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CCL7 and CXCL10 Orchestrate Oxidative Stress-Induced Neutrophilic Lung Inflammation[1]

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Oxidative stress from ozone (O_3) exposure augments airway neutrophil recruitment and chemokine production. We and others have shown that severe and sudden asthma is associated with airway neutrophilia, and that O_3 oxidative stress is likely to augment neutrophilic airway inflammation in severe asthma. However, very little is known about chemokines that orchestrate oxidative stress-induced neutrophilic airway inflammation in vivo. To identify these chemokines, three groups of BALB/c mice were exposed to sham air, 0.2 ppm O_3, or 0.8 ppm O_3 for 6 h. Compared with sham air, 0.8 ppm O_3, but not 0.2 ppm O_3, induced pronounced neutrophilic airway inflammation that peaked at 18 h postexposure. The 0.8 ppm O_3 up-regulated lung mRNA of CXCL1,2,3 (mouse growth-related oncogene-α and macrophage-inflammatory protein-2), CXCL10 (IFN-γ-inducible protein-10), CCL3 (macrophage-inflammatory protein-1α), CCL7 (monocyte chemoattractant protein-3), and CCL11 (eotaxin) at 0 h postexposure, and expression of CXCL10, CCL3, and CCL7 mRNA was sustained 18 h postexposure. O_3 increased lung protein levels of CXCL10, CCL7, and CCR3 (CCL7R). The airway epithelium was identified as a source of CCL7. The role of up-regulated chemokines was determined by administering control IgG or IgG Abs against six murine chemokines before O_3 exposure. As expected, anti-mouse growth-related oncogene-α inhibited neutrophil recruitment. Surprisingly, Abs to CCL7 and CXCL10 also decreased neutrophil recruitment by 63 and 72%, respectively. These findings indicate that CCL7 and CXCL10, two chemokines not previously reported to orchestrate neutrophilic inflammation, play a critical role in mediating oxidative stress-induced neutrophilic airway inflammation. These observations may have relevance in induction of neutrophilia in severe asthma. *The Journal of Immunology,* **2002, 168: 846–852.**

The worldwide incidence, prevalence, and mortality from asthma are increasing, but the cause of this phenomenon has not been elucidated. Previous reports suggest that the level of oxidative stress is increased in patients with asthma (1, 2). Markers of oxidative stress, such as 8-isoprostane and heme oxygenase-1, are increased in exhaled condensate and airway macrophages, respectively, of patients with asthma (1, 2). Exposure of patients to pro-oxidant environmental agents like O_3 may contribute to worsening of oxidative stress in asthma and account for the increase in the incidence, prevalence, and mortality from asthma. This hypothesis is supported by epidemiological studies, which suggest that oxidative injury from O_3 exposure can induce bronchospasm, decrease lung function, increase medication use, and increase hospitalization in patients with asthma (3–6).

Oxidative injury from O_3 exposure has been shown to induce recruitment of neutrophils and decrement in lung function in patients with asthma (7, 8). Likewise, animal studies have demonstrated that O_3 exposure potently induces recruitment and activation of neutrophils in the airways (9–12). Activation of neutrophils is likely to produce additional damage through the release of reactive oxygen species and proteolytic enzymes (7). We and others have shown that airway neutrophilia in asthma is associated with sudden onset fatality, severe asthma, and peribronchial fibrosis (13–18). These studies suggest that neutrophils may play an important role in severe asthma and support the concept that O_3 oxidative stress-induced recruitment and activation of neutrophils may contribute to exacerbation of asthma.

Chemokines belong to a family of cytokines responsible for attracting inflammatory leukocytes, and some chemokines have been shown to be up-regulated in the lungs upon O_3 exposure. Earlier studies have reported that O_3 up-regulates CXCL-8 mRNA expression and protein production in the lungs of animals and humans exposed to O_3 (19, 20). In addition to CXCL-8, O_3 has been shown to induce other CXC chemokines, such as members of the Gro family of CXCL1,2,3 and CXCL10 (21–23). Other investigators have reported that O_3 up-regulates CC chemokines such as CCL2, CCL3, and CCL11 (21, 23). Even though O_3 exposure has been shown to up-regulate these chemokines, few studies have evaluated the in vivo role of these chemokines, which mediate O_3-induced airway inflammation (12). The present study was designed to identify CC and CXC chemokines that have not been previously reported to mediate O_3-induced airway inflammation. Here, we show that CCL7 and CXCL10, two chemokines not reported to mediate neutrophilic inflammation, orchestrate oxidative stress-induced neutrophilic airway inflammation.

