

Molecular Events in Human T Cells Treated with Diesel Exhaust Particles or Formaldehyde that Underlie Their Diminished Interferon- γ and Interleukin-10 Production

Yoshinori Sasaki^a Tomoyuki Ohtani^a Yumiko Ito^a Masato Mizuashi^a
Satoshi Nakagawa^a Toru Furukawa^b Akira Horii^b Setsuya Aiba^a

Departments of ^aDermatology and ^bMolecular Pathology, Tohoku University Graduate School of Medicine, Sendai, Japan

Key Words

Diesel exhaust particles • Formaldehyde • Human T lymphocytes • Interferon- γ • Interleukin-10 • Glucocorticoid-induced leucine zipper • Growth arrest and DNA damage-inducible gene 45 α

Abstract

Background: A series of epidemiological and experimental studies have suggested that diesel exhaust particles (DEP) and formaldehyde (FA) may help trigger T helper type 2 (Th2)-mediated allergic responses. **Methods:** To identify the molecular events by which DEP and FA induce a Th2-skewed immune response, we stimulated T cells from healthy subjects with anti-CD3/anti-CD28 monoclonal antibodies and examined the effect of pretreatment with DEP or FA on mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)- κ B by Western blotting and colorimetric NF- κ B assays. We also examined the mRNA expression profiles of the T cells by microarray and real-time PCR analyses. **Results:** FA selectively suppressed interferon (IFN)- γ and interleukin-10 mRNA expression and protein production in stimulated T cells, as we previously reported with DEP. In the present study, we found that both DEP and FA suppressed NF- κ B signaling and activated MAPKs. Both also significantly suppressed the mRNA expression of T-bet, Txk and c-Maf. Microarrays revealed significant augmentation of the expression

of 2 FoxO3a-dependent genes, namely glucocorticoid-induced leucine zipper and growth arrest and DNA damage-inducible gene 45 α (Gadd45a), which are known to modulate T cell immune responses. Treatment with N-acetylcysteine reversed the augmented Gadd45a mRNA response and caused the suppressed IFN- γ mRNA response to recover. **Conclusions:** DEP and FA have similar transcriptional and nontranscriptional effects on T cell signaling that together promote a Th2-skewed immune response.

Copyright © 2008 S. Karger AG, Basel

Introduction

Recently, we demonstrated that a major environmental pollutant, diesel exhaust particles (DEP), has a unique biological ability to induce a T helper type 2 (Th2)-skewed immune response in both T cells and dendritic cells in vitro [1]. Specifically, we showed that human T cells stimulated with anti-CD3/CD28 antibody in the presence of DEP evinced significantly suppressed interferon (IFN)- γ and interleukin (IL)-10 mRNA and protein expression, while exhibiting normal IL-4 and IL-5 mRNA and protein expression. Moreover, DEP suppressed IL-12p40 and IL-12p35 mRNA expression and IL-12p40 protein production by monocyte-derived dendritic cells stimulated with CD40 ligand and/or IFN- γ , while augmenting their

expression of IL-1 β mRNA. Our observations are consistent with a series of epidemiological studies that have suggested that the increased prevalence of allergic diseases may be associated with elevated levels of environmental pollutants such as DEP in the atmosphere [2]. Animal and human models have also demonstrated that DEP has an adjuvant activity in that it enhances IgE production and promotes Th2 differentiation (reviewed by Nel et al. [3]). Thus, our in vitro studies further extended these observations by clarifying the molecular mechanism by which DEP induces Th2-dominant responses.

Like DEP, formaldehyde (FA), which is a highly prevalent indoor air pollutant and a well-known irritant, has been suggested to significantly increase the risk of asthma in young children [4]. In addition, recent experimental animal studies have indicated that FA may help trigger allergic responses [5].

In the study reported herein, we first examined the effect of FA on the cytokine production profile of T cells stimulated with microbeads coated with anti-CD3 and anti-CD28 antibodies (T cell expander). These studies revealed that FA pretreatment significantly suppressed IFN- γ and IL-10 mRNA and protein expression while having no effect on IL-4 and IL-5 mRNA and protein expression. Since both DEP and FA have similar effects on T cells in vitro despite their chemical differences, we examined their effects on T cells at both the transcriptional and non-transcriptional levels. Thus, we examined whether DEP and FA pretreatment of T cell expander-stimulated T cells influences the phosphorylation of 3 mitogen-activated protein kinases (MAPKs) and the activation of nuclear factor (NF)- κ B. In addition, we analyzed the effects of DEP and FA on the mRNA expression profile of stimulated T cells by oligonucleotide microarrays and quantitative real-time PCR. These studies revealed that DEP and FA both alter the T cell immune response by nontranscriptionally regulating NF- κ B signaling and transcriptionally regulating several transcription factors, namely T-bet, Tbx, glucocorticoid-induced leucine zipper (GILZ), growth arrest and DNA damage-inducible gene 45 α (Gadd45a) and heme oxygenase-1 (HO-1).

Methods

Isolation and Culture of T Cells

Peripheral blood mononuclear cells were obtained by Ficoll Paque (Pharmacia Corporation, Peapack, N.J., USA) gradient centrifugation of heparinized blood from different nonatopic healthy donors (age range 22–25 years). The T cells were then isolated using a pan T cell isolation kit (Miltenyi Biotec Inc., Sunny-

vale, Calif., USA). The frequencies of CD2 $^{+}$ and CD3 $^{+}$ cells in these preparations were determined by flow cytometry, and only those specimens that consisted of over 98% CD2 $^{+}$ and CD3 $^{+}$ cells were used in subsequent experiments. These T cells (2×10^6 /ml) were cultured in RPMI-1640 including 25 mM Hepes buffer (Sigma Chemical Co., St. Louis, Mo., USA) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin, streptomycin and fungizone antibiotic solution (Sigma) and 10% fetal calf serum (Bioserum, Canterbury, Vic., Australia) (complete medium). This study was approved by the ethics committee of Tohoku University Graduate School of Medicine, Sendai, Japan, and all subjects gave informed consent before recruitment.

Preparation of DEP and FA

The DEP preparation was a kind gift from M. Sagai (Faculty of Health Sciences, Aomori University of Health and Welfare, Aomori, Japan). These particles were generated by a light-duty, 4-cylinder diesel engine (4JB1 type, Isuzu Automobile) using standard diesel fuel, as previously described [6]. The diameters of the particles were measured by a low-pressure Andersen air sampler, and the mean diameter was 0.4 μ m. Scanning electron microscopy revealed that most of the particles were spherical. The DEP were suspended in PBS (pH 7.4) containing 0.05% Tween 80 and sonicated for 2 min. The concentration of DEP used throughout the experiments was 30 μ g/ml. We chose this concentration because our previous study demonstrated that T cells that were pretreated for 1 h with this concentration of DEP and then stimulated showed significantly suppressed IFN- γ mRNA and protein expression but unimpaired viability. The endotoxin content of the final dilution used was <30 pg/ml, as determined by the Limulus amoebocyte lysate assay (Seikagaku Kogyo, Tokyo, Japan). The FA solution (Wako Pure Chemicals Industries Ltd., Osaka, Japan) was dissolved in distilled water.

DEP/FA Pretreatment of T Cells and Their Stimulation with T Cell Expander

T cells were pretreated with DEP or FA for 1 h and then treated for 6 h with T cell expander, which consists of superparamagnetic polystyrene beads (4.5 μ m in diameter) coated with a highly optimized mixture of CD3 and CD28 antibodies (Dyna Beads, Oslo, Norway). In some experiments, to determine whether DEP or FA suppresses IFN- γ production by T cells by generating reactive oxygen species (ROS), we precultured T cells for 1 h with an optimal concentration (25 mM) of N-acetylcysteine (NAC; Sigma) before treating the cells with DEP or FA (for 1 h) and then with T cell expander (for 6 or 48 h depending on the experiment). All treatments occurred in the presence of NAC.

