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OZONE EXPOSURE IMPAIRS ANTIGEN-SPECIFIC IMMUNITY BUT ACTIVATES IL-7-INDUCED PROLIFERATION OF $CD4^-CD8^-$ THYMOCYTES IN BALB/*c* MICE

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It is well known that ozone (O_3), a potent reactive oxidant and air pollutant, induces respiratory inflammation and hyperresponsiveness upon inhalation. It was previously shown that O_3 exposure (0.6 ppm, 10 h/day for 15 days) not only results in local bronchial inflammation, but also affects the nervous system and thymocyte proliferation, and places mice under oxidative stress. In the present study, data showed that O_3 exposure could impair both the natural killer (NK) cell activity and the proliferation potential of spleen T cells to a specific antigen stimulus. Immunological function assays indicated that O_3 exposure attenuated the proliferation of spleen mononuclear cells induced by concanavalin A and decreased $CD4^+$ and $CD28^+$ lymphocyte subsets. However, supplementation with natural antioxidants protected mice from O_3-induced dysfunction of splenocyte proliferation. Meanwhile, O_3 exposure resulted in a decline of mitogen-induced IL-2 production in splenocytes. It was also found that O_3 exposure dramatically enhanced the proliferation of $CD4^-CD8^-$ thymocytes stimulated by recombinant mouse interleukin-7 (rmIL-7), which is usually observed during the mammal aging process. Taken together, data conclude that short-term repetitive O_3 exposure damages both innate and acquired immunity via altering the lymphocyte subset and cytokine profile, and via impact on thymocyte early development. O_3-induced oxidative damage is one of the key factors leading to immune dysfunction in this mouse model.

The health effects of atmospheric pollution are topics of increasing public concern (Schwela, 2000). Ozone (O_3), a major air pollutant, possesses a strong oxidizing potential. Accumulating epidemiological and laboratory evidence suggest that short- or long-term O_3 exposure may weaken the biological functions of exposed organs, such as skin (Thiele et al., 1997) and respiratory tract (Bhalla, 1999; Toward & Broadley, 2002), by mechanisms involving oxidative damage.

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In recent decades, the correlation of O_3 exposure and bronchial inflammation has been intensively studied (Holtzman et al., 1983; Weinmann et al., 1995; Kleeberger et al., 1997; Bhalla, 1999; Hamada et al., 1999). Acute and subacute exposure to O_3 induces bronchopulmonary inflammation and hyperpermeability and produces epithelial injury of both nasal and pulmonary conducting airways (Bhalla, 1999). Furthermore, since O_3 exposure may disrupt the airway epithelial lining barrier against penetration of exogenous gaseous or particles, coexposure to O_3 and other inhaled ambient particles enhances inflammatory and epithelial responses (Goldsmith et al., 2002; Wagner et al., 2002). In addition, inhalation of O_3 may affect the pulmonary immune function and allergic responses to antigens by increasing immunoglobulin (Ig) E synthesis in predisposed animals and humans. Repeated O_3 exposures trigger a Th2-like response and enhance the allergic immune response when combined with ovalbumin (OVA) aerosol (Neuhaus-Steinmetz et al., 2000). Ozone pollution also contributes to the development or exacerbation of allergic disorders by enhancing the underlying allergic inflammatory response (McConnell et al., 2002; Salvi, 2001).

Although a large body of evidence exists for O_3-induced oxidative stress in the respiratory tract, the long-term in vivo effects of O_3 exposure on the immune system have remained unclear (Fujimaki et al., 1987; Li & Richters, 1991; Cohen et al., 2001). The literature supports the conclusion that O_3 may react with the pulmonary surfactant system and produce oxygen/nitrogen radicals (Holtzman et al., 1983; Stevens et al., 1995), which in turn attack biomacromolecules such as proteins and lipids (Lerner & Eschenmoser, 2003; Pryor et al., 1995). Aldehydes and lipid ozonation products generated after O_3 reaction with cell membrane lipids are considered to be involved in the inflammatory response (Frampton et al., 1999). Reactive oxygen species (ROS) also play a role in this process (Doelman & Bast, 1990). It was hypothesized that cells or biological fluids containing these ozonation products could certainly move to other sites of the body by lymphocinesia or blood circulation and place other tissues under oxidative stress. Such distal or secondary damage from O_3 exposure on immune and other systems may contribute to a compromised immune response after repeated inhalation of O_3 (Fujimaki et al., 1987; Cohen et al., 2001). To investigate this, a murine oxidative stress model was established for repetitive inhalation of O_3 (Feng et al., 2001). After inhalation of O_3, the mice show increased oxidative damage levels in tissues and urine and decrease in antioxidative capacity. Inhalation of O_3 results in cytological nuclear concentration in brain neurons or thymocytes and induces thymus atrophy (Feng et al., 2001). However, treatment of the mice with antioxidants partially or completely protects against these deteriorative alterations (Feng et al., 2001).

Over the last decade, considerable progress has been made in understanding the mechanisms of T-cell activation and proliferation. CD4 and CD8 are two accessory molecules in the interactions between T cells and antigen-presenting cells (APCs) carrying peptide–MHC complexes, and thus play important roles in the recognition by T cells of the processing peptides on the APCs

and activation of T-cell proliferation. The costimulation activity of CD28 also appears to be key in the activation and differentiation of naive T cells in the immune response (McAdam et al., 1998; Lenschow et al., 1996). In the present study, the impacts of O_3 exposure were further evaluated on lymphocyte proliferation, $CD4^+$, $CD8^+$, and $CD28^+$ lymphocyte subsets, cytokine production, and specific and nonspecific immunity. The possible protection by natural antioxidants against O_3 exposure-induced immune dysfunction was also investigated. In addition, thymus is the primary organ for the production of mature αβ T cells from immature thymocyte and is thus essential for a functional immune system. Thymic epithelial cells produce the hormones thymosin and thymopoietin and in concert with interleukin (IL)-7 are important for the early development and maturation of thymocytes (He et al., 1995; Plum et al., 1996; Pallard et al., 1999). T cells differentiate from $CD4^-CD8^-$ (DN) precursors to mature $CD4^+$ or $CD8^+$ (SP) thymocytes through the $CD4^+CD8^+$ double-positive (DP) stage. During the DP stage, thymocytes are subjected for selection, either expansion/differentiation into SP cells (positive selection) or deletion (negative selection). We hypothesize that ozone exposure not only impairs the peripheral mature lymphocytes, but also affects immature thymocyte development. To probe the changes of early thymocyte development under oxidative stress, precursor thymocytes were examined and the expansion and differentiation of $CD4^-CD8^-$ thymocytes induced by IL-7 was analyzed.

