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**The Role of T Cells in the  
Regulation of Acrolein-Induced  
Pulmonary Inflammation  
and Epithelial-Cell Pathology**

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Hitesh Deshmukh, Erin Beckman, Mario Medvedovic,  
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# The Role of T Cells in the Regulation of Acrolein-Induced Pulmonary Inflammation and Epithelial-Cell Pathology

Michael T. Borchers, Scott C. Wesselkamper, Hitesh Deshmukh, Erin Beckman,  
Mario Medvedovic, Maureen Sartor, and George D. Leikauf

with a Critique by the HEI Health Review Committee

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# ABOUT HEI

The Health Effects Institute is a nonprofit corporation chartered in 1980 as an independent research organization to provide high-quality, impartial, and relevant science on the effects of air pollution on health. To accomplish its mission, the institute

- Identifies the highest-priority areas for health effects research;
- Competitively funds and oversees research projects;
- Provides intensive independent review of HEI-supported studies and related research;
- Integrates HEI's research results with those of other institutions into broader evaluations; and
- Communicates the results of HEI research and analyses to public and private decision makers.

HEI receives half of its core funds from the U.S. Environmental Protection Agency and half from the worldwide motor vehicle industry. Frequently, other public and private organizations in the United States and around the world also support major projects or certain research programs. HEI has funded more than 280 research projects in North America, Europe, Asia, and Latin America, the results of which have informed decisions regarding carbon monoxide, air toxics, nitrogen oxides, diesel exhaust, ozone, particulate matter, and other pollutants. These results have appeared in the peer-reviewed literature and in more than 200 comprehensive reports published by HEI.

HEI's independent Board of Directors consists of leaders in science and policy who are committed to fostering the public-private partnership that is central to the organization. The Health Research Committee solicits input from HEI sponsors and other stakeholders and works with scientific staff to develop a Five-Year Strategic Plan, select research projects for funding, and oversee their conduct. The Health Review Committee, which has no role in selecting or overseeing studies, works with staff to evaluate and interpret the results of funded studies and related research.

All project results and accompanying comments by the Health Review Committee are widely disseminated through HEI's Web site ([www.healtheffects.org](http://www.healtheffects.org)), printed reports, newsletters, and other publications, annual conferences, and presentations to legislative bodies and public agencies.



# ABOUT THIS REPORT

Research Report 146, *The Role of T Cells in the Regulation of Acrolein-Induced Pulmonary Inflammation and Epithelial-Cell Pathology*, presents a research project funded by the Health Effects Institute and conducted by Dr. Michael T. Borchers of the Department of Environmental Health, Division of Environmental Genetics and Molecular Toxicology, University of Cincinnati College of Medicine, Cincinnati, OH, and his colleagues. This research was funded under HEI's Walter A. Rosenblith New Investigator Award Program, which provides support to promising scientists at the early stages of their careers. The report contains three main sections.

**The HEI Statement**, prepared by staff at HEI, is a brief, nontechnical summary of the study and its findings; it also briefly describes the Health Review Committee's comments on the study.

**The Investigators' Report**, prepared by Borchers and colleagues, describes the scientific background, aims, methods, results, and conclusions of the study.

**The Critique** is prepared by members of the Health Review Committee with the assistance of HEI staff; it places the study in a broader scientific context, points out its strengths and limitations, and discusses remaining uncertainties and implications of the study's findings for public health and future research.

This report has gone through HEI's rigorous review process. When an HEI-funded study is completed, the investigators submit a draft final report presenting the background and results of the study. This draft report is first examined by outside technical reviewers and a biostatistician. The report and the reviewers' comments are then evaluated by members of the Health Review Committee, an independent panel of distinguished scientists who have no involvement in selecting or overseeing HEI studies. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, as necessary, to revise their report. The Critique reflects the information provided in the final version of the report.



# HEI STATEMENT

## Synopsis of Research Report 146

### **Role of T Cells in Mouse Airways in Response to Inhaled Acrolein**

#### **BACKGROUND**

Acrolein is a reactive aldehyde that injures the airways in humans and other species. It is an important air toxicant, one of a large and diverse group of air pollutants that, with sufficient exposure, are known or suspected to cause adverse human health effects. Even though ambient levels of air toxics are generally low, these compounds are a cause for public health concern because large numbers of people are exposed to them over prolonged periods of time. In the United States, these compounds are not regulated by the National Ambient Air Quality Standards but are subject to other rules set by the U.S. Environmental Protection Agency.

Dr. Michael Borchers, of the University of Cincinnati College of Medicine, submitted an application, "T Cell Sub-Populations Regulate Airway Inflammation and Injury Following Acrolein Exposures," under Request for Applications 03-2, the Walter A. Rosenblith Award, which was established to provide support for outstanding investigators beginning an independent research career. Dr. Borchers proposed to study the role of  $\gamma\delta$  T cells, a minor subpopulation of T cells that use the  $\gamma$  and  $\delta$  chains as their antigen-specific T-cell receptor (TCR) and are found predominantly at mucosal epithelial sites such as the airways and the gastrointestinal tract. The HEI Research Committee thought it would be valuable for Dr. Borchers to evaluate the role of both  $\gamma\delta$  T cells and  $\alpha\beta$  T cells, the major subpopulation of T cells, which uses a different two-chain molecule as its antigen-specific TCR, and Dr. Borchers agreed.

#### **APPROACH**

Dr. Borchers used 8–12-week-old wild-type (C57BL/6J) mice and mice genetically deficient in either  $\alpha\beta$  T cells or  $\gamma\delta$  T cells. Mice were exposed to 0.5 or 2 ppm acrolein vapor, or were sham-exposed

(to filtered air), for 6 hours per day, 5 days per week, for 1, 2, or 4 weeks. Immediately after exposure, bronchoalveolar lavage (BAL) was performed and total and differential cell numbers in BAL fluid were determined. The numbers of epithelial cells detected in BAL fluid were used as a marker of epithelial-cell injury. BAL supernatant was assayed for levels of mucin (Muc5ac). Lung tissue was assessed for mucous-cell metaplasia, by light microscopy, and lung cells were evaluated for expression of activated caspase 3, a marker of apoptosis (programmed cell death). The investigators also measured levels in lung cells of the cytokines interferon- $\gamma$  (IFN- $\gamma$ ) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which are produced by T cells and which activate macrophages. For biologic end points, the investigators analyzed data by using one-way analysis of variance with differences between means considered significant when  $P < 0.05$ .

To identify genes expressed differentially in  $\alpha\beta$  T cells and  $\gamma\delta$  T cells in the lung, total cellular RNA was extracted from highly purified populations of  $\alpha\beta$  T cells and  $\gamma\delta$  T cells from wild-type C57BL/6 mice exposed to 2.0 ppm acrolein or filtered air for 1 week. RNA samples were used to assess changes in gene expression by microarray analysis using slides containing more than 30,000 DNA sequences (70-base-pair probes). Borchers and colleagues used appropriate statistical tests to identify genes whose expression changed significantly. To confirm findings for some of the changes in gene expression identified by microarray experiments, the investigators performed quantitative real-time polymerase-chain-reaction (qRT-PCR) assays on RNA from 15 selected genes. They used two programs, MAPPFinder and PANTHER database, to organize the measured changes in gene expression into several discrete biologic pathways; in this way they could determine the types of biologic processes and functions that

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Dr. Michael T. Borchers at the University of Cincinnati College of Medicine, Cincinnati, OH, and colleagues. Research Report 146 contains both the detailed Investigators' Report and a Critique of the study prepared by the Institute's Health Review Committee.

had been affected in the  $\alpha\beta$  or  $\gamma\delta$  T cells by the exposure to acrolein.

### RESULTS AND INTERPRETATIONS

Acrolein exposures of wild-type mice and mice deficient in  $\gamma\delta$  T cells resulted in increased epithelial-cell sloughing (resulting in increased epithelial-cell numbers in BAL fluid), apoptosis (measured as cells stained with active caspase 3—most of which were found in distal airways and airspaces) and increases in macrophage numbers; these effects appeared to be somewhat more pronounced in mice deficient in  $\gamma\delta$  T cells than in wild-type mice. By contrast, mice deficient in  $\alpha\beta$  T cells that were exposed to acrolein did not show effects on epithelial-cell injury, apoptosis, or macrophage numbers. In the airways of all strains of mice, acrolein exposure also resulted in similar, but small, increases in the mucus-cell index (reflecting the total amount of mucus in airways and the number of airways affected) and similar increases in levels of the cytokines IFN- $\gamma$  and GM-CSF.

The expression of more than 1000 genes was altered in either  $\alpha\beta$  or  $\gamma\delta$  T cells isolated from wild-type C57BL/6J mice after a 1-week exposure to 2 ppm acrolein; about 75% of these changes were unique to either the  $\alpha\beta$  or the  $\gamma\delta$  T-cell subpopulation. Of the 15 genes selected for analysis by qRT-PCR to validate changes in gene expression observed in the microarray experiments, the investigators reported that most, but not all, qRT-PCR findings were consistent with those from the microarray experiments.

From the changes in gene expression detected in microarray experiments, the investigators singled out several genes in each subpopulation that were considered candidates for further study regarding their responses to acrolein exposure. Borchers and colleagues interpreted the data to suggest that the key changes found in  $\alpha\beta$  T cells only were in the expression of genes for cytokines and chemokines and their receptors, in particular, those associated with the activation of T cells and the consequent activation and accumulation of macrophages. They also reported that the key changes in gene expression found in  $\gamma\delta$  T cells only were associated with genes involved in host-cell recognition, affecting the clearance of damaged host tissue, cellular interactions, and certain cytokines and chemokines and their receptors.

Using the programs to link microarray data to a gene database to identify biologic processes affected

by acrolein exposure, the investigators found several intracellular pathways that were affected in  $\alpha\beta$  and  $\gamma\delta$  T cells. Some overlap between the subpopulations was noted, but some differences were also found in the pathways affected. On the basis of the most statistically significant findings, organic anion transport and mitochondrial apoptosis were concluded to be affected in  $\alpha\beta$  T cells, whereas peptide antigen binding and pathways including defense responses were concluded to be affected in  $\gamma\delta$  T cells.

### CONCLUSIONS

In its independent review of the study, the HEI Review Committee thought that Dr. Borchers and colleagues had successfully designed and conducted a preliminary descriptive study in mice genetically depleted of one or other key subpopulation of T cells—those using either  $\alpha\beta$  or  $\gamma\delta$  as their antigen-specific receptor—to study the airway response to acrolein exposure via inhalation. The study provides useful information into the host response and pathology associated with acrolein exposure; it also provides useful preliminary descriptions of the roles of two groups of T cells during the course of the host response. The findings suggest that T cells play a role in the lungs' responses to acrolein exposure, and the data generally support the view that  $\alpha\beta$  and  $\gamma\delta$  T cell subpopulations have different roles in the response to acrolein:  $\alpha\beta$  T cells primarily promote the accumulation of macrophages and  $\gamma\delta$  T cells protect the integrity of airway epithelial cells.

The Review Committee generally agreed with the investigators' interpretations of the biologic responses to acrolein exposure and thought that the results showing dissociation between inflammation (attributable to macrophages and their products) and epithelial damage were interesting and compatible with recent data from other studies showing injury responses in the absence of inflammation. However, the Committee noted caution in interpreting the results of any study based on the use of mice that are entirely genetically deficient in  $\alpha\beta$  or  $\gamma\delta$  T cells; of particular concern are uncertainties about the possibilities of unexamined developmental changes in these animals and compensatory increases in the remaining subpopulations of T cells. In addition,  $\alpha\beta$  and  $\gamma\delta$  T cells are heterogeneous, each containing numerous functionally specialized subsets; thus, mice deficient in all T cells of a given type might display a net effect of deficiencies of numerous subsets, some of which may cancel each other out.

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The Committee also thought that the studies by Borchers and colleagues of changes in gene expression pointed to potentially useful directions for future research. The Committee agreed that several genes in each T-cell subpopulation were up-regulated, and several were down-regulated, by exposure to acrolein; moreover, some genes were expressed differently in the two T-cell subpopulations. However, the Committee found the investigators' interpretations of the differences in expression of key genes

and involvement of different biologic pathways in  $\alpha\beta$  and  $\gamma\delta$  T cells after exposure to acrolein were interesting but speculative, requiring further experiments for confirmation or refutation. Nonetheless, the Committee concluded that the approaches and results of Borchers and colleagues will encourage more-mechanistic studies to explore the role of T cells in the pathology associated with exposure to acrolein.



### The Role of T Cells in the Regulation of Acrolein-Induced Pulmonary Inflammation and Epithelial-Cell Pathology

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#### ABSTRACT

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Exposure to acrolein in the ambient air in urban environments represents a considerable hazard to human health. Acrolein exposure causes airway inflammation, accumulation of monocytes, macrophages, and lymphocytes in the interstitium, mucous-cell metaplasia, and airspace enlargement. Currently, the mechanisms that control these events are unclear, and the relative contribution of T-cell subpopulations to pulmonary pathology after exposure to air toxics is unknown. In this study, we used a mouse model of pulmonary pathology induced by repeated acrolein exposure to examine whether pulmonary lymphocyte subpopulations differentially regulate inflammatory-cell accumulation and epithelial-cell pathology. To examine the role of the lymphocyte subpopulations, we used transgenic mice genetically deficient in either  $\alpha\beta$  T cells or  $\gamma\delta$  T cells and measured changes in several cellular, molecular, and pathologic outcomes associated with repeated inhalation exposure to 2.0 ppm or 0.5 ppm acrolein. To examine the potential functions of the lymphocyte subpopulations, we purified these cells from lung tissue of mice repeatedly exposed to 2.0 ppm acrolein, isolated and amplified the

messenger RNA (mRNA\*) transcripts, and performed oligonucleotide microarray analysis. Our data demonstrate that  $\alpha\beta$  T cells are primarily responsible for the accumulation of macrophages after acrolein exposure, whereas  $\gamma\delta$  T cells are the primary regulators of epithelial-cell homeostasis after repeated acrolein exposure. These findings are supported by the results of microarray analyses indicating that the two T-cell subpopulations have distinct gene-expression profiles after acrolein exposure. These data provide strong evidence that the T-cell subpopulations in the lung are major determinants of the response to pulmonary toxicant exposure and suggest that it is advantageous to elucidate the effector functions of these cells in the modulation of lung pathophysiology.

