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Evidence for the immunosuppressive role of nicotine on human dendritic cell functions

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SUMMARY

Nicotine alters a wide range of immunological functions, including innate and adaptive immune responses. To date, no studies have been reported showing the immunoregulatory effects of nicotine on dendritic cells (DCs), which are critical cells for initiation of cell-mediated immunity against infection and neoplastic diseases. In this work, we report that, in a nicotinic environment, monocyte-derived DCs manifest lower endocytic and phagocytic activities. Interestingly, although immature DCs undergo maturation in response to bacterial antigen lipopolysaccharide, they produce decreased levels of pro-inflammatory cytokines, notably interleukin-12, and reveal a reduced ability to stimulate antigen-presenting cell-dependent T-cell responses. Importantly, the reduction in T-cell responses is associated with a diminished ability of DCs to induce differentiation and expansion of type 1 T cells, as evidenced by a decreased frequency of interferon-γ-producing effector cells. These results strongly suggest that nicotine can exert its immunosuppressive effects on immune surveillance through functional impairment of the DC system.

INTRODUCTION

Cigarette smoke is a major health risk factor which significantly increases the incidence of cancers of various organs, and acute and chronic respiratory tract infections.[1–3] It is accepted that many of the health consequences of chronic inhalation of cigarette smoke may be attributable to its adverse effects on the immune system.[4] Indeed, cigarette smoking decreases the serum levels of both specific antibody and total immunoglobulin (Ig) G, IgM, IgA and IgD,[5–7] although IgE is significantly elevated in smokers.[8] In addition, a decreased ability of T cells to proliferate in response to T-cell mitogens[9] and a reduced natural killer (NK) cell activity against cultured cancer cells[7] indicate deficient cell-mediated immune responses in smokers. Alveolar macrophages from smokers secrete significantly lower levels of pro-inflammatory cytokines[10] and show a reduced ability to phagocytose and/or kill bacteria, such as *Staphylococcus aureus* and *Listeria monocytogenes*.[11,12] Taken together, these observations suggest that a smoker's ability to respond to immunologically relevant insults is compromised.

Recent evidence indicates that nicotine, one of the major components of cigarette smoke, suppresses the immune system in a manner similar to cigarette smoke.[13] In fact, animals that were treated chronically with nicotine showed a significant loss of antibody responses and T-cell proliferation.[14] Similarly, nicotine treatment prevented the relapse of ulcerative colitis in patients for several months.[15]

Dendritic cells (DCs) are highly specialized antigen-presenting cells (APCs) of the immune system. In contrast to other types of APC, DCs are potent activators of naïve T cells and are therefore regarded as important initiators of primary specific immune responses.[16,17] Depending upon microenvironmental factors that are present at the time of T-cell activation, naïve T cells differentiate into functionally distinct Th1- and Th2-type cells that amplify and shape both humoral and cell-mediated immune responses.[18] It is well documented that type 1 interferon (IFN)-γ-producing T cells are required to generate protective, cell-mediated immune responses against invading microorganisms[19–21] and growing tumors.[22] Among the factors secreted by DCs, interleukin (IL)-12 bridges innate and specific immune responses by activating NK and T cells[23] and biases the differentiation of CD4 T cells towards IFN-γ-producing Th1 cells.[24,25] Therefore, it is reasonable to speculate that the increased susceptibility of smokers to infection and cancer may reflect nicotine-induced impairment of DC functions.

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To gain further insight into the immunosuppressive action of nicotine, we have evaluated its effects on human monocyte-derived DCs. We report the first *in vitro* results demonstrating a pronounced effect of nicotinic environment on DC properties and immunostimulatory functions.

MATERIALS AND METHODS

Media and reagents

The complete culture medium (CM) used consisted of RPMI-1640, 1% L-glutamine, 1% penicillin/streptomycin, 50 μM 2-mercaptoethanol (ME), 1% sodium pyruvate, 1% non-essential amino acids and heat-inactivated 10% fetal calf serum (FCS) (GibcoBRL, Grand Island, NY) or human AB serum (Gemini Bioproducts, Calabasas, CA). Recombinant human cytokines used were IL-2 (Genzyme Co., Cambridge, MA), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Immunex, Seattle, WA), IL-4, and IL-12 (R&D System, Minneapolis, MN). 7-Aminoactinomycin D (7-AAD), cycloheximide(–)-nicotine and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO). FITC-dextran and lucifer yellow were purchased from Molecular Probes (Eugene, OR).

T-cell purification

Purified T cells were obtained from Ficoll-separated peripheral blood mononuclear cells (PBMCs) of healthy volunteers depleted of other cells using the StemSep^{TM} T-cell enrichment kit (StemCell Technologies, Vancouver, Canada) containing CD14 (RMO52), CD16 (3G8), CD19 (J4·119), CD56 (NKH-1), HLA-DR (B8·12·2), glycophorin A (D2·10) monoclonal antibodies and magnetic colloid. The purity of the enriched populations was >95%.

