In vitro Toxicity Evaluation of Diesel Exhaust Particles on Human Eosinophilic Cell

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Abstract:
Diesel exhaust particles (DEPs), comprised mainly of particles less than 2.5 μ m
(PM2.5) in aerodynamic diameter, have been assumed to enhance the response of asthma to
allergen inhalation. Although eosinophilic infiltration is remarkable in the event of bronchial
asthma induced by DEPs, the precise mechanisms leading to eosinophilia are unknown. To
examine the effect of DEPs on eosinophils, we measured the cytokine products and activity of
nuclear factor -kappa B (NF-κB) after addition of the proteasomal inhibitor MG132 in HL-60
clone 15 cells differentiated into eosinophils. We measured eotaxin-induced chemotaxis of
cells and their activity of p38 mitogen-activated protein (MAP) kinase was analyzed.
Interleukin (IL)-8 and monocyte chemoattractant protein-1 (MCP-1) were increased
markedly in DEPs-treated cells. The active form of NF-κB in cells treated with DEPs was
increased, and this effect was significantly decreased by the administration of MG132. Cell
migration in the presence of DEPs was significantly greater, and inhibited by adding N-
acetyl L-Cysteine. P38 MAP kinase activity was highly influenced by DEPs treatment. DEPs
induce MCP-1 and IL-8 production by up-regulating NF-κB activity, which is inhibited in the
presence of an inhibitor of proteasomal degradation. DEP also promotes eotaxin-induced
chemotaxis in a p38-dependent manner.

Key Words :Diesel exhaust particles, monocyte chemoattractant protein-1, IL-8, eotaxin,
chemotaxis

Abbreviations:
DEPs, diesel exhaust particles; MCP-1, monocyte chemoattractant protein-1;
NF-κB, nuclear factor -kappa B; MAP kinase, mitogen-activated protein kinase; IL-(8),
interleukin-(8); NAC, N-acetyl L-cysteine; RANTES, regulated upon activation normal T
cell expressed and secreted; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-
gamma; FCS, fetal calf serum; PBS, phosphate buffered saline; BSA, bovine serum
albumin; ATF-2, activating transcription factor-2.

Introduction

In bronchial asthma, a marked increase in eosinophils is observed at the site of inflammation and bone marrow. In addition, it is known that eosinophils which infiltrate the site of inflammation have in their cells a major basic protein that has cytotoxic properties. By releasing cytokines (Schmid-Grendelmeier et al., 2002), lipid mediators (Busse, 1998), reactive oxygen species (Albert et al., 2003), and highly charged cytotoxic granular proteins (Marguet et al., 2001), activated eosinophils contribute to airway inflammation and cause damage of the bronchial mucosa (Pegorier et al., 2006). Therefore, today eosinophils are thought to play a central role in enhancing inflammation.

The prevalence of allergic airway diseases such as bronchial asthma has increased in industrial areas and countries. Patients with bronchial asthma believed to be caused by air pollution are increasing, thus creating a social problem. The main components of air pollution are substances such as nitrogen oxides and ozone.

Recently, many studies have demonstrated that diesel exhaust particles (DEPs) exposure is linked to the incidence of pulmonary inflammation and allergic airway disease, particularly bronchial asthma. When a person is exposed to immunogens such as house dust and pollen after long-term exposure to polluted air which includes these fine particles, DEPs enhance inflammation in bronchial asthma (Brauer et al., 2003; Diaz-Sanchez et al., 2003; Holgate et al., 2003; Pandya et al., 2002).

The main components of DEPs are fine particles including heavy metals which are emitted from diesel engines when diesel oil combusts. These fine particles are 2.5 µm or less (PM 2.5) in aerodynamic diameter and include black smoke. These fine particles contribute to the adjuvant activity of DEPs on bronchial asthma. Therefore, there are calls for the reduction of DEPs in urban areas.

Recently, many researchers have reported that inhalation of DEPs induced biologic responses to bronchial asthma (Kanoh et al., 1996) (Bloemen et al., 2007; Pandya et al., 2002). DEPs promote release of specific cytokines, chemokines, immunoglobulins, and oxidants in the airway. Release of these mediators of the allergic and inflammatory response initiates a cascade that can culminate in airway inflammation, mucus secretion, serum leakage into the airways, and bronchial smooth muscle contraction. However, it is not clear if inhalation of DEPs induced biologic response on eosinophils.