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#### INTRODUCTION

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Exposure to acrolein in the ambient air in urban environments represents a considerable hazard to human health. The U.S. Environmental Protection Agency estimates that ambient concentrations of acrolein exceeded the reference concentration in > 90% of measurements in the United States in 1999 and were more than 10 times the reference concentration in > 105 measurements taken that year (Woodruff et al. 2007). On the basis of its health effects and concentrations in the environment (Caldwell et al. 1998), acrolein is proposed to be the non-cancer-causing hazardous air pollutant of greatest concern. Most recently, Woodruff and colleagues (2007) estimated that ambient concentrations of acrolein represent an important health risk, associated with decreased respiratory function, in the United States.

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This Investigators' Report is one part of Health Effects Institute Research Report 146, which also includes a Critique by the Health Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Michael T. Borchers, Ph.D., Department of Environmental Health, Division of Environmental Genetics, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0056.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award CR-83234701 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

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\*A list of abbreviations and other terms appears at the end of the Investigators' Report.

Acrolein ( $\text{CH}_2=\text{CHCHO}$ ) is a highly reactive, low-molecular-weight, unsaturated aldehyde known to cause pulmonary inflammation and respiratory tract injury and to suppress the pulmonary host defense against infection. Acrolein is both an indoor and outdoor air pollutant formed primarily by the incomplete combustion of automotive fuels, wood, tobacco, and cooking oil. Acrolein is found in high concentrations in smoke from wood fires ( $> 5.0$  ppm), industrial emissions (0.2 ppm), automobile exhaust (0.2 ppm) (Swarin and Lipari 1983), and tobacco smoke ( $> 50$  ppm) (Ayer and Yeager 1982). Lower levels of acrolein (approximately 0.005 ppm) are commonly found in ambient air; however, ambient levels of total aldehydes have reached 0.3 ppm (Marnett 1988).

Acrolein's effects on the respiratory tract involve injury to airway epithelial cells and alveolar macrophages. Experiments involving laboratory animals have provided the majority of toxicity data describing the acute and chronic effects of acrolein. The acute exposure of animals to acrolein primarily affects respiratory function by reducing the mucociliary transport rate (Kensler and Battista 1963); depressing the respiratory rate in mice, at acrolein levels  $< 1.0$  ppm (Steinhagen and Barrow 1984), and in rats, at levels  $< 3.0$  ppm (Babiuk et al. 1985); depleting epithelial glutathione, at levels  $> 0.5$  ppm (Lam et al. 1985); and inducing bronchial hyperresponsiveness and airway inflammation in guinea pigs, at levels  $\leq 1.0$  ppm (Leikauf et al. 1989).

Repeated exposure to  $\geq 1.4$  ppm acrolein causes several changes in lung structure and function that are characteristic of obstructive airway diseases. In addition, Lyon and colleagues (1970) found that repeated exposure to  $> 0.22$  ppm acrolein resulted in chronic inflammation of the bronchi and bronchioles; these effects occurred at lower doses in dogs and monkeys than in rodents, suggesting decreased sensitivity among obligate nasal-breathing species.

Feron and colleagues (1978) further characterized the effects of repeated acrolein exposure in rodents to include increases in the number of mucous cells and in the accumulation of alveolar macrophages in the airways. Borchers and colleagues (1998) have shown that mucous-cell metaplasia, accompanied by increased mucin gene expression, occurs at acrolein doses as low as 0.75 ppm in the rat and induces mucin gene expression in the mouse in a process that is partly dependent on recruitment of macrophages to the lung (Borchers et al. 1999b).

Effects of acrolein exposure have also been described in vitro in studies using cultures of airway epithelial cells and alveolar macrophages. Acrolein stimulates the release of eicosanoids from alveolar macrophages (Grundfest et al. 1982) and cultured tracheal epithelium (Doupnik and Leikauf 1990) and depletes glutathione in tracheobronchial

epithelium (Grafström et al. 1988). Additional effects of acrolein exposure include DNA-protein cross-linking (Grafström 1990), inhibition of DNA repair (Feng et al. 2006), inhibition of the release of cytokines (tumor necrosis factor  $\alpha$ , interleukin [IL]-1 $\beta$ , and IL-12) from alveolar macrophages (Li et al. 1997), and induction of expression of the mucin 5AC gene, *MUC5AC*, in airway epithelial cells (Borchers et al. 1999a).

Although it is well established that acrolein induces acute and chronic inflammation of the airways and causes epithelial damage and remodeling, the mechanisms regulating these effects are unknown. The current paradigm is that air toxics damage the epithelium lining the airways, which generates signals capable of recruiting and activating inflammatory cells (neutrophils, macrophages, and lymphocytes) that exacerbate epithelial injury. Numerous studies of several air toxics support this hypothesis, and the bulk of the data detail the functions of neutrophils and macrophages in these processes.

However, the function of effector T cells in response to acrolein exposure remains unexamined. The reasons are probably both historical and technological. T cells are traditionally viewed as mediating acquired immunity in response to pathogens. In addition, they are not an abundant cell type in the airways, nor are they easily isolated and studied. At present, the T-cell subpopulations and corresponding mechanisms directly involved in the modulation of pulmonary responses to toxicant exposure are unknown.

T cells are classified primarily on the basis of the expression of T-cell receptors (TCRs) (either  $\alpha\beta$  or  $\gamma\delta$ ) and coreceptors (either CD4 or CD8). In  $\alpha\beta$  T cells, the TCR is a heterodimer of an alpha chain and a beta chain. Each of the two chains has a variable region and a constant region. The variable regions each contain three hyper-variable regions that make up the antigen-binding site. The TCR binds a bimolecular complex displayed at the surface of an antigen-presenting cell. This complex consists of a fragment of an antigen lying within the groove of the major histocompatibility complex (MHC) molecule.

The  $\gamma\delta$  T cells differ from  $\alpha\beta$  T cells in several ways. Different gene segments encode the TCR of  $\gamma\delta$  T cells, and the  $\gamma\delta$  TCR can bind to traditional antigens as well as a variety of other types of organic molecules (often containing phosphorus atoms) not presented by MHC class I or class II molecules. The  $\gamma\delta$  T cells are found predominantly at mucosal epithelial sites, and they have the capacity to encounter antigens on the surface of surrounding cells, rather than relying on the antigen-presenting cells found in lymph nodes. Although the majority of T cells in the body are  $\alpha\beta^+$  or CD4 $^+$  or both, the majority of T cells in epithelial tissues are  $\gamma\delta^+$  or CD8 $^+$  or both.

Epithelial T cells are intriguing in the context of toxicant exposure for several reasons, in addition to the functional implications of their localization to epithelial surfaces. These T-cell subpopulations are unique in their ability to recognize non-peptide, MHC class I-related antigens expressed on transformed, stressed, or injured epithelial cells (Hayday et al. 2001). They also possess the ability to modulate epithelial-cell growth (Boismenu and Havran 1994) and express epithelial growth factors and an array of cytokines and chemokines that recruit or activate lymphocytes and macrophages (Fahrer et al. 2001; Shires et al. 2001).

Dziedzic and White (1987) first demonstrated a regulatory role for T cells in response to an air toxicant other than acrolein: ozone. Repeated exposure to ozone resulted in greater damage to the epithelium and higher inflammatory-lesion volumes in athymic mice than in intact control mice. Similar results were observed in mice treated with the immunosuppressive drug cyclosporine (Bleavins et al. 1995). Notably, the study by Bleavins and colleagues reported a 50-fold increase in neutrophil levels in the lungs of ozone-exposed, cyclosporine-treated mice. Although these studies were intriguing, the models of T-cell depletion were not specific.

Subsequently, CD4<sup>+</sup> T cells were shown to regulate inflammation after ozone exposure through a mechanism involving IL-4 production (Chen et al. 1995). Interestingly, among mice depleted of CD4<sup>+</sup> T cells by means of a monoclonal antibody, C57BL/6J mice were susceptible, and C3H/HeJ mice were resistant, to ozone effects, indicating genetic variability in the immunomodulation of pulmonary responses to toxicant exposure (Chen et al. 1995). More recently, studies using mice deficient in  $\gamma\delta$  T cells have revealed distinct roles for this cell type, as opposed to  $\alpha\beta$  T cells, in the regulation of acute pulmonary inflammation and the maintenance of epithelial integrity (King et al. 1999). Specifically, in mice deficient in  $\gamma\delta$  T cells, ozone exposure resulted in increased epithelial necrosis as compared with that in wild-type mice (King et al. 1999). On the basis of these studies, it is becoming increasingly apparent that lymphocytes are capable of responding to epithelial injury and stress in the absence of pathogenic stimuli.

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## SPECIFIC AIMS

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The purpose of this study was to examine how cells of the innate and adaptive immune systems modulate pulmonary responses to acute and repeated acrolein exposures. Few data exist regarding the role of pulmonary T cells (resident or recruited) in the initiation and perpetuation of inflammatory responses after irritant exposure. T cells

have an important role in acute inflammation and the maintenance of epithelial integrity after nonpathogenic insults (Dziedzic and White 1987; Chen et al. 1995; King et al. 1999). However, the mechanisms whereby T cells and their specific effector functions are activated in response to irritant exposure have received only limited attention. Clarifying the role of intraepithelial T cells expressing the  $\gamma\delta$  TCR is necessary because these cells are linked to the homeostasis of the lung microenvironment, localize to the epithelium (Augustin and Sim 1990), and secrete pro-inflammatory and chemotactic cytokines as well as factors that promote epithelial-cell growth and repair (Hayday et al. 2001).

The recruitment and activation of inflammatory cells (e.g., macrophages, neutrophils, and non-resident T cells), as well as the modulation of epithelial-cell integrity and morphology, by resident T cells after acrolein exposure represent an immune response serving a protective function against pulmonary injury. However, repeated activation of these T cells may culminate in lung pathology, as has been postulated to occur in the development of some features of chronic obstructive pulmonary disease (COPD) (Cosio et al. 2002). Furthermore, genetic factors contribute to individual susceptibility to the effects of air toxics (Kleeberger et al. 1993).

Using a combination of cell-sorting techniques that can produce 99%-pure cell populations and microarray technology that allows for the simultaneous analysis of > 30,000 genes, we are able to assess the effects of environmental exposure on T-cell function and their impact on the pathogenesis of and susceptibility to chronic pulmonary diseases. The long-term goals of our studies are to examine how this pathogenesis and susceptibility may contribute to the development or exacerbation of pulmonary diseases such as COPD and asthma.

In this study, we used a mouse model of pulmonary pathology induced by repeated acrolein exposure to examine whether pulmonary T-cell subpopulations differentially regulate inflammatory-cell accumulation and epithelial-cell pathology in response to acrolein exposure. The two major aims of the study were to (1) determine the role of  $\alpha\beta$  and  $\gamma\delta$  pulmonary T cells in inflammation and epithelial injury, and (2) examine the gene-expression profiles of purified pulmonary T-cell subpopulations, after repeated acrolein exposure. To address the first aim, we used transgenic mice genetically deficient in either  $\alpha\beta$  T cells or  $\gamma\delta$  T cells and measured changes in several cellular, molecular, and pathologic outcomes associated with repeated inhalation exposure to 2.0 ppm acrolein. To address the second aim and to examine the potential functions of the T-cell subpopulations, we purified T cells from the lung tissue of

mice repeatedly exposed to 2.0 ppm acrolein, isolated and amplified the mRNA transcripts, and performed oligonucleotide microarray analysis.

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### METHODS

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#### MICE

We used mice in these studies for several reasons. These include the similarity of the mouse and human genomes, the ease of designing genetically modified mice, and the availability of such mice and the reagents and probes to study the proposed mediators in mice. Wild-type mice (C57BL/6J), mice deficient in  $\gamma\delta$  T cells (B6.129P2-*Tcrd*<sup>tm1Mom</sup>/J), and mice deficient in  $\alpha\beta$  T cells (CBy.129P2(B6)-*Tcrb*<sup>tm1Mom</sup>/SzJ) were purchased from the Jackson Laboratory (Bar Harbor, ME) and have C57BL/6J as the background strain. *Tcrd*<sup>tm1Mom</sup> and *Tcrb*<sup>tm1Mom</sup> mice have been backcrossed for > 10 generations with C57BL/6J mice.

All procedures were conducted using female mice 8–12 weeks of age maintained in ventilated micro-isolator cages housed in an animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The protocols and studies involving animals were conducted in accordance with National Institutes of Health guidelines.

#### ACROLEIN EXPOSURE

Mice were exposed to either 2.0 or 0.5 ppm of acrolein; controls were sham-exposed to filtered air. Exposures were performed over 4 weeks, for 6 hours per day, 5 days per week, as previously described (Borchers 1999b). Acrolein vapor was generated by passing N<sub>2</sub> (at a flow rate of 3–15 mL per minute) over a 3-mL reservoir of liquid acrolein (Sigma-Aldrich, St. Louis, MO). This mixture was diluted (at a rate of 400 mL per minute) with air purified through a high-efficiency particulate air filter and introduced into a 0.32-m<sup>3</sup> stainless steel chamber. The exposure concentration was analyzed using a method described by Cohen and Altshuller (1961). The chamber atmosphere was sampled with a series of two glass-fitted impingers, each containing 10 mL of 96% ethanol. A fraction of each sample was mixed with 50 mM hexylresorcinol (Sigma, St. Louis, MO), 2.1 mM mercury chloride (Aldrich, Milwaukee, WI), and 29.7 M trichloroacetic acid (Fisher, Fair Lawn, NJ). Samples and known standards in equal volumes were heated (at 65°C for 15 min) and allowed to cool (at 22°C for 15 min), and the absorbance at 605 nm was measured with a spectrophotometer (DU-64; Beckman, Fullerton, CA). Exposure was measured twice each day. Fluctuations over the daily

six-hour exposure periods did not exceed 10%, and the average exposure concentrations were within 5% of the target concentrations.