Generation of DCs

Immature monocyte-derived DCs were generated from the adherent fraction of PBMCs.[26] Briefly, PBMCs were suspended in CM and allowed to adhere to plastic dishes (Falcon 6-well, Falcon, Franklin Lakes, NJ). After 2 hr of incubation at 37°, the non-adherent cells were removed, and the adherent cells were cultured in CM with GM-CSF (100 ng/ml) and IL-4 (10 ng/ml). Cultures were fed every 2 days. Cells were routinely used at day 6 and the immature DC recovery, as determined by immunofluorescence and flow cytometry, was >90% of CD1a$^+$ CD14$^-$ cells. Immature DC viability was quantified by trypan blue staining and flow cytometry using FITC-labelled annexin V and propidium iodide (PI). Briefly, a total of 2×10^5 cells were incubated with 5 μl annexin V-FITC in binding buffer for 10 min, then washed and suspended in binding buffer before the addition of 5 μg/ml PI.

Immunophenotyping of DCs

One day 6, immature DCs were suspended in CM without or with nicotine (200 μg/ml) and then activated with LPS (1 μg/ml). DC phenotypes were tested in various culture conditions: CM alone; nicotine (200 μg/ml); LPS (1 μg/ml); and LPS (1 μg/ml) plus nicotine (200 μg/ml). After 48 hr, cells were harvested, washed and stained with corresponding mAbs, CD1a (NA1/34, DAKO, Carpinteria, CA), CD14 (Tuk 4, CALTAG, Burlingame, CA), HLA-ABC (W6/32, DAKO), HLA-DR (L243, BD Biosciences, San Diego, CA), CD40 (5C3, BD Biosciences), CD80 (L307, BD Biosciences), CD86 (IT2·2, BD Biosciences), CD83 (HB15a, Beckman-Coulter, Brea, CA), mannose receptor (clone 19, BD Biosciences), and CD36 (CB38, BD Biosciences) and analysed by flow cytometry.

Fluid phase and mannose receptor-mediated endocytosis

Immature DCs were washed with phosphate-buffered saline (PBS) and re-suspended in CM containing FITC-dextran (0·5 mg/ml) or lucifer yellow (1 mg/ml) (Molecular Probe) in the presence or absence of nicotine (200 μg/ml). After 30 min of incubation at 37° or 4°, as a negative control, cells were washed four times with cold PBS containing 1% FCS prior to analysis by flow cytometry.

Phagocytosis of apoptotic cells

Apoptotic cell death was induced by prior sensitization with cycloheximide (25 μg/1 $\times$ 10^6/ml) for 2 hr followed by treatment with anti-Fas monoclonal antibody (1 μg/1 $\times$ 10^6/ml, clone CH-11, Beckman-Coulter) for an additional 12 hr. Apoptotic cells were washed with PBS and labelled with 7-AAD at a concentration of 20 μg/1 $\times$ 10^6/ml cells for 30 min at 4°. The labelled apoptotic cells were subsequently co-cultured with CD1a-labelled immature DCs preincubated with or without nicotine (200 μg/ml) at a ratio of 1 : 2.[27] After 2 hr at 37°, cells were washed, and treated with 0·05% trypsin/0·02% EDTA for 5 min to disrupt cell–cell binding. Phagocytosis was quantified by flow cytometry as the percentage of double-positive cells, CD1a$^+$7-AAD$^+$.

T-cell functional assays

Allogeneic DCs were pretreated with nicotine (200 μg/ml) for 2 hr at 37° and then co-cultured at graded doses (indicated in the Figures) in CM with 5% human AB serum with purified T cells (1×10^5/well) in round-bottomed 96-well plates (Falcon). T cells were also added to culture plates coated with anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) monoclonal antibodies (BD Biosciences). Proliferation was measured after 5 days by uptake (1 μCi/well for the last 16 hr) of tritiated thymidine. In two-step co-cultures, purified T cells (2×10^6/well) were added to culture plates coated with anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) monoclonal antibodies or co-cultured with allogeneic DCs or γ-irradiated mature DCs (2×10^5/well, 3000 rad) in the absence or presence of nicotine (200 μg/ml). After 5 days, the T cells were harvested, washed and re-stimulated for an additional 2 days with anti-CD3 and anti-CD28 or allogeneic DCs in the absence of nicotine.

Cytokine analysis

To determine DC cytokine production, 48 hr following LPS stimulation in the absence or presence of nicotine (200 μg/ml), culture supernatants were collected and the amounts of IL-1β, IL-10, IL-12, and tumour necrosis factor (TNF)-α were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available antibodies and standards according to the manufacturer's protocols (BD Biosciences). To determine T-cell cytokine production, culture supernatants from primary and secondary co-cultures were collected and amounts of IL-2, IL-4, IL-10 and IFN-γ measured using ELISA kits (BD Biosciences).

T-cell surface and intracellular cytokine staining

After secondary co-cultures had been carried out in the absence of nicotine, aliquots of T cells were stained with FITC-labelled anti-CD4 (MT310, DAKO), PE-labelled anti-CD8 (Leu-2a, BD Biosciences), and T-cell activation marker APC-labelled anti-CD25 (M-A251, BD Biosciences). The remaining T cells were washed and stimulated for 6 hr with a leukocyte activation cocktail (BD Biosciences) before staining with APC-labelled anti-CD3 (UCHT1, BD Biosciences), PE-labelled anti-IL-4 (clone 3010·211, BD Biosciences) and FITC-labelled anti-IFN-γ (clone 25723·11, BD Biosciences) according to the manufacturer's protocols. The labelled cells were then analysed by flow cytometry.

