Cellular Basis of the Role of Diesel Exhaust Particles in Inducing Th2-Dominant Response

Tomoyuki Ohtani, Satoshi Nakagawa, Masahiro Kurosawa, Masato Mizuashi, Maki Ozawa and Setsuya Aiba

J Immunol 2005; 174:2412-2419; doi: 10.4049/jimmunol.174.4.2412
http://www.jimmunol.org/content/174/4/2412

References
This article cites 36 articles, 13 of which you can access for free at:
http://www.jimmunol.org/content/174/4/2412.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Here is increasing evidence that a representative environmental pollutant, diesel exhaust particles (DEP), can influence the immunologic processes and promote the development of allergic diseases. A progressive increase in the prevalence of allergic diseases has been observed in industrialized Western countries over the course of the last century (1–3). Persistent exposure to DEP has been implicated as one of the factors that is responsible for the observed increased prevalence of atopy (4).

A series of studies using experimental animals revealed the adjuvant effect of DEP on vivo allergic responses leading to an increased total and allergen-specific IgE Ab response and the skewing of the T cell responses toward a Th2 predominant profile (reviewed by Nel et al. (5) and Heo et al. (6)). These findings in animal models have been extended in human studies. Inhalation of DEP increases the local production of IgE and IgE-secreting cells in the nasal mucosa (7, 8). Intranasal coadministration of DEP and allergen produces IgE isotype switching in the nasal mucosal B cells to induce the production of allergen-specific IgE (9–11). DEP intranasally administered together with allergen enhance the induction of a Th2-like cytokine profile (10) and augment the production of C-C chemokines such as RANTES, MCP-3, and MIP-1α in the nose (12).

In addition, in vitro studies revealed the effects of DEP on IgE production by B cells and their effects on macrophages. Namely, treatment of human B cells with polyaromatic hydrocarbons, a major active constituent of DEP, induces the production of IgE and further enhances the production of IgE induced by IL-4 and anti-CD40 Ab (8, 13). DEP inhibit NO production from murine macrophages activated with bacillus Calmette-Guérin (14), enhance the production of reactive oxygen radicals, and induce apoptosis (15). Meanwhile, DEP augment TNF-α production, but decrease IL-12p40 production by activated monocytes, which further supports that DEP can cause a Th2-biased deviation (16). In addition, IL-10 production by peripheral blood monocytes is also affected by DEP (17). However, there is a paucity of data concerning the effects of DEP on T cell function or dendritic cell function. Nel et al. (5) were not able to demonstrate that DEP affected the expression of Th2 cytokine mRNA in T cell lines.

In this study, we examined the effects of DEP on the cytokine production by T cells stimulated with microbeads coated with anti-CD3 Ab and anti-CD28 Ab and on that by monocyte-derived dendritic cells (MoDCs) stimulated with CD40L and/or IFN-γ. We examined IFN-γ, IL-4, IL-5, IL-8, and IL-10 produced by T cells and TNF-α, IL-1β, IL-10, and IL-12 produced by MoDCs using real-time PCR analysis or by ELISA. To highlight the effects of DEP, we compared the effects of DEP with those of dexamethasone (DEX) and cyclosporin A (CyA). DEP significantly suppressed IFN-γ mRNA expression and protein production, while it did not affect IL-4 or IL-5 mRNA expression or protein production. The suppressive effect on IFN-γ mRNA expression was more potent than that of DEX and comparable at 30 μg/ml with 10−7 M CyA. The suppressive effect on IFN-γ production was also more potent than that of either DEX or CyA. DEP suppressed IL-12p40 and IL-12p35 mRNA expression and IL-12p40 and IL-12p70 production by MoDCs, while it augmented IL-1β mRNA expression. Finally, by using a thiol antioxidant, N-acetyl cysteine, we found that the suppression of IFN-γ production by DEP-treated T cells was mediated by oxidative stress. These data revealed a unique characteristic of DEP, namely that they induce a Th2 cytokine milieu in both T cells and dendritic cells. The Journal of Immunology, 2005, 174: 2412–2419.
whether NAC or another antioxidant, epigallocatechin 3-gallate (EGCG), which is one of the main polyphenolic constituents of green tea (20, 21), could neutralize the suppression of IFN-γ mRNA expression and protein production by DEP. EGCG in addition to NAC significantly recovered the IFN-γ production by DEP-treated T cells, which suggested the crucial role of oxidative stress in the effects of DEP.

