

Diesel Exhaust Particle-Exposed Human Bronchial Epithelial Cells Induce Dendritic Cell Maturation¹

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Increased exposure to air pollutants such as diesel exhaust particles (DEP) has been proposed as one mechanism to explain the rise in allergic disorders. However, the immunologic mechanisms by which DEP enhance allergic sensitization and asthma remain unclear. We hypothesized that DEP act as an adjuvant for immature dendritic cell (DC) maturation via its effect on airway epithelial cell-derived microenvironment for DC. Immature monocyte-derived DC (iMDDC) failed to undergo phenotypic (CD80, CD83, CD86) or functional (T cell activation) maturation in response to exposure to DEP (0.001–100 $\mu\text{g/ml}$). In contrast, primary cultures of human bronchial epithelial cells (HBEC) treated with DEP induced iMDDC phenotypic maturation (2.6 ± 0.1 -fold increase in CD83 expression, $n = 4$, $p < 0.05$) and functional maturation (2.6 ± 0.2 -fold increase in T cell activation, $n = 4$, $p < 0.05$). Functional maturation of iMDDC was induced by conditioned medium derived from DEP-treated HBEC, and was inhibited in cultures with DEP-treated HBEC and blocking Abs against GM-CSF, or GM-CSF-targeted small interfering RNA. These data suggest that DEP induce Ag-independent DC maturation via epithelial cell-DC interactions mediated by HBEC-derived GM-CSF. Although additional signals may be required for polarization of DC, these data suggest a novel mechanism by which environmental pollutants alter airway immune responses. *The Journal of Immunology*, 2006, 176: 7431–7437.

The prevalence of atopy and asthma has increased worldwide (1). Allergic disorders result from T cell-derived immune responses that are the consequence of a complex interaction between environmental exposures and genetic susceptibility (2). Although increasing industrialization and urbanization of populations with a concomitant increase in exposure to air pollutants such as ambient particulate matter (PM)³ have been proposed as a mechanism to explain the rise in allergic disorders, the immunologic mechanisms by which ambient PM enhance allergic sensitization and asthma remain unclear (3).

Allergic asthma is characterized by a skewed polarization of T lymphocytes toward a predominant Th2 phenotype (4). The paradigm of Th1/Th2 polarization has recently been expanded to include the presence of a class of CD4⁺ regulatory T cells capable of suppressing Th1 and Th2 cell functions (5–7). Elegant studies have shown that CD4⁺ regulatory T cells inhibit Th2 cell functions such as IgE production (8) and induce tolerance to inhaled allergen

in mice (9). Thus, the possibility exists that sensitization to an allergen can be due to a biased Th2 response or the loss of CD4⁺ regulatory T cell function to a specific Ag. The T cell response however, is determined by signals provided to T cells during Ag exposure.

Ag-specific T cell responses result from engagement of naive T lymphocytes by APC (10). Dendritic cells (DC), professional APC, are abundant in the airway as a meshwork in close proximity to airway epithelial cells (AEC) (11, 12). DC traffic rapidly through the airway and have been proposed to act as sentinels in the airway, determining the response to inhaled agents such as allergens, pollutants, or bacteria (13). Although the recruitment of DC to the airway is critical for sampling and phagocytosis of allergens by DC, the polarized or suppressor T cell response to an Ag is mediated by the maturation state of DC and the polarizing signals expressed by DC (14–16). Although some allergens induce DC maturation and polarization, resulting in T cell activation, many allergens are “harmless,” and fail to induce DC maturation or polarization on their own; they may require additional signals to induce a mature DC response (14, 17). These signals have been suggested to include those derived from members of the TLR family, but may also include those derived from the tissue microenvironment.

Allergic urban asthma in children and adults is associated with sensitization to a multitude of indoor allergens including those derived from cockroaches and house dust mites (18, 19). However, the mechanisms by which local sensitization to these allergens develop and persist remain unclear; a role for adjuvant signals derived in response to ambient pollutants can be hypothesized (20). Diesel exhaust particles (DEP) are a major component of ambient PM. In animal models, DEP act as an adjuvant and induce sensitization to neoallergens (21). The possibility exists that ambient PM or DEP directly act on DC to enhance sensitization. Alternatively, because AEC are the site of first contact with environmental pollutants and are in close proximity to DC, it is reasonable to suggest that adjuvant signals may be derived from these

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³ Abbreviations used in this paper: PM, particulate matter; Treg, regulatory T cell; DC, dendritic cell(s); MDDC, monocyte-derived DC; iMDDC, immature MDDC; DEP, diesel exhaust particle(s); HBEC, human bronchial epithelial cell(s); AEC, airway epithelial cell; RISC, RNA-induced silencing complex; siRNA, small interfering RNA; CK, cytokeratin; MC, maturation cytokine.

cells. We and others have previously shown that ambient PM or DEP-treated AEC release chemokines with potential to induce DC migration, and cytokines with the potential to participate in the functional maturation of DC (22–26). We therefore hypothesized that DEP act as an adjuvant for DC maturation via an effect on AEC in the microenvironment. We demonstrated that DEP-treated human AEC induce phenotypic and functional maturation of DC and suggest that the effect of DEP on DC maturation is mediated in part by epithelial-cell derived GM-CSF.

