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Exposure to Nicotine Adversely Affects the Dendritic Cell System and Compromises Host Response to Vaccination

Mahyar Nouri-Shirazi and Elisabeth Guinet

The magnitude of Th1 cells response to vaccination is a critical factor in determining protection from clinical disease. Our previous in vitro studies suggested that exposure to the nicotine component of cigarette smoke skews the differentiation of both human and mouse dendritic cell (DC) precursors into atypical DCs (DCs differentiated ex vivo in the presence of nicotine) lacking parameters essential for the development of Th1-mediated immunity. In this study, we determined the causal relationship between nicotine-induced DC alterations and host response to vaccines. We show that animals exposed to nicotine failed to develop and maintain Ag-specific effector memory Th1 cells and Ab production to protein-based vaccine formulated with Th1 adjuvants. Accordingly, both prophylactic and therapeutic vaccines failed to protect and cure the nicotine-exposed mice from disease. More importantly, we demonstrate the nicotine-induced defects in the biological activities of in vivo DCs as an underlying mechanism. Indeed, i.v. administration of DCs differentiated in the presence of nicotine preferentially promoted the development of Ag-specific IL-4-producing effector cells in the challenged mice. In addition, DC subsets isolated from mice exposed to nicotine produced significantly less cytokines in response to Th1 adjuvants and inadequately supported the development of Ag-specific Th1 cells. Collectively, our studies suggest that nicotine-induced defects in the DC system compromises vaccine efficacy in smokers. The Journal of Immunology, 2012, 188: 2359–2370.

One of the most important contributions to improving public health has been the development of vaccines. The effectiveness of a vaccine, however, depends on the competency of the individual immune system to adequately respond to that vaccine (1, 2). For example, patients vaccinated within 2 wk before starting immunosuppressive therapy or while receiving immunosuppressive therapy are considered unvaccinated and are, therefore, advised to be revaccinated at least 3 mo after therapy is discontinued if immune competence has been restored (1). The deterioration in both innate and adaptive immunity associated with aging also leads to reduced responses to vaccines and increases morbidity and mortality from infection (2). Recently, the Centers for Disease Control and Prevention (CDC) identified smokers as a population with higher incidence and severity of infectious diseases and recommended vaccines for all identified smokers as a population with higher incidence and severity of infectious diseases and recommended vaccines for all smokers aged 19–64 y (3, 4). The benefits of such a recommendation, however, may be limited due to well-documented nicotine-induced systemic dampening of immune function (3, 5, 6). Indeed, there is compelling evidence that smokers are less responsive to vaccines. Finklea and colleagues (7) showed that smokers had lower titers and a decreased t1/2 of Abs to influenza virus after natural disease and immunization. Holt’s group (8) also reported that the longevity of the immune response to subunit vaccine was severely depressed 50wk postvaccination in smokers. Reduced protection seen in smokers compared with nonsmokers after vaccination is further supported by a study from Winter et al. (9) in which it was found that smokers who received hepatitis B vaccines at 0, 1, and 6 mo (standard booster vaccine) had lower Ab levels than nonsmokers after 3, 7, and 13 mo. Although these studies have identified the existing problem, no study has yet explained the mechanism that accounts for such a deficiency in host immunity.

Several approaches have been used (e.g., smoking machine, smoke extract, and nicotine) to evaluate the in vivo effects of cigarette smoke on the immune system. All of these approaches are regarded as model systems that imperfectly reproduce some aspect of human smoking. However, studies using these techniques in animal models have provided great insight and are likely to continue to do so (10). Cigarette smoke contains >4500 chemicals in its gaseous and particulate phases (11) including nicotine, ammonia, carbon monoxide, carbon dioxide, formaldehyde, acrolein, acetone, benzopyrenes, hydroxyquinone, nitrogen oxides, and cadmium. Many of these agents are known to be carcinogenic and toxic to the cells (10). Nicotine, however, is the only agent considered synonymous with smoking in modulating the immune system (5, 12). For example, Holt and colleagues (13) observed that exposure to smoke from high-nicotine cigarettes is more immunosuppressive than the smoke from low-nicotine cigarettes. In experiments separating the vapor phase from the particulate phase, the Sopori group (5) found that particulate phase-containing nicotine is necessary for the immunosuppression associated with cigarette smoke. Also, animals treated chronically (3 to 4 wk) with nicotine, in a manner similar to cigarette smoke, have a significant loss of Ab responses and T cell proliferation (13–15). Altogether, these studies provided the rationale for studying the impact of nicotine on host immunity and host response to vaccination.

