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Cigarette Smoke Extract Suppresses Human Dendritic Cell Function Leading to Preferential Induction of Th-2 Priming[1]

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Dendritic cells (DC) are key regulators of immune responses. In the current study, we hypothesized that cigarette smoke-induced aberrance in DC function is an important mechanism by which smokers develop cancer, infection, and allergy—diseases common in smokers. We demonstrate that cigarette smoke extract (CSE) inhibits DC-mediated priming of T cells, specifically inhibiting the secretion of IFN-γ whereas enhancing the production of IL-4 in the MLR. Conditioning with CSE did not effect cytokine (IL-10, IL-6, or IL-12) production from immature DCs, but significantly inhibited IL-12p70 release by LPS-matured DCs. In contrast, IL-10 secretion by LPS-activated CSE-conditioned DCs was enhanced when compared with control DCs. CSE also induced cyclooxygenase-2 protein levels in maturing DCs and significantly augmented endogenous PGE_2 release. Conditioning of DCs with CSE also suppressed LPS-mediated induction of CD40, CD80, and CD86, and suppressed maturation-associated CCR7 expression. Although CSE has been reported to induce apoptosis of fibroblasts and epithelial cells, the immunomodulatory effects observed with CSE were not due to diminished DC viability. The effects of CSE on DC function were not exclusively mediated by nicotine, because equivalent, or even higher concentrations of nicotine than those found in CSE, failed to suppress DC-induced T cell priming. These data provide evidence that soluble components extracted from cigarette smoke suppress key DC functions and favor the development of Th-2 immunity. ***The Journal of Immunology,*** **2005, 175: 2684–2691.**

Cigarette smoking causes lung and other cancers, is a risk factor for metastatic spread of established cancer, and increases overall mortality in cancer patients (1–4). In addition, smokers have increased vulnerability to several infections and are predisposed to allergic airway diseases (5–7). These studies suggest that by altering host immunity, smoking enables tumor cells and infectious pathogens to evade appropriate immune responses. The increased incidence of asthma and airway hyperresponsiveness in smokers suggests that smoking alters the handling of inhaled Ag by the lung, favoring the development of Th-2 cell-mediated airway inflammation in response to inhalation of Ags that normally are ignored. Interestingly, epidemiological studies suggest that the incidence of hypersensitivity pneumonitis, a Th-1-mediated hypersensitivity reaction to inhaled Ag, is very unusual in cigarette smokers (8–10). These clinical studies provide indirect evidence that smoking alters immunity by impeding Th-1 immunity while potentially favoring the development of Th-2 responses that predisposes to the development of asthma. In the current study, we postulated that many of these effects might be mediated by altered dendritic cell (DC)[3] function induced by cigarette smoke.

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[3] Abbreviations used in this paper: DC, dendritic cells; CSE, cigarette smoke extract; COX, cyclooxygenase.

Lung DC can be found in the airways where they are distributed in a subepithelial location, and in the lung parenchyma where they are located in interstitial spaces (11, 12). The lungs are continually exposed to inhaled Ag from the environment. Similar to DC in other sites, pulmonary DCs become activated and mature following exposure to Ags, or danger signals such as proinflammatory cytokines and bacterial LPS. This transformation of DCs from immature to mature cells results in an up-regulation of costimulatory receptors and a switch in chemokine receptor expression, enabling homing to draining lymph nodes where mature DCs present processed Ag and stimulate specific T cell responses (13). In addition to Ag presentation, DCs have important functions in regulating the development of Th-1 and Th-2 responses (13). Secretion of IL-12 by DCs in the early inflammatory response critically affects the features of the resulting adaptive immune response (13, 14). For instance, through IL-12 production, DCs polarize naive helper T cells to Th-1 effector cells, which mediate critical aspects of cellular immunity. Simultaneously, IL-12 suppresses the differentiation of Th-2 cells, which control humoral immunity with the production of IgG1, IgE, and IgA (15). In the current study, we postulated that smoking alters DC function leading to diminished immunity to cancer and infection while enhancing proallergic Th-2 responses. Cigarette smoke extract (CSE) generated by the passage of mainstream cigarette smoke into buffered medium was used to test the effects of mainstream cigarette smoke on human DC priming and polarization of T cell responses.

Materials and Methods

Preparation of CSE

Aqueous CSE was prepared using a modification of the method of Blue and Janoff (16). The smoking apparatus consisted of a 50-ml plastic syringe with a three-way stopcock, to which a cigarette and a sterile plastic tip were attached. One end of the plastic tip was attached to the three-way stopcock and one end was immersed in a 50-ml conical tube containing 30 ml of sterile RPMI 1640 prewarmed to 37°C for 30 min. All materials used were sterile and used only once. CSE was prepared by drawing 35 ml of cigarette smoke into the syringe and then slowly bubbling the smoke into the medium. The cigarette was smoked up to 1-cm butt length. One cigarette was

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smoked per 10 ml of medium. Following preparation of CSE, the solution was filtered through 0.2-μm filters and used immediately or stored at −80°C. CSE was prepared using four different brands of cigarettes; Camel unfiltered, Virginia Slims, Marlboro lights, and Kentucky research cigarettes 1RF4. All of the data reported in this paper were generated using CSE prepared from Camel and Kentucky 1RF4 cigarettes, although identical results were obtained using CSE prepared from other brands of cigarettes. The highest concentrations of CSE used in experiments were chosen following determination of cellular viability that was no less than 95% of control cells cultured in the absence of CSE. The content of nicotine and other metabolites in CSE was analyzed in the institutional laboratory using liquid chromatography-tandem mass spectrometry: nicotine 246.4 ± 80.5 μg/ml, cotinine 1.03 ± 0.6 μg/ml in four separate samples of 100% CSE prepared from Camel unfiltered cigarettes.