Materials and Methods

Reagents

DMEM, MEM, penicillin-streptomycin, FBS, trypsin-EDTA solution, and PBS were purchased from Invitrogen Life Technologies. Bronchial epithelial cell growth medium and bronchial epithelial cell basal medium were purchased from Cambrex BioScience. Ficoll was obtained from Amersham Biosciences, and a magnetic cell separator from MACS (Miltenyi Biotec). GM-CSF and IL-4 were obtained from PeproTech. Fluorescent reagents for FACS analyses were obtained from BD Immunocytometry Systems, BD Pharmingen, or Coulter/Immunotech. BrdU ELISA was obtained from Roche and GM-CSF ELISA kits were obtained from R&D Systems. LPS was derived from *Escherichia coli* O111:B4 and was purchased from Sigma-Aldrich.

DEP were derived from a 1.6-liter Volkswagen diesel engine (40 kW) running under standard city driving cycle conditions according to U.S. test protocol FTP 72 (U.S. Environmental Protection Agency, 1992) and were a gift of D. L. Costa (U.S. Environmental Protection Agency). Although detailed information from this automobile engine are lacking, detailed comparisons of particles derived by this protocol in automobile engines compared with those derived from a forklift generated sample (National Institute of Standards and Technology) have been published (27, 28). DEP were diluted in cell culture medium, vortexed (5 times, 10 s), sonicated (1 min), and added to cells in the defined concentrations. Because DEP sediment to the bottom of cell culture dishes, DEP concentrations were based on the available surface area ($\mu\text{g}/\text{cm}^2$). The related volume-based concentration ($\mu\text{g}/\text{ml}$) is also stated in the text, as soluble components of DEP may participate in cell responses. Endotoxin content of DEP was measured using the PyroGene Recombinant Factor C assay (Cambrex BioScience) according to the manufacturer's recommendations. Endotoxin activity in DEP (100 $\mu\text{g}/\text{ml}$), a concentration that was 10 times higher than that used in most of the experiments, was below the lower limit of detection (0.01 EU/ml). Effect of DEP (0.01–100 $\mu\text{g}/\text{ml}$, 48 h) on cell viability of HBEC, 16HBE, monocyte-derived DC (MDDC), and T cells was measured using Alamar Blue (BioSource International) according to the manufacturer's instructions.

Cells

Human bronchial epithelial cells (HBEC) were obtained by endobronchial brushing during bronchoscopy of normal human volunteers following a protocol approved by the Institutional Review Board of the New York University School of Medicine. HBEC were plated in bronchial epithelial cell basal medium supplemented with human epidermal growth factor (0.5 ng/ml), hydrocortisone (0.5 $\mu\text{g}/\text{ml}$), insulin (5 $\mu\text{g}/\text{ml}$), transferrin (10 $\mu\text{g}/\text{ml}$), epinephrine (0.5 $\mu\text{g}/\text{ml}$), tri-iodothyronine (6.5 ng/ml), gentamicin (50 $\mu\text{g}/\text{ml}$), amphotericin B (50 ng/ml), bovine pituitary extract (52 $\mu\text{g}/\text{ml}$), and retinoic acid (0.1 ng/ml) and cultured on tissue culture plates coated with human collagen (Vitrogen-100; Cohesion Technologies). All studies were performed with cells in passage 2. Confluent cultures were deprived of hydrocortisone, epinephrine, and retinoic acid for 24 h before exposure studies. 16HBE140[−] cells, a SV40 large T Ag-transformed HBEC line was generously provided by Dr. D. Gruenert (University of Vermont, Burlington, VT) (29). 16HBE140[−] cells were cultured in MEM supplemented with 10% FBS and 1% penicillin-streptomycin-fungizone solution buffered in HEPES (20 mM) and NaHCO_3 (2.2 g/L).

Monocyte-derived DC were generated from peripheral blood monocytes after isolation of PBMC by Ficoll density gradient centrifugation of heparinized blood from healthy volunteers. CD14⁺ PBMC were isolated by positive selection using magnetically labeled CD14 Abs and a magnetic cell separator (Miltenyi Biotec) according to the manufacturer's instructions. CD14⁺ PBMC were cultured in DMEM supplemented with FBS (10%, v/v), 2-ME (5 mM), and supplemented with GM-CSF (50 ng/ml) and IL-4 (10 ng/ml). Immature MDDC (iMDDC) were harvested on day 6 and were subsequently characterized by flow cytometry as lineage negative (lin[−]) cells (CD3, CD14, CD16, CD19, CD20, CD56), CD11c⁺, and

HLA-DR bright cells. Immature MDDC were identified as CD83[−]CD86[−] within the lin[−]CD11c⁺ HLA-DR⁺ population. For coculture experiments, iMDDC (1×10^5 cells/well) were added to confluent AEC (HBEC or 16HBE140[−]) and cocultured in 12-well plates. Cells were subsequently trypsinized (0.5 g/L, 5 min) for analysis. MDDC were discriminated from epithelial cells by FACS according to their expression of CD11c and cytokeratin (CK)18 and CK19.

