V γ 1+ T Cells and Tumor Necrosis Factor-Alpha in Ozone-Induced Airway Hyperresponsiveness

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$\gamma\delta$ T cells regulate airway reactivity, but their role in ozone (O₃)-induced airway hyperresponsiveness (AHR) is not known. Our objective was to determine the role of $\gamma\delta$ T cells in O₃-induced AHR. Different strains of mice, including those that were genetically manipulated or antibody-depleted to render them deficient in total $\gamma\delta$ T cells or specific subsets of $\gamma\delta$ T cells, were exposed to 2.0 ppm of O₃ for 3 hours. Airway reactivity to inhaled methacholine, airway inflammation, and epithelial cell damage were monitored. Exposure of C57BL/6 mice to O₃ resulted in a transient increase in airway reactivity, neutrophilia, and increased numbers of epithelial cells in the lavage fluid. TCR- $\delta^{-/-}$ mice did not develop AHR, although they exhibited an increase in neutrophils and epithelial cells in the lavage fluid. Similarly, depletion of $\gamma\delta$ T cells in wild-type mice suppressed O₃-induced AHR without influencing airway inflammation or epithelial damage. Depletion of V γ 1+, but not of V γ 4+ T cells, reduced O₃-induced AHR, and transfer of total $\gamma\delta$ T cells or V γ 1+ T cells to TCR- $\delta^{-/-}$ mice restored AHR. After transfer of V γ 1+ cells to TCR- $\delta^{-/-}$ mice, restoration of AHR after O₃ exposure was blocked by anti-TNF- α . However, AHR could be restored in TCR- $\delta^{-/-}$ mice by transfer of $\gamma\delta$ T cells from TNF- α -deficient mice, indicating that another cell type was the source of TNF- α . These results demonstrate that TNF- α and activation of V γ 1+ $\gamma\delta$ T cells are required for the development of AHR after O₃ exposure.

Keywords: ozone; airway responsiveness; $\gamma\delta$ T cells; TNF- α

Ozone (O₃) is a highly reactive oxidizing agent and continues to be a persistent ambient pollutant despite years of considerable effort to reduce levels in the United States (1). Toxic pulmonary effects have been demonstrated in animals and humans, and in particular in urban environments and the workplace (2). Various adverse sequelae of O₃ exposure have been documented with airway hyperresponsiveness (AHR) to nonspecific stimuli, epithelial sloughing, and neutrophil accumulation in the airways. The mechanisms leading to O₃-induced effects in the lung are not well understood, nor are there data linking a common mechanism resulting in AHR, neutrophil accumulation, and epithelial cell damage. The high reactivity of O₃ and its low solubility in water would prevent it from passing through the lung epithelial lining fluid to act directly on the underlying epithelial cells (3). Since the lung epithelial lining fluid is composed of lipids to a large extent, it has been suggested that

CLINICAL RELEVANCE

This article demonstrates for the first time the essential role of a unique subset of T lymphocytes in the development of ozone-induced airway hyperresponsiveness.

O₃ exerts its toxic effects via oxidized lipid mediators that can act as signaling molecules (3–7).

Both humans and mice vary considerably in their response to O₃, and genetic factors are important in dictating susceptibility to O₃-induced damage (8–11). Inflammatory mediators likely play a major role in the pathogenesis of O₃-induced AHR, lung inflammation, and injury. Perhaps linked to the genetic variability is the activity of these inflammatory mediators. Tumor necrosis factor (TNF)- α has been implicated in the pathogenesis of O₃-induced lung inflammation and injury. O₃ exposure enhances TNF- α release and TNF receptor expression in airway cells and tissues (12, 13). In positional cloning studies, *Tnf* was identified as a candidate susceptibility gene for lung inflammation induced by O₃ (10). These findings are supported by the protection afforded against development of O₃-induced AHR and inflammation in the absence of a TNF response (10, 13–16).

In addition to TNF- α , other factors have been implicated, including interleukin (IL)-1 β , whose levels increase in response to inhaled O₃, and where AHR, airway neutrophilia, and structural damage can be significantly reduced when the IL-1 receptor is targeted by a receptor antagonist (17). Complement activation also plays an important role in the development of O₃-induced AHR and airway neutrophilia, and in this study, the O₃-induced neutrophil response did not appear to be necessary for the O₃-induced AHR (18).

$\gamma\delta$ T cells represent a small population (1–5%) of T lymphocytes; however, they are found in greater numbers on mucosal and epithelial surfaces, and recent studies revealed the critical role of these cells in the protection against pathogens and tumor cells (19). In the development of allergen-induced AHR, it was apparent from studies of TCR δ chain-deficient mice, which lack $\gamma\delta$ T cells, that $\gamma\delta$ T cells can regulate AHR, independent of the airway inflammatory response. Moreover, specific $\gamma\delta$ T cell subsets play important regulatory roles with different activities (20). In the allergen-induced development of lung allergic responses, the V γ 1+ subset enhances the airway response to methacholine (MCh), whereas the V γ 4+ $\gamma\delta$ subset suppresses AHR without any influence on airway inflammation (21, 22). King and coworkers suggested that intraepithelial $\gamma\delta$ T cells can protect the host from O₃-induced lung damage by reducing the inflammatory response in the lung; the subset of $\gamma\delta$ T cells responsible for these effects was not determined (23).

Here, we demonstrate that $\gamma\delta$ T cells, and specifically V γ 1+ T cells, are essential to the development of O₃-induced AHR

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and that TNF- α is an important link to this $V\gamma 1$ -dependent, O_3 -induced AHR.