Statistical analysis

Values are presented as mean ± SD. The statistical significance of differences between nicotine-treated DCs and controls was calculated using a non-parametric Mann–Whitney *U*-test. A value of $P < 0{\cdot}05$ was considered statistically significant.

RESULTS

Nicotine reduces antigen uptake by DCs

Dendritic cells are commonly the first immune cells to encounter foreign antigens. DCs use several mechanisms for antigen capture, such as: receptor-mediated endocytosis, via the mannose receptor, FcγRI, and FcγRII; macropinocytosis, which allows continuous internalization of large volumes of fluid; and phagocytosis, the uptake of particles such as apoptotic and necrotic cells via CD36 and αvβ3 or αvβ5 integrins.[28] We first examined the effect of nicotine on antigen uptake by DCs. The nicotine concentration used in this study was chosen on the basis of results of dose-dependent (0–400 μg/ml) experiments (data not shown) and previous studies.[29,30] While nicotine did not affect DC viability, even at 400 μg/ml (as assessed by trypan blue staining), it significantly changed DC functional characteristics at 200 μg/ml.

To assess DC fluid phase and endocytic activities, DCs were allowed to internalize lucifer yellow or FITC-dextran in the absence or presence of nicotine for 30 min at 4° and 37°, and then analysed by flow cytometry. While DCs in the presence of nicotine maintained their antigen uptake via macropinocytosis (Fig. 1a), they internalized FITC-dextran less efficiently (up to 5-fold) than control DCs (Fig. 1b). To measure the DC phagocytic capacity, CD1a-labelled DCs were co-cultured with 7-AAD-labelled apoptotic cells in the absence and presence of nicotine for 2 hr, after which the capture of apoptotic cells by DCs was quantified by flow cytometry (Fig. 1c). In the presence of nicotine, we found a 2-fold reduction in the number of DCs that captured apoptotic cells. Taken together, these results demonstrate that both the endocytic and phagocytic abilities of immature DCs are reduced in a nicotinic environment.

Next, we examined the effect of nicotine on the expression of the mannose receptor and CD36, which are involved in the uptake of mannosylated antigens and apoptotic cells, respectively. Although no significant difference was found in the expression of the CD36 receptor (Fig. 1e), nicotine-treated DCs expressed lower level of mannose receptor than control DCs (Fig. 1d).

Effects of nicotine on DC maturation and cytokine production

The maturation of DCs is a critical process, as only mature DCs are able to induce optimal activation of naïve T cells.[16,31] *In vivo*, maturation of DCs is initiated in non-lymphoid tissues upon exposure to inflammatory molecules such as LPS and is achieved in lymph nodes upon CD40 engagement by CD40L-expressing T cells. To test whether nicotine affects DC responsiveness to maturation stimuli, DCs were treated with LPS in the presence of nicotine. After 48 hr, cells were analysed for the expression of surface molecules involved in the APC function of DCs. DC staining for MHCs, CD40, co-stimulatory molecules B7·1/CD80 and B7·2/CD86, and DC maturation marker CD83 revealed that nicotine at the highest concentration used in our assays had no effect on DC maturation (Fig. 2). Identical results were obtained when immature DCs were activated by soluble CD40L (data not shown).

In response to bacterial compounds such as LPS, DCs produce substantial amounts of pro-inflammatory cytokines, including IL-12, a key cytokine required for the development of type 1 T cells.[23,32] Here we examined the influence of nicotine on cytokine production by DCs in response to LPS. Interestingly, DCs produced significantly lower levels of IL-12 (60% reduction), and slightly lower levels of IL-1β (15% reduction), IL-10 (23% reduction), and TNF-α (25% reduction) as compared with control DCs (Fig. 3). Taken together, these results indicate that, in the presence of nicotine, DCs are able to mature in response to bacterial product, while their ability to secrete pro-inflammatory cytokines, notably IL-12, is diminished.

Nicotine diminishes APC-dependent T-cell responses

Given that DCs are initiators/controllers of immune effector cells, we next examined the nature of T-cell responses induced by DCs in the presence of nicotine. T cells isolated from human peripheral blood were co-cultured with allogeneic DCs. As expected, the purified T cells were able to proliferate in response to DCs in a dose-dependent manner. However, we found a diminished capacity of DCs to stimulate T cells when nicotine was present during the co-culture (Fig. 4a). Interestingly, this antiproliferative action of nicotine was APC-dependent as it did not affect APC-independent T-cell proliferation induced by anti-CD3/CD28 Abs (Fig. 4b).