CD4⁺CD45RA⁺CD4RO[−] T cells were isolated from PBMC using the CD4 multisort kit in combination with depletion of CD45RO⁺ cells using CD45RO-magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions.

Immunofluorescence labeling and flow cytometry

For phenotypic characterization of MDDC and epithelial cells, cells were labeled (30 min, 0°C) with FITC-, PE-, or allophycocyanin-conjugated murine mAb directed against lineage markers, CD11c, CK18, CK19, CD80, CD83, CD86, and HLA-DR, or mouse IgG isotype control. Cells were washed (cold PBS) and fixed (PBS containing 1% formaldehyde). Cytometric analyses were performed on a FACScalibur (BD Biosciences). Forward and 90° angle scattered laser light intensities were used to exclude cellular debris. Fluorescence spillover detected by inappropriate channels, including autofluorescence above the first log, was corrected by electronic compensation. Irrelevant mAbs conjugated to the same fluorophores were used to determine nonspecific cell surface binding. Expression of surface markers was determined as the product of the mean fluorescence intensity per cell multiplied by the percentage of positive cells in a given sample (weighted mean fluorescence). In some experiments, viable cells recovered from MDDC/HBEC cocultures were resuspended in PBS containing 1% BSA and labeled with CD11c-PE (30 min, 0°C). After washing, the cells were separated by flow sorting on a FACS-Vantage SE (BD Biosciences) equipped with an aerosol containment unit for handling isolates of human origin. Cells displaying a CD11c⁺ phenotype (DC) were collected, assessed for viability and used in defined experiments.

MLR assay

MLR assays were performed with naive T cells (CD4⁺CD45RA⁺CD4RO[−]) derived from allogeneic PBMC. T cells (2.5×10^5) were cultured (5 days) in 96-well round-bottom microplates in the presence of mitomycin C-treated MDDC (5×10^3). Cells were incubated with BrdU (10 μM , 24 h) and BrdU incorporation by proliferating responder cells was quantified using a BrdU ELISA according to the manufacturer's instructions.

Small interfering RNA (siRNA) gene silencing of GM-CSF

16HBE140[−] were seeded at 70% confluence. After 24 h, cells were transfected with a siRNA duplex-pool targeted against human GM-CSF (siGENOME SMARTpool M-011166; Dharmacon) using Lipofectamine (Invitrogen Life Technologies) according to the manufacturer's instructions. Additional cells were transfected with a nontargeting duplex-pool (siCONTROL; Dharmacon). After 48 h, cells were again transfected with the same siRNA duplex-pools (48 h). Transfected 16HBE140[−] were subsequently cultured with MDDC as previously described.

Data analysis

Data are presented as mean $\pm$ SD. Experiments were performed from HBEC derived from at least three individual donors. Significance was determined by Student's *t* test, with a value of *p* < 0.05 considered significant.

Results

DEP alone fail to induce DC maturation

Previous studies have suggested that DEP induce sensitization to neoallergens (21). One mechanism by which this might occur is via direct induction of DC maturation by DEP thus enabling T cell responses to an allergen. We therefore investigated whether DEP directly induced phenotypic and functional maturation of iMDDC. To address this question, we exposed iMDDC to increasing concentrations of DEP (0.001–100 $\mu\text{g}/\text{ml}$, 48 h) and monitored the surface expression of maturation markers (CD80, CD83, CD86) within the CD11c⁺ MDDC population. As shown in Fig. 1A, stimulation of iMDDC with DEP (3 $\mu\text{g}/\text{cm}^2$; 10 $\mu\text{g}/\text{ml}$) failed to up-regulate CD11c⁺ MDDC expressing CD83 compared with untreated iMDDC (*n* = 4, not significant). Similar results were seen

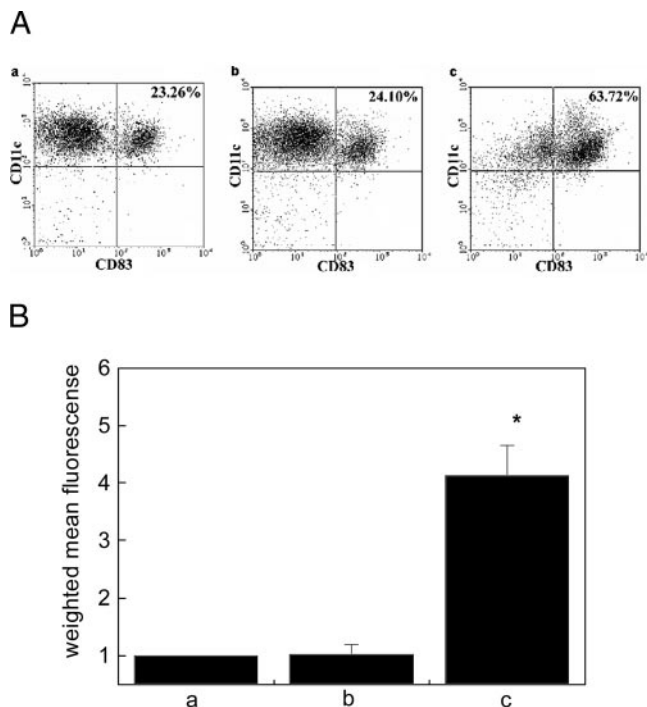


FIGURE 1. DC maturation in response to DEP. CD83 expression on iMDDC cultured for 48 h with medium (a), DEP (3 $\mu\text{g}/\text{cm}^2$) (b), or MC (c). FACS was performed as described in *Materials and Methods*. A, Dot blot is representative of four different donors; percentage of CD11c⁺ cells expressing CD83 is shown in the upper right quadrant. B, Relative CD83 expression on DC derived from multiple donors ($n = 4$, mean $\pm$ SD), cultured as described in A. *, $p < 0.05$ compared with medium.