Materials and Methods

Animal exposure to sham air or O_3

Six- to 8-wk-old BALB/c mice (Harlan Laboratories, Indianapolis, IN) were placed in individual stainless steel wire-mesh cages with free access to food and water and were exposed to either sham air or O_3 using Hinners-type 0.85-m^3 stainless steel chambers. The inlet air was passed through

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activated charcoal and HEPA filters at sufficient flow to achieve chamber turnover rates of 30 volume changes/h. O_3 was generated from 100% O_2 (medical grade) using a silent arc electrode (Sander, Uetze-Eltze, Germany) adjusted to achieve the desired chamber O_3 concentration. The total O_3/O_2 flow was introduced into the chamber-input port using mass flow controllers (Scott Specialty Gas, Pasadena, TX) to tightly regulate the flow of O_2 through the ozonizer. Chamber temperature (24.3 ± 0.4°C) and humidity (59.1 ± 1.7%) were maintained in both sham air and O_3 chambers throughout the exposure period. O_3 concentrations were continuously monitored using a model 49 O_3 analyzer (Thermo Environmental Instruments, Franklin, MD). Mice were exposed for 6 h to either a low (0.2 ± 0.03 ppm) or high (0.8 ± 0.03 ppm) dose of O_3. After exposure, sham and O_3 exposed animals were sacrificed, and bronchoalveolar lavage (BAL)[4] was performed at 0, 18, 42, and 138 h after exposure cessation (Fig. 1).

BAL and cell count

Cellular infiltration into the airway lumen was assessed by an analysis of BAL fluid. Mice were anesthetized with an i.p. injection of ketamine and xylazine. A tracheotomy was performed and the trachea was cannulated. BAL of both lungs was performed twice with 0.7 ml of sterile PBS (pH 7.3) through the trachea cannula with a syringe. The BAL was centrifuged at 4°C for 10 min at 400 × *g*, and the pellet was suspended in 750 μl of ice-cold Dulbecco's PBS (Sigma-Aldrich, St. Louis, MO). Total cell counts in BAL were determined from an aliquot of the cell suspension. Differential cell counts were done on cytocentrifuge preparations (Cytospin 3; Thermo Shandon, Pittsburgh, PA) stained with Wright-Giemsa, counting 200 cells from each animal.

Analyses of lung chemokine mRNA levels by RT-PCR

Chemokine mRNAs were assessed by semiquantitative RT-PCR. Immediately after the mice were sacrificed, the lungs were removed, frozen rapidly in liquid nitrogen, and stored at 80°C until RNA extraction. The frozen whole lung was placed into 2 ml of TRIzol solution (Life Technologies, Grand Island, NY) and homogenized using a Polytron homogenizer. Total RNA was isolated following the manufacturer's protocol. Five micrograms of total RNA was reverse transcribed using 200 U of SuperScript II Reverse Transcriptase in a buffer (Life Technologies) containing 10 mM DTT, 1 μM oligo(dT) primer (Life Technologies), 500 μM of each dNTP, and 10 U of RNasin (Promega, Madison, WI) in a total volume of 20 μl. The resultant cDNA was diluted 5-fold with diethyl pyrocarbonate-treated water to a final volume of 100 μl. To amplify the reverse-transcribed cDNA, 4.0 μl of the PCR mixture was added to give a final solution containing 1× *Taq* buffer II, 2.0 mM $MgCl_2$, 0.2 μM of each sense and antisense primer, and 0.5 U of AmpliTaq Gold polymerase (PerkinElmer/Applied Biosystems, Foster City, CA). PCR was performed in GeneAmp PCR system 9700 (PerkinElmer/Applied Biosystems). All primers were custom-designated using the Primer software program (Table I). The number of amplification cycles used ranged from 23 to 26. The number of amplification cycles was selected for each primer based on the linear portion of the PCR amplification cycle. The PCR products were electrophoresed in a 3% agarose gel (Seakem LE Agarose; FMC Bioproducts, Riceland, ME). The intensity of bands on photographs of the agarose gel was quantified by scanning the photographs with a contrast scanner (JX-330; Sharp Electronics, Mahwah, NJ) using optical software (ImageQuant, version 3.3; Molecular Dynamics, Sunnyvale, CA). The values obtained from individual cytokines were expressed as a ratio of cytokine band intensity relative to band intensity of housekeeping gene, β_2-microglobulin (β_2m).

Lung CXCL10 (IFN-γ-inducible protein (IP)-10), CCL7 (monocyte chemoattractant protein (MCP)-3), and CCR3 protein levels

Whole lung lysates of mice exposed to sham air or 0.8 ppm O_3 for 6 h were subjected to denaturing gel electrophoresis. To detect these chemokines or chemokine receptors, Western blotting was performed using rabbit anti-murine chemokine Abs (PeproTech, Rocky Hill, NJ) or rabbit anti-murine CCR3 Ab (BD PharMingen, San Diego, CA).

Immunohistochemistry of lung sections for CCL7

Mice were exposed to either air or 0.8 ppm O_3 for 6 h and the lungs were dissected out 18 h postexposure. Cryosections of the lung were fixed in methanol:acetone and blocked with goat serum. Serial sections of the lungs were incubated with either normal rabbit IgG or rabbit anti-mouse CCL7 (PeproTech, Rocky Hill, NJ). The tissue-bound anti-CCL7 was detected with Alexa 488-conjugated, highly cross-absorbed goat anti-rabbit IgG (Molecular Probes, Eugene, OR). The sections were visualized in an inverted TE200 Nikon Fluorecent microscope attached to a Photometrics Cool Snap Fx CCD digital camera and Metamorph software (Roper Scientific, Tucson, AZ).