Research identified a family of APCs called dendritic cells (DCs) as being the body’s key immune cells that are pivotal in the ini-
titiation of immune responses against invading pathogens and neoplastic cells. It was also recognized that vaccines, designed to enhance host immunity against infectious diseases and cancer, depend on functional DCs (16, 17). The observed immunosuppression in smokers combined with the importance of DCs in maintaining immunity led us to study whether the biological activities of DCs are severely affected by exposure to nicotine. Our pioneering in vitro work (18–21) revealed the nicotine-induced defects in the differentiation and biological activities of both human and mouse DCs differentiated ex vivo, particularly the ability to prime effector memory T cells as a possible mechanism for the depressed immune response reported in vaccinated smokers (7–9). In this study, we report that exposure to nicotine significantly diminishes the development of Ag-specific effector memory Th1 cells and Ab production, leading to poor host response to an otherwise protective and therapeutic vaccine. We further show how the defects in the immunobiology of the DC system in vivo underlie this poor outcome.

Materials and Methods

Cell lines and animals

Cell lines were from the American Type Culture Collection. EL4 is a thymoma cell line of H-2b haplotype, and YAC1 is an NK-sensitive cell line. The OVA-producing EG7 (OVA-EG7) cell line, an EL4 cell line stably transfected with OVA cDNA, was maintained in the presence of G418 (400 μg/ml). All experimental mice were used at 6–16 wk of age. Wild-type (WT) C57BL/6 (B6, H-2b), BALB/c (Ba, H-2a), and OVA-specific TCR-transgenic DO11.10 (DO11, H-2d) mice were purchased from The Jackson Laboratory and maintained at our animal facility. All experimental procedures were conducted in accordance with standard operating procedures for the Animal Resource Unit of the institution. The experimental procedures were conducted in accordance with the guidelines of the U.S. Department of Agriculture Animal and Plant Health Inspection Service. Systemic administration of nicotine was performed using minia-
ture osmotic pumps (Alzet Durect) filled with nicotine (1 mg/kg body weight [Bwt]/d; Sigma-Aldrich) and implanted s.c. during surgery.

Protein- and cell-based vaccination

The protein-based vaccination protocol consisted of an s.c. injection of OVA protein (2 mg/200 μl; Sigma-Aldrich) plus LPS (50 μg; Sigma-Aldrich) or CpG (50 μg; Invivogen) as a Th1 adjuvant. Age- and sex-matched WT BALB/c recipient mice adaptively transferred with 2.5 × 10^6 purified naïve DO11.10 TCR-transgenic CD4+ T cells were immunized once. Age- and sex-matched WT C57BL/6 recipient mice were vaccinated twice at weekly intervals. They were challenged with 1 × 10^6 viable OVA-EG7 tumor cells by injection in the shaved lower dorsal region before (therapeutic) or after (prophylactic) vaccinations. For the DC-based vaccination protocol, WT BALB/c recipient mice adaptively transferred with purified naïve DO11.10 TCR-transgenic CD4+ T cells were injected i.v. with 1 × 10^6 LPS-matured DC pulsed with OVA protein.

Blood, tissue, and cell isolations

Mice were anesthetized with a mixture of ketamine/xylazine. Prior to blood collection, the size of the tumor was measured with calipers in two perpendicular directions. Midline incision was made through the skin, muscle, and peritoneum from xiphoid to pubis. The intestines were placed perpendicular to expose the abdominal aorta and vena cava. A 27 ½-gauge needle syringe was inserted into the aorta, and up to a 1-ml blood sample was collected. Following the blood collection, mice were euthanized, and their spleens and lymph nodes (LNs) were removed aseptically and kept on ice. Single-cell suspensions were obtained by rubbing the tissue between two frosted glass slides and suspended in PBS. lymphoid and splenic CD4+ T cells from OVA-specific TCR-transgenic DO11.10 mice were purified using the EasySep Negative Selection CD4 T Cell Enrichment Kit (StemCell Technologies), as reported previously (22). The purity of enriched OVA-specific CD4+ T cells was >95% when analyzed by flow cytometry.

DC generation

DCs were generated by the methods reported previously with some modifications (23, 24). Femurs and tibia were removed from WT mice and the marrow flushed with PBS using a syringe with a 0.45-μm filter. Clusters within the marrow suspension were dissociated by vigorous pipetting and then filtered through a 70-μm cell strainer. Bone marrow (BM) cells were suspended in complete medium (CM): RPMI 1640, 1% t-glutamine, 1% penicillin/streptomycin, 50 μM 2-ME, 1% sodium pyruvate, 1% essential amino acids, and heat-inactivated 10% FCS. For DC administration and recall assays, 1 × 10^9/ml BM cells were plated onto a 75-cm² tissue culture flask with or without 200 μg/ml nicotine (Sigma-Aldrich). They were cultured for up to 4 d in the presence of 20 ng/ml GM-CSF and 20 ng/ml IL-4 (PeproTech) at 37°C, 5% CO2. On day 3, 100 μg/ml OVA protein (Sigma-Aldrich) was added to the cells. DCs were collected on day 4 and isolated using 14.5% in CM histodenz density gradient (Sigma-Aldrich). For the analysis of the DC subsets, BM cells were seeded at 1 × 10^9/ml on a six-well tissue culture plate. They were cultured at 37°C for up to 5 d in the presence of 200 μg/ml FLT3 ligand (PeproTech) with or without 200 μg/ml nicotine (Sigma-Aldrich). On days 2 and 4, nicotine was added to the culture. DCs were collected on day 6, analyzed by flow cytometry, and the myeloid, lymphoid, and plasmacytoid DC subsets were then collected, and the amounts of IL-12 and IFN-α were measured by ELISA according to the manufacturer’s protocol (ebiScience).