Dendritic cell preparation and effect of CSE on dendritic cell maturation

Human monocytes were isolated from buffy coats obtained from healthy non-smoking adult blood donors following approval from the institutional review board. Monocytes were isolated using commercially available depleting Ab mixtures (RosetteSep; StemCell Technologies) and further purified by adherence to plastic for 1 h in complete medium. Human monocyte-derived DCs were generated by culturing monocytes for 5–7 days in RPMI 1640 supplemented with 5% FBS, 2 mM glutamine, and 800 U of GM-CSF (Immunex) and 5 ng/ml IL-4 (R&D Systems), as described previously (17). Maturation of DCs was induced by overnight culture with 100 ng/ml (unless stated otherwise) LPS (from *Escherichia coli*; Sigma-Aldrich). To determine the effect of CSE on DC maturation, immature DCs (1×10^6/ml) were incubated with 100 ng/ml LPS in the presence or absence of CSE (0–2%) or nicotine (0–200 μg/ml) (Sigma-Aldrich) overnight at 37°C. In parallel experiments, the effect of CSE or nicotine on immature DC phenotype was determined by the addition of CSE or nicotine for the final 24 h of a 7-day culture. Subsequently, surface molecule expression was determined using flow cytometry, and supernatants from DC cultures were either stored at −20°C or assayed immediately for cytokine content.

Mixed lymphocyte reaction

To determine the effect of CSE on DC-induced T cell proliferation, we assessed the affect of transient incubation of immature DCs on allogenic T cell proliferation in the MLR. Immature DCs were plated in complete medium supplemented with IL-4 and GM-CSF (1×10^6 per ml) in the presence of varying concentrations of CSE (0–2%) or nicotine for a final 18–24 h of culture. Following this, DCs were washed in fresh medium, irradiated (25 Gy), and added as stimulator cells to 96-well plates containing 2×10^5 responder allogenic T cells. T cells were obtained from (unrelated donor) buffy coats of non-smoking adult blood donors using a commercially available negative selection Ab-depleting mixtures (RosetteSep, StemCell Technologies), according to the manufacturers instructions. Following 72 h of coculture, cells were pulsed with 1 μCi/well of tritiated thymidine for the final 16 h of culture. Thereafter, cells were harvested and radioactivity measured using scintillation counting. In other experiments, cell proliferation was determined using a BrdU cell proliferation ELISA kit (Amersham Biosciences) according to the manufacturer's instructions. To determine the production of T cell cytokines induced by CSE-conditioned DCs, supernatants were saved following 24 h for measurement of IL-2 and 48 h for measurement of IL-4, IL-10, and IFN-γ.

Measurement of cytokines

Human IL-2, IFN-γ, IL-4, and IL-10 levels in the MLR were measured using ELISA according to the manufacturer's instructions (Biosource). Similarly, human IL-12p70, IL-10, and IL-6 were measured using commercially available ELISA (BD Biosciences) according to the manufacturer's instructions.

Flow cytometry and assessment of cellular viability

Flow cytometry was used to determine the expression of cell surface molecules. Cell surface staining was performed using FITC or phycoerythrin-conjugated Abs (BD Pharmingen): anti-CD14, anti-CD1a, anti-CD83, anti-CD80, anti-CD-86, anti-CD40, anti-MHC I, anti-HLA. CCR7 Ab was obtained from R&D Systems. Before staining with relevant Ab, DCs were incubated with an excess of human IgG (20 μg per sample) (Sigma-Aldrich) for 30 min to block nonspecific binding. Staining was performed on ice for 30 min. Isotype-matched Abs were used as controls. Ten thousand cells were acquired for each sample, and dead cells were gated out on the basis of light scatter properties. Acquisition and analysis of data was performed on a FACScan (BD Pharmingen).

Due to prior reports demonstrating a reduction in epithelial and fibroblast viability with the use of CSE (18), we determined DC viability following incubation with varying concentrations of CSE from a variety of commercially available cigarettes (Camel, Marlboro lights, Virginia Slims). Determination of apoptosis and necrosis was performed using conjugated PI and annexin V in accordance with the manufacturer's instructions (BD Pharmingen). Parallel results were obtained by determination of viability using the XTT calorimetric assay (cell proliferation kit II; Boehringer Mannheim). Concentrations of CSE that caused viability to be <95% of control by any of the aforementioned assays resulted in exclusion from further analysis.

Prostaglandin E_2 determination and intracellular flow cytometry

To determine the effect of CSE on prostaglandin E_2 release, human DCs were incubated with CSE for 12–16 h and then stimulated with LPS. PGE_2 levels in the supernatant were measured using a commercial ELISA kit (R&D Systems). Intracellular determination of cyclooxygenase-2 (COX-2) levels was determined by intracellular flow cytometry. Cells were fixed and permeabilized by washing with Cytofix/Cytoperm solution (BD Pharmingen), and stained with PE-conjugated COX-2 Abs (BD Pharmingen) in Perm/Wash solution (BD Pharmingen) according to the manufacturer's recommendations. Equivalent concentrations of isotype mouse Ab was used as control.

Statistical analysis

Data from the MLR experiments are shown as a percentage of the controls. Comparison between groups was performed using the Wilcoxon rank test. All other data are shown as the mean ± SEM unless otherwise stated and were tested for statistical significance using the Student *t* test. Statistical differences were considered to be significant if p was <0.05. Statistical analysis was performed using JMP 5.0.