In this study, we examined the effects of DEPs on eosinophils by studying cytokine production when DEPs organic solvent extracts were given to a human leukocytic cell line HL-60 clone 15, which had been differentiated into an eosinophil-like cell line.

2.Materials and Methods

2.1.Eosinophil cell culture and differentiation

HL-60 clone 15 cells (CRL-1964, American Type Culture Collection, Rockville, MD) were maintained in RPMI-1640 medium (pH 7.8) (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (Sigma, St. Louis, MO) and passaged twice weekly in an atmosphere of 1.5% CO2. For experiments, cells (2 X 105 cells/ml) were induced with n-butyrate (0.5 mM; Sigma) for 5 days (differentiated HL-60 clone 15 cells; df-HL-60 clone 15 cells), as previously described (Fischkoff, 1988) (Yamaguchi et al., 1994). Df-HL-60 clone 15 cells were assessed by morphology (eosinophil granules on May-Günwald and eosinophil peroxidase staining), previously described (Lopez et al., 2003). Peroxidase positive df-HL-60 clone 15 cells induced by n-butyric acid were collected and were used for experiments.

2.2.Preparation of DEPs suspensions

The DEPs suspension (National Institute for Environmental Studies, Tsukuba, Ibaraki) was prepared just prior to use. To induce particle disaggregation, stock solutions of

particles were dispersed in phosphate buffered saline (PBS) containing 0.05% Tween 80 (Sigma Chemical, St. Louis, MO) at a concentration of 10 mg/ml and then sonicated at output 5 and duty 30 of ultrasonic disruption for 2 minutes under cooling conditions (Amakawa et al., 2003). Different concentrations of particles were then diluted with RPMI-1640. PBS containing 0.05% Tween 80 was used as a negative control in separate experiments. For cytokine array assay, DEPs (0.03 mg/ml) were treated to df-HL-60 clone 15 cells. For MCP-1 EILSA, NF-KB DNA binding assay, chemotaxis assay and p38 MAP kinase assay, DEPs (0.1 mg/ml) were treated to df-HL-60 clone 15 cells.

2.3. Analysis of cytokines by Human Cytokine Array

Df-HL-60 clone 15 cells (5×10^5 cells/ml; 1ml) in 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) were treated with DEPs (0.03 mg/ml), IL-1 (10 ng/ml), or buffer for 72 hours at 37ºC in 1.5% CO2, and cytokines and chemokines present in the supernatant were determined using the RayBioTM Human Cytokine Array III kit (RayBiotech, Inc., Norcross, GA) following the manufacturer's instructions. According to the manufacturer, 42 different cytokines and growth factors (for example, IL-2, IL-3, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, MCP-1, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-α), regulated upon activation normal T cell expressed and secreted (RANTES) could rapidly and accurately identify the expression profiles in just one experiment by using this system. One ml of each sample was used for analysis. Each signal in the membrane was detected with a chemiluminescence imaging system (AE-6972 Densitograph Lumino-CCD, ATTO, Tokyo) and quantified by image analysis using a CS analyzer (ATTO, Tokyo).

2.4. Preparation of Nuclear Extracts

Df-HL-60 clone 15 cells (5×10^5 cells/ml; 20ml) in 250ml Tissue culture flasks (Becton Dickinson Labware, Franklin Lakes, NJ) were cultured either with or without 0.1 mg/ml DEPs for 4 hours at 37°C in 1.5% CO2. Untreated cells and DEPs-treated cells were harvested by centrifugation in cold PBS. Nuclear extracts were prepared according to the method described by Lee et al. (Lee et al., 1988) with modifications(Xu et al., 1997). In brief, cells were washed twice with ice-cold PBS after incubation and suspended in one packed cell volume (PCV) of buffer A (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 1 μg/ml leupeptin and aprotinin, pH 7.9) on ice for 10 minutes and then lysed by passing the cell suspension through a 27-gauge needle 5 times. Crude nuclei were washed twice with buffer A to prevent cytosolic contamination, and the nuclear proteins were extracted with two-thirds PCV of ice-cold buffer B (20 mM HEPES, 420 mM KCl, 1.5 mM MgCl2, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and aprotinin, and 25% glycerol, pH 7.9). Two-thirds PCV of ice-cold buffer C (20 mM HEPES, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and aprotinin, 20% glycerol, pH 7.9) was added. The mixture was then centrifuged at 14,000 rpm at 4°C for 15 minutes. The nuclear proteins were transferred to new tubes as aliquots and stored at -70°C until use. The protein concentration was determined by a DC Protein Assay kit (Rio-Rad, Richmond, CA).