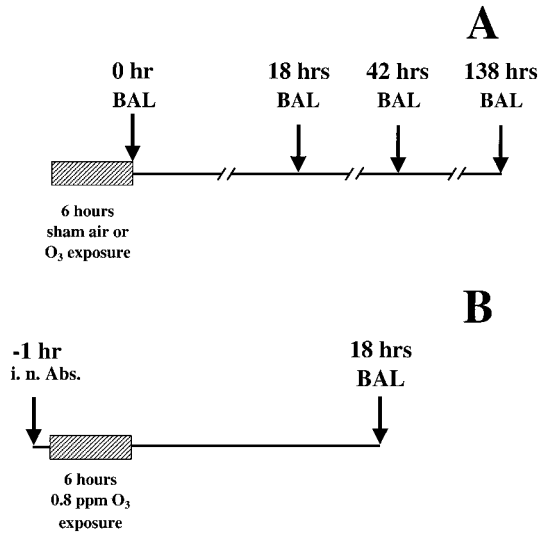

FIGURE 1. Protocol for O_3 exposure. *A*, Protocol for exposure of naive mice to O_3 or sham air. Six- to 8-wk-old BALB/c mice were placed in individual stainless steel wire-mesh cages placed in stainless steel chambers. Mice were exposed for 6 h to a low (0.2 ppm) or high (0.8 ppm) dose of O_3 or sham air. Animals were sacrificed, and BAL was performed at 0, 18, 42, and 138 h after the cessation of O_3 exposure. *B*, Protocol of anti-chemokine Ab treatment of naive mice before O_3 exposure. Ten micrograms of either normal rabbit IgG (control) or rabbit anti-murine CXCL1,2,3 (KC and MIP-2), CXCL10, CCL3, CCL7, and CCL11 was administered i.n. to naive BALB/c 1 h before O_3 exposure. The mice were exposed to 0.8 ppm O_3 for 6 h and sacrificed 18 h later to perform BAL. Animals were sacrificed and BAL was performed at 18 h after the cessation O_3 exposure.

Administration of anti-chemokine Abs

Rabbit IgG (control) and rabbit IgG Abs against CXCL1,2,3 (mouse growth-related oncogene-α (KC), macrophage-inflammatory protein (MIP)-2), CXCL10 (IP-10), CCL3 (MIP-1α), CCL7 (MCP-3), and CCL11 (eotaxin) (PeproTech) were administered to naive BALB/c. All Abs were administered intranasally (i.n.) at a concentration of 10 μg/100 μl PBS 1 h before O_3 exposure (Fig. 1*B*). We and others have previously shown that anti-chemokine Abs are very effective in preventing lung inflammation in vivo when they are administered directly into the lungs (24, 25). For this reason, we administered Abs (rabbit polyclonal IgG) directly into the lungs in this study. The mice were exposed to 0.8 ppm O_3 for 6 h and were sacrificed to perform BAL at the anticipated peak of lung inflammation, that is 18 h after the end of O_3 exposure.

Specificity of rabbit anti-murine chemokine Abs

To determine specificity of rabbit anti-mouse chemokine Abs, we performed denaturing gel electrophoresis of recombinant murine CXCL10 (IP-10), KC, CCL7 (MCP-3), and CCL5 (RANTES), followed by Western blotting using rabbit Abs directed against murine CXCL10, KC, and CCL7 (PeproTech).

Data analysis

Data are presented as mean ± SEM. The difference in outcome variables between treatment groups was analyzed by one-way ANOVA. Significant ANOVAs were further analyzed by the Bonferroni/Dunn's post hoc test.

[4] Abbreviations used in this paper: BAL, bronchoalveolar lavage; i.n., intranasal(ly); β_2m, β_2-microglobulin; IP, IFN-γ-inducible protein; MCP, monocyte chemoattractant protein; KC, mouse growth-related oncogene-α; MIP, macrophage-inflammatory protein.

Table I. *Primers for PCR*

Chemokines	Forward Primers	Reverse Primers
CXCL1,2,3 (KC)	CCA ACA CAG CAC CAT GAT CC	CCT CGC GAC CAT TCT TG
CXCL1,2,3 (MIP-2)	ACA CTT CAG CCT AGC GCC AT	GGT CAG TTA GCC TTG CCT
CXCL10 (IP-10)	CCA TCA GCA CCA TGA ACC	TCC GGA TTC AGA CAT CTC
CCL3 (MIP-1α)	CAC TCT GCA ACC AAG TCT TC	TCA GTT CCA GGT CAG TGA TG
CCL7 (MCP-3)	AGC TAC AGA AGG ATC ACC AG	CAC ATT CCT ACA GAC AGC TC
CCL11 (eotaxin)	GCT CCA CAG CGC TTC TAT TC	TTG TGG CAT CCT GGA CC
β_2m	ATG GCT CGC TCG GTG ACC ACC CTA G	TCA TGA TGC TTG ATC ACA TGT CTC G

Results

High-dose (0.8 ppm) but not low-dose (0.2 ppm) O_3 exposure induces neutrophilic and macrophagic airway inflammation