Microarray Analysis and Significant Gene Selection Criteria

T cells from 3 different nonatopic healthy donors that were pretreated with or without either DEP or FA for 1 h were further stimulated with T cell expander. Total RNA was extracted using an RNeasy Mini kit (Qiagen, Valencia, Calif., USA). After being cultured for 6 h, 9 RNA samples from these T cells were separately hybridized to the array using a Codelink Uniset Human 20K I Bioarray System (Amersham Biosciences, Piscataway, N.J., USA). Signals in the scanned image were converted to intensity values, and the values were normalized by dividing them by the median value using Codelink System Software (Amersham Biosciences). Genespring 6 (Silicon Genetics, Redwood City, Calif., USA) was

Table 1. PCR primer sequences

Target	Forward primer	Reverse primer
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
IFN- γ	TGTAGCGGATAATGGAACTCTTTTCTTGA	GACAATTTGGCTCTGCATTATTTT
IL-4	TGAAACGGCTCGACAGGAACCTC	GAAGTTTTCCAACGTACTCTGGT
IL-5	AAGAGACCTTGGCACTGCTTTCTA	ATGTACAGGAACAGGAATCCTCA
IL-8	GTGTGTAAACATGACTTCCAAGCTG	TCTTTAGCACTCCTTGGCAAAAC
IL-10	GGGAGAACCTGAAGACCCTCA	TGCTCTTGTTTTCACAGGGAAG
STAT4	CCTCTATGGCCTGACCATAGATT	TGTTGTGGGACTCAGGTTTTCTCT
Gadd45a	ACCGAAAGGATGGATAAGGTGG	AAATACCCAACTATGGCTGCAC
Gadd45b	GCAGAAGATGCAGACGGTGA	AGTGGTTTGAATAACAAGTGCAA
HO-1	GACAGCATGCCCCAGGATTT	CTTCAGTGCCCCACGGTAAGGA
GILZ	CGTTAAGCTGGACAACAGTGCCT	GGCTCAATCTCTCCCATCTGTGT
GATA3	AGCCTGTCCTTTGGACCACA	ATCGAGCAGGGCTCTAACCC
T-bet	AACCGCCTGTACGTCCACC	ATGAACTTCTGGCGCATC
c-Maf	CTGGTGACCATGTCTGTGCG	GGTGGTTCTCCATGACTGCAAA
SOCS5	AGAGGCGCTACGGCGTAAG	GCCACAGTATCCTGCAACC
SOCS3	ACCTTTCTGATCCGCGACAG	AGACTGGGTCTTGACGCTGAG

Table 2. TaqMan probe sequences

Target	TaqMan probe
GAPDH	(FAM)-TGGCAAATTCCATGGCACCGTCA-(TAMRA)
IFN- γ	(FAM)-CATTTTGAAGAATTGGAAAGAGGAGAGTGA-(TAMRA)
IL-4	(FAM)-CGGGCTTGAATTCCTGTCTGTGAAG-(TAMRA)
IL-5	(FAM)-TCATCGAACTCTGCTGATAGCCAATGAGAC-(TAMRA)
IL-8	(FAM)-TGATTTCTGCAGCTCTGTGTGAAGGTGC-(TAMRA)
IL-10	(FAM)-CTGAGGCTACGGCGCTGTCATCGAT-(TAMRA)
STAT4	(FAM)-ACACTACAGCTCTCAGCCTTGCGAAGTTTC-(TAMRA)
Gadd45a	(FAM)-AGCGGCCAAGCTGCTCAACGTC-(TAMRA)
Gadd45b	(FAM)-GCAGAAGATGCAGACGGTGA-(TAMRA)
HO-1	(FAM)-TGACCCGAGACGGCTTCAAGCT-(TAMRA)
GILZ	(FAM)-AACAGGCCATGGATCTGGTGAAGAATCAT-(TAMRA)
GATA3	(FAM)-CCTCCAGCATGGTCACCGCCA-(TAMRA)
T-bet	(FAM)-CTCCCCCAACACAGGAGCGCACT-(TAMRA)
Txk	(FAM)-AGAAGCAATCCAACACGGGCCG-(TAMRA)
c-Maf	(FAM)-TTTTTCATAACTGAGCCCACTCGCAAGTTG-(TAMRA)
SOCS5	(FAM)-CCAGCAGAACTGTAGGAAGTCGCTCTCTAA-(TAMRA)
SOCS3	(FAM)-TCGGACCAGCGCCACTTCTTCAC-(TAMRA)

used to analyze the data. The genes that were significantly up- or downregulated by treatment with DEP or FA were selected by Genespring based on the following criteria: (1) genes whose detection was 'present' in 9 experiments, (2) genes whose normalized intensity was more than 2.0, (3) genes for which the relative increase or decrease in T cells treated with T cell expander plus DEP or FA compared to T cells treated with only T cell expander was more than 2.0 or less than 0.5 and (4) genes that passed statistical analysis (one-way ANOVA, $p < 0.05$). All data are compliant with minimum information about a microarray experiment (MIAME).

Real-Time PCR

Total cellular RNA was prepared as above. Quantitative fluorescent PCR was performed on the TaqMan system (ABI 7700; Applied Biosystems, Forester City, Calif., USA) using gene-specific primers and probes (tables 1, 2) as previously described [1]. The ratio of the copy numbers of cDNA for each gene to those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; the target/GAPDH ratio) was calculated.

To compare the data from different donors, we also calculated the percentage suppression of the target/GAPDH ratio, as follows:

percentage suppression of target gene mRNA expression = $(A - B)/A$

where A is the target/GAPDH ratio from T cell expander-stimulated T cells and B is the target/GAPDH ratio from FA-pretreated and T cell expander-stimulated T cells.

The statistical significance of differences between the A and B values of each subject was analyzed by unpaired Student's t test, while the statistical significance of differences between the A and B values of multiple subjects was evaluated by paired t test. The statistical significance of differences in the percentage suppression relative to the responses of T cells stimulated with T cell expander alone was also evaluated by paired t test.

ELISA for Cytokine Production

Cytokine production was measured by ELISA kits obtained from Endogen Inc. (Woburn, Mass., USA; IL-8 and IL-10) and Peprotech EC (London, UK; IL-4, IL-5 and IFN- γ) that employed 96-well microtiter plates and were used according to the manufacturers' instructions. When the cytokine levels in the culture supernatants exceeded the upper limits of the standard curves, they were measured again after being appropriately diluted. To compare the data from different donors, we also calculated the percentage suppression of cytokine production, as follows:

percentage suppression of cytokine production by activated T cells = $(A - B)/(A - C)$

where A is the cytokine production by activated T cells, B is the cytokine production by activated T cells in the presence of FA and C is the cytokine production by untreated T cells. The statistical significance of differences in the percentage suppression relative to the responses of T cells stimulated with T cell expander alone was evaluated by paired t test.