#### BRONCHOALVEOLAR LAVAGE

Immediately after exposure, mice were anesthetized through intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight of Nembutal; Abbott Laboratories, Chicago, IL) and were killed by severing the posterior abdominal aorta. The lungs were then lavaged two times with 1 mL of Hanks balanced salt solution (HBSS). Individual bronchoalveolar lavage (BAL) samples were pooled and centrifuged at 1000 × *g* for 10 minutes. The supernatant was removed and stored at –70°C until assayed for mucin activity. The cell pellet was reconstituted in 1 mL HBSS containing 2% fetal calf serum (Gibco BRL, Carlsbad, CA).

#### CELL AND DIFFERENTIAL COUNTS

Total cell counts were determined with a hemocytometer. Differential cell counts were obtained from cytocentrifuge slides (Cytospin3, Shandon Scientific) with ≥ 300 cells per slide, prepared from 250  $\mu$ L of BAL fluid and stained with Diff-Quik (Baxter Diagnostics, McGaw Park, IL). Data are presented as the total number of cells after adjustment for the volume of fluid recovered.

#### TISSUE FIXATION, HISTOLOGY, AND IMMUNOHISTOCHEMISTRY

To obtain tissue for histologic analysis after the mice were killed, a cannula was inserted in the middle of the trachea, and the lung was instilled with 10% phosphate-buffered formalin (at 20 cm H<sub>2</sub>O for 1 min). The trachea was ligated, and the inflated lung was immersed in fixative for 24 hours. The fixed tissues were then dissected, washed with phosphate-buffered saline (PBS), dehydrated through graded ethanol solutions (30%–70%), and processed into paraffin blocks (with a Hypercenter XP tissue processor; Shandon, Pittsburgh, PA).

Activated caspase 3 protein (a marker of apoptosis) was detected with a rabbit polyclonal antibody (R&D Systems, Minneapolis, MN) in paraffin sections (5  $\mu$ m thick) of mouse airway tissue, as previously described (Borchers et al. 2007). Antigen–antibody complexes were detected with a goat IgG kit (Vectastain ABC Peroxidase Elite; Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was quenched by placing the sections in 3% H<sub>2</sub>O<sub>2</sub> in methanol (for 15 min at 22°C). They were incubated in 2% normal goat serum in PBS with 0.2% Triton X-100 blocking solution for 2 hr at 22°C and incubated for 20 hr at 4°C with primary antibody (1:15,000 dilution). The sections were washed (six times, for 5 min each time) in PBS with

0.2% Triton X-100 and incubated (for 30 min at 22°C) with biotinylated goat anti-chicken antibody (1:2000 dilution). The sections were treated (for 15 min at 22°C) with the avidin–biotin blocking kit (Vector Laboratories) to block endogenous biotin in the tissue. They were then incubated with the avidin–biotin–peroxidase complex diluted in blocking solution (for 30 min at 22°C), with nickel diaminobenzidine in 0.1 M acetate buffer (for 4 min at 22°C) for development of a colored reaction product, and with Tris–cobalt (for 4 min at 22°C) and counterstained with 0.1% nuclear fast red (for 2 min at 22°C).

#### QUANTIFICATION OF MUCOUS-CELL METAPLASIA

Mucous-cell development along the airway epithelium was quantified in paraffin-embedded tissue sections (5  $\mu$ m thick) stained with periodic acid–Schiff reagent. Parasagittal sections were analyzed by bright-field microscopy with an image-analysis software program (ImagePro Plus; Media Cybernetics, Silver Spring, MD) to derive an airway mucus index that reflects both the amount of mucus per airway and the number of airways affected. The airway mucus index was calculated by dividing the ratio of the periodic acid–Schiff–positive epithelial area to the total epithelial area per section by the number of airways in the section (Borchers et al. 2001).

#### BAL MUCIN ASSAY

The amount of Muc5ac mucin in the airways was determined using an enzyme-linked immunosorbent assay (ELISA) modified following Miller and colleagues (2002). Briefly, 100  $\mu$ L of BAL sample was distributed onto a 96-well microtiter plate (Immulon, PGC Scientific), dried overnight at 37°C, washed and incubated with a biotinylated primary anti-MUC5AC antibody (LabVision, Fremont, CA), and finally, incubated with a secondary peroxidase-conjugated antibody. A dilution curve was generated using BAL fluid from rats exposed to 2.0 ppm acrolein, 6 hr per day for 10 days.

#### LUNG-CELL ISOLATION AND FLOW CYTOMETRY

Lungs were perfused with 10 mL PBS containing 0.6 mM EDTA. Lungs were removed and diced into pieces < 300  $\mu$ L in volume. Four milliliters of HBSS containing 175 U per milliliter collagenase (Sigma), 10% fetal calf serum (Gibco BRL), 100 U per milliliter penicillin, and 100  $\mu$ g per milliliter streptomycin were added to the tissue and incubated for 30 min at 37°C in an orbital shaker. The digested lung tissue was sheared with a 20-gauge needle and forced through 40- $\mu$ m filters. Cells were washed three times and resuspended in HBSS before being counted with a hemocytometer.

#### T-CELL PURIFICATION

Briefly, single-cell suspensions from perfused, digested lung tissue (as described above) were layered onto a single-step Percoll gradient (60% Percoll [ $\rho = 1.084$ ],  $1 \times$  HBSS, 15 mM HEPES [pH 7.4]) and centrifuged (for 45 min at  $2000 \times g$  at 4°C). The buffy coat containing mononuclear cells was removed and washed twice in PBS containing 2% fetal calf serum. The cell pellet was then subjected to lysis of red blood cells, washed, and resuspended in PBS containing 2% fetal calf serum. Cell sorting with a flow cytometer (FACSVantage, Becton Dickinson, Franklin Lakes, NJ) was subsequently used to isolate T cells.

To isolate subpopulations of T cells, the cells were labeled with antibodies against CD3 (clone 145-2C11; Pharmingen, San Diego, CA), TCR- $\beta$  (clone H57-597, Pharmingen), and TCR- $\delta$  (clone GL3, Pharmingen). Cells positive for both CD3 and TCR- $\beta$  were isolated into a separate tube from the cells that were positive for both CD3 and TCR- $\delta$ . The purity of the isolated cells was determined by flow cytometry performed on a cytofluorometer (FACScan; Becton Dickinson). Data acquisition and analysis were performed using CellQuest software (Becton Dickinson).

#### RNA ISOLATION AND AMPLIFICATION

Total cellular RNA from purified lymphocyte populations was isolated with a column-based procedure using the RNEasy Micro kit (Qiagen, Valencia, CA) to obtain a maximum yield from a very small amount of starting material. To obtain enough RNA for microarray analysis, we performed two rounds of linear amplification of the total RNA using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion, Austin, TX).

In summary, first-strand complementary DNA (cDNA) was synthesized using a reverse-transcriptase enzyme and the T7-oligo(dT) primer to generate cDNA with the T7 promoter sequence attached. DNA polymerase and RNase H were added to synthesize second-strand cDNA and degrade RNA. A cDNA purification procedure involving a column-based technique was used to remove RNA, salts, primers, and enzymes.

The templates were amplified in the first round by *in vitro* transcription using the T7 polymerase and unlabeled primers. After another round of purification, the *in vitro* transcription was performed as in the first round, except amino allyl uridine triphosphate was substituted for the unlabeled uridine triphosphate, thus allowing for subsequent conjugation of cyanine 3 (Cy3) and cyanine 5 (Cy5). Finally, the amplified RNA was again purified on a column and tagged with the appropriate fluorescent dye, as detailed below.

### EXPERIMENTAL DESIGN OF MICROARRAYS

T-cell subpopulations were purified from lung tissue on six separate occasions. On each occasion,  $\gamma\delta$  T-cell samples were obtained from four controls and four acrolein-exposed mice, and  $\alpha\beta$  T-cell samples were also obtained from four controls and four acrolein-exposed mice. T cells were pooled on each occasion and represent a single RNA sample. Ten arrays (four for  $\gamma\delta$  T cells and six for  $\alpha\beta$  T cells) were run and analyzed as described in the next section. Each array contained 10  $\mu\text{g}$  of amplified RNA from controls and from acrolein-exposed mice.

### MICROARRAY HYBRIDIZATION

Microarray analysis was conducted in the Genomics and Microarray Core Facility in the Center for Environmental Genetics at the University of Cincinnati, housed in our Department of Environmental Health. A detailed protocol for the procedure has been published by Wesselkamper and colleagues (2005). Briefly, RNA quality was assessed and quantified by analysis with an Agilent Bioanalyzer (Quantum Analytics, Foster City, CA). To examine the differential gene expression of 31,775 70mer oligonucleotides, a microarray was fabricated at the Genomic and Microarray Laboratory, using a commercial library of Qiagen–Operon (Alameda, CA).

Clones (70mers) from the Operon Library were amplified by polymerase chain reaction (PCR) and printed onto glass slides (by the Omnigridd Microarrayer; GeneMachines, San Carlos, CA). Each sample from control or exposed mice was randomly, reciprocally tagged with fluorescent Cy3 or Cy5. Cy3 and Cy5 samples were co-hybridized with the printed 70mers. After hybridization, the slides were washed and scanned at 635 nm (Cy5-tagged samples) and 532 nm (Cy3-tagged samples) (with a GenePix 4000B scanner; Axon Instruments, Union City, CA). Imaging and data generation were carried out using scanners (GenePix 4000A and 4000B) and associated software (GenePix Pro version 5.0 software; Axon Instruments, Foster City, CA). The microarray slides were scanned with dual lasers with wavelength frequencies serving to excite Cy3 and Cy5 to emit fluorescence. Images were captured in JPEG and TIFF files, and DNA spots were captured by the adaptive circle segmentation method.

### MICROARRAY ANALYSIS

The data generated by the software to represent the spot intensities were analyzed, after subtraction of the background intensity, to identify differentially expressed genes. Data normalization was performed in two steps for each microarray separately. First, the background-adjusted intensities were  $\log_2$ -transformed and the differences (R) and

averages (A) of the transformed values were calculated as  $R = \log_2(X1) - \log_2(X2)$  and  $A = [\log_2(X1) + \log_2(X2)]/2$ , respectively, where X1 and X2 denote the Cy5 and Cy3 intensities, respectively, after subtraction of the local background values. Second, normalization was performed by fitting the array-specific local regression model of R as a function of A. Normalized  $\log_2$  intensities of the Cy5 and Cy3 fluorescences were then calculated by adding half of the normalized ratio of the two intensities to A for the Cy5 fluorescence and subtracting half of the normalized ratio from A for the Cy3 fluorescence.

Statistical analyses were performed for each T-cell subpopulation independently, by fitting the following mixed-effects linear model for each gene:  $Y_{ijk} = \mu + A_i + S_j + C_k + \varepsilon_{ijk}$ , where  $Y_{ijk}$  corresponds to the normalized  $\log_2$  intensity on the  $i$ th array labeled with the  $k$ th dye (with  $k = 1$  for Cy5 and  $k = 2$  for Cy3) and for the  $j$ th treatment;  $\mu$  is the overall mean  $\log_2$  intensity;  $A_i$  is the effect of the  $i$ th array;  $S_j$  is the effect of the  $j$ th treatment (with  $j = 1$  for control mice and  $j = 2$  for exposed mice);  $C_k$  is the effect of the  $k$ th dye; and  $\varepsilon_{ijk}$  is the random experimental error associated with  $Y_{ijk}$ . Effects of the arrays were treated as random effects, whereas treatment and dye effects were treated as fixed effects (Wolfinger et al. 2001). The resulting  $t$  statistics for estimating differences between exposed and control mice were modified using an empirical Bayesian moderated- $t$  method (Smyth 2004).

Estimates of the change in expression in acrolein-exposed cells as compared with control cells were calculated (as the “fold change”). Genes were considered to be differentially expressed if all three of the following criteria were met: the  $P$  value for the increase in expression was  $< 0.005$ , the fold change in expression was  $> 2.0$ , and the average intensity (an arbitrary, unitless value) was  $> 50$  (659 transcripts in  $\alpha\beta$  T cells and 626 transcripts in  $\gamma\delta$  T cells). For exposed-versus-control comparisons, the  $P < 0.005$  cutoff corresponded to an estimated false discovery rate (Benjamini and Hochberg 1995) of  $< 0.09$  in  $\gamma\delta$  T-cell subpopulations and  $< 0.11$  in  $\alpha\beta$  T-cell subpopulations. From the 1093 transcripts identified as differentially expressed in at least one of the two T-cell subpopulations, expression of a transcript was defined as having changed in both types of cells if both  $P$  values were  $< 0.05$ . Data normalization and statistical analyses were performed using SAS statistical software package (version 8.2; SAS Institute, Cary, NC).

To further analyze the microarray data set, we used the MAPPFinder program to dynamically link microarray data to the Gene Ontology hierarchy database (Salomonis et al. 2007). Genes exhibiting significant differences in the microarray analysis ( $P < 0.005$ ) were used for the input. MAPPFinder generated a gene-expression profile from this

input and categorized the data into more than 5000 classes of biologic processes, cellular components, and molecular functions that allowed for the identification of specific biologic pathways. The results, calculated using the Fisher's exact test, are expressed as a z-score with significance estimated by a permuted  $P$  value for a particular pathway, and permuted  $P$  values of  $< 0.005$  were considered to be significant.

We supplemented this analysis with a complementary approach using the PANTHERDB program (Applied Biosystems). Data complied with the minimum information about a microarray experiment (MIAME) standards and were deposited in the ArrayExpress database (accession number E-MEXP-1354).

### QUANTITATIVE REAL-TIME PCR

To obtain RNA for the validation of genes identified by microarray analysis, additional samples of  $\gamma\delta$  and  $\alpha\beta$  T-cell subpopulations were purified from lung tissue on separate occasions as described above, from three groups of four control mice and three groups of four acrolein-exposed mice for each type of T cell. The T cells from the four mice were pooled on each occasion and represent a single RNA sample. Total RNA was isolated from lung lymphocytes, and quantitative real-time PCR (qRT-PCR) was performed, using an ABI 7600 System and pre-validated TaqMan Gene Expression Assays according to the manufacturer's protocols (Applied Biosystems). Data are expressed as the fold change over the control value, as calculated by the  $2^{-\Delta\Delta CT}$  method (RQ Software, version 1.4, Applied Biosystems).

### ELISA

Protein levels in BAL fluid were determined by ELISA, according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). The limit of detection for this assay is typically 5–10 pg per milliliter.

