marrow derived dendritic cells, and dendritic cells-dependent differentiation of CD4⁺ T-cells. It was found that nicotine protected dendritic cells from apoptosis, reduced expression of MHC class II molecular on dendritic cells and the phagocytic, but not endocytic, ability. Moreover, nicotine increased surface co-stimulatory signal markers CD40, CD80 and CD86 in lipopolysaccharide-treated dendritic cells. Dendritic cell-mediated induction of CD4⁺ T-cells differentiation was evaluated by flow cytometry and q-PCR. Up-regulation of Foxp3, IL-6, IL-10, and IL-13 expression and down-regulating of INF- γ were observed in dendritic cells/CD4⁺ T-cells co-cultured with nicotine. The nicotine and LPS treatment increased mRNA expression of IL-4, IL-6, IL-13 and decreased INF- γ production and IL-1 β mRNA significantly. In contrast, losing sustained nicotine exposure during CD4⁺ T-cells differentiation, nicotine-pretreated dendritic cells failed to prime native CD4⁺ T-cells to T helper cells (Th1, Th2, or Treg) for adaptive responses. These findings provide new insights into the immunosuppressive properties of nicotine in dendritic cells, and demonstrate that sustained presence of nicotine protected dendritic cells from apoptosis and play an important role in CD4⁺ T-cell stimulation and differentiation.

Key words: Dendritic cells, CD4⁺ T-cells, nicotine, antiapoptosis

Tobacco smoke or nicotine exposure affects innate and adaptive immunity in animals and humans^[1]. Tobacco smoke increases the incidence of a number of respiratory diseases and cancers^[2,3], interestingly, cigarettes smokers have a lower risk of inflammatory enteritis, ulcerative colitis, sarcoidosis^[4] and neurodegenerative diseases, such as Parkinson disease and Alzheimer's disease^[5], suggesting that nicotine has distinct effect on the immune system. Dendritic cells (DCs) are derived from hematopoietic bone marrow progenitor cells. These progenitor cells initially transform into immature DCs in the bone marrow^[6]. Immature DCs constantly sample the surrounding environment for pathogens such as viruses and bacteria via pattern recognition receptors such as the toll-like receptors (TLRs)^[7]. TLRs recognize specific chemical signatures found on subsets of pathogens^[8]. Once DCs have come

into contact with a presentable antigen, they become activated into mature DCs and begin to migrate to the lymph nodes where they activate helper T-cells and killer T-cells, as well as B-cells by presenting them with antigens derived from the pathogen. Additionally, DCs present non-antigen co-stimulatory signals that further initiate and shape the adaptive immune response^[9,10]. Mature DCs stimulate and differentiate native T-cells into T-helper 1 (Th1), T-helper 2 (Th2), T-helper 17 (Th17), and regulatory T-cells (Treg)^[11,12].

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The role of nicotine exposure on DC function remains controversial^[13,14]. Some studies suggest that nicotine strongly suppresses DC-mediated adaptive immune responses^[15-17]. However, Vassallo *et al.*^[18] reported that cigarette smoke extract, but not nicotine, inhibited DC-mediated adaptive immune responses. Aicher *et al.*^[19] showed that nicotine activated DCs and augmented their capacity to stimulate T-cell proliferation and cytokine secretion. The inconsistency in reports may be attributed to limitations of DC isolation techniques and cell culture environment. Nicotine participates a large number of signaling pathways promoting cell proliferation, migration and invasion, and inhibited cell apoptosis^[20-24]. Not many studies were reported about the antiapoptotic effect of nicotine in DCs. Here, mouse bone marrow-derived DCs were used to systemically examine the impact of nicotine on the activation and apoptosis of DCs, and subsequent CD4⁺ T-cell differentiation to Th1, Th2 and Treg cells.

MATERIALS AND METHODS

The complete culture medium used was RPMI 1640 basic (1×) containing 1 % L-glutamine, 25 µM HEPES, and 1 % penicillin/streptomycin. All components were obtained from GIBCO (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Kybio (Tianjin, China). Complete culture media containing 10 % FBS (R-10) was used to culture DCs. Recombinant murine GM-CSF, IL-4, and recombinant TGF-β1 were obtained from Peprotech (Rocky Hill, NJ). TRIzol came from Invitrogen Life Technologies (Carlsbad, CA). RevertAid First Strand cDNA synthesis kit, Maxima SYBR Green/ROX qPCR Master Mix (2×) were purchased from Molecular Probes (Eugene, OR). (-)-Nicotine, LPS, and FITC-dextran were purchased from Sigma-Aldrich (St. Louis, MI). FITC-OVA was obtained from Solarbio (Beijing, China). CytoTox96 nonradioactive cytotoxicity assay was obtained from Promega (Madison, WI). Bradford protein assay kit and caspase 3 activity assay kit were bought from Beyotime (Shanghai, China). EasySep™ Murine CD4⁺ T-cell isolation kit, recombinant murine IL-2, and cell detachment solution were obtained from StemCell Technologies (Vancouver, Canada). Brefeldin A, antimurine CD3e and monoclonal antibodies (mAbs) including CD40 (HM40-3), CD86 (GL1), CD80 (16-X10A1), CD197 (4B12), MHC-II (1-A/I-E), CD4 (RM4-5), CD25 (PC61.5), Foxp3 (FJK-16s) and IFN-γ (XMG1.2) were purchased from eBioscience (San Diego, CA). Antimurine CD11c was purchased from

Biolegend (San Diego, CA). Red cell lysis buffer, with main composition of ammonia chloride, was obtained from Biosharp (BL503A, Shanghai, China).