Materials and Methods

Separation and culture of T cells

PBMC were obtained by Ficoll Paque (Pharmacia) gradient centrifugation of heparinized blood from different nonatopic healthy donors (age range, 22–25 years; 3 males and 2 females). These PBMC were treated with a pan T cell isolation kit (Miltenyi Biotec), according to the manufacturer’s protocol. Before culturing, we examined the percentage of CD2+ and CD3+ cells in these preparations by flow cytometry and used cell specimens containing >98% both CD2+ and CD3+ cells in the experiments. Afterward, these T cells (2 × 10^6/ml) were cultured in RPMI 1640, including 25 mM HEPES buffer (Sigma-Aldrich) supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 1% penicillin, streptomycin and fungizone antibiotic solution (Sigma-Aldrich), and 10% FCS (Bioserum) (complete medium). This study was approved by the ethics committee of Tohoku University Graduate School of Medicine, and all the subjects gave informed consent before the examinations.

Culture of MoDCs

PBMC were isolated from heparinized fresh leukocyte-enriched buffy coats from the same donors using Lymphoprep (Nycomed). After several washes with PBS, 2 × 10^6 PBMC were treated with 400 µl of CD14 microbeads (Miltenyi Biotec) in 1600 µl of MACS buffer at 4°C for 30 min. After washing with PBS supplemented with 1% BSA (<1 ng/ml detectable endotoxin) (Sigma-Aldrich) and 5 mM EDTA (MACS buffer), the cells coated with CD14 microbeads were separated by a magnetic cell separator, MACS, according to the manufacturer’s protocol. Before culturing, we examined the percentage of CD14+ cells in these preparations by flow cytometry and used cell specimens containing >98% CD14+ cells in the experiments. Afterward, these CD14+ monocytes (2 × 10^6/ml) were cultured in complete medium containing 100 ng/ml (1000 U/ml) human rGM-CSF (a gift from Kirin Brewery) and 100 ng/ml human rIL-4 (1000 U/ml) (PeproTech). One-half of the culture medium was changed on days 3 and 5. CD14+ cells in the culture medium were <30 pg/ml, as determined by the Limulus amebocyte lysate assay (Seikagaku Kogyo).

Preparation of DEP and immunosuppressive agents

DEP were the kind gift of M. Sagai (Faculty of Health Sciences, Aomori University of Health and Welfare, Aomori, Japan). These particles were generated by a light-duty, four-cylinder diesel engine (4JB1 type; Isuzu Automobile) using standard diesel fuel, as previously described (18, 19). The diameters of the particles were measured by a low-pressure type Andersen Air Sampler, and the mean diameter was 0.4 μm. Most of the particles analyzed by a scanning electron microscope were spherical. DEP were suspended in 50 nM PBS (pH 7.4) containing 0.05% Tween 80 and particles analyzed by a scanning electron microscope were spherical. DEP particles were coated with CD14 microbeads were separated by a magnetic cell separator, MACS, according to the manufacturer’s protocol. The diameters of the particles were measured by a low-pressure type Andersen Air Sampler, and the mean diameter was 0.4 μm. Most of the particles analyzed by a scanning electron microscope were spherical. DEP were suspended in 50 nM PBS (pH 7.4) containing 0.05% Tween 80 and sonicated for 2 min. The endotoxin content of the final dilution used was <30 pg/ml, as determined by the Limulus amebocyte lysate assay (Seikagaku Kogyo). Water-soluble CyA (Sigma-Aldrich) was also dissolved in distilled H2O. CyA (Sigma-Aldrich) was dissolved in DMSO.