MATERIALS AND METHODS

Animals

C57BL/6 (wild-type; WT) mice, B6.129P2-*Tcrd*^{tm1Mom}/J (TCR- δ chain-deficient; TCR- $\delta^{-/-}$) mice and B6.129P2-*Tcrb*^{tm1Mom}/J (TCR β chain-deficient; TCR- $\beta^{-/-}$) mice (background: C57BL/6 strain), were bred at National Jewish, or B6.129S-*Tnf*^{tm1Gkl}/J (TNF- α -deficient; TNF- $\alpha^{-/-}$) mice were purchased from Jackson Laboratory (Bar Harbor, ME); all were studied at ages of 8 to 12 weeks. All genotypes of the modified mice were confirmed by flow cytometry analysis as previously described (21). All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

Experimental Protocol

Mice were exposed to O_3 at a concentration of 2.0 ppm for 3 hours. The parameters were measured 6 to 8 hours after O_3 exposure. T cell depletion was achieved after injection of 200 μ g hamster anti-TCR- δ monoclonal antibodies (mAb) from clones GL3 and 403.A10 (1:1 mixture), anti- $V\gamma 1$ mAb from clone 2.11, or anti- $V\gamma 4$ mAb from clone UC3 into the tail veins of mice 3 days before O_3 exposure. This dosing regimen was optimized for T cell depletion (20, 21) and monitored routinely to confirm depletion of more than 95% of splenic $\gamma\delta$ T cells or specific subsets. Control antibody treatments were performed with the same amount of ChromePure Syrian hamster IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Throughout this article, we use the nomenclature for murine TCR- $V\gamma$ genes introduced by Heilig and Tonegawa (24).

In some experiments, mice received rat anti-mouse TNF- α (MP6-XT3; AMC 3814; Biosource, Camarillo, CA) or isotype rat IgG1 as control, just before O_3 exposure. The single dose of anti-mouse TNF- α Ab was 250 μ g based on previous results (25). All antibodies were suspended in 200 μ l of PBS at the time of intravenous injection.

O_3 Exposure

Mice were exposed to O_3 at 2.0 ppm for 3 hours in stainless steel wire cages. Cages were set inside 240-liter laminar flow inhalation chambers. HEPA-filtered room air was passed through these chambers at 25 changes/hour. Room temperature was maintained at 20 to 25°C. O_3 was generated by directing compressed medical-grade oxygen through an electrical discharge O_3 generator (Sander Ozonizer, Model 25; Erwin Sander Elektroapparatebau GmbH, Uetze-Eltze, Germany) located upstream of the exposure chamber. The O_3 -air mixture was metered into the inlet air stream with mass flow controllers (Model #1359C; MKS Instruments Inc., Andover, MA). Exposure to HEPA-filtered air was done in a separate chamber with age- and treatment-matched control animals. O_3 concentrations were continuously monitored at mouse nose levels within the chamber with a photometric O_3 analyzer (Model 400A; Advanced Pollution Instrumentation, Inc., San Diego, CA) and recorded on a strip-chart recorder. Calibration of the O_3 analyzer was performed by the Colorado Department of Public Health and Environment (Denver, CO).

Determination of Airway Resistance and Dynamic Compliance

Airway resistance (R_L) and dynamic compliance (C_{dyn}) were determined as a change in airway function after aerosolized methacholine (MCh) challenge as previously described (26). Mice were anesthetized with sodium pentobarbital (100 mg/kg), tracheostomized, and mechanically ventilated at a rate of 160 breaths/minute with a constant tidal volume of air (0.2 ml). After each MCh challenge, the data were continuously collected for 1 to 5 minutes and maximum values of R_L and minimum values of C_{dyn} were taken to express changes in these functional parameters. Data are presented as percent change from baseline values recorded after challenge with saline.

Determination of Cell Numbers in Bronchoalveolar Lavage

Immediately after the assessment of AHR, lungs were lavaged via the tracheal cannula with Hanks' balanced salt solution (HBSS, 1 ml). Total leukocyte numbers were measured (Coulter Counter; Coulter Corporation, Hialeah, FL). Differential cell counts were made from cytocentrifuged preparations (Cytospin 2; Shandon Ltd., Runcorn, Cheshire, UK), stained with Leukostat (Fisher Diagnostics, Pittsburgh, PA). As the number of macrophages, neutrophils, and epithelial cells in bronchoalveolar lavage (BAL) were good indicators of the response to O_3 exposure (27), these cell types were identified by standard hematologic procedures and at least 200 cells counted under $\times 400$ magnification in a blinded manner.

Histopathologic Study

Lungs were fixed after inflation and immersion in 10% formalin. To identify epithelial injury and airway inflammation in formalin-fixed airway tissue, sections were stained with hematoxylin/eosin.

Adoptive Transfer of $\gamma\delta$ T Cells

Mononuclear cells were isolated from spleens of WT mice, TCR- $\beta^{-/-}$ mice, or TNF- $\alpha^{-/-}$ mice. Cells were recovered by mincing the tissues and subsequently passing them through a stainless steel sieve. Cells were then washed and isolated by Histopaque-1083 (Sigma, St. Louis, MO) gradient centrifugation at 2,000 rpm for 20 minutes. For isolation of $\gamma\delta$ T cells, the cells were applied to T cell immunocolumns (Cedarlane Laboratories Ltd., Burlington, NC) according to the manufacturer's direction. For isolation of $V\gamma 1 +$ cells, the cells were incubated with biotinylated anti- $V\gamma 1$ monoclonal antibodies 2.11 (15 min, 4°C), then washed and incubated with streptavidin-conjugated magnetic beads (Miltenyi Biotec, Gladbach, Germany) for 10 minutes at 4°C. The cells were passed twice through magnetic columns to purify $V\gamma 1 +$ cells, achieving a more than 95% purity.

The recovered $\gamma\delta$ (4×10^4 or 8×10^4 cells/mouse) or $V\gamma 1 +$ T cells (5×10^4 cells/mouse) were transferred intravenously into TCR- $\delta^{-/-}$ mice 16 to 20 hours before O_3 exposure. Within 30 minutes to 1 hour, intravenously transferred $\gamma\delta$ T cells reach steady-state levels in the lung (28). Prior to and following adoptive transfer, the deficiency of surface receptors from TCR- $\delta^{-/-}$ mice or recruitment of transferred cells into lungs were monitored by flow cytometric analysis of blood and lung tissue as previously described (21). Assays of AHR and BAL were performed 6 to 8 h after completion of the O_3 exposure.