DC and T cell analysis

Cells were isolated in cold staining media (PBS, 2% FCS, and 2 mM EDTA) and stained with relevant surface markers. In vitro-generated DCs and DCs isolated from spleen of WT mice were labeled with mAbs against CD11c (N418, BD Biosciences), B220 (RA3-6b2), CD19 (1D3), MHC class II (MHC II; I-Ab, M5/114.15.2), MHC class I (MHC I; H-2K, H-2D, H-2Q), CD80 (16-10A1), CD86 (GL1), CD40 (3/23), and CCR7 (4B12) (ebiScience). Single-cell suspensions prepared from LNs and spleens of indicated mice were labeled with mAbs against CD4 (RM4-5), CD8α (53-6.7), CD49b (DX5), IFN-γ (XMGl2.1), IL-4 (11B11), and OVA-specific clo-
tonotypic TCR (K1-26) (ebiScience). Cells were also stained with the PE-Cy7-labeled (PerCP eFluor780) dendritic cell antigen (DC-AG) followed by staining with anti-CD8α (53-6.7) and anti-CD11b (1 D3) mAbs (ebiScience). Match-isotype Abs were used as controls. The labeled cells were then analyzed by flow cytometry using an FACSCalibur cytometer and FACSaria (BD Biosciences). FlowJo analysis software (Tree Star).

Ag-specific recall assay

Single-cell suspensions prepared from LNs and spleens of indicated mice were restimulated in vitro with 1 μM OVA (323–339 and/or 257–264) peptides. After 4 d, the culture supernatants and the cells were harvested; the amounts of IFN-γ and IL-4 released in the supernatants were measured by ELISA (ebiScience), and the frequency of OVA-specific, CD4+, and CD8+ T cells producing IFN-γ was measured by flow cytometry following 4 h restimulation with a leukocyte activation mixture containing brefeldin A (BD Biosciences). Lymphoid and splenic cells from tumor-challenged mice were also added to wells previously plated with OVA-pulsed DC (25:1 ratio) in six-well tissue culture plates. After incubation at 37°C for 5 d, expanding cells were collected and the frequency and functional properties of the tumor-specific immune effector cells analyzed by flow cytometry and cytotoxic assay.

Cytotoxic assay using Calcein-AM

Cells recovered from coculture with OVA-pulsed DCs by gradient centrifugation were used as effectors. Target cells consisting of OVA-EG7, EL4, and YAC1 cell lines were labeled with 15 μM Calcein-AM (Invitrogen) for up to 1 h and washed three times with CM. Effector and target cells were cocultured at 37°C for 4 h in 96-well round-bottom tissue culture plates. Supernatants were recovered, and Calcein-AM release was measured using a fluorescent microplate reader (excitation = 485 nm; emission = 530 nm). The percentage-specific lysis was calculated using the formula: percent lysis = 100 × [(experimental – spontaneous)/maximal – spontaneous]. Maximal lysis was achieved with 5% Triton X-100.

Serum cotinine, anti-OVA IgG1, anti-OVA IgG2a, and anti-OVA IgG2b levels

Cotinine levels were determined by a competitive enzyme immunosorbent assay, following the manufacturer’s instructions (Calbiotech). Briefly, samples and enzyme-cotinine conjugate were plated on a 96-well micro-
plate coated with anti-cotinine Abs and incubated for 30 min. After washing, the substrate was added to the plate. Absorbance was measured at
450 and 630 nm. For measurement of anti-OVA Abs, enzyme immunoassay plates (Costar) were coated with 15 μg/ml OVA protein (Sigma-Aldrich) in coating buffer overnight at 4°C, blocked with 10% FCS/PBS, washed, and incubated for 2 h with serum samples or standards (AntibodyShop). Plates were then washed, incubated with 2 μg/ml biotin-conjugated anti-mouse IgG1 (A85-1), IgG2a (R19-15) or IgG2b (R12-3) Abs for 2 h, followed by incubation with 1/2000 streptavidin-HRP (BD Biosciences) for 1 h at room temperature. After the final washes, a one-to-one mixture of BD OptEIA substrate reagents A and B (BD Biosciences) was added as a chromogen. The reaction was stopped with 2 N H₂SO₄. The optical densities at 490 nm were measured, and the Ab titers were determined for each sample.