Sham air exposure of BALB/c mice did not alter the number of neutrophils, eosinophils, macrophages, or lymphocytes in the recovered BAL at any time. Exposure of mice to a low concentration (0.2 ppm) of O_3 failed to induce a detectable increase in any of these cell populations within the lungs. In contrast, exposure of mice to a high concentration of O_3 (0.8 ppm) increased the total cells maximally by 18 h (air = $2.57 \pm 0.53 \times 10^4$/ml vs 0.8 ppm O_3 = $23.43 \pm 4.99 \times 10^4$/ml; $p \leq 0.01$; Fig. 2). The same high concentration of O_3 increased the number of neutrophils at 18 h from $0.02 \pm 0.0 \times 10^4$/ml in the air control group to $9.44 \pm 2.30 \times 10^4$/ml ($p \leq 0.01$, Fig. 2). Furthermore, 0.8 ppm O_3 also increased the number of macrophages at 18 h (air = $2.52 \pm 0.52 \times 10^4$/ml vs 0.8 ppm O_3 = $13.77 \pm 3.37 \times 10^4$/ml; $p \leq 0.01$; Fig. 2). These data indicate that 0.8 ppm O_3 for 6 h increased total cells, neutrophils, and macrophages as early as 18 h postexposure, and both cell types demonstrated sustained increase until 42 h postexposure.

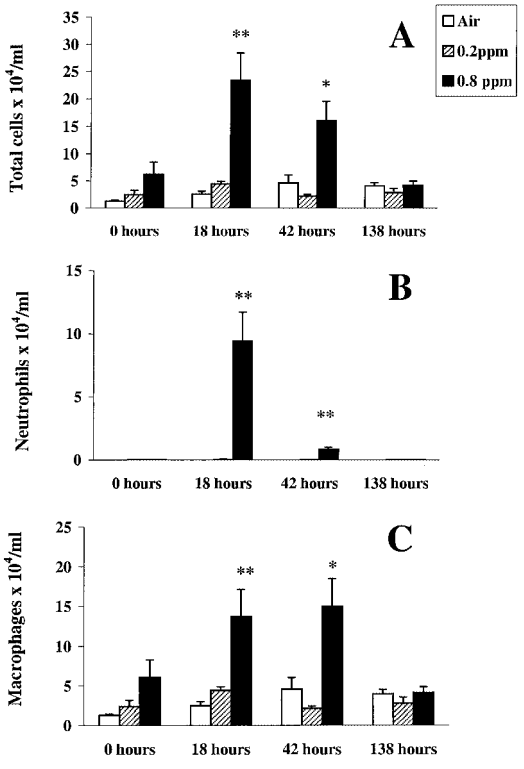

FIGURE 2. BAL cell counts in BALB/c mice exposed to O_3 or sham air. BALB/c mice were exposed to O_3 (0.2 or 0.8 ppm) or sham air for 6 h. After exposure, sham- and O_3-exposed animals were sacrificed, and BAL was performed at 0, 18, 42, and 138 h after the cessation of O_3 exposure. The numbers of cells were calculated as the product of the total cell count and the differential count of cytospin slides stained with Wright-Giemsa stain. Values shown represent mean ± SEM. Asterisks indicate significant differences (*, $p \leq 0.05$; **, $p \leq 0.01$) and represent difference in cell influx associated with clean air vs O_3 exposure.

High dose of O_3 up-regulates expression of CXCL10, CCL7, and other chemokines

RT-PCR of lung mRNA was performed to measure expression of CXC and CC chemokines, and the data of two representative chemokines, namely, CXCL10 and CCL7, are shown in Fig. 3. The ratio of intensity of chemokine band to the intensity of the housekeeping gene β_2m was determined (Fig. 4). Exposure of mice to 0.8 ppm O_3 for 6 h up-regulated some chemokine genes at 0 h postexposure, which did not persist at 18 h postexposure. These include the CXCL1,2,3 (KC, 10.5-fold increase, $p \leq 0.001$), MIP-2 (18.3-fold increase, $p \leq 0.001$), and CCL11 (9.1-fold increase, $p \leq 0.01$). Exposure of mice to the same dose of O_3 for 6 h up-regulated other genes at 0 h postexposure, but these genes remained up-regulated at 18 h postexposure. This group of chemokines includes CXCL10 (10.6-fold increase, $p \leq 0.01$), CCL3 (3.9-fold increase, $p \leq 0.05$), and CCL7 (13.1-fold increase, $p \leq 0.0001$). The expression of the second group of chemokine genes (CXCL10, CCL3, and CCL7) was up-regulated 3.3-, 2.1-, and 4.5-fold, respectively, at 18 h. These results indicate that some genes

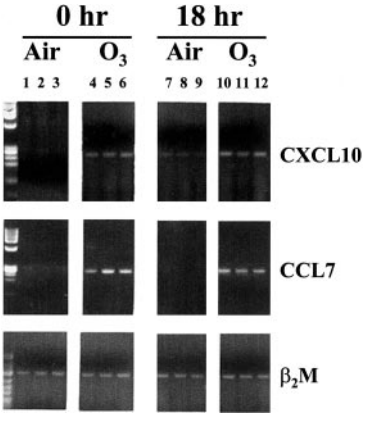

FIGURE 3. RT-PCR of total lung RNA. BALB/c mice were exposed to 0.8 ppm O_3 or sham air for 6 h. The animals were sacrificed 0 h or 18 h later. The numbers 1–12 represent individual animals. Animals 1–3 were exposed to air and sacrificed at 0 h postexposure. Animals 4–6 were exposed to 0.08 ppm O_3 for 6 h and sacrificed at 0 h postexposure. Animals 7–9 were exposed to air and sacrificed at 18 h postexposure. Animals 10–12 were exposed to 0.08 ppm O_3 and sacrificed at 18 h postexposure. Total RNA was extracted from the lung of all animals. RT-PCR was performed to amplify the chemokine and β_2m RNA (see Table I for primer sequence). This figure shows representative data of two chemokines, namely, CXCL10 and CCL7. The presence of β_2m bands in all animals indicates that mRNA extraction and cDNA synthesis was performed successfully.