Flow Cytometry Analysis

Enriched lung cells, following preincubation with naive mouse serum in staining buffer (PBS, 2% FCS, and 0.2% sodium azide), were labeled with phycoerythrin (PE)-conjugated anti-CD69 (BD Pharmingen) and biotinylated anti- $V\gamma 1$ antibodies (clone 2.11). The suspension was then incubated with FITC-coupled streptavidin. Fluorescence intensity was compared with negative controls and cells were incubated with FITC-streptavidin alone. Results were analyzed using CellQuest software, and all analyses used a light scatter gate designated to include only small lymphocytes.

Data Analysis

One-way ANOVA was used to determine the levels of differences among all groups. Comparisons for all pairs were analyzed using Tukey-Kramer honest significant difference test, and P values for significance were set at 0.05. All data were expressed as the mean $\pm$ SEM.

RESULTS

AHR and Airway Inflammation after O_3 Exposure in TCR- $\delta^{-/-}$ Mice

WT mice exposed to filtered air showed a small dose response to inhaled MCh when R_L and C_{dyn} were monitored. After exposure of WT mice to O_3 , the mice developed significant increases in R_L and decreases in C_{dyn} to inhaled MCh in a dose-dependent fashion (Figure 1A). In contrast, exposure to O_3

Figure 1. Failure of TCR- δ -deficient mice to develop airway hyper-responsiveness (AHR) after O₃ exposure. C57BL/6 (wild-type, WT) and TCR- $\delta^{-/-}$ ($\delta^{-/-}$) mice were exposed to 2 ppm O₃ for 3 hours. (A) Airway responsiveness (R_L and C_{dyn}) to inhaled MCh. The baseline values of airway function were comparable, with no significant differences among all experimental groups; in air-WT (open circles), O₃-WT (solid circles), air-TCR- $\delta^{-/-}$ (open squares), and O₃-TCR- $\delta^{-/-}$ (solid squares) mice. The baseline values were 0.67 ± 0.06 , 0.65 ± 0.04 , 0.60 ± 0.03 , and 0.72 ± 0.03 (cm H₂O/ml/s) for R_L, and were 0.036 ± 0.003 , 0.038 ± 0.003 , 0.045 ± 0.004 , and 0.042 ± 0.004 (ml/cm H₂O) for C_{dyn}, respectively. (B) Bronchoalveolar lavage (BAL) cell composition. Analyses were performed 6 hours after initiation of O₃ exposure. Data represent the mean $\pm$ SEM ($n = 8$). * $P < 0.05$ and ** $P < 0.01$ compared with air-exposed group. Open bars, WT air; dark shaded bars, $\delta^{-/-}$ air; solid bars, WT ozone; light shaded bars, $\delta^{-/-}$ ozone.

failed to trigger increases in R_L or decreases in C_{dyn} in the TCR- $\delta^{-/-}$ mice.

Despite the failure to alter airway reactivity after O₃ inhalation, exposure to O₃ elicited increases in numbers of neutrophils in the BAL of TCR- $\delta^{-/-}$ mice similar to those observed in WT mice. After O₃ exposure, the numbers of airway epithelial cells in the BAL fluid of TCR- $\delta^{-/-}$ mice were also significantly increased after O₃ exposure, similar to those in the BAL fluid of O₃-exposed WT mice, indicating comparable epithelial cell damage (Figure 1B).

Examination of lung tissue revealed that O₃ exposure caused airway epithelial injury and inflammatory cell infiltration in the airways of both TCR- $\delta^{-/-}$ and WT mice (Figure 2). These changes were observed primarily in major airways but not in small airways.

These data demonstrate that O₃ exposure, while causing airway (neutrophilia) inflammation and epithelial cell damage, failed to induce AHR in the absence of $\gamma\delta$ T cells.

Effects of Depletion of $\gamma\delta$ T Cells on O₃-Induced AHR and Airway Inflammation in WT Mice

To confirm these findings of the importance of $\gamma\delta$ T cells in the development of O₃-induced AHR and ensure that this was not an indirect consequence of genetic manipulation, we investigated the effects of depleting $\gamma\delta$ T cells on O₃-induced AHR and airway inflammation in WT mice treated with anti-TCR- δ mAb. O₃ exposure caused significant increases in R_L and decreases in C_{dyn} to inhaled MCh in WT mice. However, if WT mice were treated with anti- δ before O₃ exposure, increases in airway reactivity (R_L and C_{dyn}) to inhaled MCh failed to develop (Figure 3A). In contrast, treatment with control Ab did not affect the development of O₃-induced AHR. Anti-TCR- δ mAb treatment did not affect the numbers of neutrophils or epithelial cells in BAL fluid (Figure 3B).

These data confirmed that $\gamma\delta$ T cells were essential for changes in airway reactivity to O₃ but were not required for effects on airway inflammation response or epithelial cell damage.

Effect of Depletion of Specific $\gamma\delta$ T Cell Subsets on O₃-Induced AHR and Airway Inflammation

Both the V γ 1+ and V γ 4+ $\gamma\delta$ T cell subsets appear to be important regulators of airway reactivity, at least in response to allergen-induced AHR (21). In light of the findings demonstrated for $\gamma\delta$ T cells on O₃-induced AHR, we next determined if a specific subset was involved. Using specific antibodies, we depleted either V γ 1+ or V γ 4+ T cells before O₃ exposure of C57BL/6 mice. After depletion of V γ 1 cells, but not of V γ 4 cells, there was a significant reduction in MCh-induced airway reactivity after O₃ exposure (Figure 4A). These findings identified the important role of V γ 1+ T cells in the development of O₃-induced AHR. Depletion of either V γ 1+ or V γ 4+ T cells had no effects on the cell composition of BAL fluid (Figure 4B).