Table I. TaqMan probe

<table>
<thead>
<tr>
<th>Target</th>
<th>TaqMan Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>(TGGCAAATCTCCATGACCCGTCAC - (TAMRA)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>(CATTCTTGAAGAAGGTGGAAGGGAGTTGA - (TAMRA)</td>
</tr>
<tr>
<td>IL-4</td>
<td>(CGGCTGTACAACTTCCTCTGCTGGTGAAG - (TAMRA)</td>
</tr>
<tr>
<td>IL-5</td>
<td>(TACATGACCTTCTGAGTCAGCAACTTGAGC - (TAMRA)</td>
</tr>
<tr>
<td>IL-8</td>
<td>(TGGAGTCATCTCGCTGAACTTCGTCGTCGTC - (TAMRA)</td>
</tr>
<tr>
<td>IL-10</td>
<td>(CTTGAGACTAAGGCGCTTGATCATCGAT - (TAMRA)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>(TGTCCTAAGAAAGTAGCTGGTCTTCCTC - (TAMRA)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>(AGTGACATCATCTCTCTGGACCC - (TAMRA)</td>
</tr>
<tr>
<td>IL-12p35</td>
<td>(CAAAACCTCCTGAGCGCCTGTC - (TAMRA)</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>(CTGGCAAAACCCCTGACATCCAGTTC - (TAMRA)</td>
</tr>
</tbody>
</table>

Treatment of T cells with anti-CD3/CD28 Abs together with DEP or immunosuppressive agents and analysis of the effects of several chemicals on IFN-γ production by DEP-treated T cells

T cells, which were pretreated with DEP or immunosuppressive agents for 1 h, were further treated with uniform, superparamagnetic, polystyrene beads (4.5 μm diameter) coated with a highly optimized mixture of CD3 and CD28 Abs, CD3/CD28 T cell expander (Dynal Biotech), for 48 h for ELISA or for 6 h for real-time PCR.

To examine whether DEP suppress IFN-γ production by T cells through generating reactive oxygen species (ROS), we cultured DEP-treated T cells with CD3/CD28 T cell expander in the presence of various concentrations of NAC (Sigma-Aldrich) and EGCG, one of the main polyphenolic constituents of tea for 48 h, and analyzed IFN-γ production by ELISA.

Treatment of MoDCs with CD40L and IFN-γ together with DEP or immunosuppressive agents

MoDCs, which were pretreated with DEP or immunosuppressive agents for 1 h, were further treated with CD40L (100 ng/ml; PeproTech) and IFN-γ (1000 U/ml; PeproTech) for 48 h for ELISA or for 6 h for real-time PCR.

Real-time PCR

Quantitative, fluorescent PCR was performed using the TaqMan system (ABI 7700; Applied Biosystems). Sequences for human GAPDH, TNF-α, IL-1β, IL-4, IL-5, IL-8, IL-10, IL-12p40, IL-2p35, and IFN-γ were obtained from GenBank. We chose forward and reverse primers to span exon-intron boundaries. TaqMan probes were chosen to be used with these primers with Primer Express version 1.0 (Applied Biosystems) (Table I). Forward and reverse primers were made by Nihon Gene Research Laboratories, while the TaqMan probes were made by Applied Biosystems. The primers and probes used in these studies are shown in Table II. RNA was extracted by using the guanidinium thiocyanate method described by the manufacturer (ISOGEN) from MoDCs or T cells 6 h after stimulation.