Results

CSE inhibits DC-induced T cell proliferation

Monocyte-derived DCs were incubated with CSE for the final 18 h of a 7-day culture, irradiated, washed to remove residual CSE, and coincubated with allogenic T cells in a MLR (DC/T cell ratios of 1:5, 1:10, 1:20, and 1:40). Fig. 1*A* represents pooled data from eight individual experiments using DCs generated from eight donors. A statistically significant reduction in DC-induced T cell proliferation occurred with 2% CSE in which the nicotine concentration measured 3.8 μg/ml for Camel cigarette-derived CSE (Fig. 1*A*, $p < 0.05$ for all DC/T cell ratios, with the exception of the DC/T cell ratio of 1:40). A trend toward suppression of T cell proliferation was observed with 0.5 and 1% CSE-conditioned DCs (Fig. 1*A*), but this did not reach statistical significance due to variability in individual responses. Inhibition of DC-induced T cell proliferation occurred irrespective of the brand of cigarette used. To determine whether the inhibitory effect of CSE was caused by the nicotine component, we tested the effect of nicotine (0–200 μg/ml) on DC-mediated T cell responses to allogeneic Ags. These concentrations were selected to include a range of nicotine levels achieved in the blood stream of smokers (range of 10–100 ng/ml), as well as potentially higher nicotine levels achievable in local tissues exposed directly to cigarette smoke, such as the oral cavity and airways (19, 20). In contrast to CSE, nicotine did not inhibit DC-induced T cell priming (Fig. 1*B*). In individual experiments ($n = 2$), a small but statistically significant ($p < 0.05$) suppression of T cell proliferation occurred with nicotine concentrations ≥100 μg/ml when compared with controls (data not shown). However, the magnitude of suppression of DC-mediated T cell proliferation, even with nicotine concentrations of 200 μg/ml, was of significantly lesser magnitude than that observed with CSE (Fig. 1*B*). Identical results were obtained using nicotine from two different commercial sources (data not shown). Thus the observed inhibitory effect of CSE on T cell proliferation is unlikely to result exclusively from the nicotine present in CSE (nicotine concentrations

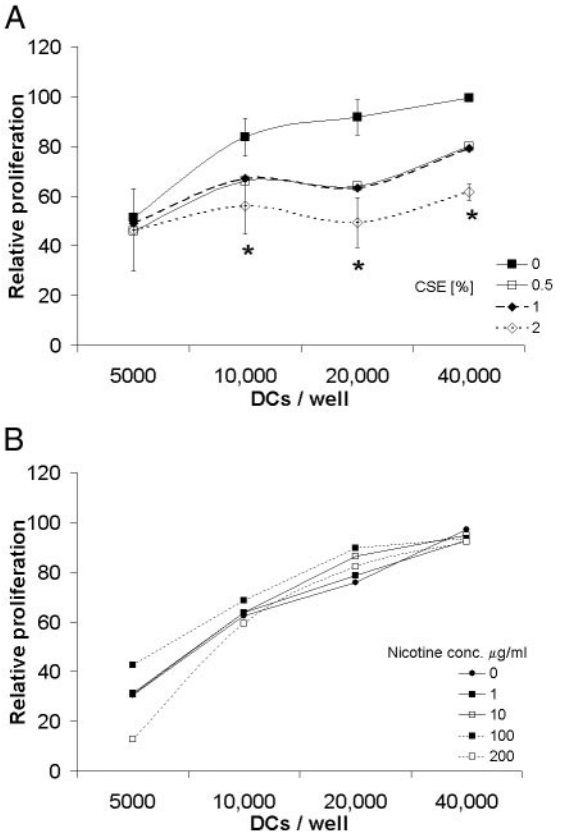

FIGURE 1. *A*, DCs were transiently incubated with CSE for 16–24 h and coincubated with allogenic T cells. Proliferation is represented as a percentage relative to control DCs without CSE (average cpm and SD for the 100% control response were $28.7 \pm 12.3 \times 10^3$). *A* represents pooled data from eight individual experiments using DCs generated from eight donors. Data represented as means together with SEM for 0 and 2% CSE groups. *B*, Pooled data from five individual experiments using nicotine-conditioned DCs generated from five donors. DCs were transiently incubated with nicotine (0–200 μg/ml) before incubation with allogenic T cells. Proliferation of nicotine-conditioned DCs is represented as percentage of the control DCs without nicotine (when measured with $[^3H]$, average cpm, and SD for the 100% control response were $54.3 \pm 0.3 \times 10^3$ (n = one experiment): measured by incorporation of BrdU, average OD, and SD for control response were 1.62 ± 1.3 (n = four experiments)).

in 0.5% and 2.0% Camel cigarette-derived CSE were 0.95 ± 0.01 μg/ml and 3.8 ± 0.04 μg/ml, respectively) (Fig. 1*A*).

CSE-modulated DCs selectively inhibit Th-1 and enhance Th-2 cytokine production from T cells

To further characterize the effect of CSE on the priming capacity of DC, we determined the effect of CSE on cytokine production in the MLR. Immature DCs were incubated with CSE (0–2%) for the final 16–24 h of a 7-day culture, washed, and coincubated with allogenic T cells in a MLR. Supernatants were taken from the MLR (2×10^5 T cells incubated with 2×10^4 DCs) and levels of IFN-γ, IL-2, IL-4, and IL-10 were measured. Transient incubation of immature DCs with CSE resulted in substantially less IFN-γ in the MLR when compared with control DCs (Fig. 2*A*; $p = <0.05$ for DCs conditioned with concentrations of CSE greater than 0.5% compared with control DCs). Interestingly, IL-2 production was not inhibited in the MLR with CSE concentrations up to 2% (Fig. 2*B*). In addition to suppressing the production of IFN-γ, CSE-conditioned DCs preferentially induced Th-2 cytokine production (Fig. 2, *C* and *D*). A significant increase in IL-4 production occurred in MLR reactions performed with CSE-conditioned DCs (Fig. 2*C*; $p = 0.037$ for IL-4 production by T cells incubated with 2% CSE-conditioned DCs compared with controls). To a lesser extent, IL-10 production was also enhanced in the MLR induced by CSE-conditioned DCs, although this did not reach statistical significance (Fig. 2*D*). Therefore, CSE-conditioned DCs preferentially inhibit Th-1 cytokine production, and stimulate Th-2 cytokine production.