### STATISTICAL ANALYSIS

Normally distributed data (excluding data from the microarrays) were analyzed for statistical significance by using one-way analysis of variance. Differences between means were considered significant if the  $P$  value was  $< 0.05$ .

## RESULTS

### ACROLEIN-INDUCED PULMONARY PATHOLOGY

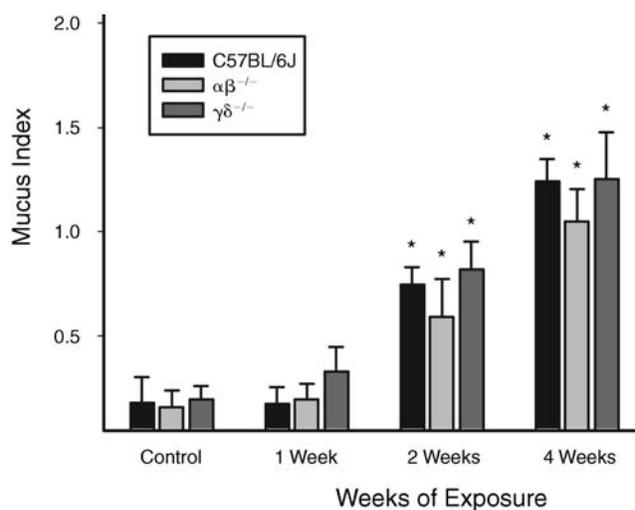
Mucous-cell development was restricted to the largest airways, and there were no differences observed among

the strains (Figures 1 and 2). These histologic findings are consistent with the results obtained from analysis of mucin glycoprotein in the BAL fluid. To determine whether marked hypersecretion occurs in response to acrolein exposure, we developed an ELISA-based method to assay Muc5ac mucin glycoprotein in BAL fluid from mice (as described in Methods). None of the BAL-fluid samples were positive for the Muc5ac mucin glycoprotein at any time point (data not shown).

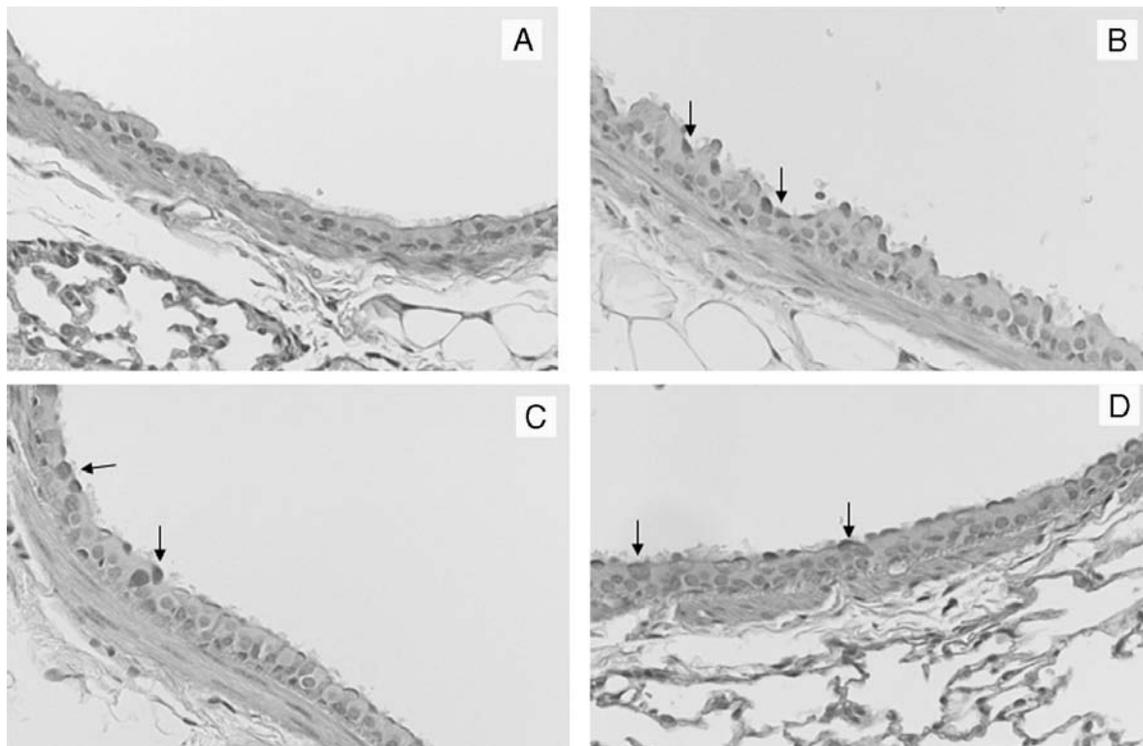
### INJURY TO BAL EPITHELIAL CELLS

Acrolein exposure caused a significant increase in epithelial-cell sloughing in the C57BL/6J mice and mice deficient in  $\gamma\delta$  T cells but not mice deficient in  $\alpha\beta$  T cells (Figure 3). Exposure to 2.0 ppm acrolein resulted in an approximately twofold increase in the number of ciliated epithelial cells recovered in the BAL fluid of C57BL/6J mice. This effect was observed after 1 week of exposure and persisted through 4 weeks of exposure.

The mice deficient in  $\gamma\delta$  T cells had a significantly higher level of epithelial cells in the BAL fluid than C57BL/6J mice at all time points examined. Exposure to 2.0 ppm acrolein led to an increase of more than eightfold in the number of ciliated epithelial cells recovered from the BAL fluid from mice deficient in  $\gamma\delta$  T cells after 1 week of



**Figure 1. Quantitation of mucous-cell metaplasia after repeated acrolein exposure.** No significant differences were detected among morphometric estimates of mucous-cell metaplasia in C57BL/6J mice and mice deficient in  $\gamma\delta$  T cells ( $\gamma\delta^{-/-}$ ) or  $\alpha\beta$  T cells ( $\alpha\beta^{-/-}$ ). The airway mucus index was calculated by dividing the ratio of the periodic acid–Schiff–positive epithelial area to the total epithelial area per section by the number of airways in the section. Values are the means  $\pm$  SEM of two independent, blinded assessments of data for eight mice per group. \* Significantly different from the mean strain-matched control value ( $P < 0.05$ ).



**Figure 2. Mucous-cell metaplasia (arrows) in the airways of mice after repeated acrolein exposure.** A sample from a C57BL/6J control mouse is shown (A) for comparison with samples obtained 4 weeks after acrolein exposure from a C57BL/6J mouse (B), a mouse deficient in  $\gamma\delta$  T cells (C), and a mouse deficient in  $\alpha\beta$  T cells (D). Each image is representative of the eight mice per group. Mucous-cell metaplasia was visualized by light microscopy of lung sections stained with periodic acid–Schiff, which stains epithelial mucosubstances (appearing as dark gray here).

exposure. The response in the mice deficient in  $\gamma\delta$  T cells was greatest after 1 week of exposure and had declined slightly by 2 and 4 weeks of exposure but was still significantly greater than that in C57BL/6J mice. In contrast, mice deficient in  $\alpha\beta$  T cells did not exhibit a significant increase in epithelial-cell sloughing after any of the exposure periods.

In response to 0.5 ppm acrolein, significant sloughing of epithelial cells in the BAL fluid was observed only in the mice deficient in  $\gamma\delta$  T cells (Figure 4), for which there were significantly more recovered epithelial cells than among control mice deficient in  $\gamma\delta$  T cells or among C57BL/6J mice exposed to 0.5 ppm acrolein, at all time points examined.

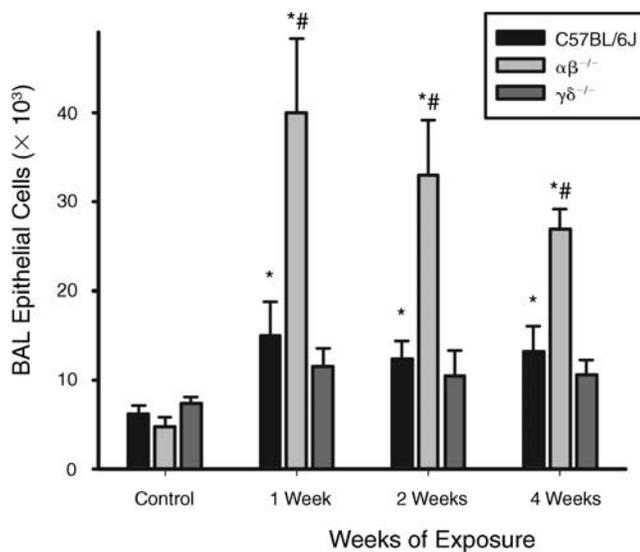
#### APOPTOSIS OF PULMONARY EPITHELIAL CELLS

Immunohistochemistry was performed on tissue sections to complement the BAL assessment of the magnitude of epithelial-cell damage in the airways. We used an antibody against active caspase 3, a marker of apoptosis, to determine the extent and localization of cellular injury.

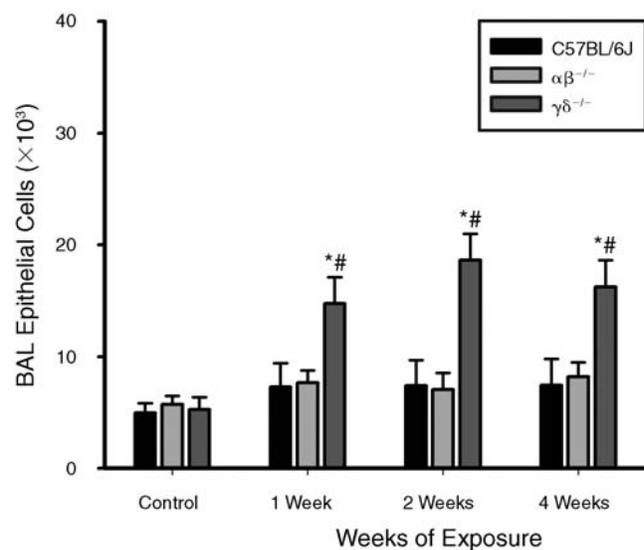
Our results demonstrate that apoptosis occurs in the terminal bronchioles and alveolar epithelium in response to exposure to 2.0 ppm acrolein (Figure 5). Consistent with the data on BAL epithelial cells, acrolein exposure caused a significant increase in apoptosis in the C57BL/6J mice and mice deficient in  $\gamma\delta$  T cells but not mice deficient in  $\alpha\beta$  T cells (Figure 6). Furthermore, mice deficient in  $\gamma\delta$  T cells had a significantly higher level of caspase 3–positive cells, and these cells increased significantly more rapidly, than in C57BL/6J mice (Figure 6).

#### PULMONARY INFLAMMATION

Exposure to 2.0 ppm acrolein resulted in a persistent increase in macrophage accumulation in the lungs of C57BL/6J mice (Figure 7). Mice deficient in  $\gamma\delta$  T cells exhibited an increase in macrophage accumulation after 2 and 4 weeks of exposure to 2.0 ppm of acrolein that was significantly lower than that in C57BL/6J mice. In contrast, mice deficient in  $\alpha\beta$  T cells did not exhibit an increase in macrophage accumulation after any of the exposure periods. In response to 0.5 ppm acrolein, C57BL/6J mice



**Figure 3. Injury to airway epithelial cells in mice after exposure to 2.0 ppm acrolein.** The number of epithelial cells (primarily ciliated) recovered in BAL fluid from C57BL/6J mice increased after exposure to 2.0 ppm acrolein. This response was attenuated in  $\alpha\beta^{-/-}$  mice but was significantly augmented in  $\gamma\delta^{-/-}$  mice at all time points. The number of epithelial cells was determined by estimating the percentage of the total number of cells recovered that were epithelial cells, by assessing Wright-stained cytocentrifuge slides with  $\geq 300$  cells per slide. Values are the means ( $\pm$  SEM) of eight mice per group. \*Significantly greater than the mean strain-matched control value ( $P < 0.05$ ). #Significantly greater than the mean exposure-matched C57BL/6J value ( $P < 0.05$ ).



**Figure 4. Injury to airway epithelial cells in mice after exposure to 0.5 ppm acrolein.** The number of epithelial cells (primarily ciliated) recovered in BAL fluid from  $\gamma\delta^{-/-}$  mice increased after exposure to 0.5 ppm acrolein. This response was not observed in C57BL/6J mice or  $\alpha\beta^{-/-}$  mice. The number of epithelial cells was determined by estimating the percentage of the total number of cells recovered that were epithelial cells, by assessing Wright-stained cytocentrifuge slides with  $\geq 300$  cells per slide. Values are the means ( $\pm$  SEM) of five mice per group. \*Significantly greater than the mean strain-matched control value ( $P < 0.05$ ). #Significantly greater than the mean exposure-matched C57BL/6J value ( $P < 0.05$ ).

exhibited a significant increase in macrophage accumulation after 2 and 4 weeks of exposure (Figure 8). This response was attenuated as compared with the response to 2.0 ppm acrolein; the increase in macrophage accumulation was only 50%–60% greater than among controls as compared with the 200%–250% increase in response to 2.0 ppm acrolein at the same time points. Nearly identical results were observed in mice deficient in  $\gamma\delta$  T cells. No significant changes in macrophage accumulation were observed in mice deficient in  $\alpha\beta$  T cells at any time points examined.

Neutrophils were the only other type of cell consistently found in the BAL fluid of acrolein-exposed mice. However, the numbers of neutrophils were negligible ( $< 2\%$ ) as compared with the numbers of macrophages at all time points and doses in all three strains. BAL fluid contained low numbers of lymphocytes (making up  $< 0.5\%$  of samples).