We further measured T-cell cytokine production from the primary and secondary co-cultures. Whereas production of IL-2, IL-4 and IL-10 was almost unchanged, T cells co-cultured with DCs in the presence of nicotine produced significantly lower levels of IFN-γ (63% reduction) than control DC/T-cell co-cultures (Fig. 5, upper panels). Importantly, the production of IFN-γ by these T cells remained significantly lower (55% reduction) than that of control T cells once re-stimulated in the absence of nicotine (Fig. 6). In contrast, while T cells stimulated with anti-CD3/CD28 Abs in the presence of nicotine in the first cultures produced lower levels of IL-2, IL-4, IL-10 and IFN-γ than their counterparts (Fig. 5, lower panels), they were able to secrete equivalent amounts of IFN-γ upon re-stimulation in the absence of nicotine (Fig. 6). These data suggest the

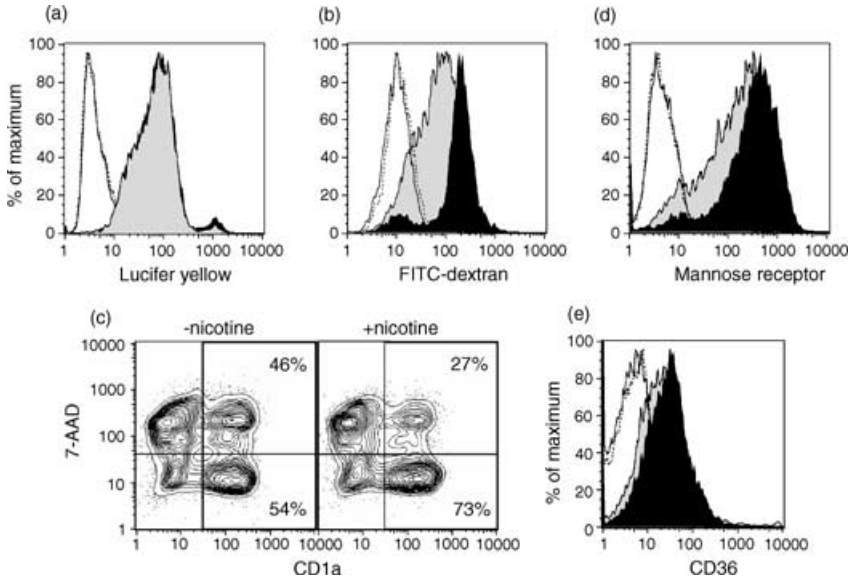

Figure 1. Effect of nicotine on DC antigen uptake. Immature DCs were cultured in medium alone or in medium containing nicotine prior to addition of (a) lucifer yellow, (b) FITC-dextran or (c) co-culture with 7-AAD-labelled apoptotic cells and (d, e) cell surface staining using specific monoclonal antibodies. After the indicated times, fluid phase uptake, FITC-dextran internalization and phagocytosis of apoptotic cells by immature DCs were analysed by flow cytometry. (a) Solid (without nicotine) and dashed (with nicotine) lines represent staining (negative control) at 4°, and black [median fluorescence index (MFI) = 66] and grey (MFI = 64) shaded areas represent fluid phase uptake of lucifer yellow at 37°, in the absence and presence of nicotine, respectively. (b) Solid and dashed lines represent staining (negative control) at 4° in the absence and presence of nicotine, respectively, and black (without nicotine, MFI = 176) and grey (with nicotine, MFI = 59) shaded areas represent endocytosis of FITC-dextran at 37°. (c) Contour plots represent the percentage of immature DCs (CD1a-positive gated events) that captured apoptotic cells. (d) Solid (without nicotine) and dashed (with nicotine) lines represent the isotype control antibodies. Black (without nicotine, MFI = 336) and grey (with nicotine, MFI = 164) shaded areas represent expression of the mannose receptor. (e) Solid (without nicotine) and dashed (with nicotine) lines represent the isotype control antibodies. Black (without nicotine, MFI = 17) and grey (with nicotine, MFI = 23) shaded areas represent expression of CD36. One representative experiment of three is shown. The background fluorescence was always subtracted.

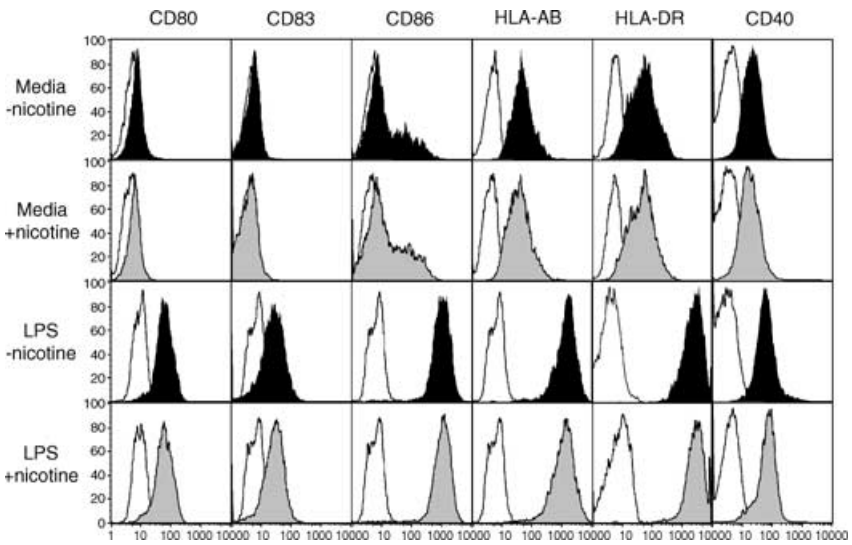

Figure 2. Flow cytometric analysis of LPS-activated DCs in the presence of nicotine. Immature DCs were exposed to various conditions: medium alone, nicotine, LPS, and nicotine plus LPS. After 48 hr, cells were stained with corresponding monoclonal antibodies. Unshaded areas under graphs represent the isotype control antibodies. Black (without nicotine) and grey (with nicotine) shaded areas represent specific staining of the indicated cell-surface markers. One representative experiment of four is shown.