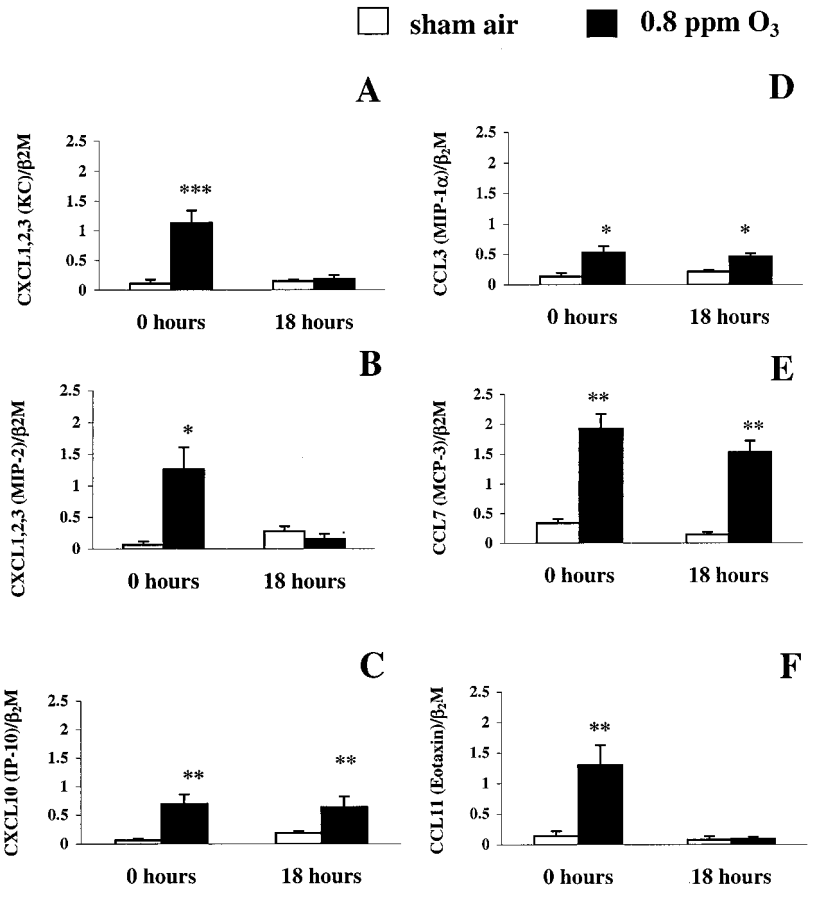

FIGURE 4. Intensity of cytokine PCR bands. The intensity of cytokine and β_2m PCR bands in the original photographs of the agarose gel was quantified by scanning the photographs with contrast scanner and analyzing intensity by ImageQuant optical software. The ratios of CXCL1,2,3 (KC) (*A*), CXCL1,2,3 (MIP-2) (*B*), CXCL10 (*C*), CCL3 (*D*), CCL7 (*E*), and CCL11 (*F*) band intensity to β_2m band intensity are shown. Values are expressed as the mean ± SEM. Asterisks indicate significant differences (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

(CXCL1,2,3 and CCL11) are only transiently up-regulated by O_3 exposure, whereas others (CXCL10, CCL3, and CCL7) are up-regulated for a prolonged period (Figs. 3 and 4).

High dose of O_3 increases CXCL10, CCL7, and CCR3 protein levels in the lungs

Because exposure of mice to sham air or 0.8 ppm O_3 for 6 h up-regulated CXCL10 and CCL7 mRNA levels, we sought to determine whether it also increased protein levels of these chemokines. As shown in Fig. 5*A*, 0.8 ppm O_3 dramatically increased lung levels of both CXCL10 and CCL7. Furthermore, 0.8 ppm O_3 also increased lung levels of CCR3, the receptor for CCL7 (Fig. 5*B*).

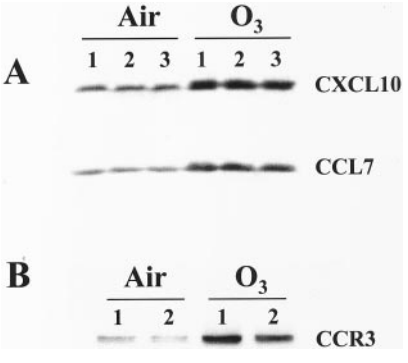

FIGURE 5. Effect of 0.8 ppm O_3 on lung CXCL10, CCL7, and CCR3 protein levels. BALB/c mice were exposed to sham air (Air) or 0.8 ppm O_3 (O_3) for 6 h, and lungs were dissected out 18 h postexposure. The whole lung lysates were prepared in radioimmunoprecipitation assay buffer and 30 μg of protein was loaded per lane for SDS-PAGE. The gels were subjected to Western blot analyses using rabbit anti-murine CXCL10 and CCL7 (*A*) or CCR3 (*B*). The numbers *1*, *2*, and *3* represent different mice within a treatment group.