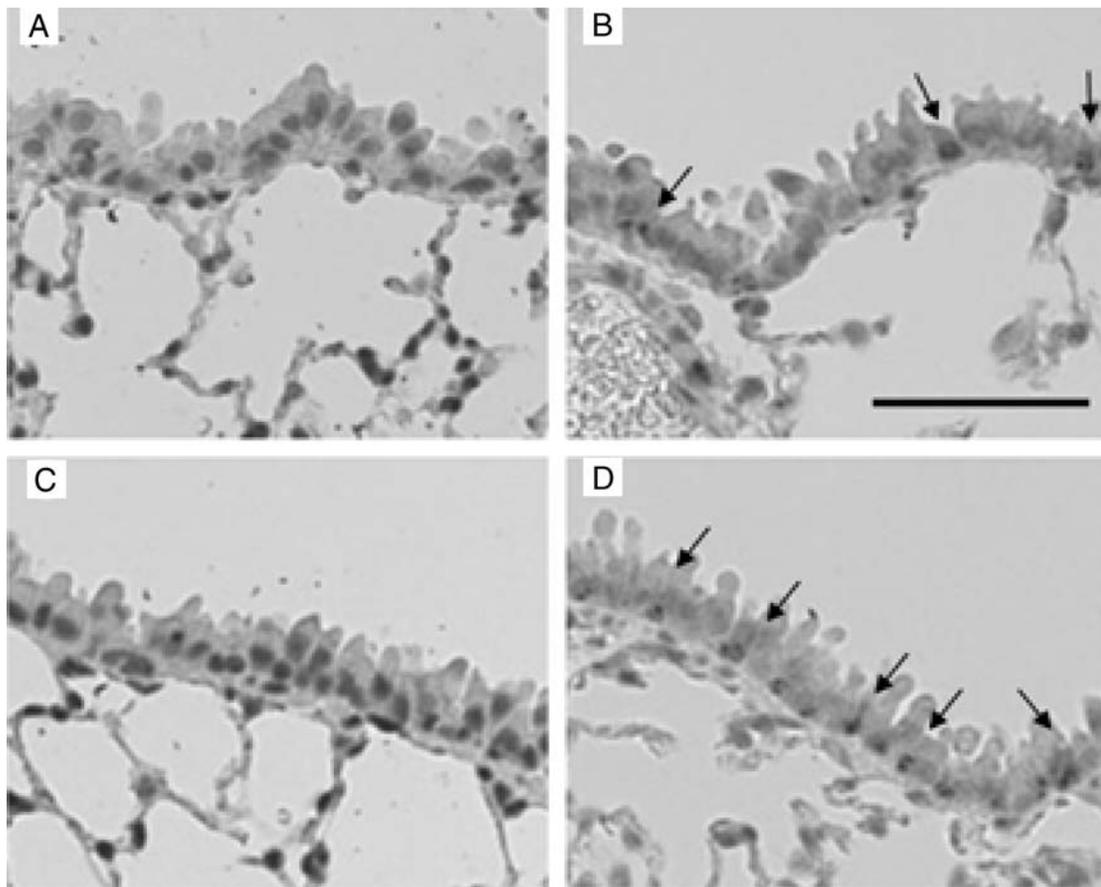
#### LEVELS OF MACROPHAGE MEDIATORS IN THE LUNG

We measured two mediators of alveolar macrophage function and activation, interferon (IFN)- $\gamma$  and granulocyte-macrophage colony-stimulating factor (GM-CSF), in the

lungs of mice exposed to 2.0 ppm acrolein. IFN- $\gamma$  expression in the lungs of control mice was significantly increased after 2 weeks of acrolein exposure but not after 1 week or 4 weeks (Figure 9). A similar response was found in mice deficient in  $\gamma\delta$  T cells. The mice deficient in  $\alpha\beta$  T cells exhibited a different profile of IFN- $\gamma$  expression, expressing less IFN- $\gamma$  at baseline than C57BL/6J mice. Similarly, mice deficient in  $\alpha\beta$  T cells expressed less IFN- $\gamma$  at 1 week and 4 weeks than C57BL/6J mice, although IFN- $\gamma$  expression did increase after 2 weeks of acrolein exposure. An identical expression pattern to that of IFN- $\gamma$  was measured for GM-CSF in response to exposure to 2.0 ppm acrolein, in all strains of mice examined (Figure 10).

#### PURIFICATION OF PULMONARY LYMPHOCYTES

To begin to examine the contributions of the individual lymphocyte subpopulations to the observed phenotypes of epithelial-cell injury and increased macrophage accumulation, we purified  $\alpha\beta$  and  $\gamma\delta$  T cells from isolated, perfused, enzymatically digested lung tissue. Purification of individual cell types was performed using a cell-sorting strategy that takes advantage of the fact that expression of  $\alpha\beta$  and  $\gamma\delta$  receptors is mutually exclusive in T-cell



**Figure 5. Apoptotic cells (arrows) in the airway epithelium of mice exposed to 2.0 ppm of acrolein.** Photomicrographs are shown for a C57BL/6J control mouse (A), as well as for mice after 4 weeks of exposure to 2.0 ppm of acrolein: a C57BL/6J mouse (B), an  $\alpha\beta^{-/-}$  mouse (C), and a  $\gamma\delta^{-/-}$  mouse (D). Apoptotic cells were identified by immunohistochemistry on paraffin-embedded sections using a rabbit antibody specific for active caspase 3. Photomicrographs (shown at 400 $\times$  original magnification) are representative of the eight mice per group. Scale bar = 100  $\mu$ m.

subpopulations. Using fluorescent-labeled antibodies against the TCR-associated protein CD3, the TCR- $\beta$  subunit, and the TCR- $\delta$  subunit, we achieved purities of approximately 99% (Figure 11). This degree of purity is critical for the accurate analysis of the mRNA transcript data because a greater level of contamination would considerably reduce the validity of the microarray studies.

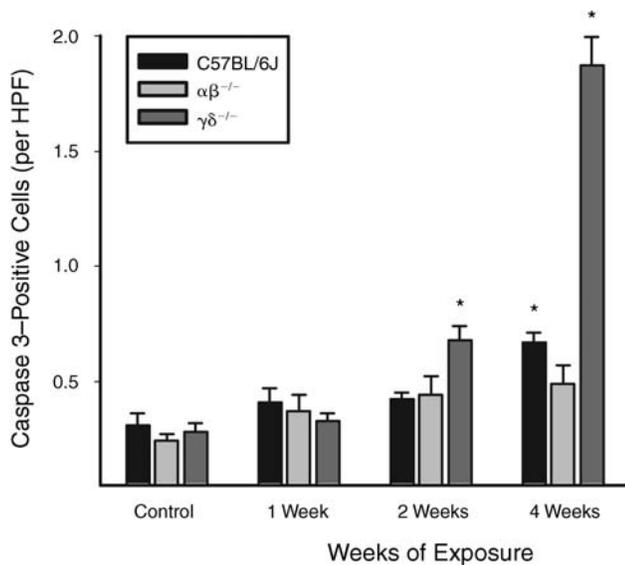
#### MICROARRAY DATA

The analysis of the microarray data consisted of several components: the evaluation of overall changes in gene expression in T cells from the lungs of control mice as compared with the same type of T cells from the lungs of mice exposed to 2.0 ppm acrolein for 1 week, both for  $\alpha\beta$  T cells and  $\gamma\delta$  T cells; the validation of changes in the expression of 14 candidate genes by qRT-PCR; the comparison of significant changes in mRNA transcripts between  $\alpha\beta$  and

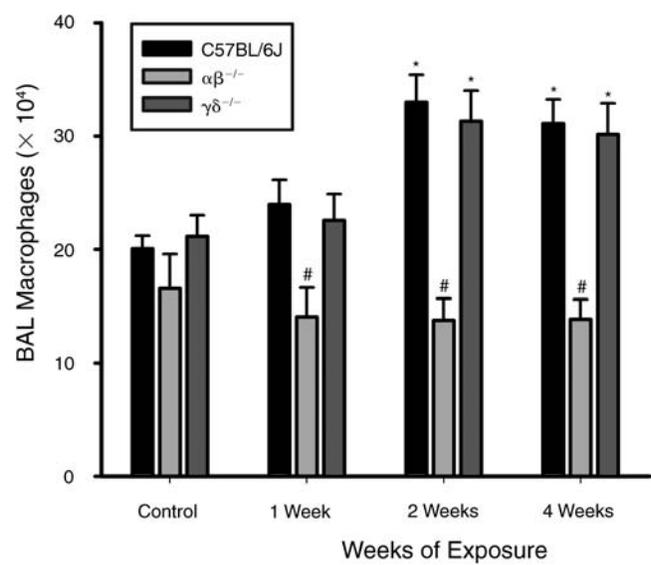
$\gamma\delta$  T cells after acrolein exposure; the assessment of biologic pathways that are significantly altered in  $\alpha\beta$  and  $\gamma\delta$  T cells after acrolein exposure; and detailed listings of the levels and changes in expression of cytokines, chemokines, and their cognate receptors in  $\alpha\beta$  and  $\gamma\delta$  T cells after acrolein exposure. We chose a 1-week exposure period for these studies because this was the earliest time point at which gene expression related to both  $\alpha\beta$  and  $\gamma\delta$  T cells was significantly changed in the C57BL/6J mice and at which corresponding alterations were first evident in the mice deficient in either type of T cell.

#### Changes in Gene Expression

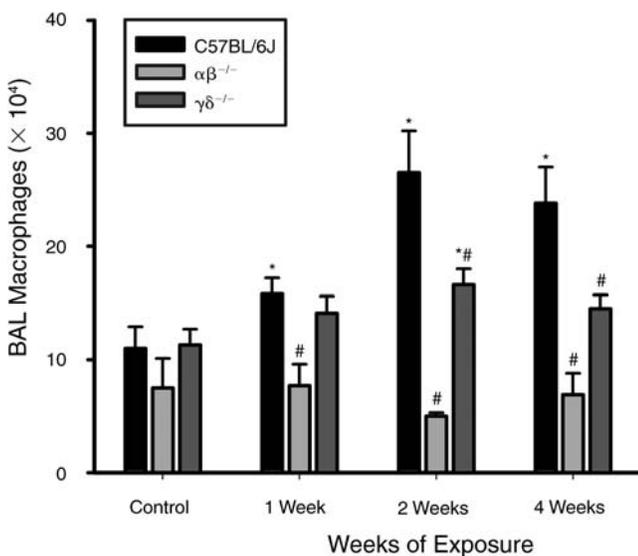
Analysis of the microarray data revealed 659 genes in  $\alpha\beta$  T cells and 626 genes in  $\gamma\delta$  T cells isolated from the lungs that exhibited significant changes in expression levels after acrolein exposure. Many of these genes may contribute to



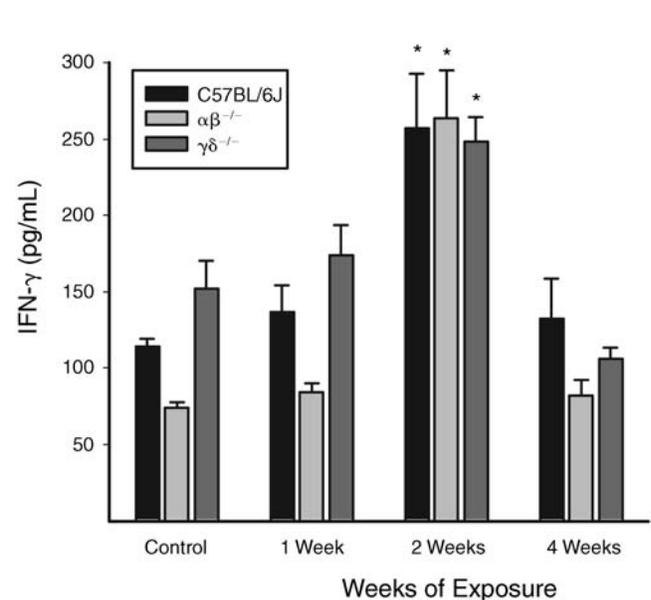
**Figure 6. Quantitation of apoptosis, as indicated by active caspase 3, in the lungs after exposure to 2.0 ppm of acrolein.** Cells positive for active caspase 3 were quantified from photomicrographs of lung sections from control mice and mice exposed to acrolein for various time periods, in the three strains: C57BL/6J mice,  $\alpha\beta^{-/-}$  mice, and  $\gamma\delta^{-/-}$  mice. Values presented are means  $\pm$  SEM of the number of positive cells per high-powered field (HPF, 400 $\times$ ). \*Significantly greater than the mean strain-matched controls ( $P < 0.05$ ).



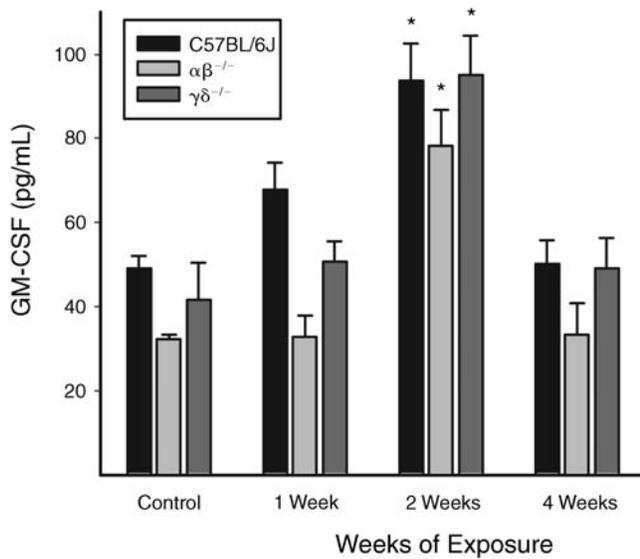
**Figure 8. Macrophage accumulation in the lungs of mice exposed to 0.5 ppm of acrolein.** The number of macrophages was determined by estimating the percentage of the total number of cells recovered from BAL fluid that were macrophages, by assessing Wright-stained cytocentrifuge slides with  $\geq 300$  cells per slide. Data are shown for C57BL/6J mice,  $\alpha\beta^{-/-}$  mice, and  $\gamma\delta^{-/-}$  mice. Values presented are the means ( $\pm$  SEM) of four or five mice per group. \*Significantly greater than the mean strain-matched control value ( $P < 0.05$ ). #Significantly different from the mean exposure-matched C57BL/6J value ( $P < 0.05$ ).