at concentrations as high as 100 $\mu\text{g}/\text{cm}^2$ (data not shown). The inability of DEP (3 $\mu\text{g}/\text{cm}^2$; 10 $\mu\text{g}/\text{ml}$) to up-regulate CD83 was confirmed in MDDC derived from four different donors (Fig. 1B). The ability of iMDDC to respond to agents known to up-regulate CD83 was confirmed with exposure to a combination of cytokines (maturation cytokines, MC) previously described to induce DC maturation (IL-1 β , 10 ng/ml; TNF- α , 10 ng/ml; IL-6, 100 ng/ml; and PGE₂, 30 μM) (30). As expected, MC induced a 4.1 ± 0.5 -fold increase in the expression of CD83 compared with cells exposed to medium alone ($n = 4$, $p < 0.05$). The ability of iMDDC to respond to engagement of TLR was confirmed by exposure of iMDDC to LPS (1 $\mu\text{g}/\text{ml}$, 18 h). As expected, expression of CD83 increased in cells exposed to LPS compared with those exposed to medium alone (3.0 ± 0.4 -fold increase; $n = 3$, $p < 0.05$). The expression of CD80 and CD86, additional surface markers associated with MDDC maturation, followed a similar pattern in DEP-treated as well as MC-treated iMDDC (data not shown). Cells exposed to DEP (10 $\mu\text{g}/\text{ml}$) remained viable ($90 \pm 12\%$ viability compared with medium alone).

To confirm that phenotypic MDDC maturation correlated with functional MDDC maturation, allogeneic MLR were performed. Immature MDDC were exposed to the defined doses of DEP, MC, or medium and were subsequently washed and treated with mitomycin C. These cells were then cultured (5 days) with allogeneic naive T cells (1:50; DC to T cell). Proliferation of T cells was measured by BrdU incorporation (24 h). As shown in Fig. 2, iMDDC treated with DEP (3 $\mu\text{g}/\text{cm}^2$, 10 $\mu\text{g}/\text{ml}$) failed to induce a significant increase in BrdU incorporation ($n = 3$; not significant). As expected, MC-treated MDDC induced a 6.8 ± 0.3 -fold induction of BrdU incorporation in T cells ($n = 3$, $p < 0.05$). MDDC treated (48 h) with both, LPS (10 ng/ml) and DEP (10

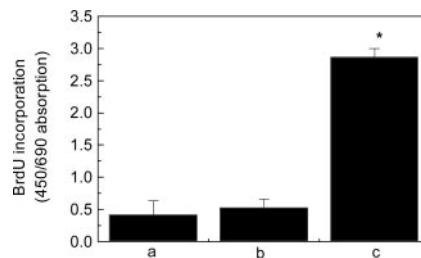


FIGURE 2. T cell proliferation in response to DEP-treated DC. CD4⁺CD45RA⁺ T cells were stimulated with mitomycin C-treated MDDC (DC to T cell ratio, 1:50; 5 days), which were pretreated 48 h(s) with medium (a), DEP (3 $\mu\text{g}/\text{cm}^2$) (b), or MC (c). T cell proliferation was determined by BrdU incorporation over the last 24 h. Data are expressed as mean $\pm$ SD ($n = 4$ different MDDC donors). *, $p < 0.05$ compared with medium.

$\mu\text{g}/\text{ml}$) induced a small increase in BrdU incorporation in T cells compared with MDDC that were exposed to LPS alone (0.3 ± 0.1 -fold increase, $n = 3$, $p < 0.05$). Although MDDC were washed after DEP exposure, we examined the potential effect of residual DEP on T cell viability. T cells were exposed to DEP (10 $\mu\text{g}/\text{ml}$) and remained viable ($88 \pm 20\%$ viability compared with medium alone). These data suggested that DEP alone were an incomplete stimulus for phenotypic or functional maturation of iMDDC.

DEP induce DC maturation in the presence of HBEC

Because the previous studies failed to demonstrate direct phenotypic or functional maturation of iMDDC by DEP, we next investigated whether the effects of DEP would be altered by additional signals derived from the tissue microenvironment and tested whether maturation of iMDDC could be induced by DEP in the presence of HBEC. To address this question, we exposed iMDDC to DEP-treated HBEC. To enable phenotypic analysis of MDDC, MDDC and epithelial cells were discriminated during FACS analysis by their expression of CD11c and CK18/19. HBEC were CD11c[−] and intracellular CK18/19⁺ (CK⁺). In contrast, MDDC were CD11c⁺ CK[−] (data not shown). Expression of the maturation marker CD83 was therefore monitored on CD11c⁺ CK[−] MDDC. HBEC exposed to DEP (10 $\mu\text{g}/\text{ml}$) remained viable ($93 \pm 14\%$ viability compared with medium alone).