MATERIALS AND METHODS

Reagents and Animals

Ovalbumin (OVA) fraction V, tetrazolium salt (MTT), bovine albumin (BSA), concanavalin A (Con A), and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO). RPMI 1640 and Freund's adjuvant complete (CFA) and noncomplete (NFA) were from GIBCO Life Technologies (Grand Island, NY). The Ficoll-Paque Plus kit was obtained from Amersham Biosciences (Uppsala, Sweden). Enzyme-linked immunosorbent assay (ELISA) quantitative kits for mouse IL-2 and interferon (IFN)-γ were purchased from Diaclone (Besancon, France). Recombinant mouse interleukin-7 (rmIL-7) was obtained from R&D (Minneapolis, MN). Polyethylene (PE)- or FITC-conjugated monoclonal antibody was available through PharMingen (San Diego, CA). Anti-CD4- and anti-CD8-conjugated microbeads were from Biosource (Camarallo, CA). [^{3}H]-TdR (high-performance liquid chromatography [HPLC] pure) was from National Institute of Atomic Energy (Beijing, China). Medium used for all cell culture work consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT), 10 m*M* HEPES, 2 m*M* L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Natural antioxidants, catechin and black tea polyphenol extract, were obtained from the Japan Institute for the Control of Aging (Haruoka, Japan) and verified for their antioxidant properties in vitro (Feng et al., 2000).

Specific-pathogen-free male BALB/*c* mice (5 wk old, 18–20 g) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), Beijing, China. The mice were kept in facilities that are specific pathogen free and environmentally controlled (21 ± 0.5°C and 45 ± 5% humidity), fed a sterile standard rodent diet, and provided water ad libitum. They were acclimatized for at least 3 days before the start of the study.

Animal Treatment Protocol

The treatment of the laboratory animals and the experimental protocols for the present study adhered to the guidelines of the Peking Union Medical College and were approved by the Institutional Authority for Laboratory Animal Care. The O_3 exposure was conducted as described previously (Feng et al., 2001). Briefly, mice in a small cage were placed in a half-open system, in which an ozonator (Ozone R&D Center, TsingHua University, Beijing, China) was placed on a rack. The ozonator–cage distance was regulated to make sure the O_3 concentration at the central point of the cage was maintained at 0.6 ppm during exposure, as measured using an ozonometer purchased from the Advisory Board for Toxic Material Detection (Beijing, China). The mice were exposed to O_3 for 10 h/day (11 p.m. to 9 a.m. in the dark) until the end of the experiment at day 15. The mice breathing the filtered air were used as the untreated control group. For the protection experiments with natural antioxidants, mice were fed via gastric intubation with catechin (64 mg/kg) or black tea extract (20 ml/kg) once per day. Two days later, the mice were placed into the O_3 exposure regimen as already described, with continuing feeding with the tested antioxidants by gastric intubation until day 15.

Lymphocyte Proliferation Assay

Mouse spleen was teased and passed through a stainless-steel mesh to obtain single-cell suspensions from which mononuclear cells (MNCs) were separated by Ficoll-Hypaque centrifugation. The MNCs of each mouse were placed into triplicate wells of a microplate and then received Con A (5 μg/ml) or LPS (20 μg/ml). The plates were then incubated at 37°C for 48 h before all wells were pulsed with [^{3}H]-TdR (1 μCi/well) for 13 h. After harvesting onto glass-fiber filters, cell radioactivity in each well was counted in a β-counter (Beckman LS8000, Fullerton, CA), and all data were expressed as mean counts per minute (CPM) ± SD.

Lymphocyte Phenotype Assay

Surface phenotypes of splenic MNCs were identified using monoclonal antibodies (mAbs) in conjunction with a single- or two-color immunofluorescence test. Cell viability (>95%) was assessed by trypan blue exclusion assay. Flow cytometric profiles were shown with cells gated on the lymphocyte population by forward and side-angle light scatter. The respective isotype-matched control immunoglobulins (Igs) or biotin-conjugated anti-mouse CD4 (L3T4)/avidin-FITC mAb, PE-conjugated anti-mouse CD8a (Ly-2), and PE-conjugated anti-mouse CD28 were used. The percentages of $CD4^+$, $CD8^+$, and $CD28^+$

cells in MNCs from each group of four mice were analyzed by a FACScan system (Coulter Co.). Ten thousand cells were assayed to create the profiles.

ELISA Measurement for Cytokines Production

Splenic MNCs (1×10^6/ml) from each mouse of each group were cultured in triplicate in 24-well plates in 1 ml medium with or without Con A (5 μg/ml). Cytokine production (IL-2 and IFN-γ) in the culture supernatants at 72 h was assayed using ELISA quantitative kits.

Natural Killer Cell Activity Assay

The natural killer (NK) cell cytolytic activity was evaluated by the MTT method as described previously by our laboratory (Yu et al., 1999). The target cells were murine lymphoma cell-line YAC-1, which is a sensitive target for mouse NK cells. The mouse splenic MNCs, separated by Ficoll-Hypaque centrifugation, were used as the effector cells. MNCs in Ca^{2+}- and Mg^{2+}-free PBS were adjusted to 4×10^6/ml; 75-μl aliquots were then placed into triplicate wells of 96-well plates and target cells were then added (75 μl volume) at an E:T ratio of 50:1. The cultures were incubated at 37°C for 4 h in a humidified atmosphere at 5% CO_2 in air. MTT assay was carried out and the absorbance at 570 nm was recorded with a reference wavelength of 630 nm. The percent cytolysis was calculated as:

$$\{[\text{T OD value} - (\text{E} + \text{T OD value})]/(\text{T OD value})\} \times 100\%$$

where T indicates target cells, E effector cells, and OD optical density.