**Statistical analysis**

Values are presented as mean ± SEM. The statistical significance of differences between samples (*−NIC versus −NIC/I and **−NIC/I versus +NIC/I) was calculated using a t-test. Statistical differences were considered to be significant if p was <0.05.

**Results**

Nicotine exposure diminishes host response to immunization

Immune responses against T cell-dependent Ags are heterogeneous with respect to the cytokines made by T cells and the class of Abs secreted by B cells (25). Immune responses dominated by T cells producing IFN-γ and B cells secreting IgG2a Ab are termed Th1 responses; those dominated by IL-4 and B cells secreting IgG1 are termed Th2 responses (25). To investigate the extent to which the presence of nicotine influences the development of Ag-specific effector memory T cells and Ab production, cohorts of WT BALB/c mice were implanted with osmotic pumps containing nicotine for up to 3 wk. The mice were then adoptively transferred with transgenic OVA-specific CD4⁺ T cells and immunized s.c. with soluble chicken OVA protein and the Th1-polarizing adjuvant LPS. At the height of the primary immune response (day 4), we monitored the in vivo proliferative capacity of OVA-specific T cells. We also measured the cytokine profile of the in vivo-polarized OVA-specific T cells following in vitro restimulation with the OVA peptide (323–339). We found that immunization with soluble OVA and the Th1 adjuvant LPS induced clonal expansion of adoptively transferred transgenic OVA-specific (detected by KJ1-26 mAbs) CD4⁺ T cells in the spleen and LNs of both control and nicotine-exposed mice (from 0.37 ± 0.08 to 2.44 ± 0.45 and 2.55 ± 0.53%, respectively) (Fig. 1A). However, nicotine exposure prior to immunization affected the development of effector T cells within proliferating cells (Fig. 1B), as evidenced by a significant reduction in the frequency of OVA-specific IFN-γ–secreting effector CD4⁺ Th1 cells (up to 4-fold) and IFN-γ released by these cells in recall response to OVA peptide (Fig. 1C). Similar results were obtained in mice exposed to nicotine for a shorter period (1 wk) or injected s.c. with nicotine twice daily 12 h apart to provide the maximum pharmacokinetics (data not shown).

![FIGURE 1.](http://www.jimmunol.org/)

**Statistical analysis**

Values are presented as mean ± SEM. The statistical significance of differences between samples (*−NIC versus −NIC/I and **−NIC/I versus +NIC/I) was calculated using a t-test. Statistical differences were considered to be significant if p was <0.05.
There is increasing evidence that Ag-specific T cells that remain in the body after the contraction of effector T cell population are then maintained indefinitely as long-lived memory T cells, which can generate a rapid recall response to secondary Ag challenge (26). To investigate the extent to which nicotine affects the natural contraction of the memory T cell pool, the response to Ag rechallenge, and the development of humoral response, cohort of mice was studied 14 d after immunization. We observed a contraction (up to 6-fold) in the frequency of transgenic OVA-specific CD4+ T cells in the spleen and the LNs of mice exposed to nicotine 2 wk postimmunization (Figs. 1A, 2A). Yet, consistent with our in vitro human study (20), we found that these memory T cells were hyporesponsive to secondary Ag challenge as evidenced by a significant reduction in the frequency of IFN-γ-secreting OVA-specific effector CD4+ T cells and the amount of IFN-γ protein released by these cells in response to OVA peptide (Fig. 2B–D). We also quantified the production of OVA-specific IgG1, IgG2b, and IgG2a in the sera of immunized mice and discovered a significant reduction in the amounts of anti-OVA IgG2a but not IgG1 or IgG2b Abs in nicotine-exposed mice (Fig. 2E).

**FIGURE 2. In vivo effects of nicotine exposure on effector memory T cell pool expansion.** LNs and spleens of unimmunized (−NIC or +NIC) or immunized (−NIC/I or +NIC/I) mice implanted with an osmotic pump containing saline (−NIC) or nicotine (1 mg/kg Bwt/d; +NIC) were isolated 14 d after the immunization and analyzed by flow cytometry. (A) Flow cytometry plots and bars indicate the frequency (left panel) and absolute number (right panel) of in vivo-expanded transgenic OVA-specific CD4+ (KJ1-26/CD4) T cells. (B–D) Aliquots of lymphoid and splenic cells were restimulated in vitro with OVA peptide (323–339, 1 μM) for 72 h. (B) Flow cytometry plots and bars indicate the frequency of expanding OVA-specific CD4+ (KJ1-26/CD4) T cells. (C) Flow cytometry plots and bars display the frequency and absolute number of OVA-specific effector memory T cells producing IFN-γ (KJ1-26/IFN-γ). (D) Bars show the amount of IFN-γ measured in the supernatant of the same cultures. (E) Titers of OVA-specific IgG1, IgG2a, and IgG2b in the serum of mice. One representative of flow cytometry data is shown. Bars are expressed as means ± SEM; n = 8 (n denotes number of individual animals). **−NIC/I versus +NIC/I: p < 0.05.