To address the question of whether DEP induced MDDC maturation in the presence of HBEC, HBEC were cultured on 12-well plates, treated with DEP (3 $\mu\text{g}/\text{cm}^2$; 10 $\mu\text{g}/\text{ml}$) and subsequently iMDDC (1×10^5 cells) were added and cultured together (48 h). Expression of surface maturation markers was examined on MDDC by FACS analysis. As shown by both a representative dot blot and data derived from four separate experiments, CD83 was minimally expressed on iMDDC cultured in the presence of untreated HBEC (Fig. 3A). In contrast, expression of CD83 was increased in iMDDC exposed to DEP-treated HBEC compared with resting HBEC (2.6 ± 0.1 -fold increase in expression, $n = 4$, $p < 0.05$) (Fig. 3Bb). This response was similar although not as great as that induced by MC and HBEC, in which a 4.3 ± 0.9 -fold increase in CD83 expression was detected (Fig. 3Bc; $n = 4$, $p < 0.05$). The expression of additional surface markers associated with MDDC maturation, CD80 and CD86 followed a similar pattern in all cases described (data not shown).

To confirm functional DC maturation, allogeneic MLRs were performed. CD11c⁺ MDDC were flow-sorted by FACS from the MDDC-HBEC cocultures, treated with mitomycin C to prevent proliferation, washed, and cultured with naive T cells (1:50; DC to T cell) as previously described. Although DC purification was

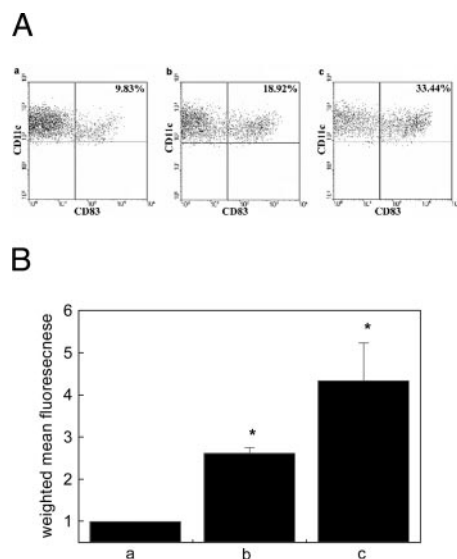


FIGURE 3. DC maturation in response to DEP-treated HBEC. CD83 expression on iMDDC cultured for 48 h with resting HBEC (a), DEP-treated HBEC (b), MC-treated HBEC (c). DEP was used at a concentration of $3 \mu\text{g}/\text{cm}^2$. FACS was performed as described in *Materials and Methods*. A, Dot blot is representative of four different donors; percentage of CD11c⁺ cells expressing CD83 is shown in the upper right quadrant. B, Relative CD83 expression on DC derived from different donors ($n = 4$). Data are expressed as mean $\pm$ SD. *, $p < 0.05$ compared with resting HBEC.

>90% (data not shown), we also assessed possible effects of residual (<10%) HBEC on T cell proliferation. As shown in Fig. 4, neither HBEC alone (Fig. 4, a) nor iMDDC that had been exposed to resting HBEC (Fig. 4, b) induced T cell proliferation. In contrast, MDDC that had been exposed to DEP-treated HBEC induced a 2.6 ± 0.2 -fold increase in T cell proliferation compared with MDDC that had been exposed to HBEC alone (Fig. 4, c; $n = 4$, $p < 0.05$). This response was similar to that induced by iMDDC that had been exposed to HBEC and MC (Fig. 4, d; 2.9 ± 0.7 fold increase in T cell proliferation, $n = 4$, $p < 0.05$). In similar experiments, stimulation of 16HBE140⁻ cells with DEP ($3 \mu\text{g}/\text{cm}^2$; $10 \mu\text{g}/\text{ml}$) resulted in a significant increase in CD83 expression on MDDC as well as an increase in BrdU incorporation in an MLR assay (data not shown).

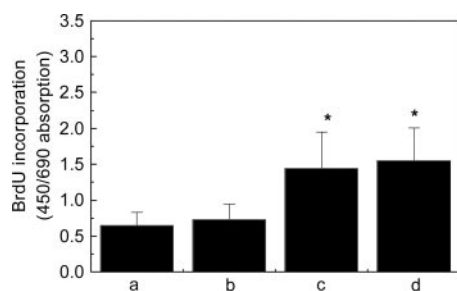


FIGURE 4. T cell proliferation in response to DC and HBEC. CD4⁺CD45RA⁺ T cells were stimulated with mitomycin C treated HBEC (a) or MDDC, which had been cocultured for 48 h with resting HBEC (b), DEP-treated HBEC ($3 \mu\text{g}/\text{cm}^2$) (c), or MC-treated HBEC (d), and isolated by flow-sorting. MDDC or HBEC-induced T cell proliferation was determined by BrdU incorporation over the last 24 h. Data are expressed as mean $\pm$ SD ($n = 4$ different donors for each, MDDC and HBEC). *, $p < 0.05$ compared with medium.