Experimental animals:

Specific pathogen-free adult C57BL/6 mice were purchased from the Changzhou Cavion Experimental Animal Co, Ltd. (license number SCXY (Su) 2011-0003). Mice were housed in a vivarium maintained on a standard 12 h light-dark cycle (lights on at 07:00 AM) with constant temperature and humidity (22° and 50 %, respectively) and *ad libitum* access to food and water. All procedures were conducted in accordance with the guidelines as described in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH publication no. 8023, revised 1978) and were approved by the Institutional Animal Care and Use Committee at Anhui University of Science and Technology (#201718).

Preparation and immunophenotyping of mBMDCs:

Femurs were collected after C57BL/6 mouse was euthanized. The precursor DCs were derived from the bone marrow, which was flushed out with phosphate buffered saline (PBS) using a syringe with a 0.50×20 mm needle. Clusters within the marrow suspension were dissociated by vigorously pipetting and filtered through a 70-µm cell strainer. Red cells were depleted using 1 ml red cell lysis buffer for 2 min at room temperature. Cells were washed with PBS. Then, the cells were cultured in R-10 and plated on 100 mm dishes at 37° in 5 % humidified CO₂. R-10 supplemented with 20 ng/ml of recombinant murine GM-CSF and 10 ng/ml of recombinant murine IL-4 was used to culture primary immature DCs, and 10 ml culture medium were added 3 or 4 d later. DCs were harvested after 7-9 d when they grew to a high confluence. DCs derived from murine bone marrow (mBMDCs) were identified with CD11c^[25]. Cell surface molecules (CD11c, CD40, CD86, CD80, CD197, MHC-II) were determined by flow cytometry.

DCs antigen uptake:

The DCs were pre-treated with 100 µg/ml nicotine for 24 h (mImNicDCs). mBMDCs or mImNicDCs (5×10⁴) were suspended in a 2 ml R-10 containing FITC-dextran (250 µg/ml) or FITC-OVA (25 µg/ml). After 30 min incubation at 37° or 4° (negative control), cells were washed three times with cold staining medium (PBS containing 0.09 % NaN₃ and 1 % FBS) and analysed by flow cytometry. Images were captured

with a Leica DFC7000T microscope and analysed by LASX 3.0.2.16120 imaging software.

Caspase-3 (CCP-3) activity assay:

Total proteins and cleaved CCP-3 were identified by using Bradford protein assay kit and caspase 3 activity assay kit, respectively. mBMDCs were plated on 12-wells and stimulated with various concentration of nicotine (25, 50, 100 µg/ml) for 72 h without or with LPS (1 µg/ml). After stimulation, cells were washed three times in 1 ml of cold physiological saline, lysed in 50 µl lysis buffer for 15 min on ice. The supernatants were then obtained after centrifuged at high speed (20 000×g) for 15 min. Assays of CCP-3 were performed on 96-well microtitre plates by incubating 25 µl protein of cell lysate per sample in 25 µl reaction buffer containing 5 µl CCP-3 substrate (Ac-DEVD-pNA; 2 mM). Lysates were incubated at 37° for 2 h. Samples were measured with an ELISA reader (ELx800) at an absorbance of 405 nm. The detail analysis procedure was followed by the manufacturer's protocol. The unit of enzyme CCP-3 in a sample was calculated according to the formula: (CCP-3: protein) × 100 %.

***In vitro* T-cell purification and differentiation:**

Spleens from C57BL/6 mice were removed aseptically and kept on ice. Single cell suspensions were obtained by grinding the tissue between two frosted glass slides and filtered through a 70-µm cell strainer. Red cells were removed by resuspending the cell pellet in 1 ml red cell lysis buffer for 2 min and cell pellets were collected by centrifugation. CD4⁺ T-cells were purified using EasySep™ Murine CD4⁺ T-cell isolation kit according to the manufacturer's protocol. The purity of the enriched CD4⁺ T-cell population was >86 %. mImNicDCs and LPS-stimulated mImNicDCs were developed by incubating mBMDCs with nicotine (100 µg/ml) or a combination of nicotine (100 µg/ml) and LPS (1 µg/ml) for 24 h. DCs were co-cultured at a ratio of 1:10 with native CD4⁺ T-cells for 3 d. Differentiation of CD4⁺ T-cells into Th1 and Treg was induced by LPS (1 µg/ml) or TGF-β1 (5 ng/ml, positive control)^[26]. Antimurine CD3e (1 µg/ml) and IL-2 (20 U/ml) were used for regulatory T cells induction and maintenance. Cytokines were analysed by flow cytometry and Q-PCR.

Quantitative real-time PCR (Q-PCR):

Total mRNA was extracted with TRIzol reagent and

stored at -80° until use. Total mRNA (100 ng) was used for cDNA synthesis in a 20 µl reaction volume containing oligo (dT)₁₈ primer (100 µmol), 20 U of RiboLock RNase inhibitor, 200 U of RevertAid M-MuLV RT, and 10 mM dNTP Mix (RevertAid first strand cDNA synthesis kit) according to the manufacturer's instructions. Q-PCR reactions were performed in a Bio-Rad iQ5 real-time thermo cycler using Maxima SYBR Green/ROX qPCR Master Mix (2×). Q-PCR reactions were set up in triplicate for each cytokine using murine GAPDH gene as a reference for normalization. The qPCR reaction volume was kept at 25 µl with 1 µl of cDNA, and reaction condition was initial 95° for 10 min, 95° for 10 s, 58° for 1 min (40 cycles) for amplification. Primers used to amplify murine INF-γ, Foxp3, IL-13, IL-4, IL-10, IL-6, IL-12p40, IL-1β, and GAPDH genes are included in Table 1. The relative abundance of transcripts for each gene was calculated according to the comparative 2^{-ΔΔCT} method.