Proliferation of $CD4^-CD8^-$ Thymocytes Stimulated by rmIL-7

The proliferation of $CD4^-CD8^-$ double-negative (DN) thymocytes induced by rmIL-7 was measured by [^{3}H]-TdR incorporation (He et al., 1995). Purification of DN cells from thymic MNCs was performed using a magnetic activated cell sorter system (MACS). Thymic MNCs were suspended in MACS buffer (PBS, pH 7.2, 5% BSA) to which anti-CD4- and anti-CD8-conjugated microbeads were added (0.2 ml/10^7 cells). The cells were incubated for 30 min at 4°C and then placed in a MACS magnetic column frame (Immunotech Co., Marseille Cedex, France) for 5 min. The entire effluent was collected and then retreated with microbeads. The final effluent was collected as the "nonmagnetic" fraction, in which $CD4^-CD8^-$ thymocytes were confirmed with flow cytometry assay.

The pooled DN cells of each mouse were cultured in triplicate in 96-well round-bottom plates in a volume of 0.2 ml (5×10^4 DN cells). Cytokine rmIL-7 was added at different concentrations (ng/ml). After being cultured for 60 h at 37°C in a humidified atmosphere of 5% CO_2 in air, the cells were pulsed with 1 μCi [^{3}H]-TdR per well and harvested 13 h later to quantify label incorporation.

Proliferation Frequency of OVA-Specific T Cells After OVA Sensitization

T cells play a central role in the initiation and regulation of the immune response to antigen. The frequencies of OVA-specific T-cells in murine spleen

MNCs were estimated by a limiting dilution assay (Chou et al., 1992). The OVA sensitization protocol used is described in Figure 1. Eight female BALB/*c* mice (10 wk old, 22.2–23 g) were never exposed to OVA and were fed OVA-free standard rodent diet (NICPBP, Beijing). In the experimental group, mice ($n = 4$) were exposed to O_3 for 5 days and then subjected to an immunization procedure. Mice (control and O_3 inhalation groups) were immunized by intraperitoneal injection of 0.2 ml of an emulsion containing 100 μg OVA and CFA. A boost immunization with 80 μg antigen in NFA was performed 7 days after initial priming. The second challenge was performed via intravenous injection with 50 μg OVA in 0.1 ml saline solution 12 days after the boost. Three days after that, spleens were removed, teased apart, and passed through number 200 wire mesh screens. The pooled splenic MNCs for each group were washed twice and applied to a nylon wool adherent column (Yuetai Sciences, Beijing, China), according to the manufacturer's instructions. The nonadherent splenic T cells were obtained by dropwise elution using complete medium (5% FCS) at a rate of 2 ml/min to a total volume of 50 ml. Cells were washed and resuspended in medium (15% FCS) for use in the experiments described next. T cells accounted for >89% of the eluent, as assessed by flow cytometry with anti-CD3.

Freshly isolated splenic MNC from normal female BALB/*c* mice were irradiated (2500 rad) and used as a source of antigen-presenting cells. The prepared enriched T cells were diluted to 2, 1, 0.5, and 0.25×10^5 cells/well, respectively. The irradiated splenic MNCs (APC) were added to the wells at dilutions of 0.125, 0.125, 0.25, and 0.5×10^5 cells/well, respectively. The total volume of each well was 0.2 ml. Ten replicate wells were cultured at each cell concentration with OVA (20 μg/well). The assay control wells were cultures of the same cell concentration that did not receive OVA. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 5 days. At 80 h, the cultures were fed by removing 75 μl supernatant and replacing it with 80 μl fresh medium without (for assay controls) or with OVA. After 5 days, proliferation was determined by [^{3}H]-TdR uptake. Individual wells were scored as

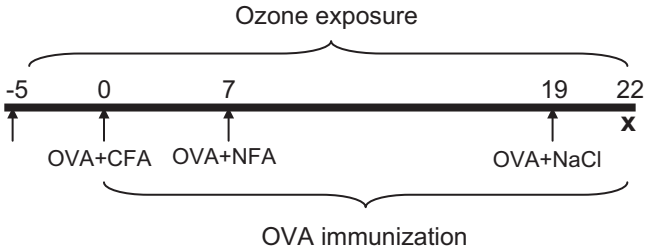

FIGURE 1. Protocol of OVA sensitization used in this study. OVA + CFA: the first immunization by intraperitoneal injection of 100 μg OVA and Freund's adjuvant complete; OVA + NFA: boost immunization with 80 μg OVA and Freund's adjuvant noncomplete; OVA + NaCl: challenge by intravenous injection of 50 μg OVA in normal saline; X: sacrifice.

antigen responders if the CPM exceeded the sum of the mean CPM + 2SD from 6 assay control wells cultured at the same cell concentration but without antigen.Using the percent non-responding wells (PNW) at each cell concentration, antigen-specific T-cell frequencies were evaluated by the following formula, where ln is the natural logarithm and *m* the antigen-specific T-cell number):

$$-\ln(\text{PNW}) = m$$

According to this formula, a straight line would be drawn on semilogarithmic coordinate paper with –ln (PNW) as the ordinate and cell dilution as the abscissa. When –ln (PNW) = 1.0, the abscissa value indicates the cell dilution in which one antigen-responder cell is present.

Statistical Analyses

Data were expressed as mean ± standard deviation (SD). Differences between two groups were compared by Student's *t*-test for independent samples. Probability (*p*) values less than .05, calculated as two-tailed *p* values, were considered statistically significant. The SPSS (version 10.0) software package was used for this purpose.

RESULTS

Proliferation of Splenic MNCs After O_3 Exposure and the Effects of Antioxidants

To determine whether O_3 exposure exerted any influence on lymphocyte proliferation, the proliferation capabilities of mitogen-stimulated splenic MNCs were determined. As shown in Table 1, LPS-induced non-T-cell proliferation was not markedly affected by inhalation of O_3 during the study period. However, O_3 exposure significantly inhibited [^{3}H]-TdR incorporation into spleen MNCs stimulated by Con A, a mitogen specific for mouse T lymphocyte proliferation. In another set of experiments, the mice were orally administered antioxidants (catechin or black tea polyphenol extract) and then treated as in the

TABLE 1. Effect of O_3 Inhalation on the Proliferation of Mouse Splenic MNCs Induced by Mitogens

Treatment groups	Con A (5 μg/ml)	LPS (20 μg/ml)
Control (n = 6)	118,262 ± 32,064	20,346 ± 2890
O_3 exposure (n = 8)	71,371 ± 12,540[a]	19,043 ± 4192

Note. Mouse splenic MNCs were cultured for 48 h at 2×10^5 cells/well in the presence of Con A or LPS. Cells were then pulsed with [^{3}H]-TdR for 13 h. Data are expressed as ΔCPM (mean of CPM in stimulated wells – mean of CPM in unstimulated wells) ± SD.