Immunoblotting

T cells from 3 different nonatopic healthy donors were pretreated with or without DEP or FA for 1 h and then further stimulated with T cell expander for different time periods. Immunoblotting of phosphorylated p42/44 extracellular signal-regulated kinases (ERKs), stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), p38 MAPK and I κ B was performed using p42/44 ERK, SAPK/JNK, p38 MAPK and I κ B immunoblotting kits purchased from New England Biolabs (Beverly, Mass., USA). The cells were washed twice in cold PBS and resuspended in 100 μ l of lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM PMSF and 1 mM sodium orthovanadate). The nuclei and insoluble cell debris were removed by centrifugation at 14,000 g for 10 min at 4°C. The postnuclear extracts were collected and used as total cell lysates. Total cell lysates were suspended in 2 $\times$ SDS sample buffer (313 mM Tris-HCl, pH 6.8, 10% SDS, 2-mercaptoethanol, 50% glycerol and 0.01% bromophenol blue) and heated at 95°C for 3 min. The protein samples were fractionated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, Mass., USA). The non-specific antibody-binding sites on the membranes were blocked with 1% BSA and 0.01% Tween 20 in saline (10 mM Tris-HCl, pH 7.4, 100 mM NaCl) for 20 min at 37°C. Immunoblotting of ERKs, SAPK/JNK, p38 MAPK and I κ B was performed according to the instruction manual. Blots were visualized by enhanced chemilu-

minescence. To ensure that there were similar amounts of MAPKs in each sample, the same membranes were stripped, reprobed with rabbit polyclonal antibodies to p42/44 ERKs, SAPK/JNK, p38 MAPK and I κ B and developed with horseradish peroxidase-conjugated secondary antibodies by enhanced chemiluminescence.

Microwell Colorimetric NF- κ B Assay

The activity of the NF- κ B family, p65 (RelA), in stimulated T cells from 3 different nonatopic healthy donors with or without 1 h of pretreatment with DEP or FA was examined using an NF- κ B transcriptional factor assay kit (Active Motif North America, Carlsbad, Calif., USA). Untreated control cells and those stimulated for 30 min, 1 h and 2 h were rinsed twice with cold PBS and centrifuged for 10 min at 1400 rpm. The pellet was then resuspended in 100 μ l of lysis buffer containing a protease inhibitor cocktail and 5 mM DTT. After 10 min on ice, the lysate was centrifuged for 20 min at 14,000 rpm. The activity of p65 in each sample was measured by a microwell colorimetric NF- κ B assay using the NF- κ B transcriptional factor assay kit. To confirm the specificity of binding, the wild-type or mutated consensus oligonucleotide was added to the corresponding wells. The assay was conducted in triplicate and the data were expressed as means $\pm$ SD.

Results

Effects of FA Pretreatment on Cytokine mRNA and Protein Expression by Activated T Cells

Since our preliminary studies showed that a concentration of FA of more than 30 nM reduced the viability of T cells (data not shown), we used $\leq$ 10 nM FA in all experiments. First, T cells were pretreated with or without 1, 3 or 10 nM FA for 1 h, and then stimulated with T cell expander in the presence of FA for 6 h. The cytokine mRNA levels in the T cells were analyzed by real-time PCR. Figure 1a shows the average suppressive effect of FA on cytokine mRNA levels for all 5 donors. FA significantly suppressed IFN- γ and IL-10 mRNA expression in stimulated T cells. In contrast, FA had no consistent effect on IL-4 or IL-5 mRNA expression (fig. 1a). The effect of FA on IL-8 mRNA expression also varied depending on the donor but tended to augment it, although this did not reach statistical significance. Using trypan blue exclusion assays or propidium iodide staining, we confirmed that 1–10 nM FA did not decrease T cell viability. When the culture supernatants of the T cells treated as described above were recovered 48 h after stimulation and analyzed for cytokine levels (fig. 1b), FA was found to have significantly suppressed IFN- γ and IL-10 production. However, it had no significant effect on IL-4, IL-5 or IL-8 production. These observations are entirely consistent with the mRNA data. Significantly, when the T cells

were pretreated with 25 mM NAC 1 h before FA treatment and stimulation, the IFN- γ production of the T cells was unaffected by 10 nM FA (data not shown).

Effect of DEP or FA on the Signal Transduction of Stimulated T Cells

Since FA closely resembles DEP in its effects on stimulated T cells [1], we examined the effect of both chemicals on the signal transduction of activated T cells. Thus, T cells were pretreated with or without 30 ng/ml DEP or 10 nM FA and then stimulated for 30 min, 1 h or 2 h. The phosphorylation of 3 MAPKs and I κ B was examined by immunoblot analysis (fig. 2a, b). T cell expander stimulation of unpretreated T cells induced strong phosphorylation of p44/42 ERKs that was first observed at 30 min and then tapered off. Pretreatment with DEP extended the strong phosphorylation of p44/42 ERKs at 30 min through to 2 h, but FA did not have this effect. Similarly, while unpretreated but stimulated T cells showed no phosphorylation of p38 MAPK or SAPK/JNK, DEP pretreatment led to the phosphorylation of both MAPKs at all time points. FA pretreatment also induced the phosphorylation of SAPK/JNK at the 1-hour time point. In contrast, while unpretreated but stimulated T cells showed upregulated phosphorylation of I κ B at all time points, pretreatment with either DEP or FA actually downregulated the baseline I κ B phosphorylation at all time points.

We also determined the binding activity of the total protein extracts of the cells to the NF- κ B p65 consensus sequence using an NF- κ B transcriptional factor assay kit. The unpretreated but stimulated cells showed marked upregulation of p65-binding activity, which was significantly downregulated by pretreatment with DEP and FA (at 1 h for DEP and at both 1 h and 2 h for FA; fig. 2c).

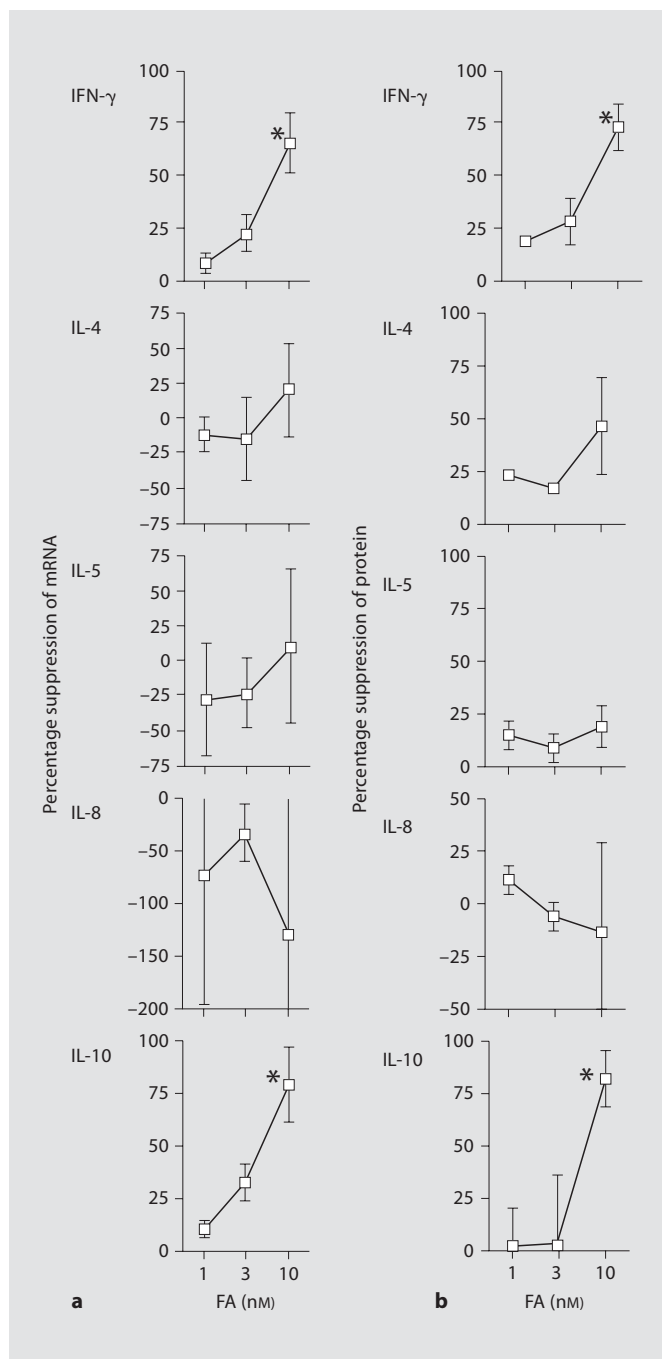


Fig. 1. Effect of FA pretreatment on cytokine mRNA and protein expression by activated T cells. T cells from 5 donors were pretreated in triplicate with FA for 1 h and then stimulated with T cell expander in the presence of FA. **a** Cytokine mRNA levels. By real-time PCR, the cytokine/GAPDH ratio was calculated and the means of these triplicate ratios for each FA concentration were determined. The mean cytokine/GAPDH ratios of the FA-untreated but stimulated T cells from the 5 donors ranged 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. The percentage suppression induced in each patient by each FA con-