Flow cytometry:

Cells were washed with cold staining medium three times to remove reagent and incubated for 15 min on ice with CD16/CD32 antibodies at a concentration of 1 µg per test for blockade of FC receptors. Cells were then treated with surface molecules mAbs for 1 h on ice without light. To detect Foxp3 and IFN-γ expression, cells were stained with antiCD4 and antiCD25 mAbs and incubated overnight at 4° in a dark box in fixation/permeabilization buffer. PE-conjugated Foxp3 mAbs and PerCP-Cy5.5-conjugated IFN-γ mAbs were used to detect Foxp3 and IFN-γ expression in cells, respectively. Brefeldin A (3 µg/ml) was added to examine IFN-γ levels 5 h before staining. Flow cytometry was done with FACSCalibur (BD, America) and data were analyzed with CellQuest software. The effect of nicotine on the endocytic and phagocytic activity of DCs was examined by adding FITC-dextran (250 µg/ml) or FITC-OVA (25 µg/ml) for 30 min.

Statistical analysis:

Data were expressed as mean±SEM. Statistical differences were determined by analysis of variance (ANOVA), followed by post hoc multi comparisons. p<0.05 was considered statistically significant.

RESULTS AND DISSICUSSION

Nicotine reduced the antigen-presenting ability of DCs and subsequent stimulation of allogeneic T-cells.

Flow cytometry and fluorescence microscopy were used to analyse the effect of nicotine (100 µg/ml) on endocytosis and phagocytosis. Nicotine at 100 µg/ml was not cytotoxic to DCs and CD4⁺ T-cells as tested by the lactate dehydrogenase-based cytotoxicity assay. There was no significant difference between the endocytic abilities of nicotine-treated and vehicle-treated DCs (fig. 1A). However, the phagocytic activity

of DCs was dramatically inhibited by nicotine (roughly two-fold increase; fig. 1B). Nicotine-treated mBMDCs also presented a lower amount of FITC staining (fig. 1C).

Nicotine exposure may alter the phenotype of DCs without or with LPS by changing the expression of stimulatory cell surface markers. To test this hypothesis, the DCs were set into 4 groups, R-10 medium

TABLE 1: CYTOKINE PRIMERS

Primer	Gene	Primer Orientation	Sequence (5'-3')	Region	Amplicon size (bp)
1	IFN-γ	F	AAATCCTGCAGAGCCAGATTAT	206-315	110
		R	GCTGTTGCTGAAGAAGGTAGTA		
2	Foxp3	F	AGAAGCTGGGAGCTATGCAG	807-906	108
		R	GCTACGATGCAGCAAGAGC		
3	IL-13	F	TTGCTTGCTTGGTGGTCTC	100-327	228
		R	TCTGGGTCCTGTAGATGGCA		
4	IL-4	F	AACGAGGTCACAGGAGAAGG	171-357	187
		R	TCTGCAGCTCCATGAGAACA		
5	IL-10	F	CCA AGC CTT ATC GGA AAT GA	315-473	159
		R	AGG GGA GAA ATC GAT GAC AG		
6	IL-6	F	TACCACTTCACAAGTCGGAGGC	169-284	116
		R	CTGCAAGTGCATCATCGTTGTTC		
7	IL-12p40	F	TTGAACTGGCGTTGGAAGCACG	720-851	132
		R	CCACCTGTGAGTTCTTCAAAGGC		
8	IL-18	F	TGG ACC TTC CAG GAT GAG GAC A	894-1041	148
		R	GTT CAT CTC GGA GCC TGT AGT G		
9	GAPDH	F	CACTGAGCATCTCCCTCACA	1707-1817	111
		R	GTGGGTGCAGCGAACTTTAT		

Primers are used in Q-PCR analysis. F stands for forward primer. R stands for reverse primer