O_3 increases CCL7 production from airway epithelium

We sought to determine the cell source of CCL7. As shown in Fig. 6, exposure of mice to air failed to induce production of CCL7. Exposure of mice to 0.8 ppm O_3 increased epithelial levels of CCL7 (Fig. 6).

CXCL10 and CCL7 mediate O_3-induced neutrophilic inflammation

To evaluate the role of the six chemokines up-regulated by O_3 in mediating airway inflammation induced by O_3, seven groups of mice were treated i.n. with normal rabbit IgG (isotype control) or polyclonal rabbit anti-mouse IgG against one of six murine chemokines, namely, CXCL1,2,3 (KC and MIP-2), CXCL10, CCL3, CCL7, and CCL11 (Fig. 7). Exposure of control IgG-treated mice to 0.8 ppm O_3 for 6 h induced a vigorous inflammatory response by 18 h postexposure. As expected, the Ab to KC decreased the number of total cells by 64% ($p \leq 0.01$) and neutrophils by 73% ($p < 0.01$). Surprisingly, the Ab to CXCL10 also decreased total cells by 58% ($p < 0.05$) and neutrophils by 72% ($p \leq 0.01$), whereas the Ab to CCL7 decreased neutrophils by 63% ($p \leq 0.05$). Abs to MIP-2, CCL3, and CCL11 did not inhibit recruitment of either neutrophils or total cells. Macrophagic inflammation was not significantly attenuated by any of these Abs (data not shown).

Specificity of anti-murine chemokine Abs

Because cross-reactivity of anti-murine CXCL10 and anti-CCL7 with KC could explain our unexpected observations of the effects of these Abs on neutrophil recruitment, we sought to examine the

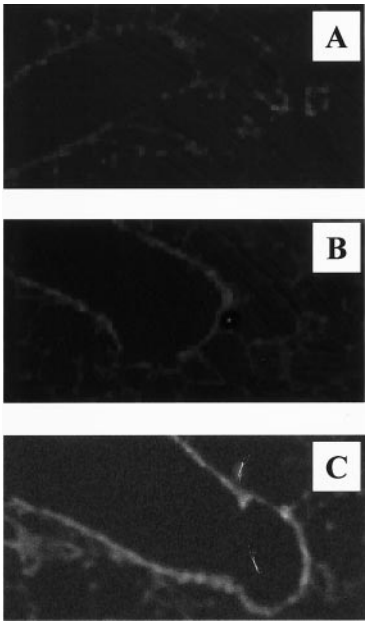

FIGURE 6. Effect of 0.8 ppm O_3 on CCL7 production by airway epithelium. BALB/c mice were exposed to sham air or 0.8 ppm O_3 for 6 h, and lungs were dissected out 18 h postexposure. Cryosections of the lungs were incubated with rabbit IgG or rabbit anti-murine CCL7. Tissue-bound Ab was detected with Alexa 484-conjugated, highly cross-absorbed goat anti-rabbit IgG. *A*, Lung section from a mouse exposed to air and incubated with anti-murine CCL7. Lung section from a mouse exposed to O_3 and incubated with either normal rabbit IgG (*B*) or anti-murine CCL7 (*C*).

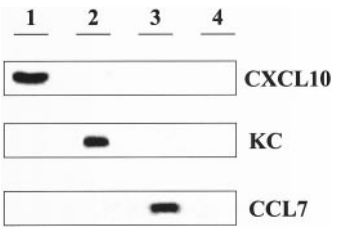

FIGURE 8. Specificity of anti-murine chemokine Abs. To determine specificity of rabbit anti-mouse chemokine Abs, denaturing gel electrophoresis was performed with 100 ng of recombinant CXCL10 (IP-10, *lane 1*), KC (*lane 2*), CCL7 (MCP-3, *lane 3*), and CCL5 (RANTES, *lane 4*). This was followed by Western blotting using rabbit IgG Abs against murine CXCL10, KC, and CCL7 (PeproTech). Anti-CXCL10 recognized CXCL10 but not KC, CCL7, and CCL5. Likewise, the Abs to CCL7 and KC specifically recognized CCL7 and KC, respectively.

specificity of these Abs. We performed denaturing gel electrophoresis of recombinant chemokines followed by Western blotting using rabbit anti-murine chemokine Abs. As shown in Fig. 8, anti CXCL10 recognized CXCL10 but not KC, CCL7, and CCL5. Likewise, the Abs to CCL7 and KC specifically recognized CCL7 and KC, respectively.