2.5. NF-κB DNA binding assay

NF-κB is a ubiquitous transcription factor that mediates the inflammatory response. The proteasome inhibitor MG132 inhibits NF-κB formation and degradation of its inhibitor I-κB. One μg of nuclear extract was used for NF-κB DNA binding assay. NF-κB activation in the nuclear extracts was quantified by an EZ-Detect NF-κB p65 assay kit (Pierce, Rockford, IL) based on an ELISA technique. Specifically, an immobilized oligonucleotide containing

the NF-κB consensus site (5'-GGGACTTTCC-3') was bound to microwell plates. The active form of NF-κB present in nuclear extracts specifically binding to this oligonucleotide was detected using a primary antibody that recognizes an epitope on p65 that is accessible only when NF-κB is activated and bound to its target DNA. Then competitive oligonucleotide (wild type) of consensus site was added to the control. Following the addition of an HRP-conjugated secondary antibody, the plates were read on an automated plate reader, and the level of nuclear NF-κB p65 was expressed as the absorbance at 450 nm (A450).

2.6. Measurements of MCP-1 by ELISA

Df-HL-60 clone 15 cells (1 X 10^5 cells/ml; 10ml) in 70ml Tissue culture flasks (Becton Dickinson Labware, Franklin Lakes, NJ) that had been preincubated with either medium or MG132 (Calbiochem, La Jolla, CA)(5 nM) for one hour were cultured either with medium or DEPs (0.1 mg/ml) for 24 hours at 37ºC in 1.5% CO2. MCP-1 protein was measured in cultured supernatants using a commercial solid phase quantitative sandwich ELISA kit for MCP-1 (R&D SYSTEMS, Minneapolis, MN) according to the manufacturer's descriptions.

2.7. Chemotaxis Assay

Df-HL-60 clone 15 cells (5 X 10^6 cells/ml; 5ml) were pretreated with either medium or N-acetyl L-cysteine (NAC) (Wako Pure Chemicals Ltd, Tokyo) (5 mM) for one hour and then stimulated with human recombinant eotaxin (R&D SYSTEMS) (100 ng/ ml) for one hour at 37ºC in 1.5% CO2. After eotaxin stimulation, the cells were further stimulated with medium or DEPs (0.1 mg/ml) for one hour at 37ºC in 1.5% CO2. Cell chemotaxis was evaluated using a microchamber technique (Falk et al., 1980). Eotaxin (100 ng/ml) in RPMI-1640 with 0.1% bovine serum albumin (BSA) was added to the lower compartment of the disposable 96-well chemotaxis chamber (KURABO, Osaka) in a volume totaling 80 µl. The cell suspension (50 µl of 2 x 106 cells/ml) was added to the upper compartment of the chamber that had been precoated with BSA for 2 hours at 37ºC in 1.5% CO2. Compartments were separated by a 5-µm pore size polycarbonate, polyvinylpyrrolidone-free filter. The chamber was incubated for 2 hours at 37ºC in 1.5% CO2. At the end of the incubation, the cells in the lower compartment were stained using the CyQUANT NF Cell Proliferation Assay Kit (Invitrogen, Eugene, OR).

2.8. P38 MAP kinase assay

Df-HL-60 clone 15 cells (5 X 106 cells/ml; 5ml) that had been preincubated either with medium or NAC (5 mM) for one hour were stimulated with eotaxin (100 ng/ ml) for an additional one hour. After eotaxin stimulation, the cells were further stimulated with medium or DEPs (0.1 mg/ml) for one hour. The activity of p38 MAP kinase was analyzed by a commercially available kit (p38 MAP kinase Assay Kit; Cell Signaling Technology, Inc., Danvers, MA). Cell lysates were prepared and incubated overnight with immobilized p38 MAP kinase (Thr180/Tyr182) monoclonal antibody. Kinase reaction was performed in the presence of 100 µM of cold ATP and 2 µg of activating transcription factor-2 (ATF-2) fusion protein. One µg of samples was separated by a 5-20% gradient SDS-PAGE and transferred to membranes. Phosphorylation of ATF-2 at Thr71 was measured by western blot using phospho-ATF-2 (Thr71) specific antibody.