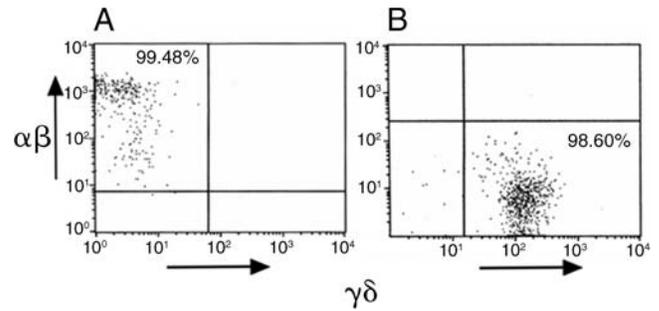
**Figure 7. Macrophage accumulation in the lungs of mice exposed to 2.0 ppm of acrolein.** The number of macrophages was determined by estimating the percentage of the total number of cells recovered from BAL fluid that were macrophages, by assessing Wright-stained cytocentrifuge slides with  $\geq 300$  cells per slide. Data are shown for C57BL/6J mice,  $\alpha\beta^{-/-}$  mice, and  $\gamma\delta^{-/-}$  mice. Values presented are the means ( $\pm$  SEM) of seven or eight mice per group. \*Significantly greater than the mean strain-matched control value ( $P < 0.05$ ). #Significantly different from the mean exposure-matched C57BL/6J value ( $P < 0.05$ ).



**Figure 9. IFN- $\gamma$  levels in the lungs of acrolein-exposed mice.** IFN- $\gamma$  levels are shown for mice exposed to 2.0 ppm acrolein and for control mice. Data are shown for C57BL/6J mice,  $\alpha\beta^{-/-}$  mice, and  $\gamma\delta^{-/-}$  mice. Levels are increased among mice exposed for 2 weeks. Levels were determined by ELISA of the whole lungs of mice. Values are the means ( $\pm$  SEM) of eight mice per group. \*Significantly greater than the mean strain-matched control value ( $P < 0.05$ ).



**Figure 10. GM-CSF levels in the lungs of acrolein-exposed mice.** GM-CSF levels are shown for mice exposed to 2.0 ppm acrolein and for control mice. Data are shown for C57BL/6J mice,  $\alpha\beta^{-/-}$  mice, and  $\gamma\delta^{-/-}$  mice. Levels are increased among mice exposed for 2 weeks. Levels were determined by ELISA of the whole lungs of mice. Values are the means ( $\pm$  SEM) of eight mice per group. \* Significantly greater than the mean strain-matched control value ( $P < 0.05$ ).



**Figure 11. Purity of pulmonary  $\alpha\beta$  T cells and  $\gamma\delta$  T cells.** The purity of the isolated cells, determined by flow cytometry, is depicted by dot plots (on a  $\log_{10}$  scale) that indicate the numbers of cells that stained positive for the  $\alpha\beta$  TCR (A) and the  $\gamma\delta$  TCR (B). Cells were isolated, by enzymatic digestion of perfused lung tissue (see Methods), from C57BL/6J control mice and those that were exposed to 2.0 ppm of acrolein for 1 week. To purify T-cell subpopulations, cells were labeled with antibodies against CD3, TCR- $\beta$ , and TCR- $\delta$ . Cells positive for both CD3 and TCR- $\beta$  were isolated into a separate tube from the cells that were positive for both CD3 and TCR- $\delta$ .

**Table 1. Changes in Expression of Candidate Genes in  $\alpha\beta$  T Cells After Acrolein Exposure**

Gene	Description of Protein	Average Intensity <sup>a</sup>	Fold Change in Expression <sup>b</sup>	P Value
<i>Ifngr2</i>	IFN- $\gamma$ receptor 2	1783	2.76	0.0005
<i>Itgb3</i>	Integrin beta 3, adhesion molecule	4424	2.07	0.003
<i>S100a4</i>	Involved in antimicrobial function	3336	3.00	0.0008
<i>Serpinh1a</i>	Serine proteinase inhibitor	329	5.87	0.0007
<i>Flt3l</i>	Anti-apoptotic, involved in T-cell proliferation	136	2.42	0.001
<i>Gstp1</i>	Detoxifying enzyme	4249	2.38	0.0005
<i>Il18r1</i>	IL-18 receptor	1613	2.53	0.0009
<i>Il3</i>	Macrophage growth and survival factor	393	2.72	0.001
<i>Il12rb1</i>	IL-12 receptor	112	2.46	0.001
<i>Cd97</i>	Involved in adhesion and activation of leukocytes	1246	2.23	0.002
<i>Il28ra</i>	IL-28 receptor	385	2.36	0.001
<i>Kit</i>	Involved in thymocyte differentiation	51	2.19	0.001
<i>Bad</i>	Apoptosis antagonist	758	2.22	0.003
<i>Lpar1</i>	Lysophosphatidic acid receptor	123	-3.56	0.002
<i>Cnbp1</i>	Cellular nucleic acid binding protein	6660	-3.02	0.0002
<i>Casp2</i>	Caspase 2	843	-2.50	0.002
<i>Klrc1</i>	Killer cell lectin-like receptor subfamily C, member 1	164	-2.66	0.003
<i>Ikbke</i>	Inhibitor of NF- $\kappa$ B kinase	226	-2.32	0.003

<sup>a</sup> Average intensity is an arbitrary, unitless value derived from the fluorescence intensity of the microarray analysis.

<sup>b</sup> Fold change in expression refers to the change in acrolein-exposed cells as compared with control cells.

**Table 2.** Changes in Expression of Candidate Genes in  $\gamma\delta$  T Cells After Acrolein Exposure

Gene	Description of Protein	Average Intensity <sup>a</sup>	Fold Change in Expression <sup>b</sup>	P Value
<i>Chi3l3</i>	Chitinase, promotes inflammation	630	13.39	0.0000001
<i>Mpeg1</i>	Involved in perforin-like activity	389	8.25	0.00001
<i>Cd44</i>	Adhesion molecule	146	8.06	0.0002
<i>Fgl2</i>	Involved in T-cell regulation	183	5.91	0.00003
<i>Egr1</i>	Involved in T-cell differentiation	253	5.51	0.00001
<i>Ptprc</i>	Involved in receptor signaling in T and B cells	12,668	5.19	0.0001
<i>Lyz1</i>	Antimicrobial enzyme	452	5.05	0.0003
<i>Serpine1a</i>	Serine proteinase inhibitor	178	4.35	0.0002
<i>Ifngr</i>	IFN- $\gamma$ receptor, alpha chain	145	3.85	0.001
<i>Cd164</i>	Adhesion molecule	1,340	3.80	0.0003
<i>Tnfrsf11</i>	Mediates cytokine-receptor cross talk	996	3.68	0.0002
<i>Ier5</i>	Mediator of growth-factor signaling	887	3.62	0.002
<i>Cxcl2</i>	Involved in neutrophil chemotaxis	4,743	3.42	0.001
<i>Hmgb1</i>	Agonist of TLRs and Rage	332	3.39	0.0007
<i>Daf2</i>	Decay accelerating factor, complement regulator	412	3.36	0.001
<i>Cd28</i>	T-cell receptor co-receptor	17,742	3.13	0.002
<i>Il4ra</i>	IL-4 receptor, alpha	323	3.11	0.004
<i>Cd14</i>	TLR signaling	400	3.00	0.003
<i>Cxcl1</i>	Chemokine	102	2.98	0.001
<i>Itgb2</i>	Adhesion molecule	180	2.79	0.004
<i>Icos</i>	Inducible T-cell co-stimulator	1,189	2.77	0.003
<i>Bpil2</i>	Antimicrobial protein	2,978	2.61	0.003
<i>Klra2</i>	MHC class I molecule	192	2.51	0.004
<i>Klra9</i>	MHC class I molecule	732	-3.04	0.002
<i>Faim2</i>	Fas apoptotic inhibitory molecule	97	-3.69	0.001
<i>Cxcl16</i>	Chemokine	55	-3.28	0.003
<i>Fgf10</i>	Growth factor	1,207	-2.63	0.003
<i>Mmp1a</i>	Matrix metalloproteinase 1a	1,486	-3.09	0.003

<sup>a</sup> Average intensity is an arbitrary value derived from the fluorescence intensity of the microarray analysis.

<sup>b</sup> Fold change in expression refers to the change in acrolein-exposed cells as compared with control cells.

the phenotypes observed in acrolein-exposed mice deficient in  $\alpha\beta$  T cells (Table 1) or  $\gamma\delta$  T cells (Table 2). Among these candidate genes are those involved in a diverse group of cellular functions including, but not limited to, cytokine-receptor signaling, chemotaxis, growth-factor production, lymphocyte activation, and apoptosis.

Specifically, several transcripts involved in macrophage-T cell interactions were up-regulated in  $\alpha\beta$  T cells. These included genes encoding components of the IFN- $\gamma$  receptor, IL-18 receptor, IL-12 receptor, and IL-3. All of the ligand-receptor interactions mediated by these genes exhibit defined effector functions involved in macrophage accumulation and activation. In  $\gamma\delta$  T cells, specific changes in levels of mRNA transcripts related to the phenotype of compromised epithelial-cell integrity included genes associated with

recognition of MHC class I molecules (*Klra2*, *Klra9*), Toll-like-receptor signaling (*Hmgb1*, *Daf2*, *CD14*), lymphocyte-effector functions (*Mpeg1*, *Cd28*), and the resolution of tissue injury and remodeling (*Cd44*, *Serpine1*).

### Validation

To verify changes observed in the microarray experiments, we isolated RNA from identical cohorts of mice and performed qRT-PCR on a subset of genes. We selected a total of 15 genes whose expression was either significantly increased in one or both of the T-cell subpopulations (*Cd97*, *Hmgb1*, *Cxcl2*, *Ifngr2*, *Egr1*, *Ptprc*, *Cd164*, *Chi3l3*, *Il18r1*, *S100a10*), significantly decreased in one or both (*Tnfrsf11*, *Egr1*, *Cd164*, *S100a4*, *S100a10*, *Klrc1*), or not significantly altered but expressed at high levels in one or both (*Irf1*,

**Table 3.** Validation of Microarray Changes by qRT-PCR

Gene	$\alpha\beta$ T Cells		$\gamma\delta$ T Cells	
	Microarray	qRT-PCR	Microarray	qRT-PCR
<i>Cd97</i>	2.23	2.92	1.54	2.59
<i>Hmgb1</i>	1.28	1.33	3.39	1.96
<i>Cxcl2</i>	1.43	11.76	3.42	4.25
<i>Tnfsf11</i>	0.64	0.68	3.68	2.33
<i>Ifngr2</i>	2.76	3.09	3.85	2.13
<i>Egr1</i>	0.64 <sup>a</sup>	4.23	5.51	2.65
<i>Irf1</i>	1.07	2.05	1.38	1.61
<i>Ccl5</i>	1.57	1.67	0.67	0.79
<i>Ptprc</i>	1.10	1.99	5.19	1.91
<i>Cd164</i>	0.61 <sup>a</sup>	1.16	3.80 <sup>a</sup>	0.81
<i>S100a4</i>	3.00	3.21	0.47	0.66
<i>S100a10</i>	1.35	0.99	0.78	0.64
<i>Chi3l3</i>	1.15	2.72	13.39 <sup>a</sup>	0.86
<i>Klrc1</i>	0.37	0.58	1.95	3.44
<i>Il18r1</i>	2.53	1.62	1.64 <sup>a</sup>	0.53

<sup>a</sup> These values were not confirmed by qRT-PCR.

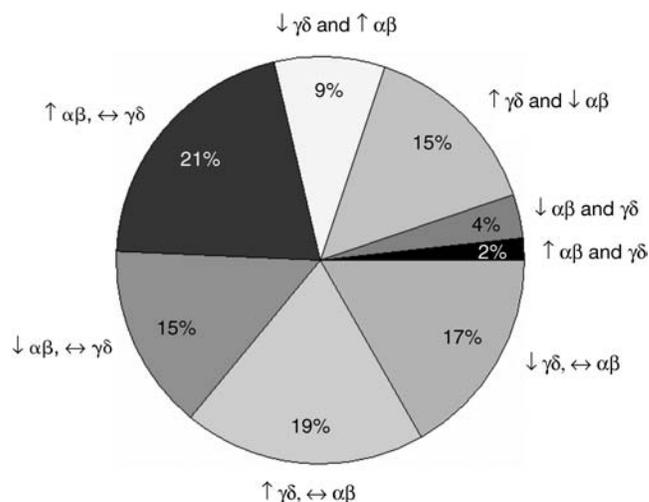
*Ccl5*) (Table 3). The results indicate that there is generally good agreement between the two methods. Of the 15 genes assayed by qRT-PCR measurements for each cell type, 25 of 30 measurements were supportive of the microarray results. These results underscore the necessity of performing a validation technique when relying on microarray data.

### Comparisons of Changes in Gene Expression

Surprisingly, of the 1285 genes that showed significant changes in expression in the two T-cell subpopulations, only 6% had significant changes in both types of cells: only 2% were genes whose expression increased in both  $\alpha\beta$  and  $\gamma\delta$  T cells, and only 4% were genes that decreased in both  $\alpha\beta$  and  $\gamma\delta$  T cells (Figure 12). A total of 15% of the significant changes were for genes whose expression was increased in  $\gamma\delta$  T cells but decreased in  $\alpha\beta$  T cells, and 9% of the significant changes were for genes whose expression was increased  $\alpha\beta$  T cells but decreased in  $\gamma\delta$  T cells. The remaining 72% of significant changes occurred in only one of the T-cell subpopulations. Representative genes from each group are listed in Table 4.

### Biologic Pathways

To further analyze the functional significance of the differences in gene expression, MAPPFinder was used to



**Figure 12.** Gene expression in  $\alpha\beta$  T cells and  $\gamma\delta$  T cells after repeated acrolein exposure. The percentages of genes with significant changes in mRNA levels are shown for  $\alpha\beta$  T cells and  $\gamma\delta$  T cells, grouped according to whether the levels were increased ( $\uparrow$ ) or decreased ( $\downarrow$ ); no significant change is also indicated ( $\leftrightarrow$ ). Oligonucleotide microarray analyses revealed that the majority of gene changes in  $\alpha\beta$  T cells and in  $\gamma\delta$  T cells were unique to the cell type.

identify molecular pathways that contained an increased number of altered transcript levels. The program generated a gene-expression profile organized according to biologic processes, cellular components, and molecular functions, which allowed for the identification of specific biologic pathways that were altered. We also analyzed the data using a similar program distributed by Applied Biosystems and obtained nearly identical results. The data presented in this report are derived from MAPPFinder.