Numbers and Activation of V γ 1+ T Cells in the Lung after O₃ Exposure

To determine if the numbers and/or activation of V γ 1+ T cells were altered after O₃ exposure, we analyzed lung cells for V γ 1, CD3, and CD69 expression, an activation-associated marker, after O₃ or filtered air exposure. As shown in Figure 4C, the total number of V γ 1+ T cells in the lungs was not altered after O₃ exposure. However, the percentage of CD69+ V γ 1+ T cells was increased in the lungs after O₃ exposure (Figure 4D).

Restoration of O₃-Induced AHR by Adoptive Transfer of $\gamma\delta$ or V γ 1+ T Cells

In parallel to the depletion experiments, we confirmed the role of $\gamma\delta$ T cells in adoptive transfer experiments in TCR- $\delta^{-/-}$ recipient mice. As described above, TCR- $\delta^{-/-}$ mice did not develop AHR after exposure to O₃ (Figure 5A). Adoptive transfer of (total) $\gamma\delta$ T cells from TCR- $\beta^{-/-}$ mice into TCR- $\delta^{-/-}$ mice before O₃ exposure reconstituted O₃-induced increases in R_L and decreases in C_{dyn} with higher numbers of transferred cells leading to greater changes in airway reactivity. Adoptive transfer of $\gamma\delta$ T cells also did result in an increase in numbers of epithelial cells, without influencing the numbers of neutrophils and macrophages in BAL fluid (Figure 5B). Adoptive transfer of V γ 1+ $\gamma\delta$ T cells (5×10^4 cells/mouse) into TCR- $\delta^{-/-}$ also reconstituted the increases in R_L and decreases in C_{dyn} after O₃ exposure (Figure 5C). O₃-exposed TCR- $\delta^{-/-}$ and WT mice had a similar BAL cell composition, including numbers of neutrophils and epithelial cells, and transfer of V γ 1+ $\gamma\delta$ T cells into

Figure 2. Histopathologic changes in WT and TCR- $\delta^{-/-}$ mice after O₃ exposure. Epithelial desquamation in major airways was identified in both WT and TCR- $\delta^{-/-}$ mice exposed to O₃. Insets represent histology of small airways without any detectable changes.

TCR- $\delta^{-/-}$ mice had little effect other than modestly increasing numbers of epithelial cells (data not shown). On histopathologic examination, O₃ caused epithelial injury in the airways of both WT and TCR- $\delta^{-/-}$ mice. The extent of

the epithelial injury in major airways was the same after O₃ exposure of TCR- $\delta^{-/-}$ mice, regardless of whether they received total V γ 1+ T cells (Figure 5D), $\gamma\delta$ T cells, or no T cells at all. No apparent changes were observed in small airways.

Figure 3. Effect of depletion of $\gamma\delta$ T cells on O₃-induced AHR and airway inflammation in WT mice. Depletion was performed using an anti- δ antibody before O₃ exposure. (A) Airway responsiveness to inhaled MCh. The baseline values of airway function were comparable among experimental groups; in air (open circles), O₃ (solid circles), air-IgG (open triangles), O₃-IgG (solid triangles), air-anti-TCR- δ (open squares), and O₃-anti-TCR- δ (solid squares) were 0.67 ± 0.04 , 0.72 ± 0.05 , 0.74 ± 0.03 , 0.71 ± 0.03 , 0.65 ± 0.03 , and 0.67 ± 0.07 (cm H₂O/ml/s) for R_L, and were 0.039 ± 0.002 , 0.040 ± 0.003 , 0.038 ± 0.002 , 0.035 ± 0.001 , 0.048 ± 0.004 , and 0.049 ± 0.006 (ml/cm H₂O) for C_{dyn}, respectively. (B) BAL cell composition. Data represent the mean $\pm$ SEM (n = 8). *P < 0.05 and **P < 0.01 compared with air-exposed group. #P < 0.05 and ##P < 0.01 compared with O₃-exposed group.

Figure 4. Effect of depletion of V γ 1+ or V γ 4+ T cells on O₃-induced AHR and airway inflammation in WT mice. Depletions were performed with specific antibodies before O₃ exposure. (A) R_L and C_{dyn} . The baseline airway function values were comparable among experimental groups; in air (open circles), O₃ (solid circles), air-IgG (open triangles), O₃-IgG (solid triangles), air-anti-V γ 1 (open squares), O₃-anti-V γ 1 (solid squares), air-anti-V γ 4 (open diamonds), and O₃-anti-V γ 4 (solid diamonds) were 0.67 ± 0.04 , 0.75 ± 0.03 , 0.75 ± 0.04 , 0.81 ± 0.03 , 0.67 ± 0.03 , 0.73 ± 0.05 , 0.75 ± 0.13 , and 0.75 ± 0.03 (cm H₂O/ml/s) for R_L , and were 0.039 ± 0.002 , 0.043 ± 0.002 , 0.045 ± 0.004 , 0.041 ± 0.003 , 0.044 ± 0.004 , 0.042 ± 0.002 , 0.043 ± 0.003 , and 0.037 ± 0.003 (ml/cm H₂O) for C_{dyn} , respectively. (B) BAL cell composition. Data represent the mean $\pm$ SEM ($n = 8$). Mononuclear cells from lungs were isolated and purified after O₃ or filtered air exposure in WT mice. The numbers of V γ 1+ T cells were not altered (C), but CD69 expression levels of V γ 1+ T cells were increased after O₃ exposure (D). * $P < 0.05$ and ** $P < 0.01$ compared with air-exposed group. # $P < 0.05$ and ## $P < 0.01$ compared with IgG-treated and O₃-exposed group.