centration was calculated as described in the Methods section, and the mean percentage suppression $\pm$ SEM of all 5 patients is shown. * $p < 0.05$ by paired t test. **b** Cytokine protein levels. The culture supernatants of 3 donors were recovered after 48 h of culture, and their cytokine levels were measured by ELISA. The cytokine levels (pg/ml) of the FA-untreated but stimulated T cells from the 3 donors ranged from 2,893 to 4,057 for IFN- γ , from 168 to 222 for IL-4, from 512 to 853 for IL-5, from 189 to 843 for IL-8 and from 3,143 to 30,512 for IL-10. The mean percentage suppression of cytokine production $\pm$ SEM is shown. * $p < 0.05$ by paired t test.

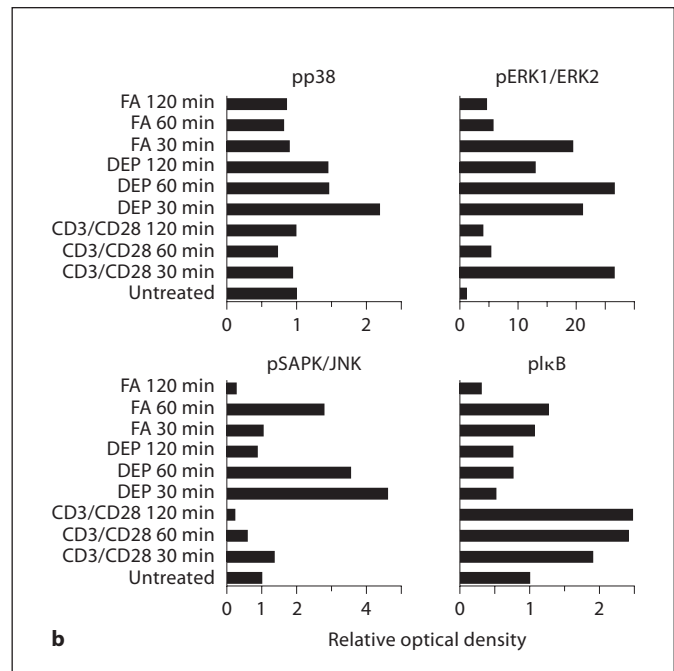
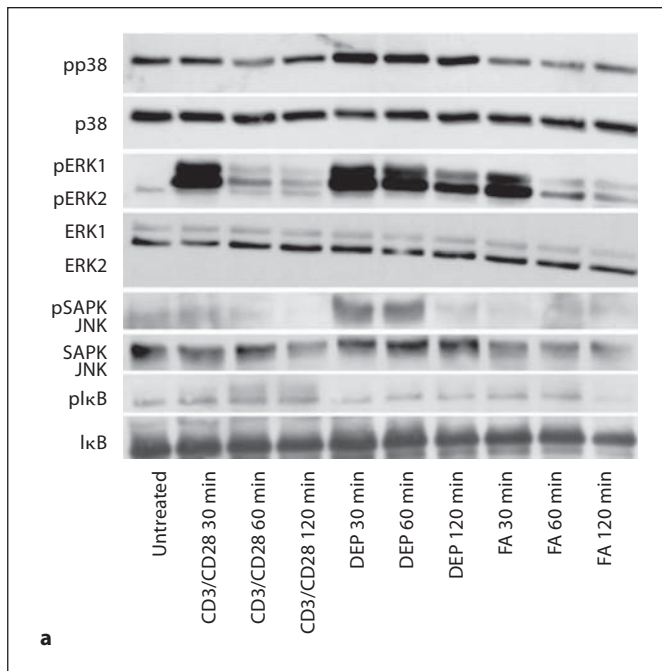
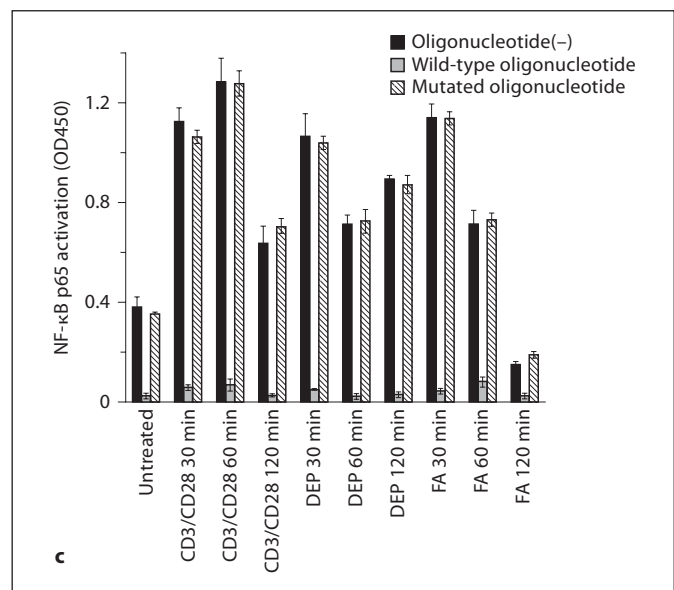


Fig. 2. Effect of DEP or FA pretreatment on the signal transduction of stimulated T cells. T cells were pretreated with or without 30 μ g/ml DEP or 10 nM FA for 1 h and then stimulated with T cell expander for 30 min, 1 h or 2 h. **a** Effect on the levels of phosphorylated and unphosphorylated p42/44 ERKs, SAPK/JNK, p38 MAPK and I κ B proteins in the T cells. Shown are representative immunoblot data from 3 independent experiments. **b** The optical densities of the signals were quantified by densitometry and National Institutes of Health image. The optical densities are expressed relative to that of the untreated control. **c** Effect on the binding activity of the total protein extracts of the cells to the NF- κ B p65 consensus sequence. The T cell extracts were analyzed using an NF- κ B transcriptional factor assay kit and the data are shown as means of the OD450 value $\pm$ SD of triplicates. To confirm the specificity of binding, the wild-type or mutated consensus oligonucleotide was added to the corresponding wells. One of 3 similar experiments is shown.



Effects of DEP or FA Pretreatment on Genes Involved in Th1/Th2 Skewing

Next, we examined the transcriptional effect of FA and DEP on T cells by real-time PCR using T cells from 3–8 healthy donors. For that purpose, we selected 8 genes that have been reported previously to be involved in Th1/Th2 skewing other than IFN- γ , IL-4, IL-5 and IL-10. These genes were T-bet, STAT4, GATA3, c-Maf (reviewed by Agnello et al. [7]), Txk [8], SOCS3 [9], SOCS5 [10] and

Gadd45b [11]. These analyses revealed that DEP and FA both suppressed IFN- γ , IL-10, T-bet, Txk and c-Maf mRNA expression in a statistically significant manner (fig. 3).