**Administration of DCs differentiated in the presence of nicotine directs inadequate Th1 immunity in vivo**

We have reported (18, 20, 21) that both human and mouse DCs differentiated ex vivo in the presence of nicotine (henceforth called nicDCs) hardly support the differentiation of IFN-γ-secreting Th1 cells, whereas they potently promote the differentiation of IL-4-secreting Th2 cells. To determine the causal relationship between nicotine-induced DC alterations in vivo and the diminished host response to immunization, we first examined whether administration of nicDCs pulsed with OVA protein and matured with LPS direct adequate Ag-specific immune responses in mice adoptively transferred with OVA-specific T cells. Two weeks after DC vaccination, the cells isolated from the LNs and spleens of vaccinated mice were evaluated for the clonal expansion and frequency of adoptively transferred transgenic OVA-specific CD4+ T cells making either IFN-γ or IL-4 following restimulation with OVA peptide. We found that administration of both control DCs and nicDCs significantly increased the frequency of OVA-specific CD4+ T cells (from 0.41 ± 0.10 to 0.97 ±
0.11 and 0.83 ± 0.13%, respectively) (Fig. 3A). However, the frequency of IFN-γ–producing OVA-specific effector cells differentiated in response to nicDCs remained profoundly low even upon recall with OVA peptide (Fig. 3B). Accordingly, we found a significant reduction in IFN-γ protein detected in the supernatant released by these cells (Fig. 3B). Interestingly, we found that nicDC injection favored the differentiation of OVA-specific CD4+ T cells into IL-4–producing effector cells, as evidenced by an increase in the amount of IL-4 released by the isolated lymphoid and splenic cells in response to OVA peptide (Fig. 3C).

Nicotine exposure impacts DC subsets in vivo

Like other cell types within the immune system, DCs are continuously produced from hematopoietic stem cells within the BM. We previously reported (18, 20, 21) that both human monocyte-derived DCs and mouse BM-derived myeloid DCs generated ex vivo in the presence of growth factors GM-CSF/IL-4 during nicotine exposure produce substantially less Th1-promoting cytokine IL-12 in response to the Th1-polarizing adjuvant LPS. Prior to establishing whether nicotine interferes with the immunobiology of developing DC subsets in vivo, including myeloid, lymphoid, and plasmacytoid DCs, we first evaluated the extent to which the presence of nicotine in the microenvironment would affect the ex vivo differentiation of BM DC precursors into DC subsets in response to FLT3 ligand, the key growth and differentiation factor for DC subsets in vivo (27–29). We found that although nicotine somewhat reduced the yield of DCs generated (data not shown), it did not affect the development or the frequency of the three separate DC subsets differentiated from their precursors: CD11c+MHC II+CD11b+ myeloid (56.5 ± 7.2 versus 50.8 ± 5.3%), CD11c+MHC II+CD11b+B220– (and CD8α+ data not shown) lymphoid (21.9 ± 4.1 versus 29.7 ± 1.8%), and CD11c+MHC II+CD11b+B220+ plasmacytoid DCs (17.8 ± 4.2 versus 16.7 ± 1.3%) (Fig. 4A). However, it significantly compromised the ability of all DC subsets to produce the key cytokines IL-12 and IFN-α in response to Th1 adjuvants LPS and CpG (Fig. 4B). In fact, myeloid and lymphoid nicDCs secreted 3-fold and plasmacytoid nicDCs secreted 7-fold less IL-12 than control DCs in response to TLR-9 ligand CpGB. In addition, we found a 2-fold reduction in IFN-α released by the plasmacytoid nicDCs in response to CpGA (Fig. 4C).

We next evaluated the direct impact of nicotine exposure on DCs developed in vivo by assessing their frequency, costimulatory and migratory phenotypes, and ability to produce Th1-polarizing cytokine IL-12 in response to the Th1 adjuvants. We also examined whether DCs isolated from nicotine-exposed mice have the capability to prime Ag-specific immune responses ex vivo. Although we found an increase in the total number of splenocytes recovered...
from nicotine-exposed mice compared with control mice as previously reported (30) (Fig. 5F), our data suggest no significant differences in the frequency and absolute numbers of total splenic CD11c+MHC II+ DCs (Fig. 5A) and DC subsets within the CD11c+MHC II+ fraction (Fig. 5B). Compared to the DCs from the control mice, the DCs isolated from mice exposed to nicotine maintained the migratory molecule CCR7 expression (Fig. 5C); however, they displayed lower levels of costimulatory molecules CD40, CD80, and CD86 (Fig. 5C) and produced lower amounts of the cytokine IL-12 in response to the Th1 adjuvant CpG (Fig. 5D). Accordingly, we observed a shortage of IFN-γ released in culture supernatant in which CD11c+ DCs isolated from mice exposed to nicotine were used to prime transgenic OVA-specific CD4+ T cells in the presence of OVA peptide (Fig. 5E).