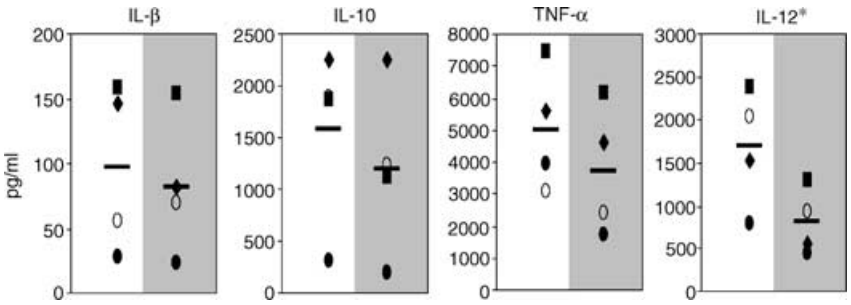

Figure 3. Effect of nicotine on cytokine release from LPS-stimulated DCs. Immature DCs were stimulated with LPS in the absence or presence of nicotine. After 48 hr, the culture supernatants from control DCs (unshaded areas) and nicotine-treated DCs (grey areas) were harvested, and cytokine production determined by ELISA. Each symbol represents an independent experiment ($*P < 0{\cdot}05$).

reduction in T-cell proliferation and IFN-γ production seen in our assays was APC-dependent.

In the presence of nicotine, DCs fail to efficiently induce type 1 T-cell polarization

To determine whether the reduction in IFN-γ production was a default mechanism attributable to an inability of T cells to produce IFN-γ or a decrease in the frequency of IFN-γ-producing cells in response to DCs, we tested the proliferative response of T cells in a two-step culture system. In these experiments, T cells were co-cultured with allogeneic DCs or added to anti-CD3/CD28 Abs coated plates as controls in the absence or presence of nicotine. After 5 days, T cells were rescued and re-stimulated in second co-cultures with the same stimulators in the absence of nicotine. We found a reduction (up to 40%) in the expansion of both CD8 and CD4 T cells, expressing T-cell activation marker CD25, which were primarily co-cultured with DCs in the presence of nicotine (Fig. 7). In contrast, T cells stimulated with anti-CD3/CD28 Abs in the presence of nicotine responded equivalently to control T cells following re-stimulation in the absence of nicotine (Fig. 7).

We finally examined the effect of nicotine on the frequency of polarized T cells generated in the first co-culture in response to DCs or anti-CD3/CD28 Abs. Intracellular staining of T cells from second cultures for IL-4 and IFN-γ revealed a significant reduction in IFN-γ-producing T cells that were originally co-cultured with DCs (33% reduction) but not anti-CD3/CD28 Abs in a nicotinic environment (Fig. 8). In addition, we found no changes in the frequency of IFN-γ-producing T cells when γ-irradiated DCs (γDCs) were used as stimulators in the first cultures in the presence of nicotine, suggesting a direct effect of nicotine on DC immunostimulatory properties during cognate DC/T-cell interaction (Fig. 8, lower panels). Taken together, these results suggest that, in a nicotinic environment, the ability of DCs to prime type 1 T-cell polarization is reduced.

DISCUSSION

Cigarette smoke has been shown to alter a wide range of immunological functions and adversely influences humoral and cellular immune responses in both humans and animals.[33] Human smokers are more likely to develop influenza and have lower antibody titres to influenza virus than non-smokers. In addition, exposure of animals to cigarette smoke has been shown to lead to a progressive decrease in resistance to transplanted tumours and increased tumour metastases and mortality.[34] The DC/T-cell interaction in the T-cell area of secondary