[a] Statistical significance ($p < .05$) vs. control mice.

O_3 exposure protocol. As shown in Figure 2, antioxidant administration significantly protected T lymphocytes against O_3 exposure-induced decrease of proliferation.

Percentages of Lymphocyte Subpopulations Under Oxidative Stress

To understand the influence of O_3 exposure on the expression of these CD molecules, the percentages of $CD4^+$, $CD8^+$, and $CD28^+$ lymphocyte subsets were measured using flow cytometry. As shown in Table 2, the percent $CD4^+$ or $CD28^+$ cells in splenic MNCs decreased significantly after the mice were exposed to O_3 for 15 days, whereas the $CD8^+$ lymphocyte subset remained

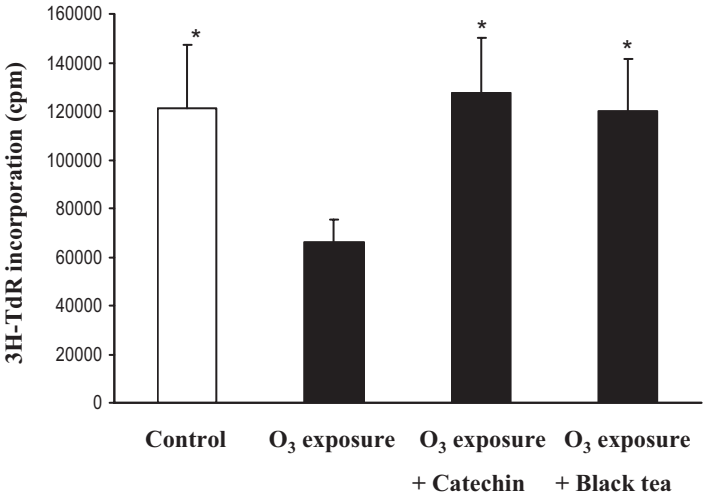

FIGURE 2. Inhibition of O_3 exposure-induced decrease of T-cell proliferation by natural antioxidants. Mice were fed orally via gastric intubation with catechin or black tea extract for 2 days, and then exposed to either filtered air (control) or O_3. Mouse splenic MNCs (2×10^5 cells/well) were cultured and treated with Con A (5 μg/ml) for 48 h. T-cell proliferation was determined with the $[^3H]$-TdR incorporation assay and expressed as ΔCPM (mean of CPM in stimulated wells – mean of CPM in unstimulated wells) ± SD ($n = 4$). Asterisk indicates statistical significance ($p < .05$) vs. O_3 exposure.

TABLE 2. Effect of O_3 Exposure on Mouse lymphocyte Subpopulations of Splenic MNCs

Treatment groups	$CD4^+$ (%)	$CD8^+$ (%)	$CD28^+$ (%)
Control ($n = 4$)	22.7 ± 2.1	8.2 ± 0.7	25.7 ± 1.2
O_3 exposure ($n = 4$)	16.9 ± 2.3[a]	7.8 ± 2.4	22.3 ± 1.2[a]

Note. The phenotypes of splenic MNCs from each group of four mice were detected with flow cytometry assay. At least three samples from each mouse were used. Results are expressed as means ± SD of the percentages of $CD4^+$, $CD8^+$, and $CD28^+$ cells in MNCs, respectively.

[a] Statistical significance ($p < .05$) versus control mice.

unchanged, indicating that O_3-induced oxidative stress may impair $CD4^+$ and $CD28^+$ lymphocytes.

Con A-Induced Cytokine Production Profiles

As a T-cell growth factor, IL-2 is a key cytokine to induce T-cell proliferation and NK cytotoxicity; IFN-γ is an important cytokine to activate inflammatory cells such as the macrophages and neutrophils. To determine the cytokine production profiles after O_3 exposure, spleen MNCs were cultured with or without the addition of Con A (5 μg/ml). The quantitation of cytokines in the supernatant was carried out with ELISA quantitative kits. As shown in Table 3, the production of mouse IL-2 in the culture supernatant was significantly reduced after O_3 exposure, whereas Con A-induced IFN-γ production increased, suggesting that O_3 exposure may promote the production of inflammatory cytokine in the mice.

NK Activity Declined After O_3 Exposure

NK cell activity plays an important role in host resistance against neoplastic and infectious diseases. To determine whether inhalation of O_3 affects innate immunity and further potentially weakens the protection against tumor antigen or viral attack, the ability of spenocytes to kill YAC-1 target cells was assessed. As shown in Figure 3, NK activity (%) significantly decreased after the repeated exposures to O_3. Compared with untreated control mice, O_3 exposure caused a 35% decrease in NK activity.

Proliferation Frequency of Antigen-Specific T Cells

To identify whether O_3 exposure may attenuate antigen-specific immunological response, after immunizing mice with OVA, the OVA-specific proliferation frequency of splenic T-cell from the hosts was analyzed using a limiting dilution assay. Table 4 shows the results for the percent nonresponding wells (PNW) and –ln(PNW) of control and O_3-treated mice, respectively. OVA-specific T-cell proliferation frequencies in O_3-treated mice were observed to have decreased by more than 60%, from 1.8×10^{-5} (control) to 6.7×10^{-6}.

TABLE 3. Effect of O_3 Exposure on Con A-induced IL-2 and IFN-γ production in Mouse Splenocytes

	Treatment	
Cytokines	Control	O_3-treated
mIL-2 (pg/ml)	360 ± 99	176 ± 101*
mIFN-γ (pg/ml)	823 ± 90	1098 ± 248*

Note. Splenic MNCs (1×10^6/ml) from each mouse were cultured in triplicate with or without Con A (5 μg/ml). After 72 h, culture supernatants were harvested and concentrations of IL-2 or IFN-γ measured by ELISA.