First-strand cDNA was synthesized from total RNA extracted in RNA-free conditions. cDNA was obtained from total RNA using a TaKaRa RNA kit (avian myeloblastosis virus) (Takara Biochemicals), as described in the manufacturer’s protocol. PCRs for GAPDH and cytokines were performed in triplicate in 30-μl total reaction volumes with 66 nM TaqMan probe, 400 nM forward primers, 400 nM reverse primers, and 2× TaqMan universal PCR Master (Applied Biosystems). Thermal cycling was performed for 2 min at 50°C for depleting contaminated RNA, 10 min denaturation at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min in the ABI Prism 7700 detection system (Applied Biosystems). The levels of cDNA for GAPDH, or each cytokine generated from cellular RNA, were calculated by using standard curves generated with bona fide human cDNAs for GAPDH or each cytokine in which there was a linear relationship between the number of cycles required to exceed the threshold and the number of copies of cDNA added. The ratio of copy numbers of cDNA for each cytokine:those for GAPDH (cytokine:GAPDH ratio) was calculated. To compare the data from different donors, we also calculated the percentage of suppression or augmentation of the cytokine:GAPDH ratio. Percentage of suppression of cytokine mRNA expression by activated T cells was determined as (A − B)/A. A: the cytokine:GAPDH ratio from T cells stimulated with CD3/CD28 T cell expander, B: the cytokine:GAPDH ratio from T cells stimulated with CD3/CD28 T cell expander + vehicle, + DEP, or + immunosuppressive agents. Percentage of suppression or augmentation of cytokine mRNA expression by activated MoDCs was determined as (A − B)/A. A: the cytokine:GAPDH ratio from MoDCs stimulated with CD40L ± IFN-γ, B: the cytokine:GAPDH ratio from MoDCs stimulated with CD40L ± IFN-γ + vehicle, + DEP, or + immunosuppressive agents.
**ELISA for cytokine production**

The culture supernatants of MoDCs and T cells were recovered 48 h after stimulation. Their production of cytokines was measured by ELISA kits obtained from Pierce (TNF-α/H9251, IL-8, IL-10, and IL-12p40), Genzyme Techne (IL-12p70), and PeproTech (IL-4, IL-5, and IFN-α/H9253), using 96-well microtiter plates, according to the manufacturer’s instructions. When the culture supernatants contained cytokines over the upper limits of the standard curves, they were measured again after optimal dilution. To compare the data from different donors, we also calculated the percentage of suppression or augmentation of cytokine production. Percentage of suppression of cytokine production by activated T cells = (A - B)/(A - C); A: the cytokine production by T cells treated with CD3/CD28 T cell expander. B: the cytokine production by T cells treated with CD3/CD28 T cell expander and DEP or immunosuppressive agents. C: the cytokine production by nontreated T cells. Percentage of suppression of cytokine production by activated MoDCs = (A - B)/(A - C); A: the cytokine production by MoDCs treated with CD40L/IFN-α/H9253. B: the cytokine production by MoDCs stimulated with CD40L/IFN-α/H9253 and DEP. C: the cytokine production by nontreated MoDCs.

**Statistical analysis**

The statistical significance of differences in the cytokine:GAPDH ratio between T cells stimulated with CD3/CD28 T cell expander and DEP or immunosuppressive agents. The mean cytokine:GAPDH ratio ± SD is shown for various concentrations of DEP and immunosuppressive agents. This is a representative data of five independent experiments.

FIGURE 1. The effect of DEP and immunosuppressive agents on cytokine mRNA expression by activated T cells. A: T cells were stimulated with CD3/CD28 T cell expander in the presence of DEP or immunosuppressive agents, as described in Materials and Methods. Six hours after stimulation, total RNA was recovered from T cells, and then reverse transcribed into cDNA. Real-time PCR was conducted to determine the number of copies of cDNA for each cytokine, and then cytokine:GAPDH ratio was calculated. The mean cytokine:GAPDH ratio ± SD is shown for various concentrations of DEP and immunosuppressive agents. This is a representative data of five independent experiments. *, Means p < 0.05 by unpaired Student’s t test. B: T cells were stimulated with CD3/CD28 T cell expander in the presence of DEP or immunosuppressive agents, as described in Materials and Methods. The cytokine:GAPDH ratio was calculated 6 h after stimulation, and then we calculated the percentage of suppression of cytokine:GAPDH ratio by the formula described in Materials and Methods. The mean percentage of suppression ± SEM is shown. *, Means p < 0.05 by paired t test. The range of the cytokine:GAPDH ratio in stimulated T cells from three different donors was from 9.4 to 67.8 for IFN-γ, from 0.74 to 5.5 for IL-4, from 0.21 to 1.8 for IL-5, from 0.17 to 1.6 for IL-8, and from 0.53 to 2.1 for IL-10.