CSE suppresses IL-12 and enhances IL-10 release by maturing DCs

The observation that CSE alters the priming capacity of DC led us to speculate that CSE might alter DC maturation. We next determined the effect of CSE in maturation of monocyte-derived DC. LPS-stimulated DCs produce substantial amounts of IL-12 (Fig. 3*A*). The production of IL-12 was markedly suppressed by concentrations of CSE as low as 0.1% and was inhibited in a dose-dependent fashion with maximal inhibition occurring with 2% CSE; 13.3 ± 15.1 pg/ml vs 802.2 ± 208.1 pg/ml IL-12 produced by the control DCs stimulated with LPS in the absence of CSE ($p = 0.009$, Fig. 3*A*). In sharp contrast, the addition of CSE to LPS-activated DC resulted in augmentation of IL-10 release (Fig. 3*B*). A statistically significant increase in IL-10 production occurred in a dose-dependent manner with increasing concentrations of CSE (Fig. 3*B*). Maximally, in the presence of 2% CSE, LPS-activated DCs produced 1201.9 ± 32.8 vs 935.3 ± 33.6 pg/ml IL-10 produced by DCs activated by LPS in the absence of CSE ($p = 0.007$, Fig. 3*B*). Although an increase in IL-10 secretion occurred with CSE conditioned DCs, this increase in IL-10 secretion was not of the same magnitude as the observed suppression in IL-12 secretion. Fig. 3*B* also demonstrates that although CSE augments LPS-induced DC production of IL-10, CSE alone does not induce IL-10 release, indicating that the effect of CSE on DC cytokine release is dependent on their state of activation. To further expand the observation that CSE inhibits specific cytokine production during the maturation of DCs, we also tested the effect of CSE on LPS-induced IL-6 release from DCs. Immature DCs produce substantial IL-6 following stimulation with LPS (Fig. 3*C*). Concentrations of CSE that completely suppress LPS-induced IL-12 release from DCs did not alter IL-6 production (Fig. 3*C*). These data indicate that CSE inhibits the production of specific cytokines during DC maturation and suggest that the effects of CSE on DCs are not due to nonspecific toxicity.

CSE does not alter immature DC phenotype, but suppresses maturation-associated costimulatory and chemokine receptor expression

Because of the observed effect of CSE on immature DC-induced T cell priming, we next examined whether CSE inhibits expression of Ag-presenting molecules and costimulatory molecules on immature and mature human DCs. To determine this, immature DCs at day 6 were incubated for a final 24 h with varying concentrations of CSE (0–2%). CSE generally did not suppress the expression costimulatory molecules including CD40, CD80, and CD86, or MHC class II expression on immature DCs (Fig. 4*A*). In addition, CSE did not alter the expression of CD83, B7-H1, B7-H2, or MHC class I on immature DCs (data not shown). These findings are consistent with the prior observation that cytokine secretion by immature DCs is not altered by CSE (Fig. 3). In parallel to the lack of effect of nicotine on human DC-mediated T cell priming, the expression of CD40, CD80, CD86, CD1a, or MHC class II expression on immature human DCs was not altered by nicotine at concentrations of 0.1–100 μg/ml (data not shown). This finding is in contrast to a recent study demonstrating that nicotine enhances

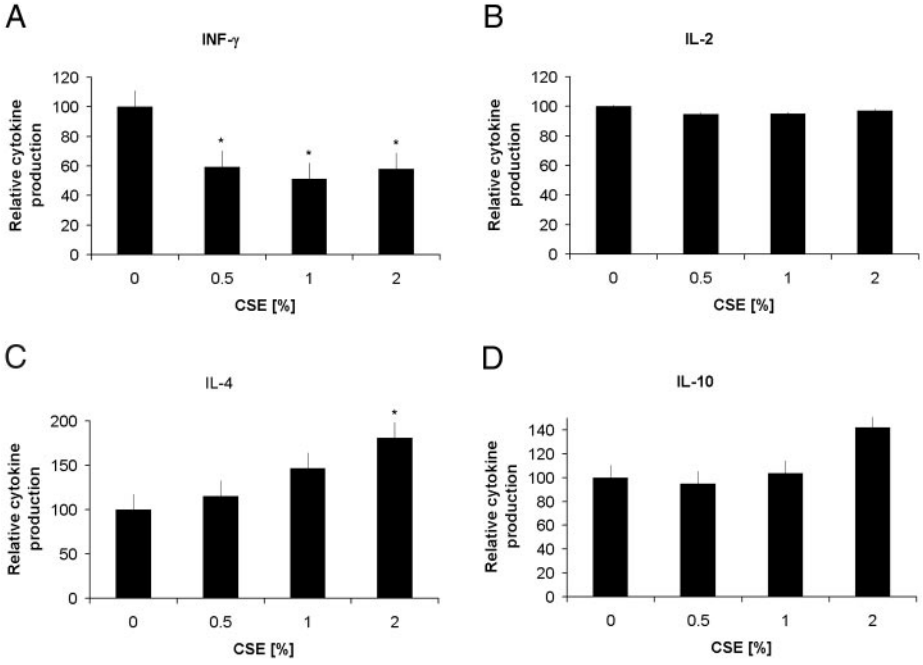

FIGURE 2. Selective inhibition of IFN-γ production in MLR. Supernatants from the MLR of 2×10^4 DC-conditioned with CSE and 2×10^5 allogenic T cells were assayed for IFN-γ, IL-2, IL-4, and IL-10 by ELISA. *A*, Transient incubation of immature DCs with CSE resulted in substantially less IFN-γ in the MLR when compared with control DCs (∗, $p < 0.01$). *B*, IL-2 production was not inhibited in the MLR. *C*, In contrast to IFN-γ, an increase in IL-4 production occurred in the MLR with CSE concentrations ≥0.5% (∗, $p < 0.01$ compared with control). *D*, Similar to IL-4 production, IL-10 production was also enhanced in the MLR.

costimulatory expression on murine bone marrow-derived DCs (21). Identical results were obtained when nicotine from two different commercial sources were used (data not shown).