Many of the pathways with significant alterations after acrolein exposure offer additional insight into the various roles of the two T-cell subpopulations (Tables 5 and 6). The analyses indicate that there is little overlap between the Gene Ontology database pathways regulated by each type of T cell (except in the MHC protein complex and antigen binding or presentation pathways) and suggests that the response of the  $\alpha\beta$  T cells was more diverse, indicated by a broader array of pathways identified, as compared with that of  $\gamma\delta$  T cells. The  $\alpha\beta$  T cells exhibited significant differences in pathways related to anion transport, mitochondrial apoptosis, helicase activity, and circulation. In contrast, significant alterations in pathways regulated in  $\gamma\delta$  T cells were largely restricted to the related pathways of peptide antigen binding, defense and immune responses, and inflammation. This is probably a reflection of the greater heterogeneity of  $\alpha\beta$  T cells as compared with  $\gamma\delta$  T cells.

**Table 4.** Gene-Expression Profiles of  $\alpha\beta$  T Cells and  $\gamma\delta$  T Cells, as Detected on Microarray

Expression Profile	Affected Gene	Description of Protein	Fold Change in Expression <sup>a</sup>	
			$\gamma\delta$ T Cells	$\alpha\beta$ T Cells
Decreased expression in both cells	<i>Aqp7</i>	Aquaporin 7	-2.06	-3.40
	<i>Zbtb805</i>	Zinc-finger and BTB-domain-containing 8 opposite strand	-2.02	-2.54
Increased expression in both cells	<i>Serpinh1a</i>	Serine proteinase inhibitor	4.35	5.87
	<i>Cd36</i>	Platelet glycoprotein IV ( <i>GPIV</i> , or CD36 antigen)	3.25	1.59
	<i>Lyz1</i>	Lysozyme	2.90	2.18
	<i>Flt1</i>	Vascular endothelial growth factor receptor 1	2.40	2.42
	<i>Alox5ap</i>	5 Lipoxygenase activating protein (Flap)	2.14	3.79
Decreased expression in $\gamma\delta$ cells, increased expression in $\alpha\beta$ cells	<i>S100a4</i>	Involved in antimicrobial function	-2.09	3.00
	<i>Hcst</i>	Hematopoietic cell-signal transducer	-3.07	2.27
	<i>H2-Q1</i>	Histocompatibility 2, Q region locus 1	-1.92	2.17
	<i>H2-M2</i>	Histocompatibility 2, M region locus 2	-2.83	1.67
Increased expression in $\gamma\delta$ cells, decreased expression in $\alpha\beta$ cells	<i>Kifc1</i>	Transporter associated with antigen processing binding protein	5.96	-2.04
	<i>Ier5</i>	Mediator of growth-factor signaling	3.62	-2.05
	<i>H2-D1</i>	H2 class I histocompatibility antigen, Q10 alpha chain	3.55	-1.76
	<i>Txn12</i>	Thioredoxin-like 2	3.26	-2.86
	<i>Tox</i>	Thymocyte selection-associated high mobility group box	3.16	-1.94
	<i>Il4ra</i>	IL-4 receptor, alpha	3.11	-2.22
	<i>Hspca</i>	Heat shock protein 90 alpha	2.92	-2.46
	<i>Fin14</i>	Fibroblast growth factor inducible 14	2.86	-1.79
	<i>Cklfsf6</i>	Chemokine-like factor superfamily 6	2.22	-3.63
	<i>Klrc1</i>	Killer cell lectin-like receptor subfamily C, member 1	1.95	-2.66
	<i>Casp2</i>	Caspase 2	1.92	-2.50

<sup>a</sup> Fold change in expression refers to the change in acrolein-exposed cells as compared with control cells.

## T Cells in Acrolein-Induced Pulmonary Inflammation

**Table 5.** Pathways in the GO Hierarchy Database Regulated in  $\alpha\beta$  T Cells After Acrolein Exposure<sup>a</sup>

GO Name	GO ID	Genes			% Changed	z-Score	P Value
		No. Changed	No. Measured	No. in GO			
Organic anion transport	15711	3	6	8	50.0	5.126	0.00001
Apoptotic mitochondrial changes	8637	4	11	14	36.4	4.844	0.00001
Protein insertion into membrane	51205	3	4	4	75.0	6.504	0.001
MHC protein complex	42611	5	20	32	25.0	4.175	0.001
Helicase activity	4386	12	108	131	11.1	3.014	0.001
Release of cytochrome c from mitochondria	1836	3	5	6	60.0	5.717	0.002
Localization within membrane	51668	3	6	6	50.0	5.126	0.002
MHC class I receptor activity	30106	5	21	32	23.8	4.025	0.002
Antigen presentation	19882	7	40	55	17.5	3.706	0.002
DNA-dependent ATPase activity	8094	5	23	27	21.7	3.752	0.003
Large ribosomal subunit	15934	5	23	34	21.7	3.752	0.004
Immunologic synapse	1772	6	32	47	18.8	3.642	0.004
ATP-dependent helicase activity	8026	9	78	90	11.5	2.734	0.004
Circulation	8015	5	25	41	20.0	3.508	0.005
Antigen presentation, peptide antigen binding	48002	3	11	12	27.3	3.445	0.005

<sup>a</sup> The MAPPFinder program links microarray data to the GO hierarchy database to generate a gene-expression profile at the level of biologic processes, cellular components, and molecular functions that allows for identification of specific biologic pathways that merit further investigation. The results are calculated using Fisher's exact test and are expressed as a "z-score" for a particular pathway. Values greater than 2.0 and  $P \leq 0.005$  were considered to indicate statistical significance.

**Table 6.** Pathways in the GO Hierarchy Database Regulated in  $\gamma\delta$  T Cells After Acrolein Exposure<sup>a</sup>

GO Name	GO ID	Genes			% Changed	z-Score	P Value
		No. Changed	No. Measured	No. in GO			
Peptide antigen binding	42605	4	7	8	57.1	6.399	0.000001
Defense response	6952	37	336	559	11.0	5.271	0.000001
Response to biotic stimulus	9607	38	354	588	10.7	5.169	0.000001
Immune response	6955	34	306	481	11.1	5.103	0.000001
Response to stimulus	50896	55	743	1254	7.4	3.275	0.000001
Organ development	48513	38	480	757	7.9	3.129	0.001
Positive regulation of protein metabolism	51247	7	34	51	20.6	4.240	0.002
Peptide binding	42277	9	69	121	13.0	3.139	0.002
MHC protein complex	42611	5	20	32	25.0	4.163	0.003
Antigen binding	3823	4	16	38	25.0	3.723	0.003
Phosphoprotein phosphatase activity	4721	12	97	135	12.4	3.419	0.003
Positive regulation of metabolism	9893	15	142	207	10.6	3.142	0.003
Response to pest, pathogen or parasite	9613	17	175	303	9.7	2.970	0.003
Organismal physiological process	50874	47	622	1078	7.6	3.160	0.005
Inflammatory response	6954	10	81	130	12.3	3.111	0.005
Positive regulation of cellular process	48522	25	297	419	8.4	2.843	0.005

<sup>a</sup> The MAPPFinder program links microarray data to the GO hierarchy database to generate a gene-expression profile at the level of biologic processes, cellular components, and molecular functions that allows for identification of specific biologic pathways that merit further investigation. The results are calculated using Fisher's exact test and are expressed as a "z-score" for a particular pathway. Values greater than 2.0 and  $P \leq 0.005$  were considered to indicate statistical significance.

## CYTOKINES AND CHEMOKINES

The regulation of cytokines and chemokines that occurs as part of the control of pulmonary inflammation and airway remodeling is of interest when considering the role of lymphocytes in pulmonary pathophysiology. Therefore, we tabulated the expression levels (average intensity) and fold changes for all genes encoding cytokines, chemokines, and cytokine or chemokine receptors that were expressed

at significant levels (average intensity of > 50) in  $\alpha\beta$  T cells (Tables 7 and 8) and  $\gamma\delta$  T cells (Tables 9 and 10). These data may reveal several novel and potentially important aspects of T-cell function after acrolein exposure and provide a comprehensive list of the detectable mediators in each cell type (see the Discussion section, Transcriptional Analysis of  $\alpha\beta$  T Cells and  $\gamma\delta$  T Cells).

**Table 7.** Cytokine and Chemokine Expression in  $\alpha\beta$  T Cells

Gene	Average Intensity <sup>a</sup>	Fold Change in Expression <sup>b</sup>
<i>Scye1</i>	5318	1.12
<i>Ccl5</i>	2013	1.57
<i>Cxcl2</i>	855	1.43
<i>Il27</i>	471	-1.87
<i>Il16</i>	417	-1.50
<i>Il17c</i>	414	-1.31
<i>Il3</i>	393	2.72
<i>Ccl3</i>	274	1.08
<i>Xcl1</i>	246	-1.37
<i>Il15</i>	224	-1.28
<i>Il17e</i>	203	1.96
<i>Il20</i>	183	1.52
<i>Il1f9</i>	171	-1.22
<i>Il18</i>	168	-1.00
<i>Il6</i>	163	1.77
<i>Flt3l</i>	136	2.80
<i>Il17b</i>	112	1.09
<i>Cxcl12</i>	83	1.56
<i>Il4</i>	83	1.49
<i>Il1f10</i>	76	-1.41
<i>Ccl7</i>	74	-1.62
<i>Il13</i>	74	1.07
<i>Il23a</i>	67	2.04
<i>Il1f5</i>	66	1.35
<i>Cxcl11</i>	58	-1.21
<i>Ccl4</i>	54	1.06
<i>Il19</i>	50	1.65
<i>Il25</i>	50	1.55

<sup>a</sup> Average intensity is an arbitrary, unitless value derived from the fluorescence intensity of the microarray analysis.

<sup>b</sup> Fold change in expression refers to the change in acrolein-exposed cells as compared with control cells.

**Table 8.** Cytokine- and Chemokine-Receptor Expression in  $\alpha\beta$  T Cells

Gene	Average Intensity <sup>a</sup>	Fold Change in Expression <sup>b</sup>
<i>Il10rb</i>	2828	1.90
<i>Ccr11</i>	2549	1.14
<i>Il7r</i>	2290	-2.20
<i>Ifngr2</i>	1783	2.76
<i>Il18r1</i>	1613	2.75
<i>Ccbp2</i>	1583	-1.23
<i>Il12rb2</i>	748	-1.20
<i>Il27ra</i>	734	1.10
<i>Cxcr6</i>	646	-2.24
<i>Ccl8</i>	625	1.52
<i>Il21r</i>	534	1.21
<i>Il15ra</i>	531	1.21
<i>Cxcr7</i>	527	1.24
<i>Il2rb</i>	438	1.02
<i>Il4ra</i>	387	-2.22
<i>Il28ra</i>	385	2.36
<i>Ccr2</i>	203	2.01
<i>Il2rg</i>	168	-1.19
<i>Ccr7</i>	136	-1.52
<i>Il17re</i>	134	-1.12
<i>Il18rap</i>	123	-1.31
<i>Il12rb1</i>	112	2.46
<i>Il17rb</i>	110	1.27
<i>Ccr12</i>	102	-1.62
<i>Cxcr4</i>	83	-1.12
<i>Ccr11</i>	78	1.20
<i>Ccr5</i>	74	-1.64
<i>Il1r1</i>	74	1.29
<i>Il1rap12</i>	72	1.03

<sup>a</sup> Average intensity is an arbitrary, unitless value derived from the fluorescence intensity of the microarray analysis.

<sup>b</sup> Fold change in expression refers to the change in acrolein-exposed cells as compared with control cells.

**Table 9.** Cytokine and Chemokine Expression in  $\gamma\delta$  T Cells

Symbol	Average Intensity <sup>a</sup>	Fold Change in Expression <sup>b</sup>
<i>Ccl5</i>	28,270	-1.49
<i>Cxcl2</i>	4,743	3.42
<i>Scye1</i>	2,168	1.07
<i>Ccl3</i>	1,908	1.62
<i>Il1b</i>	1,586	2.15
<i>Xcl1</i>	715	-2.40
<i>Il18</i>	267	1.53
<i>Ccl4</i>	186	-1.87
<i>Ccl8</i>	167	-1.71
<i>Ccl9</i>	167	1.95
<i>Il16</i>	164	1.99
<i>Ebi3</i>	148	1.55
<i>Il2</i>	120	-1.67
<i>Cxcl1</i>	102	2.98
<i>Ccl2</i>	100	1.62
<i>Il15</i>	88	1.17
<i>Il7</i>	79	1.25
<i>Cxcl16</i>	55	-3.28
<i>Il3</i>	67	1.10
<i>Cxcl11</i>	64	1.38

<sup>a</sup> Average intensity is an arbitrary, unitless value derived from the fluorescence intensity of the microarray analysis.

<sup>b</sup> Fold change in expression refers to the change in acrolein-exposed cells as compared with control cells.

## DISCUSSION AND CONCLUSIONS

This study examined the roles and potential effector functions of the  $\alpha\beta$  and  $\gamma\delta$  T-cell populations that are found in pulmonary tissues after repeated exposure to a ubiquitous air toxicant, acrolein. Using mice genetically deficient in each of these cell types, we have established a role for  $\alpha\beta$  T cells in the persistent accumulation of macrophages and  $\gamma\delta$  T cells in the protection of the pulmonary epithelium against the necrotic and apoptotic cell death observed in mice after acrolein exposure. Furthermore, we have established a comprehensive database of gene expression in each of these pulmonary-lymphocyte subpopulations and report T cell-specific changes in gene expression that will lead to more intensive studies of the mechanisms involved in protection of the lung mucosa after repeated exposure to irritants. The data reported here are among the first to identify the roles and responses of individual lymphocyte populations after exposure to air toxics as opposed to pathogens.