**Table II. Sequence of PCR primer**

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GAAAGTGGAGGGTCGGAGTC</td>
<td>GAAGATGATGGTAGGATTC</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>TGATAGCTAGATTGAGACTCTTTCT</td>
<td>GAGACAATTTGGGCTGTCATTATTTTT</td>
</tr>
<tr>
<td>IL-4</td>
<td>TGAACCGGTCGACAGGAACTTC</td>
<td>GAAGTTTCTCAAAGTACTCTTGGT</td>
</tr>
<tr>
<td>IL-5</td>
<td>AAGGAGCTCTGGACCTGCTTTCTTA</td>
<td>TATGTACAGGAGAGGACTCTCA</td>
</tr>
<tr>
<td>IL-8</td>
<td>GTGTGTAACAGTACCTCCAAGCTG</td>
<td>TCTTTAGACACTCTTGCAGAAAC</td>
</tr>
<tr>
<td>IL-10</td>
<td>GGAGAACTGAGCTGACTGCTCCA</td>
<td>TGCTCTGTGTTCTCACGGAAGAG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GAGAGTGGTCTGTGTCTTGAAGCTG</td>
<td>AGAGGGCAAGGGTCCAGGT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TTCTCATATACGGCCTCTGCCCCAG</td>
<td>TACAACATGGGCTACAGCTGTTTCAC</td>
</tr>
<tr>
<td>IL-12p35</td>
<td>TTTCATATGCCTTCCTTACCACT</td>
<td>TGTCGCTGCTCTGTGAGCA</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>CCAGACGATGGTCTTTAGGC</td>
<td>GGCCAGATCTCCAAACTCTTT</td>
</tr>
</tbody>
</table>
stimulated with CD3/CD28 T cell expander + DEP or immunosuppressive agents in each subject was analyzed using unpaired Student’s t test. Similarly, the statistical significance of the differences in the cytokine:GAPDH ratio between MoDCs stimulated with CD40L and/or IFN-γ and those stimulated with CD40L and/or IFN-γ + DEP or immunosuppressive agents in each subject was analyzed using unpaired Student’s t test. The statistical significance of differences in the percentage of suppression or augmentation of the cytokine mRNA expression by activated T cells or MoDCs with DEP or immunosuppressive agents and that with vehicle treatment was evaluated by paired t test. The statistical significance of differences in the percentage of suppression or augmentation of the cytokine production between T cells stimulated with CD3/CD28 T cell expander or those stimulated with CD3/CD28 T cell expander + DEP or immunosuppressive agents or between MoDCs treated with CD40L and/or IFN-γ or treated with CD40L and/or IFN-γ + DEP in each subject was evaluated by paired t test.

Results

The effect of DEP and immunosuppressive agents on cytokine mRNA expression by activated T cells

T cells were stimulated with CD3/CD28 T cell expander or pretreated with DEP or immunosuppressive agents for 1 h and then stimulated with CD3/CD28 T cell expander in the presence of DEP or immunosuppressive agents. Six hours after stimulation, total RNA was recovered from the T cells and then reverse transcribed into cDNA. Real-time PCR was conducted to determine the number of copies of cDNA for GAPDH or each cytokine, and then the cytokine:GAPDH ratio was calculated. Fig. 1 shows one of the representative data from five different donors and the summarized data, respectively. The data from each donor were obtained by the triplicate assays, and then the mean ± SD was calculated. In the summarized data, the mean ± SEM was obtained from the means of the data from five different donors. At first, we determined the viability of the T cells after DEP treatment. In the concentration ranges we used, DEP did not decrease the viability of T cells by trypan blue exclusion assay and propidium iodide staining. DEP significantly suppressed IFN-γ and IL-10 mRNA expression by T cells stimulated with CD3/CD28 T cell expander, while DEP did not affect the IL-5 mRNA expression by stimulated T cells. The effect of DEP on IL-8 mRNA expression was variable depending on the donors, and DEP rather augmented IL-8 mRNA expression. IL-4 mRNA expression was slightly, but significantly suppressed by DEP in one donor, although there was no dose-response relationship. In addition, the summarized data did not show a significant suppression of IL-4 mRNA expression by DEP.