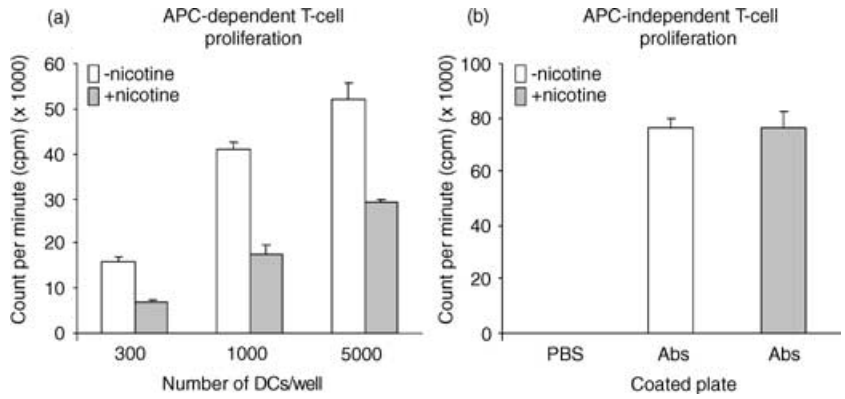

Figure 4. APC-dependent and -independent T-cell responses in the presence of nicotine. (a) Immature DCs were co-cultured at graded doses with purified T cells in the absence or presence of nicotine. (b) Purified T cells were added to plates coated with anti-CD3/CD28 Abs in the absence or presence of nicotine. T-cell proliferation is represented as the mean ± SD of [^{3}H]thymidine uptake from triplicate wells. One representative experiment of four is shown.

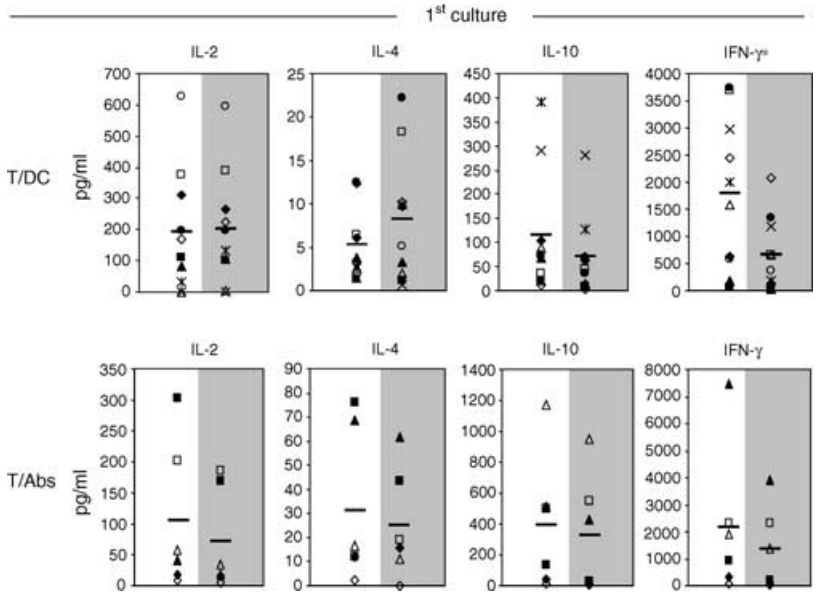

Figure 5. Analysis of cytokine release from primary T-cell cultures. Upper panels represent cytokine profiles of T cells stimulated with DCs in primary co-cultures without (unshaded areas) or with (grey areas) nicotine. Lower panels represent cytokine profiles of T cells in primary cultures activated with anti-CD3/CD28 Abs without (unshaded areas) or with (grey areas) nicotine. Each symbol represents an independent experiment ($*P < 0{\cdot}05$).

lymphoid organs is a key event in the initiation of immune responses. The amount of signal that T cells receive from DCs is dependent on DC antigen capturing, processing and presentation, expression of co-stimulatory molecules and cytokine production.[17] This report provides the first evidence of an immunosuppressive effect of nicotine on DC functions, such as antigen capturing, cytokine production, and eventually T-cell priming and polarization.

Capturing of antigens by tissue-resident APCs is crucial for the induction of immunity against invading pathogens and neoplastic cells.[28] Previous reports indicate that alveolar macrophages from smokers have reduced ability to phagocytose and/or kill bacteria.[11,12] The present data suggest that, in a nicotinic environment, DCs are less potent in taking up FITC-dextran and apoptotic cells (Figs 1b,c). Given that the receptor-independent macropinocytic activity[35] remained intact (Fig. 1a), the defect in FITC-dextran uptake can be correlated in part with the lower expression of mannose receptor on DCs exposed to nicotine (Fig. 1d). The decreased phagocytosis of apoptotic bodies was attributable neither to a toxic effect of nicotine at the optimal dose (200 μg/ml) used in our assays on DC viability (data not shown) nor to significant changes in the surface expression of CD36 on DCs exposed to nicotine (Fig. 1e). Although a decrease in other undefined receptors is possible, the mechanisms by which nicotine impairs DC antigen capture remain to be defined.

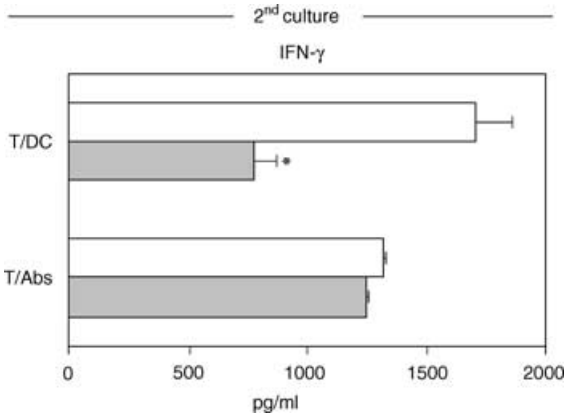

Figure 6. Analysis of IFN-γ release from secondary T-cell cultures. The panel displays secondary IFN-γ secretion by T cells rescued from primary cultures and further cultured with the same stimulators in the absence of nicotine. Unshaded bars indicate T cells originally stimulated in the absence of nicotine. Grey bars indicate T cells originally stimulated in the presence of nicotine. Cytokine levels were determined by ELISA. One representative experiment of four is shown ($*P < 0{\cdot}05$).