Upon maturation, DCs undergo profound changes resulting in up-regulation of costimulatory molecules, secretion of IL-12, and up-regulation of CCR7. Because our prior experiments demonstrated marked suppression in LPS-induced DC IL-12 release by CSE, we speculated that one of the mechanisms by which CSE suppresses DC-mediated T cell priming is by suppressing the maturational response of DCs. To determine this, DC maturation was induced with LPS in the presence of varying concentrations of CSE and nicotine. The coincubation of DCs with LPS and CSE resulted in significant inhibition in costimulatory molecule up-regulation (Fig. 4*B*). Although different donor DCs varied in the degree of suppression of costimulatory molecule up-regulation, the trend was consistent among all donors tested (Fig. 4*C*). Interestingly, the inflammatory up-regulation of CD40 and CD80 were the most consistently suppressed by CSE, while up-regulation of CD86 was least affected (Fig. 4*C*). Consistent with our prior observation that nicotine does not significantly suppress DC-induced T cell proliferation, we found that nicotine failed to suppress the inflammatory up-regulation of CD40, CD80, or CD86 expression (Fig. 4*B*). DCs incubated with nicotine concentrations ranging from 1 to 100 μg/ml resulted in no alteration in costimulatory or MHC class I or class II expression, with the exception of occasional mild suppression in CD40 and CD80/86 expression with the highest concentrations of nicotine used (100 μg/ml; data not shown). These findings were reproduced using DCs derived from four independent donors.

Upon maturation, up-regulation of DC CCR7 occurs, a change that confers to the DC enhanced migratory capacity through endothelial cells eventually to secondary lymphoid organs expressing corresponding ligands. We speculated that CSE suppresses LPS-induced up-regulation of CCR7, thereby impairing another critical function of the maturational process of DCs. CSE did not alter the low-level expression of CCR7 on immature DCs, but substantially inhibited LPS-induced up-regulation of CCR7 (Fig. 4*D*). This also occurred in a dose-dependent fashion with maximal inhibition occurring with higher concentrations of CSE. Consistent with the lack of effect on costimulatory molecules, nicotine also failed to alter expression of CCR7 on either immature or LPS-matured DCs (data not shown).

CSE-induced suppression of DC function is not a consequence of cell death

To ensure that the observed suppression of DC function by CSE did not result from nonspecific cellular toxicity and death, we determined viability following incubation of DCs with varying concentrations of CSE. Following 7 days in in vitro culture, ~15 ± 5% of cells undergo spontaneous apoptosis (Fig. 5). The addition of CSE at concentrations observed to suppress DC functions (0–2%) did not increase either apoptosis or necrosis following overnight culture (Fig. 5). These observations are also consistent with the fact that DC secretion of IL-10 and IL-6, as well as expression of MHC class II is unaffected by CSE at the concentrations used in this current study, indicating that the observed suppressive effects of CSE on DC functions are not due to nonspecific cellular toxicity. Identical results were obtained when viability was measured using the commercially available XTT calorimetric assay (Roche) or trypan blue exclusion (data not shown).

CSE enhances PGE_2 release by LPS-matured DCs through induction of COX-2

Cyclooxygenase-2 (COX-2) is the inducible, rate-limiting enzyme in prostaglandin synthesis (22). Because COX-2-mediated PGE_2 production has been demonstrated to induce IL-10 and suppress bioactive IL-12p70 production in DCs (23, 24), we tested whether CSE induced PGE_2 release by maturing DCs. Immature DCs (day 5–6) were incubated with CSE and LPS for 12–16 h, and PGE_2 levels were measured using ELISA. CSE dose dependently increased PGE_2 production by LPS-activated DCs (Fig. 6*A*). The increased secretion of PGE_2 by activated CSE-conditioned DCs correlated with diminished IL-12p70 release (data not shown). Determination of intracellular COX-2 demonstrated a parallel increase in inducible COX-2 protein levels in CSE-conditioned DCs compared with control DCs. Whereas COX-1 is constitutively expressed in DCs, COX-2 is induced by a variety of inflammatory stimuli, including LPS (Fig. 6*B*). Conditioning of DCs with CSE