In the culture of T cells from the same donors, CyA strongly suppressed IFN-γ, IL-4, IL-5, and IL-10 mRNA in a dose-dependent manner, while it suppressed IL-8 mRNA at only the highest concentration. In contrast, DEX suppressed the mRNA expression of all cytokines, although significant suppression was observed in IFN-γ and IL-10 mRNA at one concentration and in IL-4 and IL-5 mRNA at every concentration. Moreover, the magnitude of the suppression by DEX was smaller than that by CyA.

The effect of DEP and immunosuppressive agents on cytokine production by activated T cells

In the next experiment, the culture supernatants of T cells treated as described above were recovered 48 h after stimulation and analyzed for cytokine production (Fig. 2). Consistent with the results of the mRNA expression, DEP significantly suppressed IFN-γ and IL-10 production, while it did not significantly suppress either IL-4 or IL-5 production. IL-8 production was rather augmented by DEP, but not to a statistically significant extent. DEX also significantly suppressed IL-4 and IL-5 production, but did not suppress IFN-γ, IL-8, or IL-10 production significantly. Unexpectedly, although CyA significantly suppressed IL-4, IL-8, and IL-10 production, it did not suppress IFN-γ or IL-5 production significantly.

FIGURE 2. The effect of DEP and immunosuppressive agents on cytokine production by activated T cells. The culture supernatants of T cells from three donors, which were treated with CD3/CD28 T cell expander and/or DEP or immunosuppressive agents, were recovered 48 h after culture. Their cytokine production was measured by ELISA. The mean percentage of suppression of cytokine production ± SEM is shown. *, Means p < 0.05 by paired t test. The range of each cytokine in the culture supernatants from three different donors (pg/ml) was from 2,893 to 4,057 for IFN-γ, from 168 to 222 for IL-4, from 512 to 853 for IL-5, from 581 to 9,520 for IL-8, and from 3,143 to 30,512 for IL-10.
The effect of DEP and immunosuppressive agents on cytokine mRNA expression by activated MoDCs

To analyze the effect of DEP or immunosuppressive agents on the cytokine mRNA expression by MoDCs, MoDCs were stimulated with CD40L or CD40L and IFN-γ, or pretreated with the DEP or immunosuppressive agents for 1 h and then cultured with CD40L or CD40L and IFN-γ in the presence of the DEP or immunosuppressive agents. The cytokine mRNA expression 6 h after stimulation was assessed by real-time PCR, and the cytokine:GAPDH ratio was calculated. Again, we determined the viability of MoDCs after DEP treatment. In the concentration ranges we used, DEP did not decrease the viability of MoDCs by trypan blue exclusion assay and propidium iodide staining. Fig. 3 shows the summarized data of the cytokine mRNA expression by MoDCs with or without CD40L stimulation from five different donors. Fig. 4 shows one of the representative data and the summarized data of the cytokine mRNA expression by MoDCs stimulated with CD40L and IFN-γ, respectively. The data from each donor were obtained by the triplicate assays, and then the mean ± SD was calculated. In the summarized data, the mean ± SEM was obtained from the means of the data from five different donors. Without stimulation or with CD40L stimulation, DEP significantly augmented TNF-α and IL-1β mRNA expression, while it significantly suppressed IL-12p40 mRNA expression. MoDCs without stimulation or even with CD40L stimulation did not express a measurable amount of IL-12p35 mRNA. When stimulated with CD40L and IFN-γ, DEP significantly increased IL-1β mRNA, while it significantly suppressed the IL-12p40 and IL-12p35 mRNA expression in a dose-dependent fashion. In contrast, DEX suppressed TNF-α and IL-1β mRNA expression significantly, while its effect on IL-12p40 and IL-12p35 mRNA expression was not significant. CyA also significantly suppressed IL-1β and TNF-α mRNA expression, but the effect of CyA was less potent than that of DEX at all concentrations. CyA suppressed the IL-12p40 mRNA expression significantly only at the lowest concentration, although its magnitude was small. The IL-10 mRNA expression was not affected by any treatment.