2.9. Statistical Analysis

For in vitro studies, six independent replicate analyses were performed for each experimental condition and time point unless otherwise noted and the data were expressed as the mean ± SD. Groups were compared using the two-way ANOVA followed by post hoc

Dunnett's test. Statistical significance was set at p < 0.05.

3.Results

3.1.DEPs Induce MCP-1 and IL-8 Production in HL-60 clone 15 cells

We examined effect of DEPs in df-HL-60 clone 15 cells by cytokine antibody array method. As shown in Figure 1A, MCP-1 (C6 and D6) and IL-8 (E12 and F12) were detected as positive signals under each condition. MCP-1 levels in the DEP treated groups tended to markedly increase by 1.9-fold over those in the untreated group. IL-8 levels in the DEP treated groups tended to slightly increase by 1.1-fold over those in the untreated group. IL-1-treated df-HL-60 clone 15 cells exhibited 2.2-fold and 1.5-fold increases in MCP-1 and IL-8, respectively. We examined the effect of DEPs on MCP-1 production. The concentration of MCP-1 was increased in a dose-dependent manner after treatment with DEPs (0–0.1 mg/ml) and was 6.5-fold greater than that of the control at the zenith (data not shown).

3.2. DEPs up-regulate NF- κ B activation in df-HL-60 clone 15 cells

NF-κB activation was measured in the nuclear extracts from df-HL-60 clone 15 cells. NF- κ B activity of 1 μg protein was shown in Figure 2. NF- κ B activity was increased after stimulation with DEPs (Wild - DEP +); in contrast, NF- κ B activity was significantly decreased following administration of competitive oligonucleotide (Wild + DEP -, Wild + DEP + X)(p < 0.05).

3.3.MG132 inhibits DEPs-induced MCP-1 production

We examined DEPs-induced MCP-1 production after administration of MG132 in df-HL-60 clone 15 cells.As shown in Figure 3, MCP-1 was increased after stimulation with DEPs (MG132 – DEP +); in contrast, MCP-1 was significantly decreased following administration of MG132 (MG132 + DEP -, MG132 + DEP +)(p<0.01).

3.4.Effect of p38 MAP kinase activation on df-HL-60 clone 15 cell chemotaxis

We examined DEPs-induced chemotaxis after administration of NAC in df-HL-60 clone 15 cells. As shown in Figure 4, cell migration was significantly increased in the DEPs-stimulated clone 15 cells (NAC-DEP+) compared to media-stimulated control cells (NAC - DEP -); in contrast, cell migration was significantly decreased following administration of NAC (NAC + DEP -) (p<0.01).

3.5.DEPs affect p38 MAP kinase (Thr180/Thr182) activity

We examined DEPs affect of p38 MAP kinase (Thr180/Thr182) activity in df-HL-60 clone 15 cells. As shown in Figure 5, levels of the phosphorylated form of the ATF-2 protein of p38 MAP kinase were greater in DEPs-treated cells (lane 2) than in untreated cells (lane 1). In contrast, ATF-2 phosphorylation levels in NAC-pretreated cells (lane 3) and in NAC + DEPs-treated cells (lane 4) were significantly low (p<0.01), indicating that pretreatment of cells with NAC resulted in the inhibition of DEPs-induced p38 MAP kinase activation.

4.Discussion

We have examined a biologic response of DEPs leading to eosinophilia in bronchial asthma. To elucidate the mechanisms, an in vitro approach was used in which human eosinophilic cells were exposed to DEPs. We described in this paper the effect of DEPs on a myeloid cell line differentiated into eosinophils. We showed that DEPs induced MCP-1 and IL-8 production by up-regulating NF- κ B activity, which is inhibited in the presence of an inhibitor of proteasomal degradation. DEPs also promoted eotaxin-induced chemotaxis in a

p38 MAP kinase dependent manner.

The above findings indicate a mechanism that DEPs enhance inflammation by the following process. In the late stage of bronchitis due to DEPs, eosinophils are involved in neutrophil and macrophage recruitment. These cells build up and secrete eotaxin which further recruits eosinophils, leading to enhanced inflammation.