Soluble mediators induce iMDDC maturation by DEP-treated HBEC

Because iMDDC maturation was induced by DEP in the presence of HBEC, we investigated whether DEP-induced maturation of iMDDC required direct cell contact, or could be induced by soluble mediators derived from HBEC. We examined whether DEP-induced maturation of iMDDC could be mediated by culture supernatants derived from DEP-treated HBEC. Supernatants were collected from HBEC cultured in the absence or presence of DEP ($3 \mu\text{g}/\text{cm}^2$; $10 \mu\text{g}/\text{ml}$, 18 h). Supernatants were added to iMDDC and replenished after 24 h. After 48 h, MDDC were harvested and functional maturation was monitored by MLR. MDDC treated with supernatants derived from DEP-treated HBEC (Fig. 5, b) induced 1.8 ± 0.4 -fold increase in T cell proliferation compared with MDDC treated with supernatants derived from resting HBEC (Fig. 5, a; $n = 3$, $p < 0.05$). This increase was similar to the effect of MDDC cultured with supernatant derived from HBEC and MC (Fig. 5, c; 1.8 ± 0.1 -fold increase $n = 3$, $p < 0.05$). These studies suggested that soluble mediators derived from DEP-treated HBEC were capable of inducing DC maturation.

GM-CSF induced DC maturation by DEP-treated HBEC

Our data suggested that soluble compounds derived from DEP-treated HBEC were able to induce MDDC maturation. We and others (22–26) have shown that AEC up-regulate the release of GM-CSF upon stimulation with ambient PM or DEP. We therefore tested whether maturation of iMDDC was induced by GM-CSF. We first confirmed that the low levels of DEP used for studies of iMDDC maturation induced GM-CSF release by HBEC. DEP ($3 \mu\text{g}/\text{cm}^2$, 18 h) induced levels of GM-CSF that were 2.6 ± 0.3 -fold above those of resting cells ($n = 4$, $p < 0.05$; 142 ± 11 pg/ml in resting HBEC; 370 ± 13 pg/ml in DEP-treated HBEC). We then investigated whether functional maturation of MDDC induced by DEP-treated HBEC required GM-CSF. HBEC were treated with DEP ($3 \mu\text{g}/\text{cm}^2$) in the absence or presence of a GM-CSF blocking mAb or isotype control and cocultured with iMDDC. MDDC were harvested and functional maturation of MDDC was monitored by MLR. In these experiments, MDDC derived from iMDDC exposed to DEP-treated HBEC induced a 2.1 ± 0.1 -fold increase in T cell proliferation compared with MDDC exposed to untreated HBEC (Fig. 6; $n = 4$, $p < 0.05$). In contrast, MDDC derived from HBEC treated with DEP in the presence of an anti-GM-CSF mAb failed to increase T cell proliferation (Fig. 6, c). An isotype-matched control Ab had no effect (Fig. 6, d; $n = 4$, not significant).

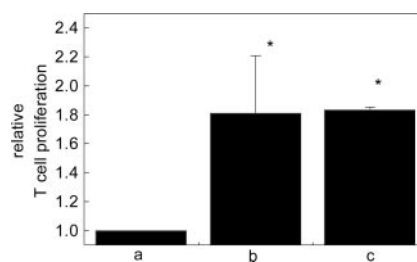


FIGURE 5. Induction of DC maturation by medium derived from DEP-treated HBEC. CD4⁺CD45RA⁺ T cells were stimulated with mitomycin C-treated MDDC (DC to T cell ratio, 1:50; 5 days), which were pretreated for 48 h with conditioned medium from resting HBEC (a), DEP-treated HBEC ($3 \mu\text{g}/\text{cm}^2$, 18 h) (b), or MC-treated HBEC (c). T cell proliferation was determined by BrdU incorporation over the last 24 h. Data were normalized to T cell proliferation in response to DC exposed to conditioned medium obtained from resting HBEC and are expressed as mean $\pm$ SD ($n = 3$ different donors for each, MDDC and HBEC). *, $p < 0.05$ compared with conditioned medium from resting HBEC.

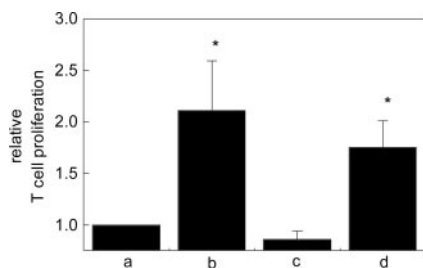


FIGURE 6. Effect of anti-GM-CSF mAb on DC maturation induced by DEP-treated HBEC. CD4⁺CD45RA⁺ T cells were stimulated with mitomycin C treated MDDC (DC to T cell ratio, 1:50; 5 days), which were cocultured for 48 h with resting HBEC (a), DEP-treated HBEC (b), DEP-treated HBEC + anti-GM-CSF mAb (10 μ g/ml) (c), or DEP-treated HBEC + isotype control Ab (10 μ g/ml) (d), and isolated by flow sorting. T cell proliferation was determined by BrdU incorporation over the last 24 h. Data are expressed as mean $\pm$ SD (n = 4 different donors for each, MDDC and HBEC). *, p < 0.05 compared with resting HBEC.