The effect of DEP on cytokine production by activated MoDCs

The cytokine production by MoDCs 48 h after stimulation was also examined by ELISA (Fig. 5). In the analysis of the cytokine production, because the differences in the effects on the cytokine mRNA expression between the DEP and immunosuppressive agents were so striking, we measured the cytokine production by only MoDCs treated with DEP. TNF-α or IL-10 production by MoDCs with or without stimulation of CD40L and/or IFN-γ was not affected significantly by the DEP at any concentration. Both IL-12p40 and IL-12p70 productions were significantly decreased by DEP when the MoDCs were stimulated with CD40L and IFN-γ. Most culture supernatants of MoDCs with or without the stimulation did not contain measurable amounts of IL-1β. IL-12p70 was not detected in the culture supernatants of nontreated MoDCs or those treated with CD40L. IL-10 production was not detected from the culture of MoDCs stimulated with CD40L and IFN-γ.

The effect of antioxidants on the suppression of IFN-γ production by DEP-treated T cells

Because it already has been demonstrated that ROS play a principal role in the adjuvant effects of DEP (5, 15, 18, 19, 22), we next examined whether NAC and EGCG could neutralize the suppression of IFN-γ production by DEP-treated T cells. In this study, we selected EGCG because it is well known that EGCG, one of the main polyphenolic constituents of green tea, can be ingested without any significant side effects in the long-term. NAC significantly, but partially recovered the IFN-γ production by DEP-treated T cells (Fig. 6). In addition, although the magnitude was much smaller than that by NAC, EGCG also could recover significantly the IFN-γ production by DEP-treated T cells (Fig. 6).
**Discussion**

A series of epidemiological studies have suggested a link between the increase in the prevalence of allergic diseases and the levels of environmental pollutants such as DEP in the atmosphere (4). Animal and human models have also demonstrated the adjuvant activity of DEP in enhancing IgE production and promoting Th2 differentiation, which enhances allergic inflammation. In contrast, however, the mechanism by which DEP enhances allergic inflammation is not clear. To address this question, in this study, we examined the effect of DEP on purified T cells or MoDCs. Using this culture system, we tried to determine the effects of DEP on these cells without them being affected by the interaction between T cells and MoDCs. We stimulated T cells via CD3 and CD28 and MoDCs via CD40L or IFN-γ receptors, mimicking the physiological stimulation provided by APCs and T cells, respectively. In this study, to highlight the characteristics of DEP, we also examined the effect of immunosuppressive agents, DEX and CyA, on the cytokine mRNA expression and production by activated T cells or MoDCs at the same time. Through these studies, we succeeded in demonstrating the unique effect of DEP on the immune response.

As anticipated based on epidemiological studies and animal and human models, DEP significantly suppressed IFN-γ mRNA expression and protein production, while it did not affect IL-4 or IL-5 mRNA expression and protein production. The suppressive effect of DEP on IFN-γ mRNA expression was more potent than that of DEX and almost comparable at 30 μg/ml with 10⁻⁷ M CyA. The suppressive effect of DEP on IFN-γ production was also far more potent than either DEX or CyA at all examined concentrations. In addition, DEP suppressed IL-12p40 and IL-12p35 mRNA expression and IL-12p40 and IL-12p70 production by MoDCs, while it augmented their IL-1β mRNA expression. We now know that Th1 differentiation is regulated by transcription factors such as T-bet, STAT1, and STAT4, as well as cytokines such as IL-12, IL-23, IL-27, type I IFNs, and IFN-γ. In contrast, Th2 differentiation is promoted by the transcription factors STAT6, GATA-3, c-Maf, and NF-AT5, and the cytokine IL-4 (reviewed by Agnello et al. (23)). Accordingly, it is reasonable to speculate that the cytokine milieu induced by DEP, i.e., the decreased IFN-γ and IL-12 production and the preserved IL-4 and IL-5 production, skews the T cell immune response to a Th2-dominant pattern. In this study, we tried to stimulate T cells or MoDCs by mimicking physiological stimuli. Therefore, it is speculated that as long as humans live in an environment polluted with DEP, T cell responses favoring a Th2-dominant pattern can be induced whenever T cells or dendritic cells are activated by infectious microorganisms. Obviously, however, most healthy subjects can mount effective Th1-mediated immunity to common viral pathogens in such an environment, which suggests that genetic predisposition plays a crucial role in leading to the clinical appearance of the Th2-dominant phenotype.