[a] Statistical significance ($p < .05$) vs. control mice.

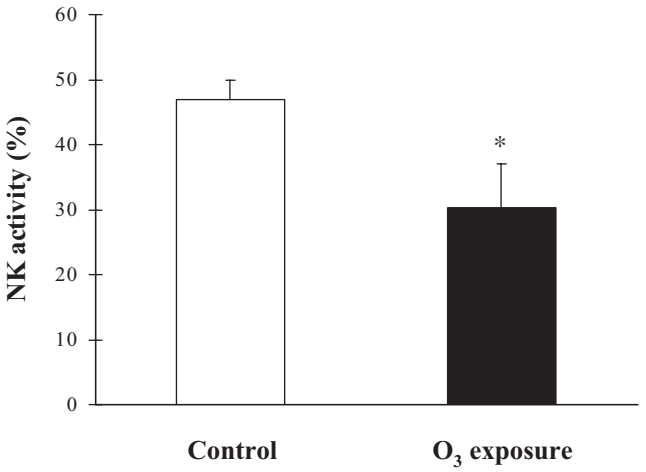

FIGURE 3. The effect of O_3 exposure on splenic NK activity. Mice were exposed to filtered air (control, $n = 6$) or O_3 (O_3 exposure, $n = 6$) for 15 days. NK activity was measured as the ability of splenic MNCs to lyse target YAC-1 cells. The splenic MNCs were mixed with the target cells at a ratio of 50:1 and incubated for 4 h. MTT assay was carried out and the absorbance (A_{570nm}) was recorded with a reference wavelength of 630 nm. Results were expressed as percent specific lysis (%). Values are means ± SD ($n = 6$). Asterisk indicates statistical significance ($p < .05$) vs. control mice.

TABLE 4. Calculation of OVA-specific T-cell Proliferation Frequencies in Mice, Measured by the Limiting Dilution Assay

	Treatment							
Parameter	Control				O_3-treated			
Cells per well ($\times 10^4$)	20	10	5	2.5	20	10	5	2.5
PNW	10	50	80	80	60	80	90	100
–ln (PNW)	2.3	0.7	0.22	0.22	0.51	0.22	0.11	0
Proliferation frequency	1.8×10^{-5}				6.7×10^{-6}			

Note. Mice were immunized according to the OVA-sensitization protocol (Figure 1). T cells were then isolated from pooled splenic MNCs from control or O_3-exposed mice, and proliferation frequencies of T cells responsive to OVA (in the presence of irradiated APCs) were determined with limiting dilution assay. PNW: percent nonresponding wells.

Enhancement of IL-7-Induced Proliferation of $CD4^-CD8^-$ Thymocytes by O_3 Exposure

It is well known that thymus is the primary organ for the production of mature T cells and is thus essential for a functional immune system. To explore the effect of O_3-induced oxidative stress on thymocyte development, studies focused on one of the development stages, the proliferation of $CD4^-CD8^-$ precursor thymocytes stimulated by their specific cytokine IL-7. The proliferative

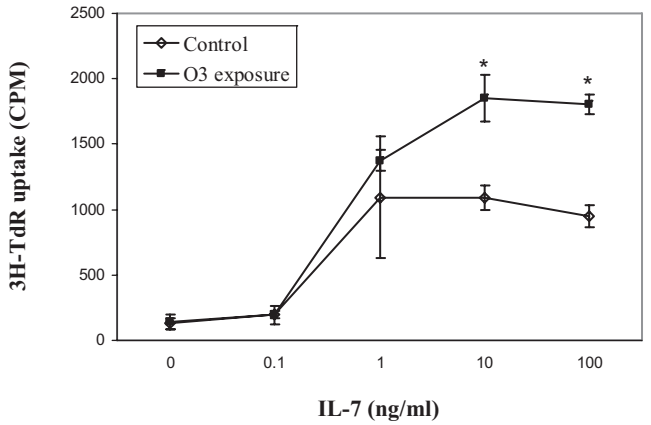

FIGURE 4. Modulation of IL-7-induced proliferation of $CD4^{-}CD8^{-}$ thymocytes by O_3 exposure. Mice were exposed to filtered air (control) or O_3 (O_3 exposure) for 15 days. The $CD4^{-}CD8^{-}$ thymocytes isolated from thymus MNCs were treated with cytokine rmIL-7 at the indicated concentrations. After culture for 60 h at 37°C, the proliferation was determined by $[^3H]$-TdR uptake. Results are expressed as means (CPM) ± SD ($n = 4$). Asterisk indicates statistical significance ($p < .05$) vs. control mice.

response of mouse DN ($CD4^{-}CD8^{-}$) cells to rmIL-7 was determined using the $[^3H]$-TdR uptake method. As shown in Figure 4, the proliferation of DN thymocytes was dependent on the dose of IL-7. IL-7 induced significant proliferation of control DN cells at about 10 ng/ml. Unexpectedly, $[^3H]$-TdR uptake of DN cells from O_3-treated mice increased over control mice, especially when IL-7 was added at a concentration of 10 ng/ml (69% increase) or 100 ng/ml (90% increase). This indicates that O_3 exposure may enhance the proliferative response of DN thymocytes to IL-7.

DISCUSSION

The present study demonstrates that repetitive O_3 exposure not only inhibited splenocyte NK cell activity but, more importantly, attenuated the proliferation of splenic T cells to a specific antigen OVA, suggesting that O_3 inhalation alone may impair innate and acquired immune functions. Ozone exposure also dramatically enhanced DN thymocyte proliferation in response to IL-7 stimulation, indicating that the early development of thymocytes was also affected by inhalation of O_3. The blocking effect of natural antioxidants on O_3-induced spleen lymphocyte dysfunction supports the conclusion that O_3 may react with biomacromolecules via an oxidative damage mechanism (Feng et al., 2001). These findings indicate that O_3 exposure not only induces airway-tract inflammation and bronchial hyperreactivity (primary effects) (Bhalla, 1999; Toward & Broadley, 2002; Holtzman et al., 1983; Weinmann et al., 1995; Kleeberger et al., 1997), but also affects other systems not in

direct contact with inhaled O_3, such as the primary and secondary lymphoid organs (secondary effects).