Inhibitory Effect of Anti-TNF- α on V γ 1+ T Cell-Dependent O₃-Induced AHR

Given the reconstitution of AHR in TCR- $\delta^{-/-}$ mice that received V γ 1+ T cells and the potential role of TNF- α in the overall response to O₃ (13), we evaluated the consequences of neutralizing TNF- α using antibody administered after adoptive

transfer of V γ 1+ T cells into TCR- $\delta^{-/-}$ mice. Transfer of V γ 1+ cells to TCR- $\delta^{-/-}$ mice restored O₃-induced increases in R_L and decreases in C_{dyn} (Figure 6A). Treatment with anti-TNF- α mAb just before O₃ exposure but after V γ 1+ T cell transfer completely suppressed development of AHR in the V γ 1-recipient TCR- $\delta^{-/-}$ mice (Figure 6A). However, treatment with anti-

Figure 5. Adoptive transfer of total $\gamma\delta$ T cells or $V\gamma 1 + \gamma\delta$ T cells restores AHR in $\delta^{-/-}$ mice after O_3 exposure. (A) The baseline values of airway function were comparable among experimental groups; in air-WT (open circles), O_3 -WT (solid circles), air- $\delta^{-/-}$ (open triangles), O_3 - $\delta^{-/-}$ (solid triangles), air- $\delta^{-/-}$ - $\gamma\delta$ 4×10^4 cells/mouse (open squares), O_3 - $\delta^{-/-}$ - $\gamma\delta$ 4×10^4 cells/mouse (solid squares), air- $\delta^{-/-}$ - $\gamma\delta$ 8×10^4 cells/mouse (open diamonds), and O_3 - $\delta^{-/-}$ - $\gamma\delta$ 8×10^4 cells/mouse (solid diamonds) were 0.87 ± 0.04 , 0.92 ± 0.03 , 0.60 ± 0.03 , 0.82 ± 0.05 , 0.75 ± 0.04 , 0.94 ± 0.03 , 0.94 ± 0.02 , and 0.96 ± 0.04 (cm H_2O /ml/s) for R_L and in a air-WT, O_3 -WT, air- $\delta^{-/-}$, O_3 - $\delta^{-/-}$, air- $\delta^{-/-}$ - $\gamma\delta$ 4×10^4 cells/mouse, O_3 - $\delta^{-/-}$ - $\gamma\delta$ 4×10^4 cells/mouse, air- $\delta^{-/-}$ - $\gamma\delta$ 8×10^4 cells/mouse, O_3 - $\delta^{-/-}$ - $\gamma\delta$ 8×10^4 cells/mouse were 0.030 ± 0.002 , 0.046 ± 0.005 , 0.058 ± 0.004 , 0.044 ± 0.003 , 0.040 ± 0.002 , 0.059 ± 0.011 , 0.031 ± 0.002 , and 0.057 ± 0.009 (ml/cm H_2O) C_{dyn} , respectively. (A, C) R_L and C_{dyn} and (B) BAL cell composition. Purified total $\gamma\delta$ T cells (A, B) or isolated $V\gamma 1 + \gamma\delta$ T cells (C) were injected intravenously into $\delta^{-/-}$ mice 16 hours before O_3 exposure. (D) Epithelial desquamation in major airways was observed in all groups after O_3 exposure. Insets represent histology of small airways without any detectable changes. Data represent the mean $\pm$ SEM ($n = 8$). (A, C) $*P < 0.05$ and $**P < 0.01$ compared with air-exposed WT group; and $##P < 0.01$ compared with O_3 -exposed WT group; $\dagger P < 0.05$ and $\dagger\dagger P < 0.01$ compared with O_3 -exposed $\delta^{-/-}$ group. (B) $*P < 0.05$ and $**P < 0.01$ compared with air-exposed group.

TNF- α did not have any effect on BAL cell composition, including numbers of epithelial cells (Figure 6B) or airway damage (data not shown).

$\gamma\delta$ T Cells Are Not the Source of TNF- α

To determine if $\gamma\delta$ T cells were themselves the source of TNF- α , we examined the consequences of reconstituting TCR- $\delta^{-/-}$ mice with $\gamma\delta$ T cells from TNF- $\alpha^{-/-}$ mice. Adoptive transfer of $\gamma\delta$ T cells from TNF- $\alpha^{-/-}$ or WT mice into TCR- $\delta^{-/-}$ mice before O_3 exposure reconstituted O_3 -induced increases in R_L and decreases in C_{dyn} (Figure 7A), suggesting that TNF- $\alpha^{-/-}$ was derived from another cell type activated by O_3 , possibly damaged

epithelium. BAL cell composition was similar after transfer of $\gamma\delta$ T cells from either TNF- $\alpha^{-/-}$ or WT mice (Figure 7B).

DISCUSSION

Acute exposure of susceptible mice to O_3 leads to the rapid development of AHR, airway neutrophilia, increased numbers of epithelial cells in BAL fluid, and histologic evidence of epithelial sloughing. These changes tend to peak at 6 to 12 hours, with resolution over the ensuing 24 to 48 hours (17). Since subacute O_3 exposure (0.3 ppm O_3 for 48–72 h) was shown to induce airway inflammation but not AHR (13), the protocol followed here, exposure to 2 ppm O_3 for 3 hours is

Figure 5. (continued).

similar to that used by others and one in which a genetic susceptibility locus linked to TNF- α has been delineated (10, 13, 29). One of the primary responses to O₃, the development of AHR in response to the nonspecific bronchoconstrictor MCh was virtually eliminated in mice genetically deficient in $\gamma\delta$ T cells. This finding was confirmed in WT mice treated with a $\gamma\delta$ T cell-depleting antibody. Thus, it appeared that $\gamma\delta$ cells were

essential to the development of O₃-induced AHR, and this was confirmed in experiments in which transfer of $\gamma\delta$ T cells restored the AHR response in TCR- $\delta^{-/-}$ mice.