The following are caused when human peripheral blood lymphocytes are exposed to DEPs: mRNA expression of IL-8, MCP-1, and RANTES increases, eotaxin mRNA level of bronchial epithelial cells increases (Fahy et al., 2000), ICAM-1 production increases (Takizawa et al., 2003), and histamine mRNA of nasal mucosal cells increases (Terada et al., 1999). Accumulation of neutrophils, eosinophils, and macrophages occurs at the inflammation site due to DEPs. At the same time, inflammatory cytokines and chemical mediators are released from cells which developed inflammation. This sequence of events is thought to enhance inflammation. A recent study has reported neutrophil migration by IL-8 possibly leads to eosinophilia in severe asthma (Kobayashi et al., 2007). In this study, IL-8 production by eosinophils increased by 10% due to DEPs exposure. Our data may suggest the mechanism which changes the acute stage of neutrophil predominance into the late stage of the eosinophils predominance in bronchial asthma.

An increase in IL-8 and MCP-1 production is inhibited by the antagonist of MAP kinase (Fahy et al., 2000). Therefore, DEPs are thought to enhance inflammation through the activation of the MAP kinase pathway. DEPs activate the NF-κB activation of bronchial epithelial cells, increase IL-8 production, and stimulate the migration of neutrophils. At the same time, DEPs induce the production of the antibacterial peptide beta-defensin 2 (Nam et al., 2006). It is believed that the defense system against bacterial infection is used to repair and to remove DEPs incorporated into the body. Furthermore, unoxidized DEPs reduce IFN-γ of antigen-specific T-cells of dendritic cells, which are derived from mouse marrow, and increase IL-10. Such DEPs activate the nuclear factor-erythroid 2 (NF-E2)-related factor 2-mediated signaling pathway and inhibit IL-12 production (Chan et al., 2006). Therefore, DEPs inhibit the Th1 differentiation and are thought to be involved in the shift to Th2 differentiation.

According to a recent report, when macrophages phagocytize ultra fine particles contained in DEPs, phagocytic function decreases (Lundborg et al., 2006). In addition, the dendritic cells (DCs) derived from immature monocytes are unable to differentiate into functional T-cells due to DEPs exposure (Bleck et al., 2006), suggesting possibly to a difficult state of foreign body removal. The results of our experiment showed that there was a 190% increase of MCP-1 through NF-κB activation due to DEPs exposure of eosinophil. The exposed eosinophils strongly affected macrophage infiltration. Furthermore, it would be interesting to examine whether or not normally functioning macrophages will mature from monocytes that accumulated in the bronchial tubes due to MCP-1, which is produced by eosinophils induced by DEP exposure.

DEPs exposure in human bronchial epithelial cells improved eotaxin gene expression (Bree et al., 2007). In this study, DEPs exposure in eosinophil also promoted eotaxin-induced chemotaxis in a p38-dependent manner. From these findings, it was found that DEP further accelerates the eosinophil infiltration by p38 MAP kinase activation of eosinophil.

Figure Legends

Figure 1. Detection of cytokines and chemokines on membrane antibody arrays by chemiluminescence. Df-HL-60 clone 15 cells (5 × 105 cells/ml) were treated with DEPs (0.03 mg/ml), IL-1 (10 ng/ml), or buffer for 72 hours at 37ºC, and cytokines and chemokines present in the supernatant were determined using a RayBio Human Cytokine Antibody Array. (A) Image obtained with a CCD camera after an exposure of 90 seconds using a Light Capture

AE-6972 imaging system. Each cytokine and chemokine was represented by duplicate spots on the membrane. G1, H1, A11 and B11 were spotted with HRP-conjugated antibody (positive control), G2 and H2 were spotted with bovine serum albumin (negative control). (B) Average net light intensity for each pair of cytokine spots detected on the basis of gray-scale imaging.

Figure 2. Effect of DEPs on NF-κB activation to df-HL-60 clone 15 cells by ELISA. Df-HL-60 clone 15 cells were treated with 0.1 mg/mL DEPs for 4 hours. After incubation, nuclear fractions were prepared and used for the analysis of NF-κB p65 by ELISA. The results were expressed as the means $\pm$ SD of six different experiments. Statistical analysis of difference was performed by two-way ANOVA. Statistically significant difference as compared to the value of the group without DEPs, ** $p<0.01$. Statistically significant difference as compared to the value of the group without DEP, ##$p<0.01$.