Nicotine exposure compromises the effectiveness of both prophylactic and therapeutic vaccines

We have demonstrated that exposure to nicotine impairs host ability to induce the development of effective immune responses by severely affecting their DC properties (Figs. 1–5). Vaccines, designed to enhance host immunity against infectious disease and cancer, depend on functional DCs. Thus, we next evaluated the amplitude and nature of the humoral and cell-mediated immunity developed in vivo in response to both prophylactic and therapeutic vaccines in nicotine-exposed mice. We implemented the OVA-EG7 immunogenic tumor model (31) used by others in preclinical studies testing vaccine formulation for cancer and infections (32–39). To study the effects of nicotine on prophylactic vaccination, WT C57BL/6 mice were implanted with osmotic pumps containing nicotine and then were vaccinated twice s.c. at the base of the tail with OVA protein mixed with the Th1 adjuvants LPS or CpG. One week later, the mice were challenged with OVA-EG7 immunogenic tumor cells. We subsequently measured the expansion of endogenous OVA-specific CD8+ T cells (detected by MHC I/OVA peptide pentamer), the percentage of effector cells, and their cytotoxic activity against target cells following a period of in vitro restimulation with OVA-pulsed DCs or OVA peptides. We also quantified the production of OVA-specific IgG in the sera. We observed that the administration of the vaccine to the mice resulted in control of tumor growth (0.75 ± 0.09 cm²) as compared with control mice (1.93 ± 0.16 cm²) (Fig. 6A). In contrast, the same vaccine was less protective, and the solid tumor grew steadily, reaching 1.47 ± 0.30 cm² in mice exposed to nicotine (Fig. 6A). Although the frequency of total CD4+, CD8+, and NK cells remained almost unchanged in these mice (Supplemental Fig. 1), we found a correlation among the tumor size, frequency,
The Journal of Immunology

FIGURE 5. Impact of nicotine exposure on DCs in vivo. WT mice were left intact (−NIC) or exposed to nicotine (1 mg/kg Bwt/d, +NIC) for up to 2 wk. (A) Gates and bars identify the CD11c/MHC II DCs, their frequency, and absolute number within total splenic cells. (B) Flow cytometry plots and bars identify the myeloid (mDC; CD11b+, B220−), lymphoid (LyDC; CD11b−, B220+), and plasmacytoid (pDC; CD11b−, B220+) DC subsets and their percentages within the CD11c/MHC II-positive fraction. Total DCs (CD11c+ fraction) were enriched from spleen tissue and analyzed by flow cytometry (C), activated with Th1 adjuvant (D), or loaded with OVA peptide (323-339, 1 μM) (E) and cocultured with OVA-specific CD4+ T cells isolated from OVA-transgenic mice. (C) Histograms display the expression levels of costimulatory and migratory molecules on DCs isolated from control (bold line) or nicotine-exposed (shaded area) mice and isotype control (dashed line). Bars show the mean fluorescence intensity of indicated cell-surface markers with isotype control subtracted. (D) Bars represent the amount of cytokine IL-12 released in the supernatant by DC from control or nicotine-exposed mice after 24 h in culture with the Th1 adjuvant CpG. (E) Bars depict the amount of Th1 cytokine IFN-γ released in culture supernatant after 3 to 4 d of coculture. (F) Bars show the absolute number of splenic cells recovered from control or nicotine-exposed mice. One representative of flow cytometry data is shown. Bars are expressed as means ± SEM; n = 9 (n denotes number of individual animals). **−NIC versus +NIC, p < 0.05.

and absolute number of endogenous OVA-specific CD8+ T cells in the draining lymphoid tissues (Fig. 6B). Additionally, in vaccinated mice in which tumor growth was contained, there was a significant increase in the percentage of OVA-specific memory CD8+ T cells, which could generate a rapid recall response to secondary Ag challenge when compared with control mice (Fig. 7A, Supplemental Fig. 2A). In contrast, the percentage of OVA-specific memory CD8+ T cells in nicotine-exposed mice remained low (Figs. 6B, 7A, Supplemental Fig. 2A). Interestingly, lymphoid cells isolated from vaccinated mice exposed to nicotine produced >60% less IFN-γ in response to OVA challenge (Fig. 7B, 7C) and exhibited markedly reduced CTL killing activity against target tumor cells when compared with unexposed vaccinated mice (Fig. 7D, Supplemental Fig. 2B). Accordingly, we found a significant reduction in the amount of OVA-specific IgG2a, a Th1 Ig in the sera of nicotine-exposed mice. However, the serum levels of Th2 Ig IgG2b but not IgG1 were increased in these mice (Fig. 7E).