Discussion

A notable novel finding in our study is that oxidative stress from O_3 exposure up-regulated CCL7 mRNA expression and protein production in the lungs. The airway epithelium was a cell source of CCL7 in the lungs. Exposure of humans to low concentrations of O_3 has recently been shown to increase allergen-induced eosinophil inflammation, an observation that may explain why O_3 exposure worsens asthma (26, 27). We have previously reported that allergen challenge induces CCL7 production from the airway epithelium and that CCL7 mediates eosinophilic inflammation in a mouse model of asthma (25). The O_3 exposure-induced production of CCL7 may explain how O_3 exposure increases allergen-induced eosinophil inflammation in allergic subjects and worsens asthma (26). Another unexpected novel finding in our study is that the Ab to CCL7 inhibited O_3-induced neutrophilic lung inflammation. This observation is quite surprising because CCL7 has been reported to bind to CCR3, a CCR that is reportedly present on Th2 cells and eosinophils, but not on neutrophils (28–30). This paradox may be partially explained by a recent report that even though neutrophils did not express CCR3 under basal condition, they expressed high levels of this receptor when they are activated (28). Although we did not examine CCR3 expression on lung neutrophils, our data indicate that CCR3 protein level is dramatically increased in the lungs 18 h after O_3 exposure, at a time point when the lungs are heavily infiltrated with neutrophils. Alternatively,

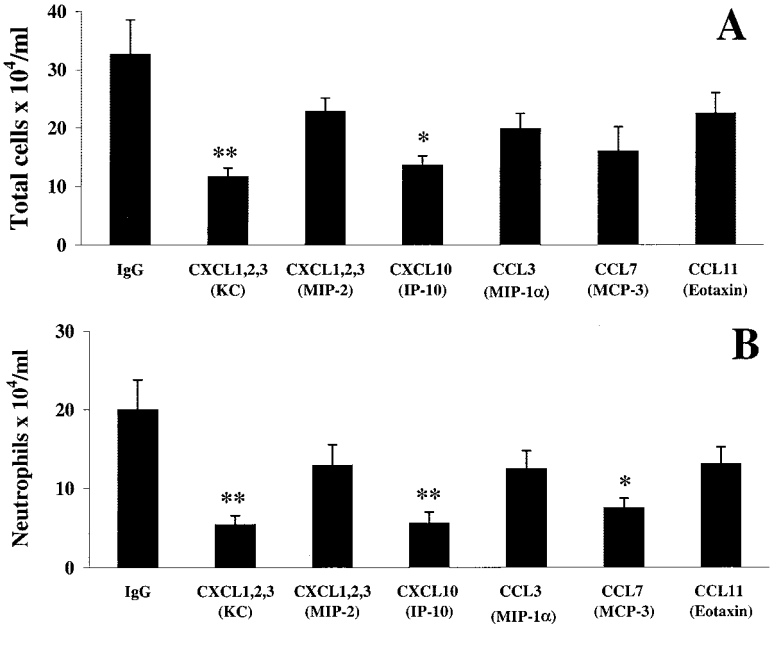

FIGURE 7. Effect of anti-chemokine Abs on cell recruitment. Following the protocol from Fig. 1*B*, a BAL was performed. This figure shows the total number of BAL cells (*A*) and total number of BAL neutrophils (*B*). Abs to CXCL10 ($p < 0.05$) and CXCL1,2,3 (KC) ($p < 0.01$) significantly decreased the total number of BAL cells. In addition, Abs to CXCL10 and CXCL1,2,3 (KC) ($p < 0.01$) and CCL7 ($p < 0.05$) decreased the total number of neutrophils.

CCL7 may facilitate neutrophil chemoattraction by a second chemokine, similar to the facilitatory role of CCL7 in neutrophil chemoattraction induced by the chemokine ragakine-1 (31).

Few investigators have evaluated the in vivo role of chemokines in mediating O_3-induced airway inflammation. Koto et al. (12) reported that in vivo treatment with an Ab to the Gro family (anti-cytokine-induced neutrophil chemoattractant analogous to MIP-2) inhibited neutrophilic inflammation but not other cell types in rats. Consistent with the observations of Koto et al., we found that an Ab to the Gro family (KC) inhibited neutrophil recruitment. Unexpectedly, we also found that Abs to CCL7 and CXCL10 significantly inhibited O_3-induced neutrophilic inflammation. The role of CXCL10 in neutrophil recruitment has not been described previously, but CXCL10 can be produced by neutrophils, and therefore can influence chemotaxis of CXCR3-expressing cells such as Th1 cells, which in turn may influence and regulate neutrophilic inflammation (32).

In the present study Abs directed against multiple chemokines, namely, CXCL10, CCL7, and KC, significantly inhibited ozone-induced neutrophil accumulation in the lung. An understanding of the specific roles of chemokines in orchestrating airway inflammation has been complicated by a number of different factors (33). First, simultaneous expression of multiple chemokines with partially overlapping functions is often observed. Second, chemokines often bind more than one chemokine receptor, and chemokine receptors typically bind more than one class of chemokine. Third, a likely scenario is that coordinated chemokine actions may have contributed to the reduced neutrophilic lung inflammation, as suggested by a study examining the roles of chemokines in allergic asthma. In that study, neutralization of several chemokines including CCL2, CCL3, CCL11, CCL12, and CCL22 was effective in inhibiting cellular recruitment (34). In this study, the chemokines were found to act in a coordinated fashion in contributing to the complex pathophysiology of allergic inflammation. In the current study, anti-chemokine Abs were administered 1 h before ozone exposure, and lung neutrophil accumulation was assessed 18 h after ozone exposure. During this interval, different chemokines may have influenced distinct processes underlying ozone-induced neutrophil accumulation in a framework of a coordinated chemokine network. The protocol of administration of the Abs in this study does not allow assessment of variable chemokine temporal activity or spatial activity because the Abs were administered before ozone exposure and ultimately to many sites in the lung via the i.n. route.