Although AHR, airway neutrophilia, and epithelial sloughing all result from acute O₃ exposure, only airway function and not the neutrophilia or epithelial damage primarily in major airways was affected by the absence or depletion of the $\gamma\delta$ T

Figure 6. Effect of anti-TNF- α on O₃-induced AHR and airway inflammation in $\delta^{-/-}$ mice recipients of transferred V γ 1+ $\gamma\delta$ T cells. Anti-TNF- α was injected just before initiation of O₃ exposure, and V γ 1+ $\gamma\delta$ T cells were injected intravenously into $\gamma^{-/-}$ mice 16 hours before O₃ exposure. (A) R_L and C_{dyn} and (B) BAL cell composition. Data represent the mean $\pm$ SEM ($n = 8$). ** $P < 0.01$ compared with O₃-exposed WT group. ## $P < 0.01$ compared with O₃-exposed $\delta^{-/-}$ group. †† $P < 0.01$ compared with IgG-treated, V γ 1+ T cell recipients, and O₃-exposed $\delta^{-/-}$ group.

Figure 7. TNF- α -deficient $\gamma\delta$ T cells reconstitute O₃-induced AHR in $\gamma^{-/-}$ mice. TNF- α -sufficient and TNF- α -deficient $\gamma\delta$ T cells were injected intravenously into $\delta^{-/-}$ mice 16 hours before O₃ exposure. (A) R_L and C_{dyn} and (B) BAL cell composition. Data represent the mean $\pm$ SEM ($n = 8$). There were no significant differences between the groups.

cells, suggesting a dissociation in the pathways leading to these events. A similar dissociation may be observed in individuals with asthma, in whom asthma control (AHR) may be difficult to achieve, and acute exacerbations remain despite significant improvement in airway eosinophilia or other biomarkers after inhaled corticosteroid therapy (30). We also previously showed that after complement depletion or treatment with an IL-1 receptor antagonist, AHR, airway neutrophilia, and epithelial damage were suppressed in parallel, but one could dissociate a requirement for airway neutrophilia in the development of O₃-induced AHR (17). Koohsari and colleagues showed that the severity of airway epithelial injury after chlorine gas exposure is greater in TCR- $\delta^{-/-}$ mice, but that the inflammatory response and the change in airway responsiveness to MCh were reduced (31). These findings also demonstrate the dissociation of AHR and airway epithelial injury. The dissociation of $\gamma\delta$ T cell effects on development of AHR and airway inflammation is very characteristic of the effects of this population of T cells in the response to allergen challenge of sensitized mice, in which AHR regulation appeared to be independent of airway eosinophilia (20, 32).

Another parallel between O₃-induced and allergen-induced AHR is in the role of V γ 1+ T cells. Under both conditions, V γ 1+ $\gamma\delta$ T cells appear essential to the development of AHR (21). Whereas V γ 4+ $\gamma\delta$ T cells down-regulated allergen-induced AHR, their role in O₃-induced AHR remains uncertain. In WT mice, depletion of V γ 4+ $\gamma\delta$ T cells was without effect on O₃-induced AHR whereas depletion of V γ 1+ $\gamma\delta$ T cells abrogated AHR. Further, supporting the role of this $\gamma\delta$ T cell subset, activation of V γ 1+ $\gamma\delta$ T cells in the lungs of O₃-exposed

mice, identified by CD69 expression, was increased and in reconstitution experiments transfer of V γ 1+ $\gamma\delta$ T cells into TCR- $\delta^{-/-}$ mice restored O₃-induced AHR. The location of V γ 1+ $\gamma\delta$ T cells has been studied in the mouse lung, and they are found in the alveoli, the lamina propria/smooth muscle layers, and around blood vessels, including locations within the blood vessel wall (33). Dziedzic and White reported on the response to O₃ in mice lacking T cells (34). They suggested that the site of the interactions may be on epithelial cells that line the alveolar duct and the alveoli. Taken together, the interaction of V γ 1+ $\gamma\delta$ T cells and damaged alveolar epithelial cells may be critical to the development of AHR after O₃ exposure.

How V γ 1+ $\gamma\delta$ T cells exert their effects on airway tone is not defined at present. Possibilities include direct interactions with airway smooth muscle or indirectly through release of mediators that themselves affect airway tone. One candidate is TNF- α . TNF- α has been implicated in O₃-induced AHR (13) and the genetic link with a locus on mouse chromosome 17 close to the *Tnfa* gene in defining susceptibility to O₃-induced inflammation and lung injury supports this concept (10). Further, TNF receptor-mediated signaling through NF- κ B and MAPK/AP-1 were shown to be essential for O₃-induced pulmonary toxicity (35). In the studies reported here, the reconstitution of AHR in TCR- $\delta^{-/-}$ mice by V γ 1+ $\gamma\delta$ T cells was completely prevented by administration of anti-TNF- α Ab before O₃ exposure. Administration of anti-TNF- α Ab, although preventing AHR, did not prevent lung inflammation and injury in response to O₃. These seemingly disparate results (10) need further evaluation, but are in agreement with the findings of Shore and coworkers (14), who demonstrated that TNF receptor deficiency prevented

O₃-induced AHR but not neutrophil accumulation in the airways. Since V γ 1 + $\gamma\delta$ T cells only impacted AHR, one possibility is that O₃ exposure induced TNF- α release, possibly from damaged airway epithelium, which in turn leads to the activation of V γ 1 + $\gamma\delta$ T cells. We previously showed that TNF- α may activate $\gamma\delta$ T cells leading to AHR (36) and that TNF- α regulates allergen-induced AHR through $\gamma\delta$ T cells, specifically through the TNF- α R2 receptor (p75) (32). Since the cell transfer and anti-TNF- α administration were before O₃ exposure, this effect of TNF- α on the activation of V γ 1 + T cells remains a possibility. Less likely is that the transferred V γ 1 + $\gamma\delta$ T cells were themselves the source of TNF- α leading to the changes in AHR. Indeed, in this study, adoptive transfer of $\gamma\delta$ T cells from TNF- α -deficient mice into TCR- $\delta^{-/-}$ mice fully reconstituted the development of AHR, indicating that they were not a primary source of TNF- α . Other possibilities now being pursued are interactions between $\gamma\delta$ T cells and dendritic cells, since $\gamma\delta$ T cells can induce the maturation of dendritic cells and dendritic cells are known to produce TNF- α (19, 37).