Figure 3. Effect of DEPs on MCP-1 production to df-HL-60 clone 15 cells by ELISA. Df-HL-60 clone 15 cells that had been preincubated with either medium or MG132 (5 nM) for one hour were cultured either with medium or DEPs (0.1 mg/ml) for 24 hours at 37ºC. The results are expressed as means $\pm$ SD of six different experiments. Statistical analysis of difference was performed by two-way ANOVA. Statistically significant difference as compared to the value of the group without DEPs, **$p<0.01$. Statistically significant difference as compared to the value of the group without MG132, #$p<0.05$.

Figure 4. Effect of DEPs on p38 MAP kinase activation to df-HL-60 clone 15 cells chemotaxis. Cells were pretreated with either medium or NAC (5 mM) for one hour and then stimulated with eotaxin (100 ng/ ml) for one hour. After eotaxin stimulation, the cells were further stimulated with medium or DEPs (0.1 mg/ml) for one hour. The results are expressed as means $\pm$ SD of six different experiments. Statistical analysis of difference was performed by two-way ANOVA. Statistically significant difference was obtained compared to the value of the group without DEPs, ** $p<0.01$. Statistically significant difference as compared to the value of the group without NAC, ##$p<0.01$.

Figure 5. Effect of DEPs on p38 MAP kinase activation to df-HL-60 clone 15 cells by Western blot. Df-HL-60 clone 15 cells that had been preincubated either with medium or NAC (5 mM) for one hour were stimulated with eotaxin (100 ng/ ml) for an additional one hour. After eotaxin stimulation, the cells were further stimulated with medium or DEPs (0.1 mg/ml) for one hour. The lysates from df-HL-60 clone 15 cells were separated by a 5-20% gradient SDS-PAGE and transferred to membranes. Phosphorylation of ATF-2 at Thr71 was measured by western blot using phospho-ATF-2 (Thr71) specific antibody (upper panel). The cells were cultured with medium (lane 1), DEPs (lane 2), NAC (lane 3), and DEPs and NAC (lane 4). The fold increase in amount of phospho-ATF-2 protein is indicated (lower panel). The amounts of phospho-ATF-2 protein were presented as relative to control cells (NAC – DEP -)(1.0). Six identical experiments independently performed gave similar results. The results are expressed as means $\pm$ SD of six different experiments. Statistical analysis of difference was performed by two-way ANOVA. Statistically significant difference was obtained compared to the value of the group without DEPs, ** $p<0.01$. Statistically significant difference as compared to the value of the group without NAC, ##$p<0.01$.

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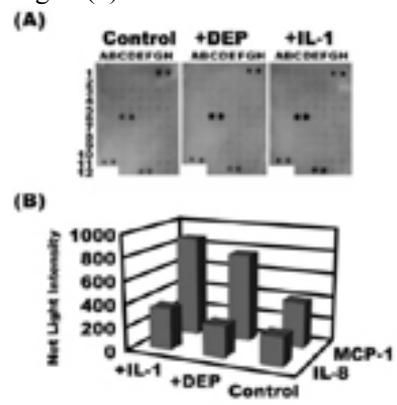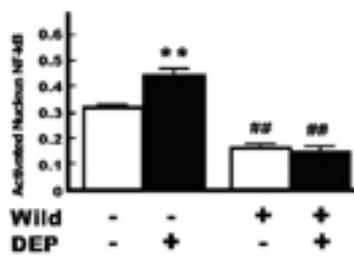
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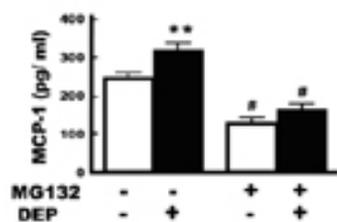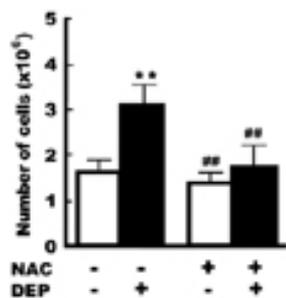
(A)

(B)

Figure 1.

Figure(2)

Figure2.

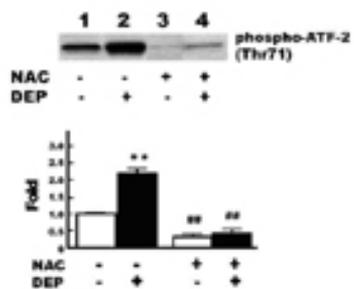Figure(3)

Figure 3.

Figure(4)

Figure 4.

Figure(5)

Figure 5.