Following *in vitro* or *in vivo* exposure to LPS or other bacterial products, DCs down-regulate antigen-processing properties and undergo maturation, which facilitates both the interaction with and the stimulation of lymphocytes.[36] DC maturation entails the increased expression of CD40, B7·1/CD80, B7·2/CD86, MHC class I and class II gene products, and DC maturation marker CD83, and the decreased expression of receptors for antigen capture.[28,31] Interestingly, we found that, while DCs exposed to nicotine showed impaired endocytic and phagocytic activities, they were able to fully mature in response to bacterial antigen LPS (Fig. 2). It has been reported that a high density of antigen-carrying DCs and high levels of B7 molecules sustain stimulation of specific T cells and lead to their rapid commitment to proliferation and differentiation.[37] Therefore, it can be envisaged that in the nicotinic environment antigen-specific T cells will encounter DCs undergoing maturation without being charged, or adequately charged, for optimal antigen delivery to T cells. This may suggest a possible

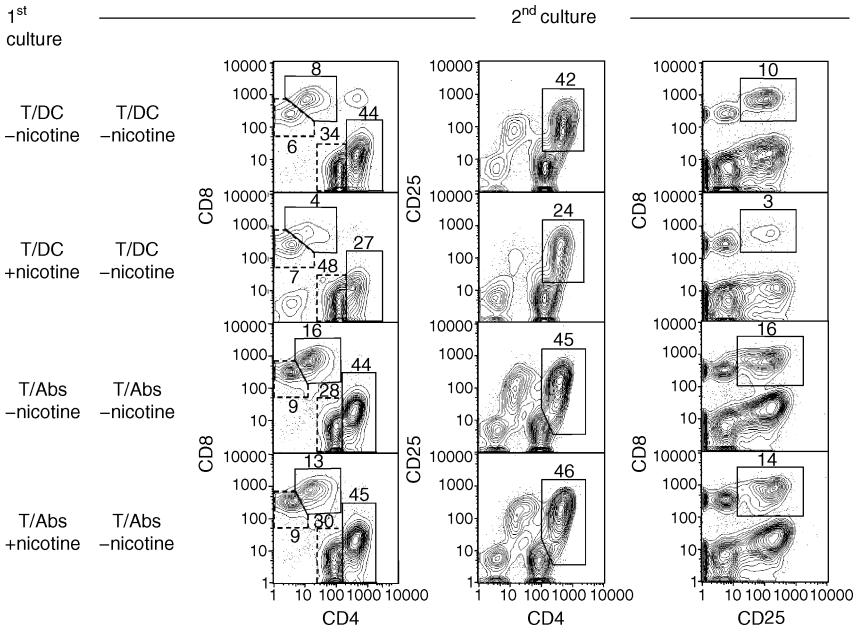

Figure 7. Flow cytometric analysis of T cells from secondary cultures. Upper panels represent surface staining of T cells rescued from primary co-cultures with DCs in the absence or presence of nicotine and re-challenged with DCs in the absence of nicotine. Lower panels represent surface staining of T cells rescued from primary cultures with anti-CD3/CD28 Abs in the absence or presence of nicotine and re-challenged with same stimulators in the absence of nicotine. Gates indicate the frequency of unactivated (dashed lines) and activated (solid lines) CD4 and CD8 T cells. One representative experiment of four is shown.