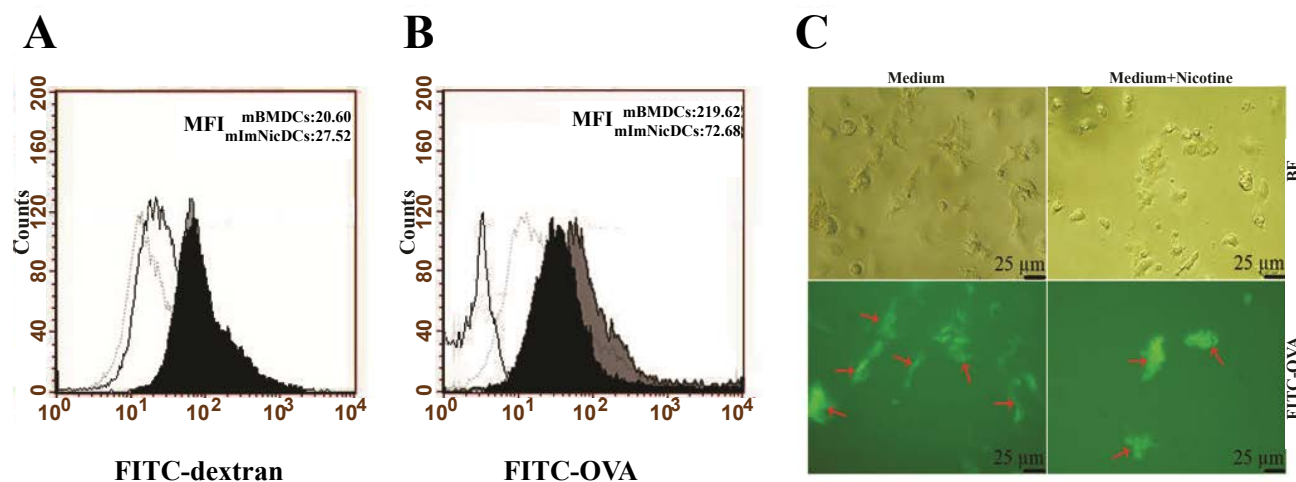


Fig. 1: Effect of nicotine on antigen uptake by mouse bone marrow-derived DC
Murine dendritic cells in the absence (mBMDCs) or presence of nicotine (100 µg/ml, mImNicDCs) were incubated with FITC-dextran (250 µg/ml) or FITC-OVA (25 µg/ml) for 30 min. (A) endocytic and (B) phagocytic activity were quantified by flow cytometry. Dashed or solid lines represent staining at 4° of mBMDCs or mImNicDCs as the negative control, whereas grey or black histograms represent staining at 37° of mBMDCs or mImNicDCs, respectively. The median fluorescence intensity (MFI) at 37° is shown after subtracting the 4° MFI value. (C) mBMDCs and mImNicDCs were incubated with 25 µg/ml FITC-OVA for 30 min and analyzed by fluorescence microscopy using bright field light (BF, upper panels) or FITC (low panels) at 37° and 5 % CO₂. Data are representative of 3 experiments. Scale bar: 25 µm

(mBMDCs), 100 µg/ml nicotine (mImNicDCs), 1 µg/ml LPS (LPS-mBMDCs), 1 µg/ml LPS with 100 µg/ml nicotine (LPS-mImNicDCs), all groups were treated for 24 h. Flow cytometry results showed that CD11c positive cells went up to 84.39 % of the total (fig. 2A). mImNicDCs and LPS-mImNicDCs presented more branched projections compared with DCs and LPS-mBMDCs respectively (fig. 2B). The median fluorescence intensity of mImNicDCs have higher levels of CD40 and CD197, and lower levels of CD86 and MHC-II, but CD80 expression was not significantly difference between nicotine-treated and vehicle-treated groups (Table 2). LPS-mImNicDCs lead to an increase in expression of the co-stimulatory molecules CD40, CD86, and CD80, and a decreased expression of CD197 and MHC-II compared to LPS-mBMDCs group (Table 2).

To explore the exact effect of nicotine on the apoptosis of DCs, mBMDCs were treated with nicotine (25, 50, 100 µg/ml) for 72 h. In fig. 3A, cleaved CCP-3 level was significantly inhibited by nicotine ranging from 25 to 100 µg/ml compared with that in the blank groups ($p < 0.01$). Nicotine treatment at 100 µg/ml showed an increase of CCP3 level relative to 50 µg/ml nicotine group, but lower level of CCP3 when compared to the control. To test whether nicotine influences LPS-induced apoptosis, LPS (1 µg/ml) combined with various concentration of nicotine (25, 50, 100 µg/ml) were tested in DC culture for 72 h. LPS-mImNicDCs showed significant reduction of CCP-3 release at 25, 50, 100 µg/ml compared with that in the LPS groups, as shown in fig. 3B. Taken together, this revealed that nicotine treatment had antiapoptotic effects in mBMDCs.

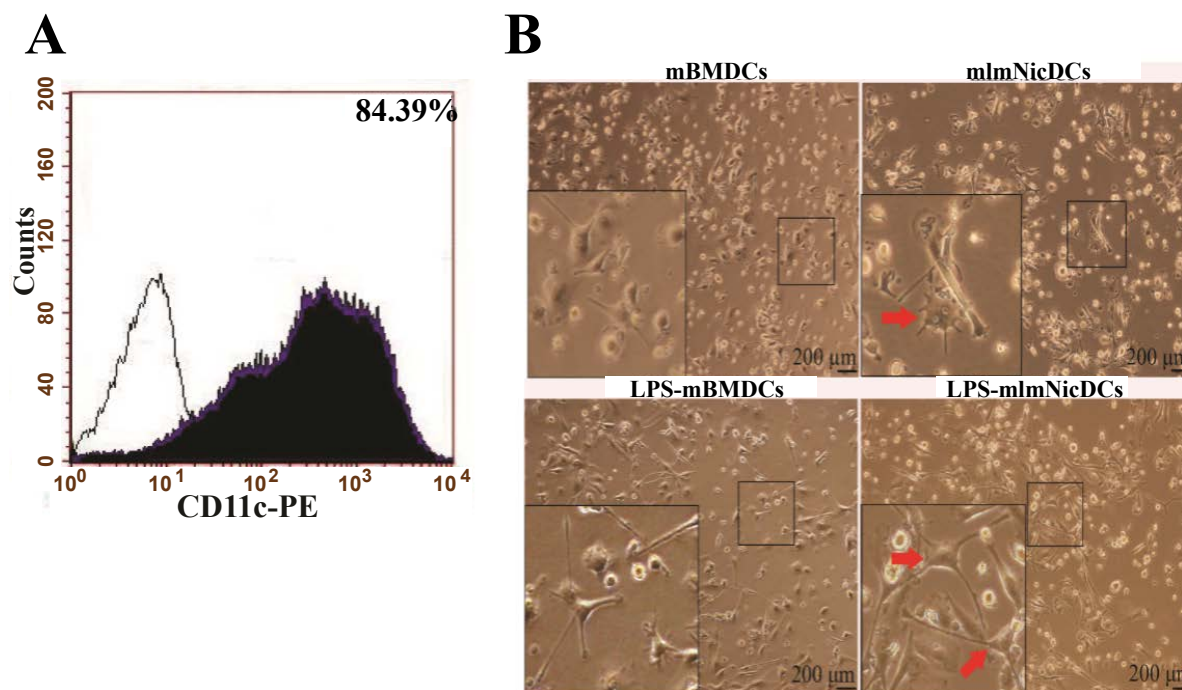


Fig. 2: Morphology of mBMDCs and LPS-mBMDCs without (-) or with (+) nicotine
 (A) primary mBMDCs were identified by CD11c with flow cytometry. Cells were treated without or with 100 µg/ml nicotine (mBMDCs versus mImNicDCs), 1 µg/ml LPS (LPS-mBMDCs), 1 µg/ml LPS and 100 µg/ml nicotine (LPS-mImNicDCs) for 24 h. (B) the morphology was observed by phase contrast microscopy. Data represent 3 independent experiments. Scale bar: 200 µm