NK activity is a primary line of defense to virally infected cells as well as certain neoplasms; therefore, testing the effect of ozone exposure on NK activity is important in understanding the immunotoxic potential of ozone. Burleson et al. (1989) reported that continuous O_3 exposures attenuated pulmonary NK activity, and that several cell types and/or products of these cells were involved. It is also known that NK cells are susceptible to ROS, which may alter surface charge and decrease the adherence of NK cells to target cancer cells in vivo and in vitro (Nakamura & Matsunaga, 1998). Ozone exposure induces oxidative stress in animals (Stevens et al., 1995; Pryor et al., 1995; Frampton et al., 1999; Feng et al., 2001; Lerner & Eschenmoser, 2003). Oxidant-products derived from O_3 exposure might impair the adherence of NK cells to target YAC-1 cells and affect the release of granule contents. In addition, NK cell activity could be induced or improved by IL-2 through the transcription of perforin. Thus, the inhibition of inductive expression of IL-2 in O_3-treated mice (Table 3) may also contribute to the decline of NK activity.

Over the last decade, considerable progress was made in understanding the mechanisms of T-cell activation and proliferation. Both the engagement of the T-cell antigen receptor (TCR) with major histocompatibility complex (MHC)/antigen and a second costimulatory signal are needed for the complete activation of the T cell. In this process, co-receptor CD4 and other accessory molecules also play critical roles in the sensitivity of T-cell recognition (Krogsgaard et al., 2003). Thus, inhibition or blockage of so-called immunological synapse impairs the response activation of T cells to an exogenous protein antigen. While O_3 exposure may impair the T-lymphocyte function in the spleen and thymus and suppress the delayed hypersensitivity reaction (Fujimaki et al., 1984; 1987; Cohen et al., 2001), it remains unclear how O_3 affects T-cell early development and the antigen-specific response of mature T cells. The present study shows that the response capacity of T cells to a specific antigen declined after O_3 exposure. To explore the underlying mechanism, other immunological alterations induced by O_3 exposure were further measured. It was found that the percentages of $CD28^+$ and $CD4^+$ T cells in spleen MNCs dropped more than 13% and 25%, respectively. In contrast, O_3 exposure has no significant influence on percentage of $CD8^+$ T cells in spleen MNCs (Table 2). In addition, O_3 inhalation mainly impaired the activation pathway of T cells but not other MNCs (mainly B cells) induced by LPS (Table 1). This is consistent with other reports that T cells were more susceptible to oxidative damage than B cells owing to less antioxidant content in T cells (Becker et al., 1991; Hendricks & Heidrick, 1988). It is acknowledged that triggering of TCR in the absence of CD28 costimulation is not sufficient to activate the T cell, and may lead to T-cell anergy or tolerance. Signals from both the TCR and CD28 integrate at some point within the T cell to provide full activation of the genes required to induce T-cell proliferation and specific T-cell effector functions (Appleman & Boussiotis, 2003). The reduction of $CD28^+$ T cells in O_3-treated

mice would attenuate the antigen presentation and antigenic peptide recognition, leading to the deficiency of responsive T cells to a specific antigen. So the findings here suggest that the pool of activated-potential T cells is reduced after O_3 exposure, which results in the reduction of T-cell proliferative capacity. On the other hand, the suppression of T-cell proliferative response to Con A suggests that O_3 may inhibit or impair the activation cascade events in T cells (Dziedzic & White, 1986).

IL-2 is a specific T-cell growth factor and mainly produced by $CD4^+$ T cells. The CD28 molecule delivers a costimulatory signal for IL-2 production by antigen-specific T cells (Jenkins et al., 1991). The reduction of Con A-induced IL-2 in our model mice is partly associated with the decrease of $CD4^+$ T cells and CD28 expression (Table 2). Decrease of IL-2 production is believed to be one of the important factors of antigen-specific T-cell anergy (Appleman & Boussiotis, 2003). The present data indicated that both the insufficiency of $CD28^+$ T-cells and IL-2 production may contribute to O_3-induced decrease in T-cell activation. IFN-γ, a mediator of local inflammatory reaction by modulating intracellular ROS and the expression of adhesion molecules that participate in inflammatory process, is mainly produced by NK cells, Th1 $CD4^+$ T cells, and all $CD8^+$ T cells. The present data indicated that the alteration pattern of IL-2/IFN-γ was homologous to that of the ratio of $CD4^+/CD8^+$ cells resulted from ozone exposure (Tables 2 and 3), suggesting that IL-2 synthesis by $CD4^+$ T cells was attenuated whereas IFN-γ production by $CD8^+$ T cells may not be damaged. On the other hand, IL-2 is known to enhance IFN-γ production by NK cells. From the present data of IL-2 production and NK activity, we may suppose that IFN-γ production is not from NK cells in our system. Further studies are needed to clarify the mechanism through which ozone exposure increases Con A-induced IFN-γ production.

It is generally accepted that O_3 exposure markedly decreases thymus weight index and thymocyte proliferation in response to mitogens (Peterson et al., 1978; Fujimaki et al., 1984; Dziedzic & White, 1986; Feng et al., 2001). However, no report was found so far concerning the effect of O_3 exposure on the composition or development of thymocytes in an animal exposure model. IL-7 is a key cytokine for the early development of thymocytes, especially the proliferation of DN thymocytes (He et al., 1995; Plum et al., 1996). The present results, obtained by monitoring [^{3}H]-TdR uptake, show that O_3 exposure promotes thymus DN cell proliferation in response to IL-7, suggesting that O_3 may increase DN cells in the atrophic thymus and change the thymocyte composition. Therefore, in our animal model, O_3 exposure results in two conflicting results: a potent proliferative potential of DN cells, and thymus atrophy (Feng et al., 2001). The explanation may be that the intrathymic maturation is inhibited by O_3 exposure at a key transitional stage, such as from DN to DP thymocytes. According to the oxidative stress theory of aging, an increase of DN cells and a decrease of DP cells were associated with immunosenescence, showing that thymocyte development is blocked at the stage where DN cells differentiate into DP cells in aged individuals (el Demellawy & el Ridi, 1992; Fridkis-Hareli et al., 1994; Capri et al., 2000). In fact, our and other reports of

O_3 exposure (Kleeberger et al., 1997; Globerson & Effros, 2000; Feng et al., 2001) showed several features of immunosenescence: (1) decrease in thymus weight and IL-2 production, (2) increase in IL-6/IFN-γ/TNF-α production, (3) fall in T-cell mitogen response and the ratio of $CD4^+/CD8^+$ T cells, and (4) decline in $CD28^+$ T cells. It was also found that antigen-specific T-cell proliferative response gradually decreases during aging or under oxidative stress (Negoro et al., 1986; Otsuji et al., 1996). However, whether or not O_3-induced oxidative stress is the same as oxidant damage associated with aging remains to be elucidated.