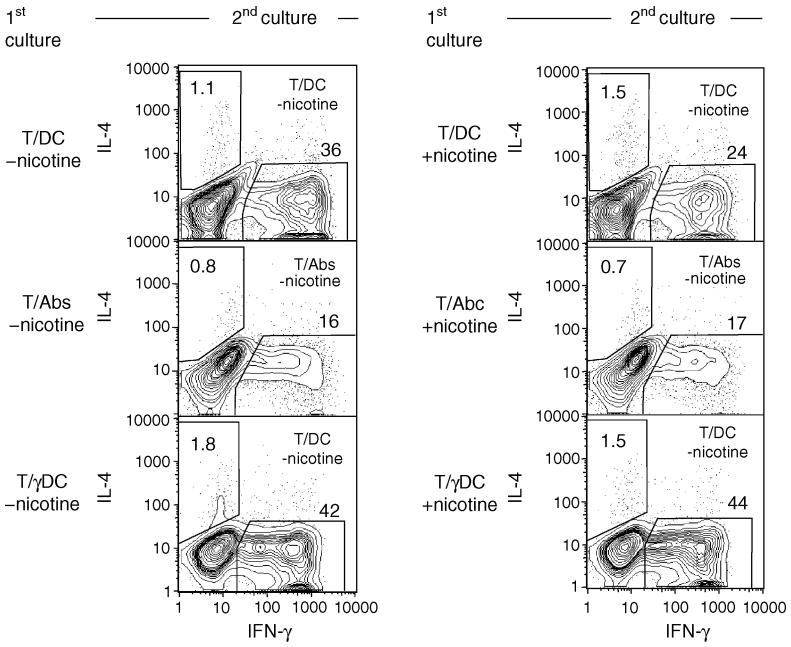

Figure 8. Flow cytometric analysis of IL-4 and IFN-γ producing T cells from secondary cultures. The T cells rescued from primary co-cultures with DCs, anti-CD3/CD28 Abs or γ-irradiated DCs (γDCs) without (left panels) or with (right panels) nicotine were re-challenged with the indicated stimulators in the absence of nicotine. The numbers in each gate indicate the respective frequency of T cells producing IL-4 and IFN-γ. One representative experiment of four is shown.

mechanism by which nicotine exerts its immunosuppressive effect.

Pro-inflammatory cytokines are also crucial for early responses to pathogens and the up-regulation of local host defences. Several cytokines such as IL-1β, IL-10, IL-12 and TNF-α are produced by DCs in response to bacterial antigens. Our data provide evidence that DCs exposed to nicotine produce lower levels of IL-1β, IL-10, TNF-α and, most notably, IL-12 (Fig. 3). IL-12, a potent pro-inflammatory cytokine, plays a central role in the initiation and control of cell-mediated immunity.[24,38,39] Importantly, IL-12 produced by DCs during early antigenic stimulation has been reported to be a powerful inducer of Th1 responses, and defects in its production have been suggested to be a factor contributing to immune depression.[40–42] Hence, the direct effect on DC cytokine production, particularly that of IL-12, may suggest another mechanism by which nicotine affects host defences against infection and cancer.

It is well established that DCs are APC specialized for naïve T-cell activation *in vitro* and *in vivo*.[16,17,31] T cells from smokers have shown a decreased ability to proliferate in response to T-cell mitogens.[9] Our data revealed a reduction in T-cell proliferation induced by DCs in the nicotinic environment (Fig. 4a), which was supported by the decreased number of activated CD4 and CD8 T cells (Fig. 7). However, the T-cell hyporesponsiveness seen in our assays was not a result of T-cell anergy. Indeed, T cells co-cultured with DCs in the presence of nicotine produced equal amounts of IL-2 (Fig. 5) compared with control T cells. Moreover, the addition of exogenous IL-2 did not reverse this stage of hyporesponsiveness (data not shown). Finally, we did not observe any changes in the viability of T cells obtained from co-cultures, suggesting that the functional inactivation of T cells was the result of a non-deletional mechanism mediated by nicotine. Interestingly, nicotine did not inhibit APC-independent T-cell proliferation induced by anti-CD3/CD28 Abs (Fig. 4b), confirming no direct effect of nicotine on T-cell responses.

From our data, it appears that nicotine has some inhibitory effect on both APC-dependent and -independent IFN-γ production by primary T cells (Fig. 5). Whereas T cells stimulated with anti-CD3/CD28 Abs were able to produce similar amounts of IFN-γ once re-stimulated in the absence of nicotine, T cells stimulated with DCs produced significantly lower levels of IFN-γ upon re-stimulation (Fig. 6). Importantly, these results were correlated with a reduction in the frequency of IFN-γ-producing T cells when DCs were used as stimulators (Fig. 8). These data indicate that nicotine had a direct effect on the ability of DCs to polarize Th1 cells. Interestingly, supplementation of IL-12 alone in the primary co-cultures could not restore DC capacity to the control level (data not shown). We surmised that, during DC/T-cell interaction, nicotine may affect the expression of accessory molecules on DCs that are critical for T-cell responsiveness to IL-12 and final Th1 polarization. Indeed, we found that, in a nicotinic environment, fully matured irradiated DCs were able to provide the necessary signals for expansion of IFN-γ-producing T cells (Fig. 8). Considering the data outlined above, it is reasonable to conclude that, prior to or upon DC/T-cell interaction, nicotine specifically impairs DC immunostimulatory functions through its effects on DC antigen capture/presentation and IL-12 shortening, and other possible mechanisms.

The concentration of nicotine in local tissue (i.e. the respiratory tract), where tissue-resident DCs encounter antigens, may be higher than the level of nicotine reported in the plasma of heavy smokers.[43] It is estimated that daily nicotine intake and intake per cigarette from nominal brands smoked are 18·6 and 1·28 mg, respectively.[44] It has also been stated that the average cigarette contains approximately 10 mg of nicotine,[45] and between 1 and 2 mg of nicotine is delivered to the lungs when a cigarette is smoked.[46] In addition, Russell *et al.*[47] reported that the salivary concentration of nicotine reaches 1560 μg/ml in smokeless-tobacco users. From these studies, one may infer that, after a cigarette has been smoked, the concentration of nicotine in the local tissue reaches the level used in this work and hence that this is physiologically relevant.

The present work provides new information on the immunosuppressive effect of nicotine on the DC system and adds to our understanding of the ways in which exposure to cigarette smoke may increase the risks of infection and cancer.

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