TABLE 2: FLOW CYTOMETRY ANALYSIS OF SURFACE MARKERS ON NICOTINE OR NICOTINE/LPS-TREATED MURINE DENDRITIC CELLS

Surface markers	mBMDCs	mImNicDCs	T value	N	LPS-mBMDCs	LPS-mImNicDCs	T value	N
CD40	152.45±7.68	296.69±15.08*	-14.762	3	1632.81±58.88	2444.60±484.16*	-2.883	3
CD86	74.70±5.37	33.40±2.84*	11.784	3	74.66±4.90	130.41±6.90*	-11.412	3
CD80	110.01±4.33	103.10±8.22	1.289	4	213.92±15.15	412.22±11.46*	-18.080	3
CD197	13.61±1.40	21.77±3.17*	-4.079	3	20.73±1.45	14.54±1.20*	5.696	3
MHC-II	902.9±18.09	432.23±11.58*	37.985	3	1329.14±64.54	969.44±27.16*	8.897	3

Flow cytometry analysis was performed to identify surface marker on murine dendritic cells. MFI is median fluorescence intensity. N is number of experiments. Data are representative of three or four experiments. Significant p value (* $p < 0.05$) were obtained by Student's t test analysis. Values were mean±SEM

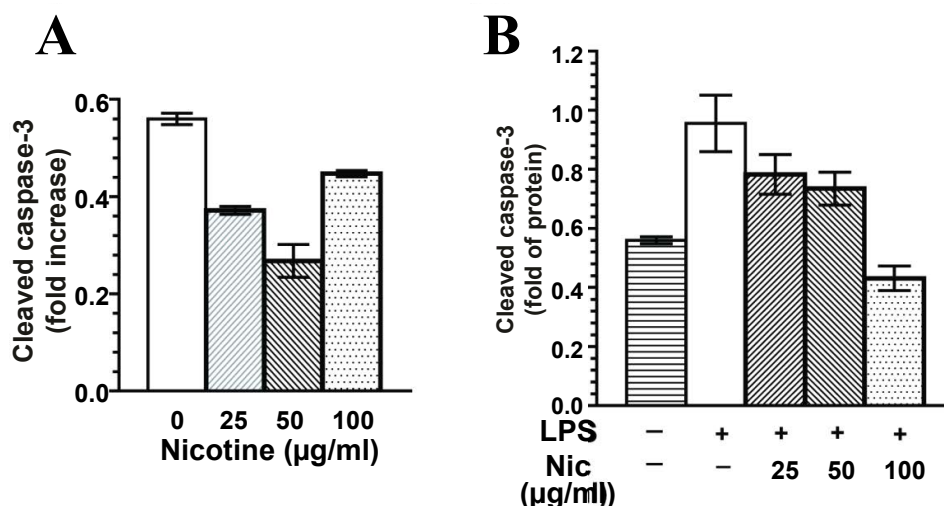


Fig. 3: Suppressive effect of nicotine on mBMDcs apoptosis and LPS-stimulated mBMDcs apoptosis

(A) mBMDcs were treated with various concentration of nicotine (25, 50, 100 µg/ml) for 72 h and (B) were administrated with LPS (1 µg/ml) and various concentration of nicotine (25, 50, 100 µg/ml) for 72 h. CCP-3 Level was measured. The data are presented as mean±SEM, n=3 per group

To investigate the effect of nicotine on DCs-induced CD4⁺ T-cell priming, mBMDcs were co-cultured with CD4⁺ T-cells at a DC/T-cell ratio of 1:10 for 3 d in the presence of nicotine (100 µg/ml), as shown in fig. 4A. Nicotine alone increased Foxp3, a Treg associated marker, protein (1.24-fold) and mRNA levels (1.85-fold). mRNA levels of Th2 associated markers IL-10 (2.34-fold), IL-6 (1.59-fold), and IL-13 (3.06-fold) were increased as well. Additionally, nicotine treatment decreased protein (0.52-fold) and mRNA (0.58-fold) levels of Th1 marker IFN-γ. Similarly, nicotine treatment decreased mRNA levels of IL-1β (0.59-fold) compared to control cells. Treatment with TGF-β1 was used as a positive control and increased expression of Foxp3 (1.25-fold in protein level, 2.17-fold in mRNA level) compared with the control (p<0.05). There was no statistical difference in the IL-4 expression in mRNA levels (p>0.05; fig. 4B).

Nicotine may affect LPS-mediated DCs activation of CD4⁺ T-cells. To test this, mBMDcs were treated with LPS (1 µg/ml) or nicotine (100 µg/ml)/LPS and analysed by flow cytometry and qPCR. Compared to LPS treatment alone, the combination of nicotine and LPS reduced mRNA levels of Th1-associated markers IFN-γ (0.46-fold) and IL-1β (0.68-fold) and increased mRNA levels of Th2-associated markers IL-6 (2.11-fold), and IL-4 mRNA (2.57-fold). There was no statistical difference in IL-10 and Foxp3 mRNA levels or in IFN-γ and Foxp3 protein expression between treatment groups (fig. 4B). LPS, as positive control, increased IFN-γ protein (1.76-fold) and mRNA (1.64-fold) expression (fig. 4B) compared to control (p<0.01).