Suppression of both humoral and cellular immunity occurs following in vivo generation of ROS (Koner et al., 1997) or in exercise-induced oxidative stress (Vider et al., 2001), indicating a possible nexus between ROS generation and immune dysfunction. Numerous factors, including O_3-induced stress, are capable of producing ROS, modulating immune responsiveness, and disrupting physiological homeostasis. The present study reveals that repetitive exposure to O_3 may contribute to the immunomodulation and suggests a possible link between in vivo redox status and immunosenescence (compromised immune response) under O_3-induced oxidative stress.

REFERENCES

Appleman, L. J., and Boussiotis, V. A. 2003. T cell anergy and costimulation. *Immunol. Rev*. 192:161–180.

Becker, S., Quay, J., and Koren, H. S. 1991. Effect of ozone on immunoglobulin production by human B cells in vitro. *J. Toxicol. Environ. Health* 34:353–366.

Bhalla, D. K. 1999. Ozone-induced lung inflammation and mucosal barrier disruption: Toxicology, mechanisms, and implications. *J. Toxicol. Environ. Health B* 2:31–86.

Burleson, G. R., Keyes, L. L., and Stutzman, J. D. 1989. Immunosuppression of pulmonary natural killer activity by exposure to ozone. *Immunopharmacol. Immunotoxicol*. 11:715–735.

Capri, M., Quaglino, D., Verzella, G., Monti, D., Bonafe, M., Cossarizza, A., Troiano, L., Zecca, L., Pasquali-Ronchetti, I., and Franceschi, C. 2000. A cytofluorimetric study of T lymphocyte subsets in rat lymphoid tissues (thymus, lymph nodes) and peripheral blood: A continuous remodelling during the first year of life. *Exp. Gerontol*. 35:613–625.

Chou, Y. K., Bourdette, D. N., Offner, H., Whitham, R., Wang, R. Y., Hashim, G. A., and Vandenbark, A. A. 1992. Frequency of T cells specific for myelin basic protein and myelin proteolipid protein in blood and cerebrospinal fluid in multiple sclerosis. *J. Neuroimmunol*. 38:105–113.

Cohen, M. D., Sisco, M., Li, Y., Zelikoff, J. T., and Schlesinger, R. B. 2001. Ozone-induced modulation of cell-mediated immune responses in the lungs. *Toxicol. Appl. Pharmacol*. 171:71–84.

Doelman, C. J., and Bast, A. 1990. Oxygen radicals in lung pathology. *Free Radical Biol. Med*. 9:381–400.

Dziedzic, D., and White, H. J. 1986. Thymus and pulmonary lymph node response to acute and subchronic ozone inhalation in the mouse. *Environ. Res*. 41:598–609.

el Demellawy, M., and el Ridi, R. 1992. Age-associated decrease in proportion and antigen expression of CD8+/CD4+ thymocytes in BALB/c mice. *Mech. Ageing Dev*. 62:307–318.

Feng, R., He, W., and Ochi, H. 2000. Experimental studies on antioxidation of extracts from several plants used as both medicines and foods. *J. Chin. Med. Mater*. 23:690–693.

Feng, R., He, W., and Ochi, H. 2001. A new murine oxidative stress model associated with senescence. *Mech. Ageing Dev*. 122:547–559.

Frampton, M. W., Pryor, W. A., Cueto, R., Cox, C., Morrow, P. E., and Utell, M. J. 1999. Ozone exposure increases aldehydes in epithelial lining fluid in human lung. *Am. J. Respir. Crit. Care Med*. 159:1134–1137.

Fridkis-Hareli, M., Mehr, R., Abel, L., and Globerson, A. 1994. Developmental interactions of CD4 T cells and thymocytes: Age-related differential effects. *Mech. Ageing Dev*. 73:169–178.

Fujimaki, H., Ozawa, M., Imai, T., and Shimizu, F. 1984. Effect of short-term exposure to O_3 on antibody response in mice. *Environ. Res*. 35:490–496.

Fujimaki, H., Shiraishi, F., Ashikawa, T., and Murakami, M. 1987. Changes in delayed hypersensitivity reaction in mice exposed to O_3. *Environ. Res*. 43:186–190.

Globerson, A., and Effros, R. B. 2000. Ageing of lymphocytes and lymphocytes in the aged. *Immunol. Today* 21:515–521.

Goldsmith, C. A., Ning, Y., Qin, G., Imrich, A., Lawrence, J., Murthy, G. G., Catalano, P. J., and Kobzik, L. 2002. Combined air pollution particle and ozone exposure increases airway responsiveness in mice. *Inhal. Toxicol*. 14:325–347.

Hamada, K., Goldsmith, C. A., and Kobzik, L. 1999. Increased airway hyperresponsiveness and inflammation in a juvenile mouse model of asthma exposed to air-pollutant aerosol. *J. Toxicol. Environ. Health A* 58:129–143.

He, W., Zhang, Y., Deng, Y., and Kabelitz, D. 1995. Induction of TCR-gamma delta expression on triple-negative ($CD3^-4^-8^-$) human thymocytes. Comparative analysis of the effects of IL-4 and IL-7. *J. Immunol*. 154:3726–3731.

Hendricks, L. C., and Heidrick, M. L. 1988. Susceptibility to lipid peroxidation and accumulation of fluorescent products with age is greater in T-cells than B-cells. *Free Radical Biol. Med*. 5:145–154.