For therapeutic vaccination, mice were injected in the midback with OVA-EG7 tumor cells. When the appearance of the tumor was
verified, the mice were vaccinated s.c. at the base of the tail with OVA protein mixed with the Th1 adjuvant LPS or CpG. We found that vaccination had a significant curative effect against tumor growth in vaccinated mice as compared with the naive mice (Fig. 8A, 8B). In mice exposed to nicotine, however, the same vaccine was less effective, and the solid tumor grew steadily (Fig. 8A, 8B). We also observed a significant reduction in the frequency and absolute number of endogenous OVA-specific CD8+ T cells in the lymphoid tissues of nicotine-exposed mice (Fig. 8C). When the lymphoid and splenic cells isolated from the nicotine-exposed vaccinated mice were rechallenged in vitro with OVA Ag, we found a significant reduction in the frequency of OVA-specific effector memory CD8+ T cells (Fig. 8D) and in their ability to produce the effector cytokine IFN-γ (Fig. 8E). Accordingly, these cells exhibited a reduced killing activity against target tumor cells when compared with the lymphoid cells obtained from control mice (Fig. 8F).

Discussion

Vaccines ensure global health security and are aimed at all target populations. Understanding the mechanism by which nicotine, a known immunosuppressive component of cigarette smoke, influences the immune system is important for understanding the reduced vaccine efficacy observed in smokers. In this study, we demonstrate that animals exposed to nicotine produced an inadequate effector/memory T cell population and Abs in response to protein-based vaccine formulated with Th1 adjuvants. In addition, the prime-boost vaccination recalled insufficient memory response and failed to protect the animals from an otherwise prophylactic and therapeutic vaccine. These observed immune alterations were due in part to the adverse effects of nicotine on DC function and polarization.

Our data suggest that following immunization, DCs were able to prime the adoptively transferred OVA-specific T cells, and the exposure of mice to nicotine had no effect on their proliferative response (Fig. 1A). Indeed, DCs differentiated in vivo under the influence of nicotine displayed a normal level of CCR7 expression (Fig. 5C) required for their entry into lymphoid tissues (40), which is consistent with a previous study showing that CCR7 expression in the DC population did not differ between smoke-exposed and control mice (41).

Feng et al. (42) reported that cigarette smoke-exposed mice had markedly reduced numbers of Ag-specific IFN-γ–producing T cells in response to immunization. Our data indicate that exposure to nicotine significantly compromised host immunity in response to immunization with the model Ag plus Th1 adjuvant by precluding the accumulation of OVA-specific IFN-γ–producing Th1 cells and Th1 helper function for OVA-specific IgG2a production (Figs. 1, 2). Additionally, our data suggest that nicotine exposure negatively influenced the quantity and quality of the generated memory pool in response to immunization. Indeed, we observed that the remaining memory T cell pools were unable to fully differentiate into IFN-γ–producing effector T cells in response to a secondary Ag challenge, which was more pronounced after the contraction phase (day 14) (Fig. 2C, 2D). These new data are consistent with our previous in vitro reported studies in which human nicDCs failed to support the progressive differentiation of naive T cells beyond central memory T cells (20). The low expression of costimulatory molecules CD40, CD80, and CD86 (Fig. 5C) and IL-12 (Fig. 5D) production in the DCs of nicotine-
exposed mice could have contributed to less accumulation of effector cells and hyporesponsiveness of the generated memory pool. Indeed, the Jenkins laboratory (43) demonstrated that the Th1 adjuvant-induced IL-12 was necessary to promote the generation of IFN-γ-producing T cells, which provide help for IgG2a production. Furthermore, DCs that express low surface expression of costimulatory molecules induce suboptimal T cell polarization (44).

Conflicting results have been reported with respect to the effects of cigarette smoke/nicotine on T cell response to mitogens. Several studies (45–48) showed no changes, whereas others (5, 13, 49, 50) showed decreased mitogen-induced T cell proliferation. We reported that nicotine did not affect T cell proliferation induced by anti-CD3/CD28 Abs (19). The reduction in the frequency of Ag-specific IFN-γ–producing T cells (Fig. 3B) and in the amount of IFN-γ produced (Fig. 5E) in response to in vitro and in vivo DCs in this study further support a DC-mediated impairment of immune responses.

In mice exposed to nicotine, prime-boost vaccination also failed to generate adequate tumor-specific CD8+ CTLs and OVA-specific Abs, allowing the transplanted tumors to continue to grow (Figs. 6–8). Impairment in DC characteristics and consequently CD4+ Th1 cell help may have resulted in lowered frequency of endogenous OVA-specific CD8+ effector T cells in these mice. The fact that DCs expressed less costimulatory molecules, particularly CD40 (Fig. 5C), could have impaired the necessary help for CD8+ T cell development. Indeed, early studies using immunization regimens suggested that CD4+ T cell help consisted of CD40L-mediated signals, conditioning DCs to become effective stimulators of naive CD8+ T cells (51). Additionally, in response to Th1 adjuvants, all DC subsets differentiated in vitro under the influence of nicotine (Fig. 4), and DCs isolated from nicotine-exposed mice (Fig. 5D), produced very low levels of IFN-α and IL-12 known to enhance IL-12 receptor β expression and IFN-γ production and killing activity in responding CD8+ T cells, respectively (52). Of note, IFN-γ has been shown to enhance CD8+ effector cells expression if highly expressed during the immune response (52). Thus, the low frequency of IFN-γ-producing CD4+ T cells in nicotine-exposed animals might have also contributed to the poor CD8+ effector T cell expansion and OVA-specific IgG2a Ab
production, known to enhance Ab-dependent cellular cytotoxicity in a tumor model (53).