Native CD4⁺ T-cells have more than 3 different fates, Th1, Th2, and Tregs. The chosen path is determined primarily by signals and antigens presented by DCs in addition to the local microenvironment. To the extent that nicotine-treated DCs may be used in vaccines in the future, we examined how DCs affect CD4⁺ T-cell differentiation without nicotine in the culture medium (fig. 5A). Nicotine or nicotine/LPS-treated DCs were co-cultured with CD4⁺ T-cells at a DC/T-cell ratio of 1:10 for 3 d in the absence of nicotine. LPS, as positive control, induced CD4⁺ T-cells into Th1 cells with increased IFN-γ protein (1.85-fold) and mRNA (1.42-fold) expression compared with the control group (fig. 5B). However, mRNA expression levels, or protein of Foxp3 and IFN-γ didn't show statistical difference (p>0.05) between nicotine group and LPS group (fig. 5B). Additionally, IL-13 and IL-12p40 mRNA levels were not significantly different between groups either (p>0.05). TGF-β1, as positive control, increased Foxp3 mRNA expression 3.2-fold compared with the control (p<0.01, fig. 5B), and 1.28-fold in protein levels (p<0.05, fig. 5B).

Nicotine has antiinflammatory and neuron-protective effects^[27-29]. In this study, it was confirmed that nicotine altered the functional activity of DCs. Moreover, nicotine impaired the phagocytic, but not endocytic activity of DCs. Nicotine increased the expression of co-stimulatory molecules on the membrane of DCs, protects DCs from apoptosis, leading to an increase in T-cell activation and differentiation.

DCs are the antigen processing cells (APCs) of the mammalian immune system. Their main function

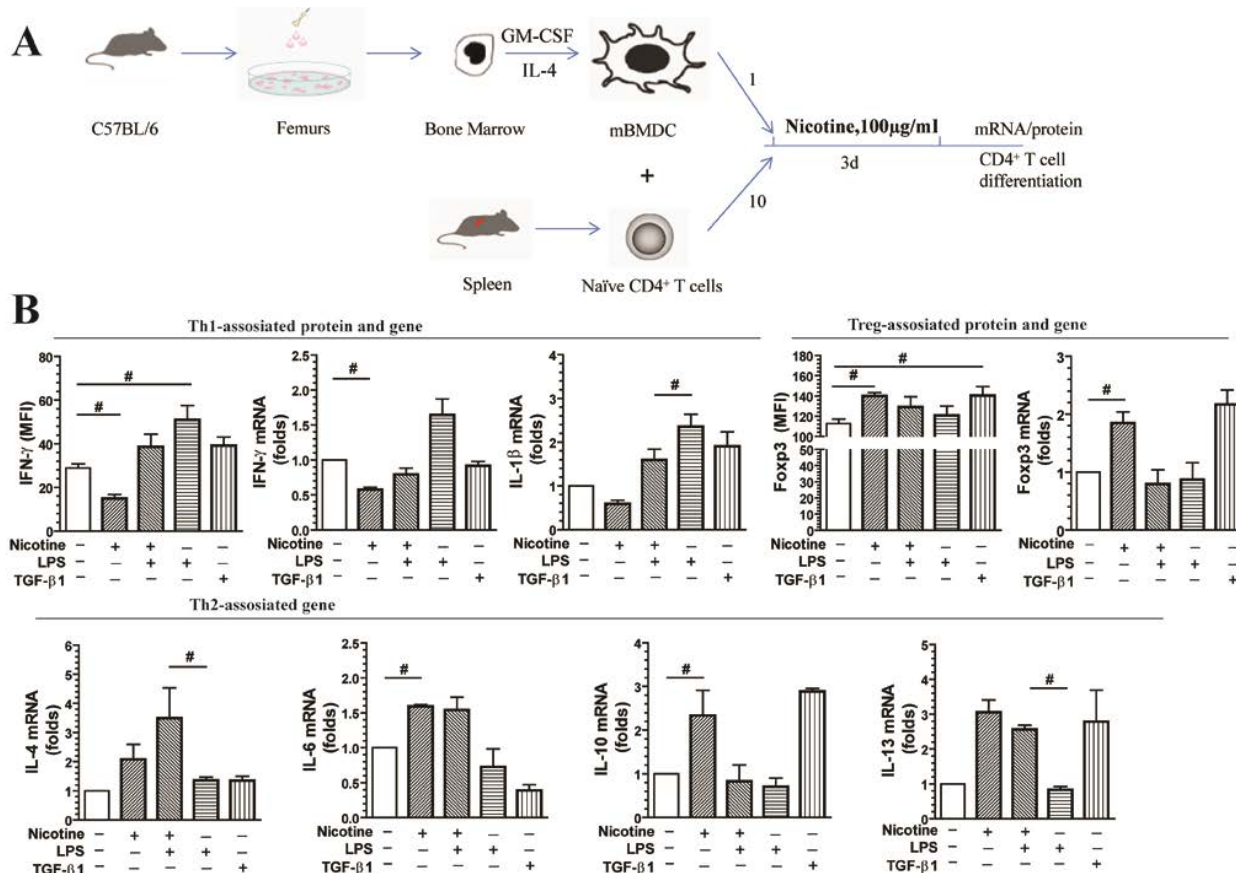


Fig. 4: Effect of nicotine or nicotine/LPS-treated mBMDCs on CD4⁺ T-cells priming in the presence of nicotine
 (A) mBMDCs were obtained from femurs of C57BL/6 mouse, and native CD4⁺ T-cells were purified from mouse spleen. mBMDCs were co-cultured with CD4⁺ T-cells at a DC/T-cell rate of 1:10 for 3 d. (B) mBMDCs and CD4⁺ T-cells were treated with 100 µg/ml nicotine without or with LPS (1 µg/ml). The expression levels of Th1-associated genes or protein (IFN-γ, IL-1β), Th2-associated genes (IL-4, IL-6, IL-10, IL-13), Treg-associated genes and protein (Foxp3) were determined via flow cytometry or q-PCR analysis. For flow cytometry, bars show the MFI value of indicated markers, which are subtracted by MFI values of isotype control. The data are presented as mean±SEM, n=3 or 4 per group, *p<0.05. For q-PCR, bars show the fold changes in mRNA expression levels. The data are presented mean±SEM, n=3 per group, *p<0.05. This figure is representative of 3 similar experiments performed on different mice