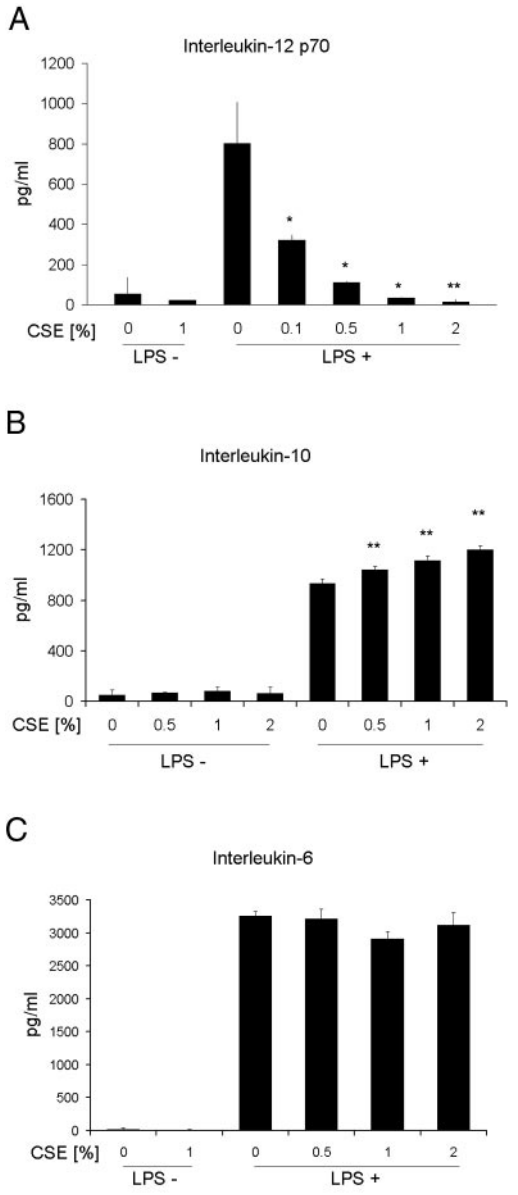

FIGURE 3. CSE selectively suppresses IL-12 production, and enhances IL-10 release by maturing DCs. Immature DCs (1×10^6/ml) were incubated with 100 ng/ml LPS in the presence or absence of CSE (0–2%) overnight at 37°C. Cytokines in the supernatants were measured by ELISA. *A*, LPS-stimulated DCs produce substantial amounts of IL-12 that was suppressed in a dose-dependent fashion by increasing concentrations of CSE (*, $p < 0.05$ and **, $p < 0.01$ compared with control). *B*, In contrast, CSE-conditioned DCs produced more IL-10 than controls (*, $p < 0.05$ compared with control). *C*, CSE does not alter LPS-induced IL-6 production. Experiments show mean and SD from a representative of six independent experiments (for IL-12 and IL-10) and two independent experiments (for IL-6).

before stimulation with LPS for 6 h resulted in substantial increase in inducible COX-2 levels (Fig. 6*B*).

Discussion

In this article, we provide evidence that CSE causes specific defects in DC function by suppressing DC-mediated priming of T cells and specifically inhibiting key Th-1 cytokine production and favoring development of Th-2 responses. CSE also suppresses maturation-associated up-regulation of costimulatory molecules and CCR7, resulting in a state of arrested DC maturation. Our results support the notion that smoking has important inhibitory effects on DC functions and skews immunity by suppressing DC-mediated Th-1 responses.

Transient exposure of immature DCs to CSE significantly impaired their capacity to induce T cell proliferation and specifically inhibited the production of IFN-γ in the MLR. The mechanisms by which this effect occurs are not fully understood. However, the current study suggests two important mechanisms by which CSE suppresses DC-mediated T cell priming; the suppression of maturation-associated IL-12p70 release and diminished costimulatory molecule expression. The suppression of IL-12 production and costimulatory molecule expression was only observed on activated DCs, which implies that CSE alters the ability of DCs to react in the face of danger, rather than in the immature or steady state. In addition to the effect of cytokines and accessory surface receptors, it is possible that CSE diminishes the priming capacity of DCs by other mechanisms, such as depletion of intracellular glutathione stores (25), suppression of NO release (26, 27), and induction of inhibitory cell surface receptors such as ILT4 or novel members of the B7 family (28–30). Oxidative stress induced by reactive oxygen species in CSE may also impair DC-T cell communication. However, oxidized protein products have been reported to enhance, rather than suppress DC function (31–33). The diminished priming capacity of CSE-conditioned DCs may also result from alterations in the activity of transcription factors like NF-κB or AP-1 (34–36).

An important effect of CSE on DC function is the preferential induction of Th-2 responses. Epidemiologic, laboratory, and clinical studies clearly demonstrate that cigarette smoking is a risk factor for the development of Th-2-mediated diseases like asthma (5, 6, 37–41). The ability of DCs to generate Th-1 or Th-2 immunity is regulated by many factors, including Ag type and dose, the level of costimulatory molecules expressed, the presence of polarizing cytokines and other mediators, and the presence of innate receptor stimulants at the time of Ag exposure. The current study suggests that CSE-conditioned DCs may preferentially polarize to Th-2 immunity because of suppressed signaling through the CD40 pathway. In macrophages, CD40 signaling has an important role in regulating the secretion of Th-1 vs Th-2 polarizing cytokines (42). Suboptimal stimulation of the macrophage CD40 receptor induced the ERK-1/2 MAPK pathway leading to induction of IL-10, whereas more robust stimulation induced the p38 MAPK-dependent IL-12 pathway (42). Although the effect of dose-dependent activation of the DC CD40 pathway is not established, it is possible that CSE polarizes to Th-2 immunity by diminishing activation though DC CD40-CD40L pathways. The enhanced production of PGE_2 by CSE-conditioned DCs represents another mechanism by which Th-2 priming is favored. In the current study, we demonstrate that endogenous DC PGE_2 release is enhanced by CSE and is associated with induction of the COX-2 pathway. PGE_2 effects many DC functions (22). PGE_2 alters DC polarizing capacity directly through suppression of IL-12 release and augmentation of IL-10 production, and indirectly through effects on T cell function (22). Our findings are consistent with the observation that inhibition of COX-2 and its downstream metabolite PGE_2 in mice leads to an increase in IL-12 production and decreases IL-10 release by APCs (24).

The effects of PGE_2 on DC functions are not fully understood. Most studies concur regarding the role of PGE_2 as a promoter of Th-2 responses through it's effects on IL-10 and IL-12 release (23, 43, 44). However, others have reported that exogenous PGE_2 added to human DC cultures promotes differentiation of naive T