Gene Expression Profiling of Stimulated T Cells Pretreated with or without DEP or FA

Since these studies suggested that both DEP and FA have similar effects on the expression of 5 different genes

involved in Th1/Th2 skewing despite their chemical differences, we next subjected the RNAs of 30 ng/ml DEP/10 nM FA-pretreated and stimulated T cells to microarray analysis to further clarify the mechanism shared by them. A number of genes that met the selective criteria described in the Methods section were identified. Thus, of the 20,000 genes examined, 26 and 48 were upregulated and 25 and 188 were downregulated by DEP and FA, respectively (not shown). DEP-upregulated genes included ferritin heavy polypeptide I, HO-1, glutamate cysteine ligase and thioredoxin reductase 1, which have been reported to be induced by ROS and depend on the Nrf2-Keap1 pathway (reviewed by Kobayashi and Yamamoto [12]). Similarly, the FA-upregulated genes included glutathione S-transferase M3 and cathepsin L, which are also activated by oxidative stress [13]. Both DEP and FA pretreatment upregulated GILZ, Gadd45a, period homolog 1, pre-B cell leukemia transcription factor interacting

Fig. 3. Real-time PCR expression of genes involved in T cell skewing in DEP/FA-pretreated and stimulated T cells. T cells from 3–8 normal donors were pretreated with DEP or FA for 1 h and subsequently stimulated with T cell expander in the presence of DEP or FA for 6 h. The total RNA from the T cells was examined by real-time PCR for the expression of the 11 genes. The target/GAPDH ratio was calculated for each gene, and the data of the different donors are expressed as means $\pm$ SEM. The numbers of donors tested are as follows: n = 8 for IFN- γ ; n = 7 for IL-10; n = 6 for T-bet and Txk; n = 4 for IL-4, GATA3, SOCS3, SOCS5, STAT4 and Gadd45b, and n = 3 for c-Maf. * p < 0.05: significant difference compared to T cells treated with CD3/CD28 T cell expander alone (paired Student's t test).

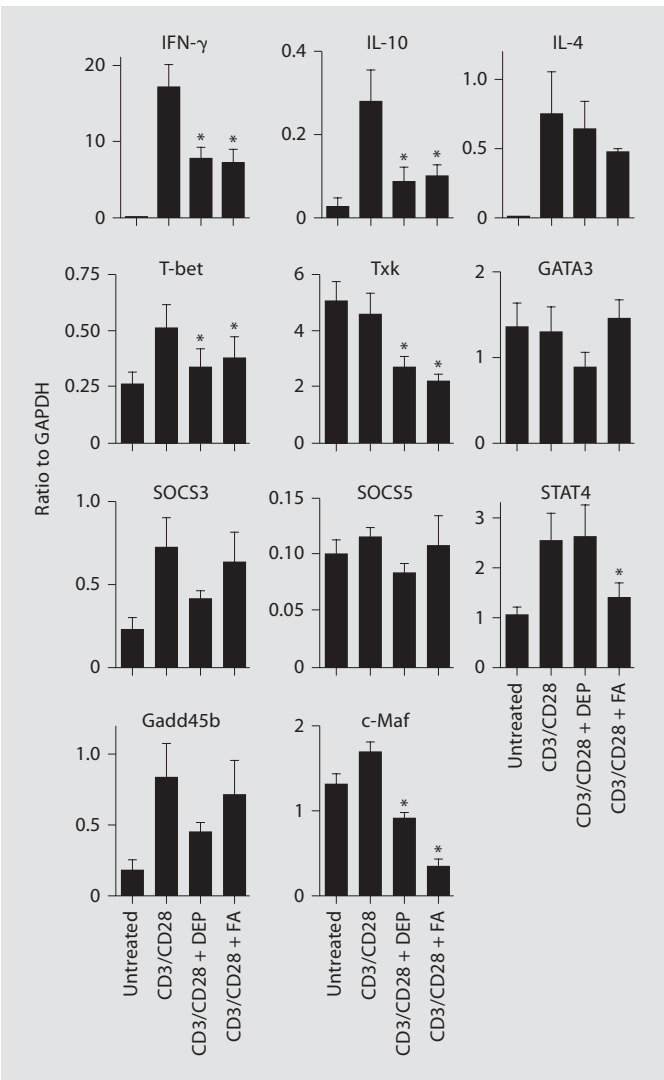


Table 3. Genes that were upregulated in T cell expander-stimulated T cells by DEP or FA pretreatment

Gene bank accession No.	Gene name	Relative increase					
		DEP			FA		
		donor 1	donor 2	donor 3	donor 1	donor 2	donor 3
NM_004089	GILZ	3.0	3.5	4.2	2.9	4.2	3.6
U60873	trophoblast-derived noncoding RNA	2.9	3.0	3.4	2.5	2.3	3.2
NM_001924	Gadd45a	1.5	3.3	2.3	2.4	2.7	2.0
NM_002616	period homolog 1 (<i>Drosophila</i>)	1.7	2.9	2.7	2.0	2.3	2.6
NM_020524	pre B cell leukemia transcription factor interacting protein 1	2.3	1.8	2.7	2.4	2.1	2.4

T cells from 3 donors were pretreated with or without DEP or FA and then stimulated with T cell expander. Genes that were upregulated by DEP or FA were selected by Genespring based on the criteria described in the Methods section. The relative increase for each gene was calculated by dividing the normalized intensity of DEP- or FA-pretreated and stimulated T cells by that of unpretreated but stimulated T cells.

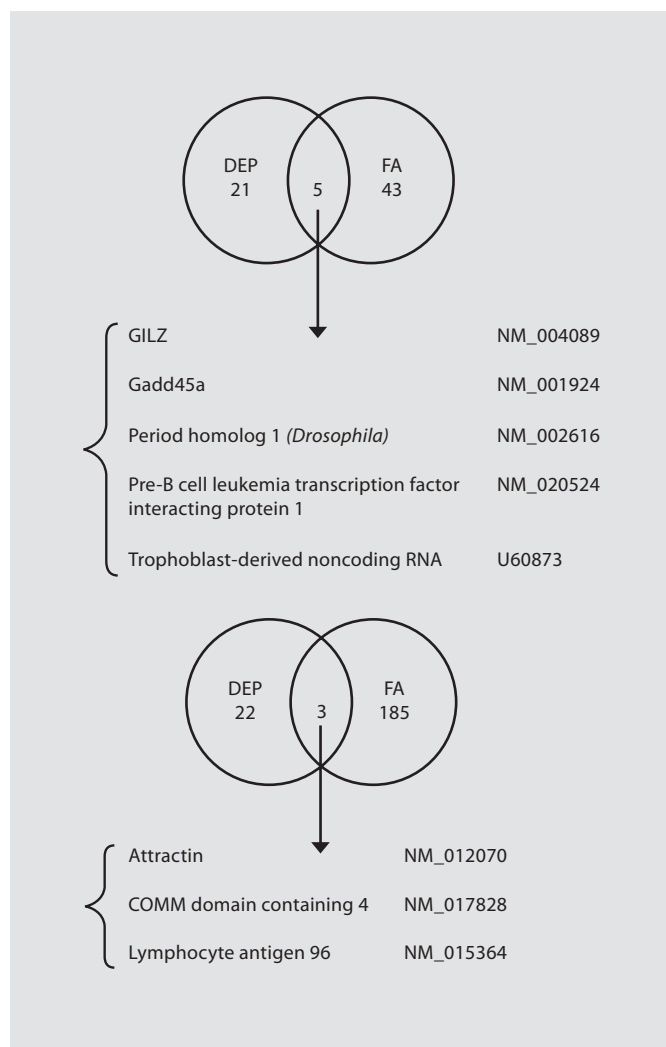


Fig. 4. Overview of the microarray experiment results. T cells from 3 nonatopic healthy donors were pretreated with or without DEP or FA and then stimulated with T cell expander for 6 h. Their cRNAs were then separately hybridized to the array. The results from the 3 different donors were pooled for statistical analysis with one-way ANOVA. Additional criteria used to select genes are described in the Methods section. The upper diagram shows the names and numbers of genes that were significantly upregulated by DEP and/or FA, while the lower diagram shows the genes that were downregulated by DEP and/or FA. Gene bank accession numbers are shown next to the gene names.