is to process foreign antigen material and present to T-cells. They act as messengers between the innate and the adaptive immune systems^[30]. Nicotine reduced the antigen-presenting ability to stimulate T-cells. It was observed that nicotine-treated DCs reduced antigen uptake and MHC-II molecular, which failed to efficiently induce type 1 interferon (IFN)-γ-producing T-cell polarization in DC/T-cell co-culture, whereas nicotine-treated DCs decreased CD86 expression while CD80 is not changed significantly. It also suggested that a decrease in CD86 expression, not CD80 expression on nicotine-treated DC was relative to nicotine-induced Treg differentiation.

DCs play a critical role in the development of a competent immune system by recognizing, processing, and presenting antigens to T-cells. DCs acquire exogenous antigens/pathogens by a variety of different endocytic processes including endocytosis and

phagocytosis. Endocytosis requires antigen binding to a variety of receptors leading to internalization of the typically small foreign molecule; however, phagocytosis mediates the internalization of a wide-variety of relatively large insoluble particulate antigens originating from necrotic/apoptotic cells and opsonized pathogenic organisms/viruses^[31]. Data obtained in this investigation revealed that nicotine did not affect the endocytic abilities but did impair the phagocytic activity of DCs. This is consistent with the published data from Ween *et al.*^[32], who showed that nicotine reduced phagocytosis of macrophages derived from THP-1 by affecting phagocytic recognition molecules. Considering that DCs are the most powerful APCs, its endocytic and phagocytic processes are principally responsible for uptake and presentation of pathogenic and pathogen-associated antigens. Dysregulation of these processes can be detrimental to the host defense^[30,31].