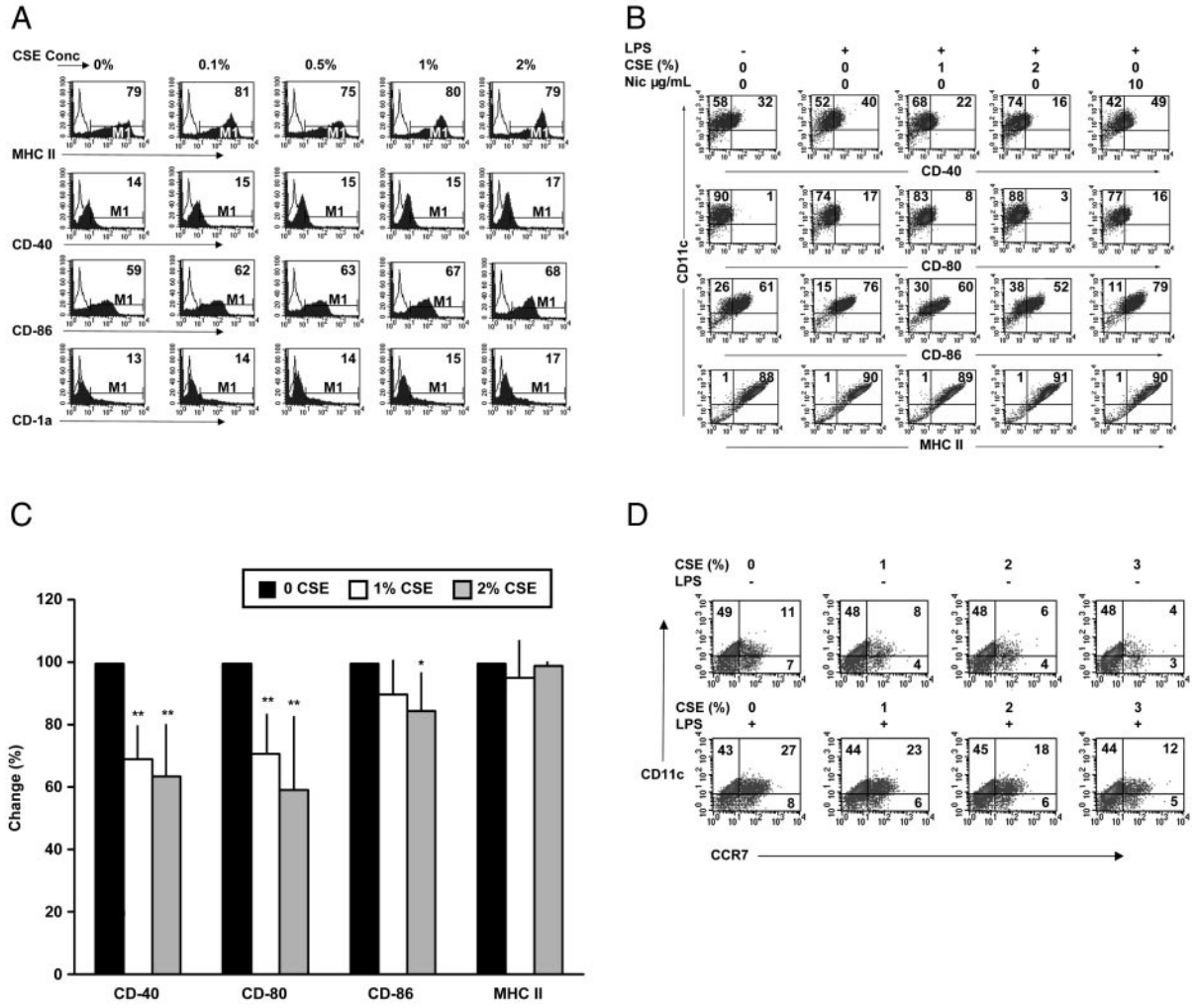

FIGURE 4. *A*, Immature DCs were incubated with varying concentrations of CSE for the final 24 h of a 7-day culture. Surface receptor expression was determined with flow cytometry. The numbers in the histograms refer to the percentage of positive cells. The experiment shown is representative of six independent experiments. *B*, Immature DCs were coincubated with varying concentrations of CSE (0–2%) and LPS (100 ng/ml) for the final 24 h of a 7-day culture. Surface receptor expression was subsequently determined with flow cytometry. The numbers in the quadrants refer to the percentage of cells in that quadrant. *C*, Although different donor DCs varied in the degree of suppression of costimulatory molecule up-regulation, the trend was consistent among all donors tested ($n = 4$, *, $p < 0.05$ and **, $p < 0.01$ compared with control). *D*, Immature DCs were incubated with varying concentrations of CSE (0–2%) in the presence or absence of LPS (100 ng/ml) for the final 24 h of a 7-day culture. Cells were then double stained with PE-labeled CD11c and FITC-labeled CCR7 Abs. The numbers in the quadrants refer to percentage of positive cells in each quadrant. The experiment shown is representative of three independent experiments.

cells into Th-1 cells (45), and contributes to the maturation of human DCs through the induction of IL-12 production (46). Although enhanced PGE_2 release by CSE-conditioned DCs may explain their preferential Th-2 priming, PGE_2 is unlikely to be accountable for the diminished T cell priming capacity or CCR7 up-regulation observed in CSE-conditioned DCs. Indeed, PGE_2 has become a standard component of maturation mixtures used to generate functional DCs in cancer vaccine trials and is considered a critical factor for the acquisition of CCR7 expression upon maturation (47, 48). It is possible that prostaglandins other than PGE_2 or other downstream products of the COX-2 pathway may be involved in mediating the effects of CSE on DCs.

The active component/s in CSE responsible for inhibition of DC function are unknown. Nonetheless, the observed inhibitory effects are not mediated by the nicotine component, at least not exclusively. In two experiments using DC generated from two separate donors, significant reduction in T cell proliferation occurred with DC incubated with nicotine concentrations greater than 100 μg/ml (data not shown). The physiological relevance of this is unclear because blood nicotine levels in smokers are in the range of 10–100 ng/ml (20). Although nicotine has been reported to suppress T cell function in rodents (49, 50), its effect on DC function is controversial. Two independent groups almost simultaneously reported contrasting effects of nicotine on a variety of DC functions. Nouri et al. (51) reported that, in the presence of nicotine, monocyte-derived DCs manifest lower endocytic and phagocytic activities, produce decreased levels of proinflammatory cytokines, notably IL-12, and have a reduced ability to stimulate Ag-presenting cell-dependent T cell responses. In contrast, Aicher and colleagues (21) reported that nicotine dose dependently enhanced DC costimulatory molecule expression, enhanced IL-12 and IL-10 release, and augmented the T cell priming ability of DCs. Our findings are consistent with Nouri et al. (51), although in contrast to their study, we found that nicotine inconsistently inhibited DC priming. In addition, the inhibitory effect was of much smaller magnitude than that achieved with CSE containing corresponding