protein 1 and trophoblast-derived noncoding RNA (5 genes; table 3), while both downregulated attractin, COMM domain containing 4 and lymphocyte antigen 96 (3 genes; fig. 4). The remaining genes were altered by only one of the pretreatments. We confirmed by real-time PCR that both DEP and FA significantly upregulated

Gadd45a and GILZ mRNA, while HO-1 mRNA was up-regulated by DEP only (fig. 5).

Effect of NAC on mRNA Expression by DEP/FA-Pretreated and Stimulated T Cells

We next examined the effects of NAC on the DEP/FA-altered mRNA expression of IFN- γ , IL-10, T-bet, Txk, STAT4, c-Maf, HO-1, GILZ and Gadd45a in stimulated T cells (fig. 5). NAC pretreatment significantly reversed the DEP/FA-induced suppression of IFN- γ mRNA expression in stimulated T cells, although it was less effective in the DEP-treated T cells (fig. 5). NAC also caused a partial but significant recovery of DEP-suppressed IL-10 mRNA expression but had no effect on the FA-suppressed IL-10 mRNA expression levels. NAC had no effect on DEP- and FA-suppressed T-bet and Txk expression (fig. 5). In addition, NAC significantly suppressed the augmentation of Gadd45a mRNA by DEP and FA pretreatment and that of HO-1 mRNA by DEP, as well as tending to suppress the FA-induced augmentation of GILZ mRNA levels.

Discussion

In this study, we demonstrated that FA suppresses Th1 immune responses in human T cells in vitro. Recently, we used similar experiments to show that another representative environmental pollutant, DEP, promotes Th2-skewed immune responses in T cells [1]. It is interesting that two such chemically different substances as DEP and FA, which are both believed to cause atopic disorders, have the same effects on T cells in vitro. These observations also suggest that FA and DEP may impair the immune system via a similar mechanism. Therefore, we sought in this study to clarify the mechanism underlying the Th2-skewed immune responses induced by DEP and FA.

We showed that on the one hand, DEP and FA act in a nontranscriptional manner, as DEP augmented p44/42 ERK, p38 MAPK and SAPK/JNK activation, while FA activated only SAPK/JNK and both suppressed NF- κ B signaling. Both p38 MAPK and SAPK/JNK are required for IFN- γ production by Th1 cells, while the activation of the Ras/ERK pathway is required for IL-4 receptor function (e.g. STAT6 phosphorylation) and the differentiation of CD4⁺ Th2 cells in vivo and in vitro (reviewed by Rincon [14]). Thus, in our studies where DEP activated 3 MAPKs while FA activated only SAPK/JNK, the role of their effects on Th2 skewing of the immune response in DEP/

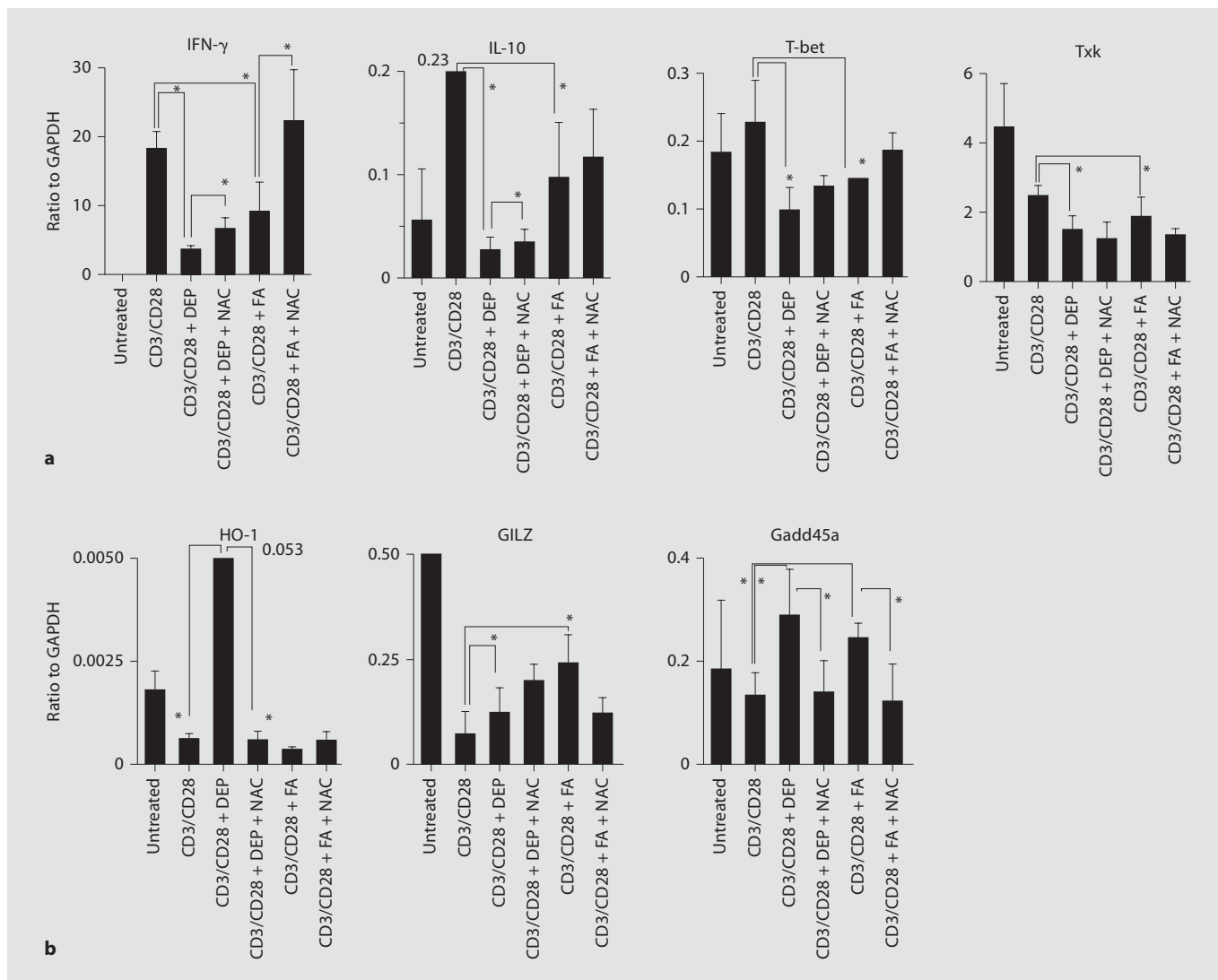


Fig. 5. Evaluation of the effects of NAC on DEP/FA-altered gene expression using real-time PCR. T cells from 3–6 normal donors were pretreated with or without 25 mM NAC for 1 h, then treated with DEP or FA for 1 h and subsequently stimulated with T cell expander in the presence of DEP or FA for 6 h. The total RNA from the T cells was examined by real-time PCR for the expression of the IFN- γ , IL-10, T-bet, Txk, HO-1, GILZ and Gadd45a

genes. The target/GAPDH ratio was calculated for each gene, and the data of the different donors are expressed as means $\pm$ SEM. The numbers of examined samples were as follows: $n = 3$ for IFN- γ , IL-10, T-bet and Txk; $n = 4$ for GILZ and HO-1, and $n = 6$ for Gadd45a. * $p < 0.05$: significant difference (paired Student's t test).