Results were similar for iMDDC exposed to DEP-treated 16HBE140⁻ cells (data not shown).

The possibility existed that the maturation of iMDDC in the presence of HBEC could be due to GM-CSF in the medium, or that produced by the MDDC themselves. To confirm that DEP-treated HBEC induced MDDC maturation via epithelial-cell derived GM-CSF, we silenced mRNA encoding for GM-CSF in 16HBE140⁻ cells and tested whether these cells retained the ability to induce MDDC maturation in response to DEP. 16HBE140⁻ cells were transfected with a GM-CSF targeting siRNA duplex-pool via Lipofectamine. In addition, cells were treated with Lipofectamine alone (vehicle control) or transfected with a control nontargeting siRNA duplex-pool (siCONTROL), which activates the RNA-induced silencing complex (RISC) pathway but does not target GM-CSF mRNA. Cells were subsequently treated with DEP as described previously and iMDDC were added to the culture (48 h). MDDC were harvested and functional maturation of MDDC was monitored by MLR. As shown in Fig. 7, MDDC that had been exposed to DEP-treated 16HBE140⁻ cells and vehicle control (Fig. 7, b) induced a significant increase in T cell proliferation compared with MDDC exposed to resting 16HBE140⁻ (Fig. 7, a) (2.0 ± 0.6 fold increase, n = 4, p < 0.05). In contrast, MDDC exposed to DEP-treated 16HBE140⁻ cells that had been transfected with siGM-CSF duplex pools (Fig. 7, d) failed to induce T

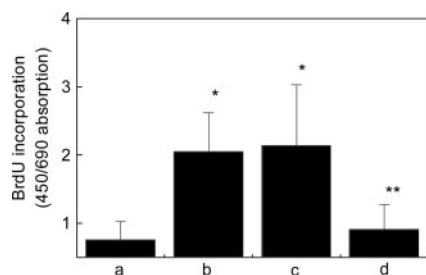


FIGURE 7. T cell proliferation in response to DC exposed to GM-CSF silenced 16HBE140⁻ cells. CD4⁺CD45RA⁺ T cells were stimulated with mitomycin C-treated DC (DC to T cell ratio, 1:50; 5 days), which were cocultured for 48 h with resting 16HBE140⁻ cells (a) 16HBE140⁻ cells plus DEP (3 μ g/cm²) plus Lipofectamine (b), 16HBE140⁻ cells plus DEP + siCONTROL (c), or 16HBE140⁻ cells + DEP + siGM-CSF (d), and isolated by flow sorting. T cell proliferation was determined by BrdU incorporation over the last 24 h. Data are expressed as mean $\pm$ SD (n = 3 different MDDC donors). *, p < 0.05; ** was not significant, compared with resting 16HBE140⁻ cells).

cell proliferation. MDDC exposed to DEP-treated 16HBE140⁻ cells that had been transfected with control nontargeting siRNA duplex-pools (Fig. 7, c) retained the ability to induce T cell proliferation to a level similar to that of untransfected cells. These studies confirmed the ability of DEP-induced epithelial cell-derived GM-CSF to stimulate functional MDDC maturation. Inhibition was not due to off-target effects by activation of the RISC pathway because a nontargeting RISC-activating duplex-pool failed to reduce MDDC-induced T cell proliferation.

Discussion

Although murine and human models have suggested that DEP alter airway immune responses, the mechanisms by which ambient PM such as DEP modify respiratory immune responses are incompletely understood. We now demonstrate that DEP induce phenotypic and functional DC maturation. Moreover, we show that DEP did not directly induce DC maturation, but acted via an effect on HBEC. Furthermore, we show that DC maturation is in part a result of DEP induction of epithelial cell-derived GM-CSF. These data begin to elucidate mechanisms by which DEP alter airway immunity, and highlight the important interaction of the tissue microenvironment with DC responses. The effect of DEP on DC maturation may be one mechanism by which urban pollutants promote immune responses to inhaled allergens and thus potentiate adverse respiratory health effects.

T cell activation and differentiation in response to an Ag presented in the context of MHC complex molecules depends on the nature of the ligand and costimulatory signals derived from DC (15). Presentation of Ag by immature DC rather than by mature DC results in T cells that promote tolerance (31, 32). In contrast, presentation of Ag by mature DC can result in a polarized T cell response that may result in either Th1 or Th2 sensitization (16). Maturation of DC is thus a first step for polarized Ag presentation. Airway DC reside adjacent to AEC (13). As such, it is likely that airway DC have the ability to directly sample ambient particles. Indeed, the ability of mucosal DC to sample through the epithelium has been demonstrated for intestinal DC (33). We therefore first evaluated whether DEP directly induce DC maturation. Several processes can be envisioned by which DEP directly induce DC maturation. Members of the TLR family, pattern recognition receptors, are targeted by agents such as LPS and unmethylated CpG and have been well described to induce DC maturation (15, 34). The possibility exists that ambient PM or DEP activate pattern recognition receptors via contaminant microbial products. Indeed, microbial components have been implicated in the macrophage-derived inflammatory response to ambient PM via TLR2 up-regulation of heat shock protein 70 (35, 36). Ambient PM and DEP activate MAPK pathways (ERK1/2, p38), which can be induced downstream of TLR signaling and are associated with DC maturation (25, 37, 38). Alternatively DEP may directly act on immature DC by unknown 'orphan' pattern recognition receptors. However, we were unable to measure detectable levels of LPS in our DEP samples. More importantly, we were unable to detect phenotypic or functional maturation of immature DC induced directly by DEP, over a wide range of DEP concentrations. Our data therefore suggested that DEP alone did not provide sufficient stimuli for immature DC maturation and that additional signals were required.