Moreover, DEP significantly suppressed the IL-10 mRNA expression and production by stimulated T cells. IL-10 was originally described as a mouse Th2 cell factor that inhibits cytokine synthesis by Th1 cells (24, 25). However, increasing evidence suggests that IL-10 also acts as an inhibitor of Th2 cell responses both in vitro and in vivo (26–28). In particular, IL-10 was found to down-regulate IL-5 production by human resting T cells (29).
oxidative stress directly impaired IFN-γ production by DEP-treated T cells. We cultured DEP-treated T cells from four different donors with CD3/CD28 T cell expander in the presence of various concentrations of NAC and EGCG for 48 h, and analyzed IFN-γ production by ELISA. *p < 0.05 by paired t test. IFN-γ production by DEP-treated T cells, although the magnitude of the recovery was far lower than that by NAC. If the suppression of IFN-γ production by DEP-treated T cells is one of the causative mechanisms of atopic diseases, EGCG might be used for the prevention of atopic diseases, because EGCG can be easily and safely taken in the form of green tea or as a supplement.

The other significant effects of DEP in our experiments were the augmentation of TNF-α and IL-1β mRNA expression, the suppression of IL-12p40 mRNA expression, and protein production by MoDCs with or without stimulation. Similar effects have also been reported in monocytes and macrophages (13, 16, 17). Recently, Whitekus et al. (19) reported that oxidative stress is a key mechanistic component in the adjuvant effect of DEP in vivo. In addition, it has also been demonstrated that the glutathione levels in murine APCs play a central role in determining through the level of IL-12 production which of the Th1 and Th2 cytokine responses predominate in vivo (33, 34). Accordingly, the effect of DEP on MoDCs in vitro is also speculated to result from the oxidative stress induced by DEP on MoDCs.

Interestingly, DEX and CyA suppressed both IFN-γ and IL-4 mRNA potently, although they did not significantly affect IL-12 mRNA. These findings suggest that they have minimal effects on the Th1/Th2 balance. Therefore, the comparison between the effect of DEP and that of DEX or CyA further highlighted the unique activity of DEP to induce a Th2-dominant response. In addition, DEX significantly augmented the IL-1β mRNA expression, although it did not increase the IL-1β production. The secreted IL-1β might be consumed by the MoDCs themselves, which resulted in their activation.

In this study, we also found clear differences between DEX and CyA. CyA was more potent than DEX in suppressing the cytokine production by T cells, while DEX suppressed the expression of proinflammatory cytokine mRNA by MoDCs more significantly than CyA. CyA suppressed IFN-γ, IL-4, and IL-5 mRNA expression in a dose-dependent manner, and abrogated their expression almost completely at the highest concentration. In contrast, DEX partially suppressed their cytokine mRNA expression. Consistent with the mRNA expression, IL-4 production was suppressed by both DEX and CyA. Unexpectedly, however, IFN-γ production was not significantly suppressed by either DEX or CyA, and IL-5 production was only suppressed by DEX. We have no clear answer for this discrepancy between the cytokine mRNA expression and production. Because we collected the culture supernatants 48 h after stimulation, during culture T cells might have recovered from the effects of the immunosuppressive agents. Interestingly, IL-10 production was suppressed significantly only by CyA, but not by DEX. Considering the role of IL-10 in the control of regulatory T
cell function, they may induce different immune states in terms of tolerance, corresponding to differences in the modulation of regulatory T cell function.

Finally, our present study demonstrated the cellular basis of the role of DEP in the induction of Th2-dominant responses. By using this in vitro system, we can further examine the molecular mechanisms by which DEP cause an enhancement of atopic diseases. In this study, however, we used whole T cells instead of purified CD4⁺ T cells or CD8⁺ T cells. Recently, in addition to the key role of Th2 cells, it has been suggested that T cytotoxic 2 cells also significantly contribute to airway inflammation and airway hyper-responsiveness in an allergen-specific mouse model (35, 36). So, it is also crucial to clarify the difference between the effects of DEP on CD4⁺ cells and those on CD8⁺ cells.

References