FA-pretreated T cells is beyond speculation. In addition, the fact that DEP and FA suppress NF- κ B signaling is very clear evidence that these environmental pollutants promote Th2 T cell responses, as 2 studies have strongly suggested that NF- κ B signaling is needed for the Th1 skewing of immune responses, as follows. Firstly, NF- κ B-binding sites have been found in the human IFN- γ promoter [15]. Secondly, mice transgenic for a mutated form

of I κ B α that is resistant to signal-induced degradation and thus functions as a transdominant inhibitor of NF- κ B/Rel induction exhibit severely impaired Th1 responses [16]. Thus, the downregulation of NF- κ B signaling in DEP- or FA-treated T cells is likely to play a pivotal role in suppressing their IFN- γ responses.

Our observations also showed that DEP and FA affect the expression of several transcription factors in stimu-

lated T cells. Both DEP and FA suppressed the mRNA levels of T-bet, c-Maf and Txk [8], which are known to play a crucial role in the Th1/Th2 balance [7, 8]. FA also suppressed STAT4 mRNA expression, which is known to participate in Th1 skewing [7, 11]. Thus, DEP and FA not only downregulate IFN- γ and IL-10 mRNA expression, they also alter the expression of a number of significant transcription factors that regulate the Th balance.

Among them, a particularly interesting gene whose expression is disturbed by DEP/FA is T-bet. T-bet is a potent transactivator of the IFN- γ gene and has been demonstrated to be the master regulator of Th1 lineage commitment [17]. Similarly, the other Tec family protein kinase, Txk, has been demonstrated to be intimately associated with the development of Th1/Th0 cells and plays a significant role in IFN- γ production [8]. Recently, it was found that T-bet contributes to the pathology of allergic asthma [18]. Moreover, T-bet $^{-/-}$ mice spontaneously develop several features of acute and chronic asthma that resemble human asthma, and these pathological changes could be adoptively transferred with T-bet $^{-/-}$ Th cells [18]. Thus, the suppression of T-bet mRNA by DEP or FA may account, at least in part, for the association between chronic DEP/FA exposure and the prevalence of allergic asthma.

The fact that DEP and FA suppress c-Maf mRNA expression is not consistent with the known roles c-Maf plays in Th2 skewing (reviewed by Agnello et al. [7]). Since neither DEP nor FA significantly affected IL-4 mRNA or protein expression, the significance of the suppression of c-Maf transcription by both DEP and FA is unclear.

Our microarray study also revealed that both DEP and FA significantly augmented the mRNA expression of Gadd45a and GILZ, which are both reported to be induced by FoxO3a [19, 20], while DEP augmented HO-1 mRNA expression, which is well known to be induced by the Nrf2-Keap1 pathway [12]. Gadd45a, GILZ and HO-1 are stress-induced genes that have recently been shown to affect T cell function. With regard to HO-1, it appears to mediate the anti-inflammatory effects of IL-10, and HO-1 knockout mice demonstrate a marked Th1 response after T cell stimulation [21]. These mice also develop chronic inflammation characterized by hepatosplenomegaly, lymphadenopathy, leukocytosis, hepatic periportal inflammation and occasionally glomerulonephritis [22].

With regard to Gadd45a, it has been reported to be a negative regulator of activation-induced T cell proliferation, and Gadd45a $^{-/-}$ mice spontaneously develop an autoimmune disease similar to human systemic lupus ery-

thematosus [23]. In addition, Salvador et al. [24] have demonstrated that Gadd45a inhibits the T cell alternative p38 MAPK activation pathway, in which the T cell receptor-activated tyrosine kinase Zap70 phosphorylates p38 MAPK on Tyr323. Several lines of evidence have indicated that the activity of this p38 MAPK is positively linked to IFN- γ production in activated T cells as well as Th1 T cell development [25]. Therefore, we initially speculated that the DEP/FA-augmented expression of Gadd45a mRNA suppresses IFN- γ production by inhibiting the p38 MAPK activation pathway. However, as mentioned above, we found DEP and FA augmented p38 MAPK phosphorylation, at least at the 30-min, 1-hour and 2-hour time points after T cell stimulation. Nevertheless, it remains possible that DEP and FA might influence these signal transduction pathways at a later time point.

With regard to GILZ, it has been shown to inhibit both T cell receptor-induced IL-2/IL-2 receptor expression and NF- κ B activity [26]. Since NF- κ B signaling is needed for the development of a Th1 response [15, 16], the augmented expression of GILZ mRNA in DEP/FA-treated T cells may also contribute to the reduced expression of IFN- γ mRNA and protein by interfering with NF- κ B signaling.

We speculated that DEP and FA may induce ROS, and that this is at least partially responsible for the plethora of transcriptional changes described above. To test this, we pretreated T cells with NAC before exposing them to DEP/FA and then stimulating them. Indeed, NAC significantly reversed both the DEP- and FA-induced suppression of IFN- γ mRNA expression; while it almost completely reversed the suppression of IFN- γ mRNA expression by FA-treated T cells, its effects on DEP-induced suppression were only partial. NAC had particularly profound effects on DEP- and FA-induced augmentation of Gadd45a mRNA. It also significantly neutralized the DEP-induced augmentation of HO-1 mRNA expression. NAC is a thiol and a precursor of L-cysteine and reduced glutathione. In addition, it is a source of sulfhydryl groups in cells and a scavenger of free radicals, as it interacts with ROS such as OH $\cdot$ and H $_2$ O $_2$ [27]. Accordingly, it is suspected that the suppression of IFN- γ production and the augmentation of Gadd45a mRNA by DEP/FA-treated T cells and the augmentation of HO-1 mRNA expression by DEP-treated T cells are, at least in part, mediated through the oxidative stress produced by DEP or FA.

Finally, the concentrations of DEP and FA used in our experiments were very high. It is true that in daily life, humans are exposed to much lower concentrations of these chemicals. For example, the US 24-hour ambient

2.5- μm particulate matter standard is 35 $\mu\text{g}/\text{m}^3$ [28], while in Sweden, the 24-hour ambient FA standard is 12–16 $\mu\text{g}/\text{m}^3$ [29]. However, humans are exposed to DEP and FA continually and for a long period of time. In contrast, we cultured T cells with these chemicals at most for 48 h in these experiments. Therefore, it might be necessary to use high concentrations of these chemicals in order to reveal their potential immunological effects. In addition, in contrast to real-time PCR analyses, our microarray study could not demonstrate significant effects of DEP and FA on the expression of genes involved in the Th1/Th2 balance. We think that this is mainly due to the low sensitivity of microarray studies and the limited numbers of our experiments.

In summary, our studies reveal that DEP and FA exert transcriptional and nontranscriptional effects on T cell signaling that promote Th2-skewed immune responses. Interestingly, DEP and FA had very similar effects on T cells, which suggests that DEP and FA may have additive effects on the immune system when present together in a polluted environment. Given that the suppression of IFN- γ expression by DEP/FA-pretreated T cells was already

seen 6 h after stimulation, the nontranscriptional effects of DEP/FA on NF- κB signaling appear to be the main mechanism behind the suppression of IFN- γ mRNA expression. However, the suppression of T-bet and Tbx mRNA and the augmentation of Gadd45a, GILZ and HO-1 mRNA may also contribute to the induction or exacerbation of allergic disorders. Of particular interest are Gadd45a, GILZ and HO-1, which are induced by stress and have recently been shown to significantly modulate T cell functions. Further investigation of the roles these molecules play in T cell immune responses is needed, as they may represent, at least in part, the missing link between environmental pollutants such as DEP and FA and the increased prevalence of allergic disorders.