IL-17 is a potent proinflammatory cytokine and its role in the pathogenesis of diseases including asthma has been intensively investigated. Pichavant and colleagues showed that IL-17 from NKT cells may play a key role in O₃-induced but not in allergen-induced AHR (38). $\gamma\delta$ T cells were also shown to be a source of IL-17; however, as Roark and coworkers demonstrated in a collagen-induced arthritis model, V γ 4 + $\gamma\delta$ T cells but not V γ 1 + $\gamma\delta$ T cells are more likely a source of IL-17 (39). The role of IL-17 and its interaction with TNF- α and $\gamma\delta$ T cell requires further investigation.

In summary, these data identify an essential role for TNF- α and V γ 1 + $\gamma\delta$ T cells in O₃-induced AHR, and clearly dissociate alterations in airway function from other consequences of O₃ exposure, airway neutrophilia and epithelial cell damage. Ozone and other air pollutants are thought to increase the risk of morbidity and acute exacerbations of asthma (40). The observation that a small subset of $\gamma\delta$ T cells is critical to the development of altered airway function after O₃ exposure highlights their functional capacity in response to a common inhaled environmental toxin. Further investigation into the role of $\gamma\delta$ T cells in the development AHR and their manipulation may lead to novel approaches for controlling O₃-related asthma exacerbations.

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References

- Greenberg MI. Occupational, Industrial, and Environmental Toxicology, Philadelphia, Mosby, 2003.
- National Institute of Environmental Health Sciences. Ozone alerts. Research Triangle Park, NC: National Institutes of Environmental Health Sciences: 1986. NIH Publications No. 99-4671.
- Pryor WA. How far does ozone penetrate into the pulmonary air/tissue boundary before it reacts? *Free Radic Biol Med* 1992;12:83-88.
- Pulfer MK, Taube C, Gelfand EW, Murphy RC. Ozone exposure in vivo and formation of biologically active oxysterols in the lung. *J Pharmacol Exp Ther* 2005;312:256-264.
- Postlethwait E, Cueto R, Velsor LW, Pryor WA. O₃-induced formation of bioactive lipids: estimated surface concentrations and lining layer effects. *Am J Physiol Lung Cell Mol Physiol* 1998;274:L1006-L1016.
- Kafoury RM, Pryor WA, Squadrito GL, Salgo MG, Zou X, Friedman M. Lipid ozonation products activate phospholipases A₂, C and D. *Toxicol Appl Pharmacol* 1998;150:338-349.
- Uhlson C, Harrison K, Allen CB, Ahmad S, White CW, Murphy RC. Oxidized phospholipids derived from ozone treated lung surfactant extract reduce macrophage and epithelial cell viability. *Chem Res Toxicol* 2002;15:896-906.
- Zhang LY, Levitt RC, Kleeberger SR. Differential susceptibility to ozone-induced airways hyperreactivity in inbred strains of mice. *Exp Lung Res* 1995;21:503-518.
- Prows DR, Shertzer HG, Daly MJ, Sidman CL, Leikauf GD. Genetic analysis of ozone-induced acute lung injury in sensitive and resistant strains of mice. *Nat Genet* 1997;17:471-474.
- Kleeberger SR, Levitt RC, Zhang LY, Longphre M, Harkema J, Jedlicka A, Eleff SM, DiSilvestre D, Holroyd KJ. Linkage analysis of susceptibility to ozone-induced lung inflammation in inbred mice. *Nat Genet* 1997;17:475-478.
- Kleeberger SR, Reddy S, Zhang LY, Jedlicka AE. Genetic susceptibility to ozone-induced lung hyperpermeability: role of toll-like receptor 4. *Am J Respir Cell Mol Biol* 2000;22:620-627.
- Arsalane K, Gosset P, Vanhee D, Voisin C, Hamid Q, Tonnel AB, Wallaert B. Ozone stimulates synthesis of inflammatory cytokines by alveolar macrophages in vitro. *Am J Respir Cell Mol Biol* 1995;13:60-68.
- Cho HY, Zhang LY, Kleeberger SR. Ozone-induced lung inflammation and hyperreactivity are mediated via tumor necrosis factor- α receptors. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L537-L546.
- Shore SA, Schwartzman IN, Le Blanc B, Murthy GKG, Doerschuk CM. Tumor necrosis factor receptor 2 contributes to ozone-induced airway hyperresponsiveness in mice. *Am J Respir Crit Care Med* 2001;164:602-607.
- Young C, Bhalla DK. Effects of ozone on the epithelial and inflammatory responses in the airways: role of tumor necrosis factor. *J Toxicol Environ Health* 1995;46:329-342.
- Pearson AC, Bhalla DK. Effects of ozone on macrophage adhesion in vitro and epithelial and inflammatory responses in vivo: the role of cytokines. *J Toxicol Environ Health* 1997;50:143-157.
- Park JW, Taube C, Swasey C, Kodama T, Joetham A, Balhorn A, Takeda K, Miyahara N, Allen C, Dakhama A, et al. IL-1 receptor antagonist attenuates airway hyperresponsiveness following exposure to ozone. *Am J Respir Cell Mol Biol* 2004;30:830-836.
- Park JW, Taube C, Joetham A, Takeda K, Kodama T, Dakhama A, McConville G, Allen C, Giclas P, Holers VM, et al. Complement activation is critical to airway hyperresponsiveness following acute ozone exposure. *Am J Respir Crit Care Med* 2004;169:726-732.
- Komori HK, Meehan TF, Havran WL. Epithelial and mucosal gamma delta T cells. *Curr Opin Immunol* 2006;18:534-538.
- Lahn M, Kanehiro A, Takeda K, Joetham A, Schwarze J, Köhler G, O'Brien R, Gelfand EW, Born W. Negative regulation of airway responsiveness that is dependent on gammadelta T cells and independent of alphabeta T cells. *Nat Med* 1999;5:1150-1156.
- Hahn Y-S, Taube C, Jin N, Sharp L, Wands JM, Aydtung MK, Lahn M, Huber SA, O'Brien RL, Gelfand EW, et al. Different potentials of $\gamma\delta$ T cell subsets in regulating airway responsiveness: V γ 1+ cells, but not V γ 4+ cells, promote airway hyperreactivity, Th2 cytokines, and airway inflammation. *J Immunol* 2004;172:2894-2902.
- Jin N, Miyahara N, Roark CL, French JD, Aydtung MK, Matsuda JL, Gapin L, O'Brien RL, Gelfand EW, Born WK. Airway hyperresponsiveness through synergy of $\gamma\delta$ T cells and NKT cells. *J Immunol* 2007;179:2961-2968.
- King DP, Hyde DM, Jackson KA, Novosad DM, Ellis TN, Putney L, Stovall MY, Van Winkle LS, Beaman BL, Ferrick DA. Protective response to pulmonary injury requires $\gamma\delta$ T lymphocytes. *J Immunol* 1999;162:5033-5036.
- Heilig JS, Tonegawa S. Diversity of murine gamma genes and expression in fetal and adult T lymphocytes. *Nature* 1986;322:836-840.
- Herring AC, Lee J, McDonald RA, Toews GB, Huffnagle GB. Induction of interleukin-12 and gamma interferon requires tumor necrosis factor alpha for protective T1-cell-mediated immunity to pulmonary cryptococcus neoformans infection. *Infect Immun* 2002;70:2959-2964.
- Takeda K, Hamelmann E, Joetham A, Shultz L, Larsen GL, Irvin CG, Gelfand EW. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell deficient mice. *J Exp Med* 1997;186:449-454.
- Krishna MT, Madden J, Teran LM, Biscione GL, Lau LC, Withers NJ, Sandström T, Mudway I, Kelly FJ, Walls A, et al. Effects of 0.2 ppm ozone on biomarkers of inflammation in bronchoalveolar lavage fluid and bronchial mucosa of healthy subjects. *Eur Respir J* 1998;11:1294-1300.
- Hahn Y-S, Taube C, Jin N, Takeda K, Park JW, Wands JM, Aydtung MK, Roark CL, Lahn M, O'Brien RL, et al. V gamma 4+ gamma