Holtzman, M. J., Fabbri, L. M., O'Byrne, P. M., Gold, B. D., Aizawa, H., Walters, E. H., Alpert, S. E., and Nadel, J. A. 1983. Importance of airway inflammation for hyperresponsiveness induced by ozone. *Am. Rev. Respir. Dis*. 127:686–690.

Jenkins, M. K., Taylor, P. S., Norton, S. D., and Urdahl, K. B. 1991. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol*. 147:2461–2466.

Kleeberger, S. R., Levitt, R. C., Zhang, L. Y., Longphre, M., Harkema, J., Jedlicka, A., Eleff, S. M., DiSilvestre, D., and Holroyd, K. J. 1997. Linkage analysis of susceptibility to ozone-induced lung inflammation in inbred mice. *Nat. Genet*. 17:475–478.

Koner, B. C., Banerjee, B. D., and Ray, A. 1997. Effects of in-vivo generation of oxygen free radicals on immune responsiveness in rabbits. *Immunol. Lett*. 59:127–131.

Krogsgaard, M., Huppa, J. B., Purbhoo, M. A., and Davis, M. M. 2003. Linking molecular and cellular events in T-cell activation and synapse formation. *Semin. Immunol*. 15:307–315.

Lenschow, D. J., Walunas, T. L., and Bluestone, J. A. 1996. CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol*. 14:233–258.

Lerner, R. A., and Eschenmoser, A. 2003. Ozone in biology. *Proc. Natl. Acad. Sci. USA* 100:3013–3015.

Li, A. F., and Richters, A. 1991. Ambient level ozone effects on subpopulations of thymocytes and spleen T lymphocytes. *Arch. Environ. Health* 46:57–63.

McAdam, A. J., Schweitzer, A. N., and Sharpe, A. H. 1998. The role of B7 co-stimulation in activation and differentiation of $CD4^+$ and $CD8^+$ T cells. *Immunol. Rev*. 165:231–247.

McConnell, R., Berhane, K., Gilliland, F., London, S. J., Islam, T., Gauderman, W. J., Avol, E., Margolis, H. G., and Peters, J. M. 2002. Asthma in exercising children exposed to ozone: A cohort study. *Lancet* 359:386–391.

Nakamura, K., and Matsunaga, K. 1998. Susceptibility of natural killer (NK) cells to reactive oxygen species (ROS) and their restoration by the mimics of superoxide dismutase (SOD). *Cancer. Biother. Radiopharm*. 13:275–290.

Negoro, S., Hara, H., Miyata, S., Saiki, O., Tanaka, T., Yoshizaki, K., Igarashi, T., and Kishimoto, S. 1986. Mechanisms of age-related decline in antigen-specific T cell proliferative response: IL-2 receptor expression and recombinant IL-2 induced proliferative response of purified Tac-positive T cells. *Mech. Ageing Dev*. 36:223–241.

Neuhaus-Steinmetz, U., Uffhausen, F., Herz, U., and Renz, H. 2000. Priming of allergic immune responses by repeated ozone exposure in mice. *Am. J. Respir. Cell. Mol. Biol*. 23:228–233.

Otsuji, M., Kimura, Y., Aoe, T., Okamoto, Y., and Saito, T. 1996. Oxidative stress by tumor-derived macrophages suppresses the expression of CD3 zeta chain of T-cell receptor complex and antigen-specific T-cell responses. *Proc. Natl. Acad. Sci. USA* 93:13119–13124.

Pallard, C., Stegmann, A. P., van Kleffens, T., Smart, F., Venkitaraman, A., and Spits, H. 1999. Distinct roles of the phosphatidylinositol 3-kinase and STAT5 pathways in IL-7-mediated development of human thymocyte precursors. *Immunity* 10:525–535.

Peterson, M. L., Rommo, N., House, D., and Harder, S. 1978. In vitro responsiveness of lymphocytes to phytohemagglutinin. *Arch. Environ. Health* 33:59–63.

Plum, J., De Smedt, M., Leclercq, G., Verhasselt, B., and Vandekerckhove, B. 1996. Interleukin-7 is a critical growth factor in early human T-cell development. *Blood* 88:4239–4245.

Pryor, W. A., Squadrito, G. L., and Friedman, M. 1995. The cascade mechanism to explain ozone toxicity: the role of lipid ozonation products. *Free Radical Biol. Med*. 19:935–941.

Salvi, S. 2001. Pollution and allergic airways disease. *Curr. Opin. Allergy Clin. Immunol*. 1:35–41.

Schwela, D. 2000. Air pollution and health in urban areas. *Rev. Environ. Health* 15:13–42.

Stevens, W. H., Conlon, P. D., and O'Byrne, P. M. 1995. Ozone-induced oxygen radical release from bronchoalveolar lavage cells and airway hyper-responsiveness in dogs. *J. Physiol*. 486:257–265.

Thiele, J. J., Podda, M., and Packer, L. 1997. Tropospheric ozone: An emerging environmental stress to skin. *Biol. Chem*. 378:1299–1305.

Toward, T. J., and Broadley, K. J. 2002. Airway function, oedema, cell infiltration and nitric oxide generation in conscious ozone-exposed guinea-pigs: Effects of dexamethasone and rolipram. *Br. J. Pharmacol*. 136:735–745.

Vider, J., Lehtmaa, J., Kullisaar, T., Vihalemm, T., Zilmer, K., Kairane, C., Landor, A., Karu, T., and Zilmer, M. 2001. Acute immune response in respect to exercise-induced oxidative stress. *Pathophysiology* 7:263–270.

Wagner, J. G., Hotchkiss, J. A., and Harkema, J. R. 2002. Enhancement of nasal inflammatory and epithelial responses after ozone and allergen coexposure in Brown Norway rats. *Toxicol. Sci*. 67:284–294.

Weinmann, G. G., Liu, M. C., Proud, D., Weidenbach-Gerbase, M., Hubbard, W., and Frank, R. 1995. Ozone exposure in humans: Inflammatory, small and peripheral airway responses. *Am. J. Respir. Crit. Care Med*. 152:1175–1182.

Yu, S., He, W., Chen, J., Zhang, F., and Ba, D. 1999. Expansion and immunological study of human tumor infiltrating gamma/delta T lymphocytes in vitro. *Int. Arch. Allergy Immunol*. 119:31–37.