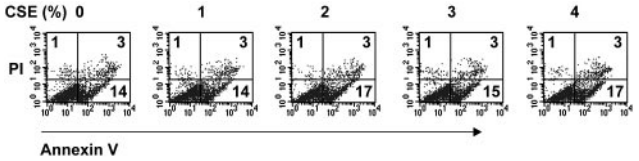

FIGURE 5. Immature DCs were incubated with varying concentrations of CSE (0–4%) for the final 24 h of a 7-day culture. Cells were then double stained with PI and annexin V and analyzed by flow cytometry. The percentage of positive cells is indicated in the quadrants. The experiment shown is representative of four independent experiments.

levels of nicotine. Although nicotine may suppress certain DC responses, the physiological relevance is uncertain, because concentrations of nicotine in excess of 100 μg/ml are probably never attained in vivo. Nevertheless, our findings do not indicate a role for nicotine as a potent inducer of DC maturation as reported by Aicher et al. (21). It is possible that although physiologically relevant concentrations of nicotine have minimal effects on DC function, interactions or synergy of nicotine with other chemicals in cigarette smoke such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (52), carbon monoxide, or benzo[*a*]pyrene may be responsible for significant inhibitory effects on DC function. Understanding potential effects of nicotine on host immunity is important clinically because nicotine replacement therapy is used in the management of smoking cessation and is also under investigation as a pharmacological agent for the treatment of Alzheimer's disease (53), Parkinson's disease (54), and autoimmune diseases (55). Based on the findings by Nouri et al. (51) and our data, we believe that at concentrations usually achievable by nicotine replacement therapy, it is unlikely that nicotine therapy exerts important inhibitory effects on DC functions.

Although the physiological significance of CSE may be challenged, it remains a useful model to study alterations in cellular function induced by smoking. The nicotine content of CSE enables correlation with the in vivo dissolution and absorption of soluble cigarette smoke components that occur during cigarette smoking. The effects reported in this study are unlikely to result from nonspecific down-regulation of DC function by CSE, because production of other critical DC cytokines such as IL-6 are unaffected, or in the case of IL-10, are actually enhanced. Although CSE is reported to induce apoptosis in epithelial cells and fibroblasts (56, 57), our data also argue against this possibility, because assessment of cellular viability demonstrate that CSE did not induce cellular death at the concentrations used in the current study. The majority of nicotine absorbed from the lungs is converted to cotinine by hepatic enzymes. Accordingly, the steady-state levels of nicotine in the blood of smokers may be substantially lower than those achieved locally in the airways. For this reason, even though the levels of nicotine achieved in CSE preparations that suppressed DC activity are slightly higher than what is generally achieved in the blood of active smokers, we believe that our findings are physiologically relevant.

The defects in DC function described in the current study are relevant to the pathogenesis of cancer, in which impaired DC function is increasingly recognized (30, 58). In the current study, we propose cigarette smoking-induced suppression of host DC functions as an important mechanism by which smokers develop cancer. Through an imbalance in IL-12/IL-10 release, smoking may promote a tolerogenic state to tumors, thereby increasing the occurrence of not only lung cancer, but also a variety of other systemic malignancies. Immune therapies aimed at reversing the effects of smoking on DC function may be beneficial in the management of cancers occurring in smokers. The data reported in the current study provide additional motives for all patients with cancer to refrain from smoking. Understanding specific defects in immunity of smokers may ultimately provide novel opportunities for management and prevention of disease that afflicts smokers.

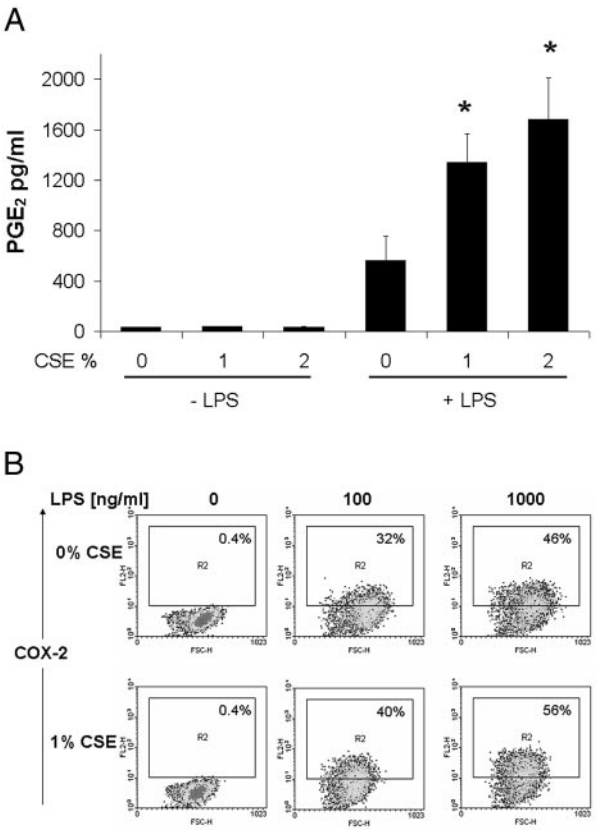

FIGURE 6. CSE induces COX-2 and enhances PGE_2 production by maturing DCs. DCs (1×10^6/ml) were incubated with CSE (0–2%) for 16 h before stimulation with LPS. *A*, CSE dose dependently increased PGE_2 production by LPS-activated DCs (*, $p < 0.01$). PGE_2 levels in the supernatant were measured by ELISA. Data represented as means and SD. Data representative of three independent experiments. *B*, Determination of intracellular COX-2 protein levels demonstrated a parallel increase in inducible COX-2 protein levels in CSE-conditioned DCs compared with control DCs. Intracellular COX-2 levels were determined by flow cytometry. The numbers in the upper right corners refer to the percentage of positive cells. The experiment shown is representative of two independent experiments.

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Disclosures

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