- delta T cells regulate airway hyperreactivity to methacholine in ovalbumin-sensitized and challenged mice. *J Immunol* 2003;171:3170–3178.
29. Kleeberger SR, Hudak BB. Acute ozone-induced change in airway permeability: role of infiltrating leukocytes. *J Appl Physiol* 1992;72:670–676.
30. Kaminsky DA, Irvin CG. New insights from lung function. *Curr Opin Allergy Clin Immunol* 2001;3:205–209.
31. Koohsari H, Tamaoka M, Campbell HR, Martin JG. The role of $\gamma\delta$ T cells in airway epithelial injury and bronchial responsiveness after chlorine gas exposure in mice. *Respir Res* 2007;8:21.
32. Kanehiro A, Lahn M, Makela MJ, Dakhama A, Fujita M, Joetham A, Mason RJ, Born W, Gelfand EW. Tumor necrosis factor (TNF)- α negatively regulates airway hyperresponsiveness through $\gamma\delta$ T cells. *Am J Respir Crit Care Med* 2001;164:2229–2238.
33. Wands JM, Roark CL, Aydintug MK, Hahn Y-S, Sharp L, Yin X, Dalporto J, Lahn M, Hyde DM, Gelfand EW, et al. Distribution and leukocyte contacts of $\gamma\delta$ T cells in the lung. *J Leukoc Biol* 2005;78:1086–1096.
34. Dziedzic D, White HJ. Response of T-cell-deficient mice to ozone exposure. *J Toxicol Environ Health* 1987;21:57–71.
35. Cho H-Y, Morgan DL, Bauer AK, Kleeberger SR. Signal transduction pathways of tumor necrosis factor-mediated lung injury induced by ozone in mice. *Am J Respir Crit Care Med* 2007;175:829–839.
36. Lahn M, Kalataradi H, Mittelstadt P, Pflum E, Vollmer M, Cady C, Mukasa A, Vella AT, Ikle D, Harbeck R, et al. Early preferential stimulation of gamma delta T cells by TNF-alpha. *J Immunol* 1998;160:5221–5230.
37. Langhorne J, Albano FR, Hensmann M, Sanni L, Cadman E, Voisine C, Sponaas AM. Dendritic cells, pro-inflammatory responses, and antigen presentation in a rodent malaria infection. *Immunol Rev* 2004;201:35–47.
38. Pichavant M, Goya S, Meyer EH, Johnston RA, Kim HY, Matangkasombut P, Zhu M, Iwakura Y, Savage PB, DeKruyff RH, et al. Ozone exposure in a mouse model induces airway hyperreactivity that requires the presence of natural killer T cells and IL-17. *J Exp Med* 2008;205:385–393.
39. Roark CL, French JD, Taylor MA, Bendele AM, Born WK, O'Brien RL. Exacerbation of collagen-induced arthritis by oligoclonal, IL-17-producing gamma delta T cells. *J Immunol* 2007;179:5576–5583.
40. Eggleston PA. The environment and asthma in US inner cities. *Chest* 2007;132:782S–788S.