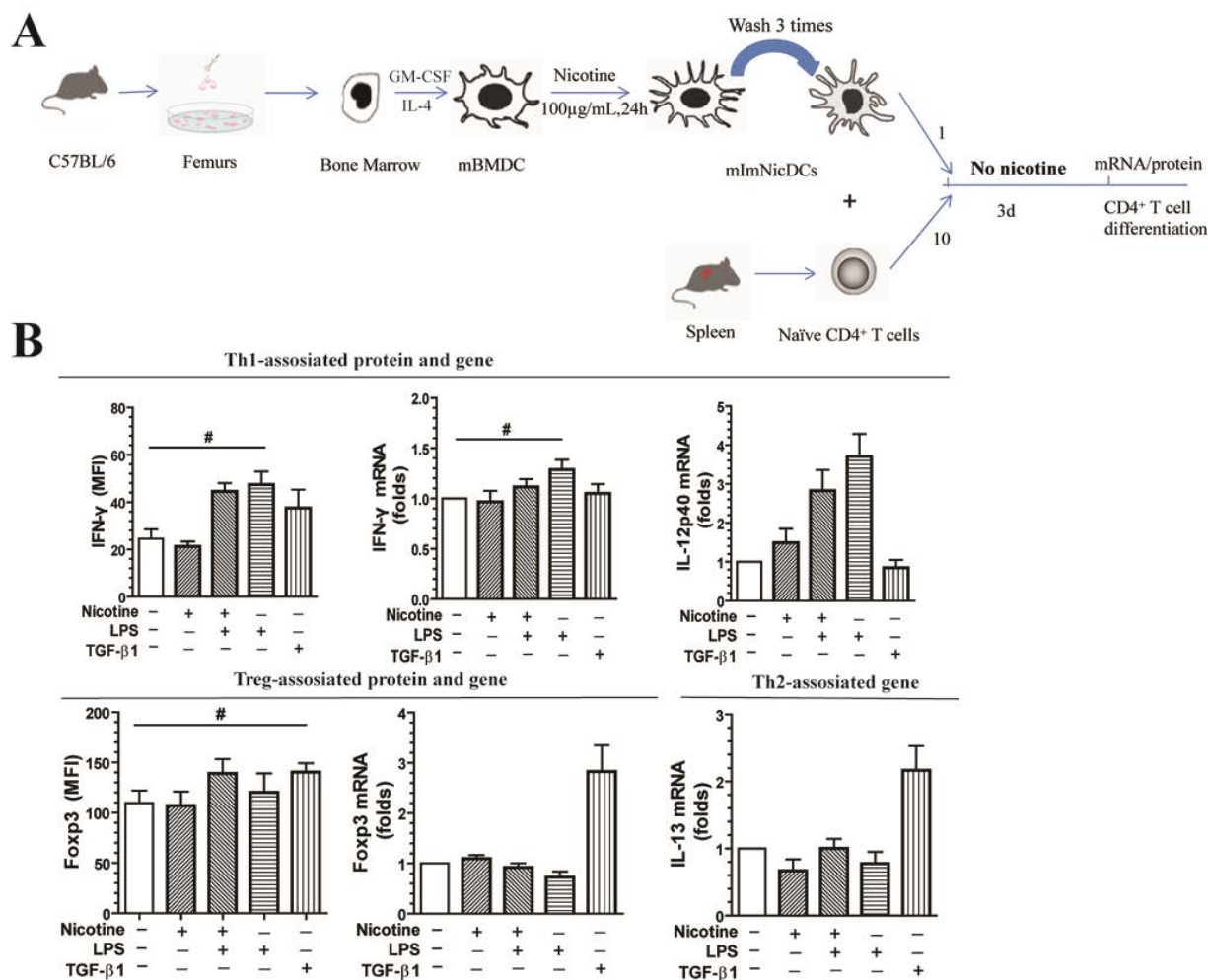


Fig. 5: Effect of nicotine or nicotine/LPS-treated mBMDCs on CD4⁺ T-cells priming in the absence of nicotine
 mBMDCs were treated with 100 µg/ml nicotine without or with LPS (1 µg/ml) for 24 h, then washed with 1×PBS 3 times to remove reagent. CD4⁺T-cells and DCs co-culture experiment was shown in a. A DC/T-cells rate of 1:10 was performed for 3 d. (b). The expression levels of Th1-associated genes or protein (IFN-γ, IL-12p40), Th2-associated gene (IL-13), Treg-associated gene and protein (Foxp3) were determined *via* flow cytometry or q-PCR analysis. For flow cytometry, bars show the MFI of indicated markers. For q-PCR, bars show the fold changes in mRNA expression levels. The data are presented as mean±SEM, n=3 per group, #p<0.05. Data are representative of 3 experiments

Nicotine increased the expression of co-stimulatory molecules on the surface of DCs as well as increases cell priming and polarization^[19,33] in addition to affecting DC-mediated immune responses^[15]. Previous studies showed that nicotine enhanced DC maturation, reduced antigen uptake, and suppressed DC-mediated T-cell priming^[15,34]. Present data revealed that nicotine-treated DCs did not alter CD80 levels, but showed increased expression of CD40 and CD197. Meanwhile, nicotine-treated DCs have a decreased expression of CD86 and MHC-II.

LPS can function as a microbial endotoxin deriving utility as a maturation stimulus for DCs and an activator of toll-like 4 receptor (TLR4) signaling. LPS treatment increased expression of co-stimulatory molecules CD80 (B7-1), CD86 (B7-2), CD40, and CD11c on the

DC membrane^[33,35] and induce DC-dependent priming of T-cells^[36,37]. Current data indicated that LPS-treated DCs increased the expression of CD40, CD86, and CD80. However Nouri-Shirazi *et al.*^[15] reported that nicotine (100 µg/ml) did not affect DCs maturation. Differences in the isolation, preparation, and treatment may account for these discrepancies. The concentrate of nicotine used in this work was chosen according to previous studies^[12,15], and correlated with nicotine levels achievable in local tissues such as the oral cavity and the lungs, where tissue resident DCs encounter antigens^[38].

Some reports showed that nicotine decreased cytotoxicity after oxidative stress occurred and have protective effect in primary cultured cortical and cerebellar granule cells through antiapoptotic effect^[39-41].

In this study, it was found that nicotine protected DCs from apoptosis in the absence or presence of LPS. Studies showed that nicotine bidirectionally regulated the proliferation of human alveolar bone marrow-derived mesenchymal stem cells^[42], mouse embryonic stem cells^[43], mouse hippocampal neuronal and glia cells^[44]. The present study showed that antiapoptosis by nicotine in DCs might play an important role in T-cell priming, in addition to surface molecular alternation in DCs. Several signalling pathways regulating nicotine-mediated antiapoptosis in different cells, such as Wnt/beta-catenin^[45], Notch^[12], PI3K/AKT, JAK/STAT, Rb/E2F1^[46]. Akt play vital role in nicotine-increased surface molecules expression in human peripheral blood mononuclear cell-derived DCs, whereas ribosomal protein S6 was involved in regulating growth and proliferation of cells^[47].

Nicotine-treated DCs had been reported to have potential antitumor and antiviral effects^[48-50] and a DC-based vaccine has been investigated in a clinical trial^[51,52]. Moreover, nicotine treated-DCs have been reported to mediate cytotoxic T lymphocyte priming^[53]; however, the role of nicotine stimulated-DC in CD4⁺ T-cell priming and the adaptive immune response had yet to be explored.

Guinet *et al.*^[17] found nicotinic environment affects the differentiation and functional maturation of monocytes derived DCs. Nicotine-treated DC expressed low level of antigen-presenting molecule MHCs and chemotactic cytokine receptor CCR7 in respond to bacterial antigen LPS, which finally decreased Th1 differentiation in DC/T-cells co-culture^[17]. When nicotine-pretreated DCs were cultured with CD4⁺ T-cell in the presence or absence of nicotine, it was surprising to find that neither nicotine-treated DCs or LPS and nicotine-treated DCs were able to prime CD4⁺ T-cells differentiate into Th1, Th2 or Treg cells in the absence of nicotine treatment, whereas CD4⁺ T-cells reduced Th1 differentiation and increase Treg and Th2 differentiation after co-cultured with DCs in the presence of nicotine. This suggested that direct impact of nicotine on CD4⁺ T-cells differentiation play a more important role than nicotine-pretreated DC. Indeed, Kawashima *et al.*^[54] reported that nicotinic acetylcholine receptor (nAChR) was highly expressed in T-cells and B-cells and these receptors may induce nicotine response, but it needs further study in our future. Considering the small size and lipophilicity properties of nicotine, it can passively move across cell membranes; however, its primary effects are receptor mediated^[55].

Nicotine produced profound effects on DCs and DC-mediated immune responses. In this study, the effect of nicotine on DCs, on CD4⁺ T-cell differentiation with or without LPS was examined. DCs express various nAChR subunits, including $\alpha 2$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 10$ and $\beta 2$ subunits. Further studies are necessary to identify the role of individual nACh receptor and signalling pathway in nicotine-mediated immunity response.

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Conflict of interest:

The authors declare that they have no conflict of interest.

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