Acknowledgements

This study was supported in part by the 21st Center of Excellence program of Tohoku University, by grants-in-aid for scientific research from the Japan Society for the Promotion of Science (17790749 and 18659318) and by the New Energy and Industrial Technology Development Organization.

References

- Ohtani T, Nakagawa S, Kurosawa M, Mizuashi M, Ozawa M, Aiba S: Cellular basis of the role of diesel exhaust particles in inducing Th2-dominant response. *J Immunol* 2005;174:2412–2419.
- Diaz-Sanchez D, Proietti L, Polosa R: Diesel fumes and the rising prevalence of atopy: an urban legend? *Curr Allergy Asthma Rep* 2003;3:146–152.
- Nel AE, Diaz-Sanchez D, Ng D, Hiura T, Saxson A: Enhancement of allergic inflammation by the interaction between diesel exhaust particles and the immune system. *J Allergy Clin Immunol* 1998;102:539–554.
- Rumchev KB, Spickett JT, Bulsara MK, Phillips MR, Stick SM: Domestic exposure to formaldehyde significantly increases the risk of asthma in young children. *Eur Respir J* 2002;20:403–408.
- Sadakane K, Takano H, Ichinose T, Yanagisawa R, Shibamoto T: Formaldehyde enhances mite allergen-induced eosinophilic inflammation in the murine airway. *J Environ Pathol Toxicol Oncol* 2002;21:267–276.
- Sagai M, Saito H, Ichinose T, Kodama M, Mori Y: Biological effects of diesel exhaust particles. I. In vitro production of superoxide and in vivo toxicity in mouse. *Free Radic Biol Med* 1993;14:37–47.
- Agnello D, Lankford CS, Bream J, Morinobu A, Gadina M, O'Shea JJ, Frucht DM: Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights. *J Clin Immunol* 2003;23:147–161.
- Kashiwakura J, Suzuki N, Nagafuchi H, Takeno M, Takeba Y, Shimoyama Y, Sakane T: Txx, a nonreceptor tyrosine kinase of the Tec family, is expressed in T helper type 1 cells and regulates interferon gamma production in human T lymphocytes. *J Exp Med* 1999;190:1147–1154.
- Seki Y, Inoue H, Nagata N, Hayashi K, Fukuyama S, Matsumoto K, Komine O, Hamano S, Himeno K, Inagaki-Ohara K, Cacalano N, O'Garra A, Oshida T, Saito H, Johnston JA, Yoshimura A, Kubo M: SOCS-3 regulates onset and maintenance of $\text{T}_\text{H}2$ -mediated allergic responses. *Nat Med* 2003;9:1047–1054.
- Seki Y, Hayashi K, Matsumoto A, Seki N, Tsukada J, Ransom J, Naka T, Kishimoto T, Yoshimura A, Kubo M: Expression of the suppressor of cytokine signaling-5 (SOCS5) negatively regulates IL-4-dependent STAT6 activation and Th2 differentiation. *Proc Natl Acad Sci USA* 2002;99:13003–13008.
- Chi H, Lu B, Takekawa M, Davis RJ, Flavell RA: GADD45beta/GADD45gamma and MEKK4 comprise a genetic pathway mediating STAT4-independent IFNgamma production in T cells. *EMBO J* 2004;23:1576–1586.
- Kobayashi M, Yamamoto M: Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxid Redox Signal* 2005;7:385–394.
- Chanas SA, Jiang Q, McMahon M, McWalter GK, McLellan LI, Elcombe CR, Henderson CJ, Wolf CR, Moffat GJ, Itoh K, Yamamoto M, Hayes JD: Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione S-transferase Gsta1, Gsta2, Gstm1, Gstm2, Gstm3 and Gstm4 genes in the livers of male and female mice. *Biochem J* 2002;365:405–416.
- Rincon M: MAP-kinase signaling pathways in T cells. *Curr Opin Immunol* 2001;13:339–345.
- Sica A, Dorman L, Viggiano V, Cippitelli M, Ghosh P, Rice N, Young HA: Interaction of NF-kappaB and NFAT with the interferon-gamma promoter. *J Biol Chem* 1997;272:30412–30420.

- 16 Aronica MA, Mora AL, Mitchell DB, Finn PW, Johnson JE, Sheller JR, Boothby MR: Preferential role for NF-kappa B/Rel signaling in the type 1 but not type 2 T cell-dependent immune response in vivo. *J Immunol* 1999;163:5116–5124.
- 17 Szabo SJ, Sullivan BM, Stemmann C, Satoskar AR, Sleckman BP, Glimcher LH: Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science* 2002;295:338–342.
- 18 Finotto S, Neurath MF, Glickman JN, Qin S, Lehr HA, Green FH, Ackerman K, Haley K, Galle PR, Szabo SJ, Drazen JM, De Sanctis GT, Glimcher LH: Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* 2002;295:336–338.
- 19 Asselin-Labat ML, David M, Biola-Vidament A, Lecoecue D, Zennaro MC, Bertoglio J, Pallardy M: GILZ, a new target for the transcription factor FoxO3, protects T lymphocytes from interleukin-2 withdrawal-induced apoptosis. *Blood* 2004;104:215–223.
- 20 Tran H, Brunet A, Grenier JM, Datta SR, Fornace AJ Jr, DiStefano PS, Chiang LW, Greenberg ME: DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. *Science* 2002;296:530–534.
- 21 Lee TS, Chau LY: Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat Med* 2002;8:240–246.
- 22 Brusko TM, Wasserfall CH, Agarwal A, Kapurczak MH, Atkinson MA: An integral role for heme oxygenase-1 and carbon monoxide in maintaining peripheral tolerance by CD4+CD25+ regulatory T cells. *J Immunol* 2005;174:5181–5186.
- 23 Salvador JM, Hollander MC, Nguyen AT, Kopp JB, Barisoni L, Moore JK, Ashwell JD, Fornace AJ Jr: Mice lacking the p53-effector gene Gadd45a develop a lupus-like syndrome. *Immunity* 2002;16:499–508.
- 24 Salvador JM, Mittelstadt PR, Belova GI, Fornace AJ Jr, Ashwell JD: The autoimmune suppressor Gadd45alpha inhibits the T cell alternative p38 activation pathway. *Nat Immunol* 2005;6:396–402.
- 25 Lu B, Yu H, Chow C, Li B, Zheng W, Davis RJ, Flavell RA: GADD45gamma mediates the activation of the p38 and JNK MAP kinase pathways and cytokine production in effector TH1 cells. *Immunity* 2001;14:583–590.
- 26 Ayroldi E, Migliorati G, Bruscoli S, Marchetti C, Zollo O, Cannarile L, D'Adamio F, Riccardi C: Modulation of T-cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor kappaB. *Blood* 2001;98:743–753.
- 27 Zafarullah M, Li WQ, Sylvester J, Ahmad M: Molecular mechanisms of N-acetylcysteine actions. *Cell Mol Life Sci* 2003;60:6–20.
- 28 National ambient air quality standards for particulate matter. *Fed Regist* 2006;71:61144–61233.
- 29 Gustafson P, Barregård L, Lindahl R, Sällsten G: Formaldehyde levels in Sweden: personal exposure, indoor, and outdoor concentrations. *J Expo Anal Environ Epidemiol* 2005;15:252–260.