Oxidative stress from O_3 exposure has been reported to induce a kinetically differential chemokine expression. For example, Zhao et al. (21) reported that 2 ppm O_3 induced CXCL1,2,3 (MIP-2) mRNA expression that peaked 4 h postexposure, whereas CCL2 mRNA levels peaked at 24 h postexposure. In the present study, the CXCL1,2,3 (KC and MIP-2), CXCL10, CCL3, CCL7, and CCL11 were up-regulated at 0 h postexposure, whereas only CCL3, CCL7, and CXCL10 were still up-regulated 18 h postexposure, indicating differences in the kinetics of different chemokine expression. Because our data indicate that KC, CXCL10, and CCL7 orchestrate O_3-induced neutrophilic lung inflammation, the kinetics of chemokine expression suggest that KC may have initiated neutrophil recruitment and that this process is later sustained by CXCL10 and CCL7. In contrast to our study, Johnston et al. (23) reported that 2.5 ppm O_3 increased mRNA expression of induced CXCL1,2,3 (MIP-2), CXCL10, CCL3, and CCL11 in C57BL/6 mice as early as 2 h and that the up-regulation persisted for 24 h. These differences between the findings of investigators may reflect differences in the mouse strain (C57BL/6 in other studies vs BALB/c in the present study) used in different studies and suggest that genetic differences that appear to reside as a quantitative trait locus on chromosome 17 may be a factor that determines the inflammatory response to O_3 (35).

Several investigators have attempted to elucidate the mechanism of O_3-induced macrophage recruitment to the lungs. Exposure of animals to O_3 has been shown to increase monocyte chemotactic activity in bronchoalveolar fluid (21). This activity was inhibited by an Ab directed against CCL2 in a monocyte chemotaxis assay (21). The in vivo role of chemokines was assessed by Koto et al. (12), who reported that in vivo administration of an Ab to cytokine-induced neutrophil chemoattractant inhibited neutrophilic but not macrophagic inflammation. Our findings are similar to the observations of Koto et al., and the Abs to the three chemokines that decreased neutrophil recruitment failed to decrease macrophage recruitment. These findings cannot be explained by the intrapulmonary route of administration of Abs because we and others have previously shown that anti-chemokine Abs are very effective in preventing lung inflammation in vivo when they are administered via this route (24, 25). An alternative explanation is that the dosage of administered Abs did not influence O_3-induced macrophagic inflammation to the same extent as it influenced neutrophilic inflammation, or that the chemokines tested in this study do not regulate macrophage recruitment. Future studies may require administration of larger doses of each Ab and a larger repertoire of Abs to various chemokines to address these questions.

Oxidative stress from O_3 exposure has been shown to induce airway hyperesponsiveness in humans (36, 37). These authors have suggested that because of these effects, O_3 exposure is likely to contribute to asthma morbidity. Oxidative injury from O_3 exposure has also been shown to induce recruitment of neutrophils in humans (7). Because severe and sudden-onset asthma is associated with airway neutrophilia, O_3 exposure is likely to contribute significantly to asthma morbidity and mortality in this subset of patients (13–18). Based on the data presented in this study, it is tempting to speculate that CXCL10 and CCL7 contribute to airway neutrophilic inflammation and asthma exacerbation in these patients after exposure to high O_3-polluted air.

The Office of Air Quality Planning and Standards has set the National Ambient Air Quality Standards "criteria pollutants," including O_3. The 1-h standard is 0.12 ppm, whereas the 8-h standard is 0.08 ppm. These levels are considerably lower than the dose that induced airway inflammation in the present study (0.8 ppm) and in previous studies involving exposure of mice to O_3 (9, 35). These differences may represent a greater response of humans to O_3 than mice, rats, and guinea pigs, as suggested by the observation that humans demonstrate an increase in neutrophilic inflammation even when they are exposed to 0.2 ppm O_3 (8). These findings suggest that caution should be exercised when extrapolating murine data to humans. Nonetheless, the results of the present study suggest that future studies in humans should be directed at evaluating the role of CCL7 and CXCL10 in O_3 oxidative stress-induced lung inflammation.

In summary, our results indicate that high concentrations of O_3 increase recruitment of neutrophils and macrophages into the lungs, associated with an increase in expression of CXCL1,2,3 (KC and MIP-2), CXCL10, CCL3, CCL7, and CCL11. Exposure to 0.8 ppm O_3 also increased lung protein levels of CXCL10, CCL7, and CCR3. In addition to the expected inhibition of neutrophil recruitment by the Ab to KC, administration of neutralizing Abs to murine CCL7 and CXCL10 before O_3 exposure inhibited O_3-induced neutrophil recruitment. These findings indicate that CCL7 and CXCL10 orchestrate oxidative stress-induced neutrophilic lung inflammation. This is the first report demonstrating an in vivo role of these chemokines in neutrophilic inflammation.

These observations may have relevance to induction of neutrophilia in severe asthma.

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