Our data indicate that exposure to nicotine induces DC polarization that preferentially supports Th2-type responses. One DC polarization model posits that low costimulatory expression and absence of IL-12 production by DCs result in a default Th2 response (54). In addition, DC expression of the Notch ligands Jagged-1 and Jagged-2 can also contribute to a Th2 polarization (55). First, we found that nicDC administration preferentially induces Ag-specific effector T cells producing the Th2 cytokine IL-4 (Fig. 3C). Second, we observed (M. Nouri-Shirazi and E. Guinet, unpublished observations) that nicDCs express higher levels of Jagged genes, which may additionally add to their ability to preferentially drive Th2-type polarization. Finally, mice exposed to nicotine had altered DCs (i.e., reduced levels of the co-stimulatory molecules and IL-12) (Fig. 5C, 5D) and produced significantly more of the Th2 Ig isotypes IgG1 and IgG2b following immunization (Figs. 2E, 7E). Of note, a significant increase in the serum level of IgG2b observed in vaccinated C57BL/6 mice inoculated with OVA-EG7 tumor cells as compared with IgG1 isotype found in immunized BALB/c mice (Figs. 2E, 7E) could be the result of the genetic predisposition and/or the tumor environment. Indeed, it is reported that OVA-EG7 and its parental cell line thymoma EL4 secrete TGF-β (56) a cytokine known
to direct switch recombination toward the IgG2b isotype (57). Observations made in smokers further support a possible DC-associated Th2 polarization. For example, cigarette smoking has been associated with both increases in serum levels of total IgE and an increased risk of developing allergic-like symptoms (58, 59). Furthermore, IL-4 production by PBMCs of smokers is significantly higher than that of nonsmokers (60). Altogether, it is most likely that by altering DC function and polarization, nicotine reduced the host response to vaccination.

Pioneering works on the effects of nicotine on the immune system used several modes of nicotine delivery including s.c./i.p. injections (45, 61), nasal administration (61), nicotine patch (62), and the most widely used miniosmotic pump (14, 15, 63–67). We chose the osmotic pump mode of nicotine delivery because it mimicked the tendency of smokers to maintain a constant blood level of nicotine throughout the day (68) and had a human correlate: nicotine transdermal patches. The average cigarette contains ~10 mg of nicotine (69), and between 1 and 2 mg of nicotine is absorbed when a cigarette is smoked, with highly variable amounts achieved in the smoker’s blood (70, 71). It is reported that a dose of nicotine up to the levels of 2mg/kg Bwt/d in rodents produces plasma nicotine levels similar to those reported in active smokers (66, 72, 73). Of note, the concentration of up to 2 mg/kg Bwt/d also takes into consideration the rapid nicotine metabolism in mice compared with humans. Indeed, plasma nicotine 1/2 in rodents is generally shorter than in primates (i.e., 6 to 7 min in mice versus 2 h in humans and nonhuman primates), which necessitates the use of higher daily doses of nicotine in mouse models to achieve the blood nicotine concentrations comparable to those seen in smokers (74). In the current study, the dose of nicotine (1 mg/kg Bwt/d) was determined based on the published studies (14, 15, 65, 75, 76) and blood cotinine levels (±10 ng/ml) reported in smokers (77). The amount of cotinine measured in the serum of the animals in our study was 12.36 ± 3.76 ng/ml and therefore equivalent to what has been reported in human smokers (77) (Centers for Disease Control and Prevention, 2005 and 2010, http://www.cdc.gov/exposurerreport).

Despite the limitations in interpreting the data presented in this study to what happens in smokers due to species-specific variables such as genetic, allometry, and metabolism, our in vivo observations together with the decreased humoral Ab response reported in vaccinated smokers (7–9) indicate that exposure to nicotine could have significant and potentially adverse effects on the vaccination efficacy of a number of vaccines currently on the market and on experimental vaccines in the pipeline. Finally, this study lays the groundwork to test remedial strategies to correct the nicotine-induced defects in the DC system and improve vaccine efficacy through the use of immunological adjuvants. This idea is supported by our previous in vitro study reporting that an adjustment in adjuvant formulation corrects the nicDC ability to drive the progressive differentiation of naïve T cells toward effector Th1 cells with memory T cells capable of generating a rapid recall response to secondary Ag challenge (20).

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Disclosures

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References


The Journal of Immunology 2369