Although DC have been well described to mature in response to Ag presented in the context of additional signal(s), the signals that allow sensitization to many harmless Ags remain unknown. The possibility exists that this additional signal is delivered from cells in the microenvironment. Thus we hypothesized that DEP enhanced DC maturation via activation of adjacent cells. Because AEC are the first target for most inhaled agents, and DC reside in

close association with AEC, we investigated the role of AEC in the induction of DC maturation upon DEP exposure. Our experiments, using either primary HBEC from healthy volunteers or a transformed cell line demonstrated that stimulation of immature DC with DEP-treated AEC induced phenotypic and functional maturation of immature DC. DC maturation did not require additional exogenous stimuli or Ag exposure. These data suggested that AECs provided the "additional signal" for DC maturation.

The signal provided by DEP-treated HBEC for DC maturation could be mediated by direct cell contact, or by the release of soluble molecules. Our ability to induce functional DC maturation with conditioned medium derived from DEP-treated HBEC, suggested that cell contact was not required. DEP induce the release of a variety of cytokines by AECs, such as IL-8, RANTES, and GM-CSF (22, 39). GM-CSF is a critical factor for the functional maturation of DC and transgene expression of GM-CSF in murine bronchial epithelial cells results in sensitization to aerosolized Ag (40). Transgene expression of GM-CSF by AECs induces sensitization to aerosolized OVA with a concomitant increase in mature DC (41–43). We as well as others have shown that ambient PM or DEP induce GM-CSF in HBEC (22, 23, 25) via the induction of reactive oxygen species and activation of MAPK pathways (ERK1/2, p38) (39, 44). We therefore hypothesized that GM-CSF was a likely candidate for DC maturation induced by DEP-treated HBEC. Our ability to inhibit functional MDDC maturation by DEP-treated HBEC with anti-GM-CSF supported a role for GM-CSF. Moreover, our studies with siRNA suggested that MDDC maturation was induced by AEC-derived GM-CSF.

These data support the importance of GM-CSF as an epithelial-cell derived cytokine that is involved in DC maturation and function, and suggest that DEP effects on DC are mediated by this cytokine. Our ability to induce immature DC maturation with HBEC-conditioned medium, to block HBEC-induced immature DC maturation with anti-GM-CSF mAb and with GM-CSF siRNA, all suggest a direct role for HBEC-derived GM-CSF in the DEP-induced maturation of immature DC. However, the possibility exists that whereas GM-CSF is necessary, increased expression of GM-CSF alone may not be sufficient to induce DC maturation or to polarize the DC response. Thus GM-CSF derived from DEP-treated AEC may be only one component in the complex process of DC maturation and polarization.

AECs are targets for repeated exposure to DEP. We used concentrations of DEP that were lower than those used in most in vitro studies of DEP. In vitro doses of 0.2–20 $\mu\text{g}/\text{cm}^2$ can be reached over a 24 h period of exposure as shown by elegant dosimetric evaluations of PM deposition in an exposed adult (45). Our concentration of 3 $\mu\text{g}/\text{cm}^2$ (10 $\mu\text{g}/\text{ml}$) falls well within this range. Despite these low doses of DEP, we were able to detect phenotypic and functional DC responses, suggesting that DEP effects on DC may not require the high levels of DEP exposure that have been used to demonstrate inflammatory responses or cellular apoptosis. DC and HBEC remained viable at the levels of DEP used for our studies, suggesting that these DEP-effects are not due to cell toxicity. Furthermore, DEP used in our studies were derived from an automobile source, the physicochemical properties and toxicity of which may differ from the National Institute of Standards and Technology standard reference material, which is derived from a forklift engine (27, 28).

We used monocyte-derived DC for our studies. The possibility exists that these cells may not be representative of the subclass of DC found in the airway. However, in murine models, the functions of DC subsets have been demonstrated to be flexible, and their preferential induction of a Th1 or Th2 response defined by the conditions of immune stimulation (46). DC plasticity has also been

described in humans (47). Our data reinforce the importance of the microenvironment for DC responses and suggest that associated cells have the potential to determine DC phenotype and function.

Our data suggest that DEP induce Ag-independent DC maturation via heterotypic cell-cell interactions and HBEC-derived GM-CSF. Additional signals may be required for Ag-specificity and polarization of DC and thus regulation of polarized airway immune responses. Our studies suggest a novel mechanism by which environmental pollutants have the potential to alter airway immune responses.

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Disclosures

The authors have no financial conflict of interest.

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