**Table 10.** Cytokine- and Chemokine-Receptor Expression in  $\gamma\delta$  T Cells

Gene	Average Intensity <sup>a</sup>	Fold Change in Expression <sup>b</sup>
<i>Il2rb</i>	19,443	1.27
<i>Crlf3</i>	15,755	2.00
<i>Ccr2</i>	2,571	2.36
<i>Il10rb</i>	2,009	2.03
<i>Il7r</i>	1,381	1.58
<i>Il18r1</i>	1,290	1.64
<i>Il12rb1</i>	563	-1.36
<i>Cxcr5</i>	424	-1.01
<i>Cxcr6</i>	344	2.26
<i>Il4ra</i>	323	3.11
<i>Il18rap</i>	307	1.15
<i>Il15ra</i>	240	1.22
<i>Ccr1</i>	227	-2.27
<i>Il17rc</i>	192	-1.35
<i>Il27ra</i>	153	1.29
<i>Cxcr4</i>	135	1.76
<i>Il2rg</i>	134	2.40
<i>Il1rl1</i>	117	1.38
<i>Tlr1</i>	87	-1.28
<i>Il17re</i>	82	1.12
<i>Il2ra</i>	73	1.14
<i>Il17ra</i>	69	1.35
<i>Il12rb2</i>	67	-1.22
<i>Il1r1</i>	57	2.86

<sup>a</sup> Average intensity is an arbitrary, unitless value derived from the fluorescence intensity of the microarray analysis.

<sup>b</sup> Fold change in expression refers to the change in acrolein-exposed cells as compared with control cells.

## EFFECTS OF ACROLEIN ON PULMONARY PATHOLOGY

Numerous studies have reported the effects of acrolein exposure on several species, including mice and rats. Acrolein penetrates the upper respiratory passages and deposits throughout the lower respiratory tract (Ayer and Yeager 1982). Acrolein and other reactive aldehydes are considered important mediators of cell damage because of their ability to covalently modify macromolecules, which disrupt critical cellular functions and cause mutations (Izard and Libermann 1978; Esterbauer et al. 1991; Cohen et al. 1992). The direct health effects of acrolein exposure are difficult to assess because acrolein is usually encountered in a pollutant mixture. However, case reports demonstrate that accidental acrolein exposure results in the development of COPD consisting of bronchiectasis, bronchitis, and

emphysema (Champeix et al. 1966; Bauer et al. 1977; Mahut et al. 1993). In laboratory animals, acrolein exposure induces lesions associated with COPD. We and others have demonstrated that acrolein induces the hallmark features of COPD, including increased macrophage accumulation, epithelial damage, airspace enlargement, and mucus hypersecretion (Lyon et al. 1970; Feron et al. 1978; Costa et al. 1986; Borchers et al. 1999b). These studies were not comprehensive (i.e., no individual study examined all end points) and not all used mice.

The present study expands previous findings of epithelial-cell hypertrophy and mucous-cell development by examining two end points of epithelial-cell injury. We have assessed numbers of epithelial cells in BAL fluid and immunoreactivity to active caspase 3 (a marker of apoptosis) in formalin-fixed, paraffin-embedded sections of mouse lung. The data derived from examination of the BAL fluid is most likely indicative of necrotic cell death in the proximal airways because the majority (> 90%) of the cells are ciliated and failed to exclude trypan blue in a dye-exclusion assay.

Direct injury to cells and subsequent cell death at this level of the respiratory tract is probable, because the dose of toxicant is greatest in the upper airways and decreases toward the distal respiratory epithelium. The mechanism of cell death in the distal airways is more likely mediated by apoptotic processes initiated by either direct injury to the epithelium or indirect mechanisms encompassing efficient removal of injured cells by the immune system. This suggestion is supported by the fact that the majority of caspase 3-positive cells in the lung after acrolein exposure are localized to the terminal bronchioles.

We have recently developed and characterized an acrolein exposure model that induces the hallmark features of COPD in mice (Borchers et al. 2007). The pathologic features of the model include airspace enlargement and hypertrophy of airway epithelial cells with mucous-cell metaplasia. Furthermore, we have assessed the effects of chronic acrolein exposure on several end points characteristic of COPD, including monocyte inflammation, production of type 1 helper cytokine production, and matrix metalloproteinase (MMP) activation. Acrolein exposure resulted in increased macrophage accumulation in the BAL, IFN- $\gamma$  production, and MMP2 and MMP9 activity (Borchers et al. 2007). These data are important because they demonstrate that our model of toxicant-induced COPD faithfully replicates the primary features of this disease.

To determine whether the absence of T cells affects the development of airspace enlargement in a mouse model of toxicant-induced COPD, wild-type (C57BL/6J) mice and mice genetically deficient in T and B cells (*Rag2*<sup>-/-</sup>, on a C57BL/6J genetic background) underwent chronic exposure

to acrolein (2.0 ppm, 6 hr per day, 5 days per week, for 12 weeks) (data not shown). Although airspace enlargement occurred in both the wild-type mice and *Rag2*<sup>-/-</sup> mice in a time-dependent manner, the magnitude of airspace enlargement was significantly inhibited in the lymphocyte-deficient mice (data not shown). These studies demonstrate that lymphocytes contribute to the development of toxicant-induced airway destruction. These findings are important to our project because they provide an essential observation for future studies investigating the role of air toxics as mediators of the immune system, in terms of contributing to the morbidity and mortality associated with COPD.

### ROLES OF $\alpha\beta$ T CELLS AND $\gamma\delta$ T CELLS

The effect of acrolein exposure on mucous-cell metaplasia and epithelial-cell injury in rats deficient in  $\gamma\delta$  T cells suggests that this type of cell is critical in the protection of epithelial-cell integrity after exposure to toxicants. This suggestion is supported by the data showing that mice genetically deficient in  $\gamma\delta$  T cells exhibited a significant increase in the number of epithelial cells sloughed from the airways, as compared with C57BL/6J mice or mice genetically deficient in  $\alpha\beta$  T cells. This response was most evident after exposure to 2.0 ppm acrolein yet was evident even after exposure to 0.5 ppm acrolein, which did not cause significant epithelial-cell death in wild-type mice.

This conclusion is bolstered by the results obtained from the caspase 3 immunohistochemistry. Counts of caspase 3-positive cells increased initially (by approximately two-fold) in the mice deficient in  $\gamma\delta$  T cells after 2 weeks of acrolein exposure and increased further (by approximately eightfold from the control count) after 4 weeks. In contrast, C57BL/6J mice exhibited a significant increase in the number of caspase-positive cells after only 4 weeks of exposure to 2.0 ppm acrolein, and mice deficient in  $\alpha\beta$  T cells, although exhibiting a trend of increasing counts, did not have a significant increase in the number of caspase 3-positive cells at any time point.

The role of individual T-cell populations in the development of mucous cells in mice after acrolein exposure is equivocal. Although in the present study, the number of mucous cells detected in the airways of mice increased over time, the number was nominal as compared with that observed in robust models of mucous-cell metaplasia, such as those in which allergen sensitization and challenge (Borchers et al. 2001) or lipopolysaccharide inhalation (Vernooy et al. 2002) was used. The failure of these assays to elucidate the role of T cells in mucous-cell metaplasia is most likely related to issues of toxicant-induced mucous-cell metaplasia in mice, as previously described (Borchers et al. 1999b). These issues include more-effective

removal of the vapor by the nasal passages and upper airways, as well as the presence of an abundance of detoxifying enzymes lining the lungs.

In contrast to the requirement of  $\gamma\delta$  T cells for epithelial-cell homeostasis,  $\alpha\beta$  T cells appear to be required for acrolein-induced macrophage accumulation. Mice deficient in  $\gamma\delta$  T cells exhibited a significant decrease in the number of accumulated macrophages, as compared with C57BL/6J mice, after 2 and 4 weeks of exposure to 2.0 ppm acrolein, but the two mouse models had similar numbers of accumulated macrophages after 1 week of exposure to 2.0 ppm acrolein and at all time points of exposure to 0.5 ppm acrolein. Strikingly, no increases in macrophage numbers were observed in mice deficient in  $\alpha\beta$  T cells at any time point of exposure to either level of acrolein. The reasons for this finding are not clear but may reflect the lack of critical cytokines and chemokines that are elaborated by  $\alpha\beta$  T cells in response to a variety of inflammatory stimuli and that affect macrophages. These results were the impetus to concentrate additional studies on the transcriptomes of the individual T-cell populations.

Preliminary studies did not indicate any significant changes in the numbers of the lymphocyte populations evaluated in the lung after acrolein exposure (data not shown). This is not altogether surprising, for several reasons. First, there is some contamination of the lung-digest fluid by peripheral blood, simply because of inconsistent or incomplete perfusion of the lung, an inherent limitation of the method. Second, there will also be incomplete digestion of tissue that will result in a degree of variability in the yields of the total cells from these preparations. However, this does not preclude the use of the lung-digest method to isolate tissue-resident lymphocytes for other purposes. For example, this method can be used, with flow cytometry, to assess lymphocyte size and granularity, since the method is sensitive enough to overcome any effect of background contamination and is not dependent on total yield but only on the types of cells isolated. Furthermore, this method is particularly amenable to the purification of T-cell subpopulations and analysis of their expression profiles. T cells from any contaminating peripheral blood should be relatively quiescent as compared with those in tissues and will probably serve to minimize the magnitudes of change in gene expression.

A major conclusion from our studies is that there is no direct association between inflammation and epithelial-cell pathology in response to acrolein exposure. The maximum accumulation of BAL leukocytes was observed in the wild-type C57BL/6J mice, but maximal epithelial-cell sloughing (up to four times that in the C57BL/6J mice) was observed in the mice deficient in  $\gamma\delta$  T cells. This result is somewhat surprising, as we would expect inflammation,

especially that related to macrophage accumulation, to increase with increasing cellular damage in the airway lumen. The caspase 3 staining revealed that the majority of cells undergoing apoptosis are in the distal airways and the airspaces. This is consistent with the BAL epithelial-cell data demonstrating that the majority of the epithelial cells are ciliated, meaning they are derived primarily from the proximal airways. Furthermore, the fact that it takes two weeks to develop significant numbers of apoptotic cells in the peripheral lung suggests that a low, constant dose delivered to the distal airspaces can have deleterious effects that are not detectable at earlier time points. Regardless, these data suggest that epithelial-cell pathology represents the major end point in terms of determining the function of  $\gamma\delta$  T cells in the response to acrolein exposure.

Along these lines, it is notable that the mice deficient in  $\alpha\beta$  T cells did not demonstrate a significant increase in epithelial-cell injury as compared with wild-type mice. The finding is even more interesting given that the mice deficient in  $\alpha\beta$  T cells have increased numbers of  $\gamma\delta$  T cells in the pulmonary interstitium (data not shown). These results suggest that the increase in the  $\gamma\delta$  T-cell subpopulation may contribute to the increased protection from airway injury through the same mechanisms that account for excessive epithelial sloughing in mice deficient in  $\gamma\delta$  T cells.

As with any genetically modified animals, the data and conclusions derived from mice genetically deficient in  $\alpha\beta$  or  $\gamma\delta$  T cells must be interpreted carefully. For example, we observed that mice deficient in  $\alpha\beta$  T cells have a significant increase in the number of  $\gamma\delta$  T cells in the lung. The consequences of this phenomenon are not well documented, but these alterations may subsequently affect the phenotype and, possibly, the function of these cells as well as other lymphocytes, such as natural killer cells. This chain of events is especially important in the mice deficient in  $\alpha\beta$  T cells, in which a small change in the number of  $\gamma\delta$  T cells could in effect be amplified, because these cells normally represent only 1%–2% of the T-cell population. Furthermore, it is important to note that in the absence of  $\gamma\delta$  T cells,  $\alpha\beta$  T cells can become more activated in terms of cytotoxicity and cytokine production (Lahn 2000).

### TRANSCRIPTIONAL ANALYSIS OF $\alpha\beta$ T CELLS AND $\gamma\delta$ T CELLS

The goal of the microarray study was to characterize the differential response of tissue-derived  $\alpha\beta$  and  $\gamma\delta$  T cells in the lungs on exposure to acrolein. We examined T-cell subpopulations isolated from the digested pulmonary tissue as opposed to peripheral blood, because the effects of the local microenvironment can have profound effects on the phenotypes and effector functions of leukocytes. The transcriptional analyses provided insight into the potential

contributions of several genes to the regulation of inflammation and epithelial-cell homeostasis.

The primary end point observed in the mice deficient in  $\alpha\beta$  T cells was a lack of increased macrophage accumulation. This phenotype was observed after exposure to 0.5 ppm acrolein as well as 2.0 ppm acrolein. Global expression analysis of purified  $\alpha\beta$  T cells after acrolein exposure revealed that a number of genes involved in the accumulation and differentiation of macrophages were induced in this cell type. We hypothesize that the coordinated activities of IFN- $\gamma$ , IL-12, and IL-18 and additional chemokines are critical components mediating the cross talk between T cells and tissue macrophages in response to acrolein-induced airway inflammation.

IL-12 and IL-18 are cytokines produced predominantly by macrophages and act to stimulate T cells to, among other things, generate cytokines such as GM-CSF and IFN- $\gamma$ , which activate and enhance the survival of macrophages (Okamura et al. 1998). IFN- $\gamma$  has proliferative effects on T cells and induces IL-12 and IL-18 from macrophages in a process shown to control inflammation and tissue destruction (Trinchieri 1998; Fantuzzi et al. 2000).

Our hypothesis about coordinated cytokine and chemokine activity is further supported by data demonstrating that patients with genetic deficiencies in *Ifngr2* display increased susceptibility to mycobacterial and *Listeria monocytogenes* infections, suggesting that this receptor is part of a critical pathway for the elimination of pathogens and protection of the airways (Remus et al. 2001). Additionally, T cells from *Ifngr2*<sup>-/-</sup> mice have a defect in type 1 helper T-cell differentiation and produce reduced amounts of IFN- $\gamma$  in response to antigen challenge (Lu et al. 1998). Moreover, Il18r1 has previously been shown to play a role in the development of inflammatory lung disease. Specifically, IL-18 and IL-18r1 signaling pathways are critical in the pathogenesis of cigarette smoke-induced disease, partly through the activation of pulmonary macrophages (Kang et al. 2007).

*Il3* represents another important gene up-regulated in  $\alpha\beta$  T cells after acrolein exposure, as it is a potent stimulator of macrophage differentiation and activation (Martinez-Moczygemba and Huston 2003). Furthermore, Ccl5 (RANTES) and Scye1, potent mediators of macrophage accumulation and activation after tissue injury (Murdoch et al. 2004; Keepers et al. 2007), were among the most highly expressed chemokines or cytokines detected in  $\alpha\beta$  T cells in our microarray experiments (Table 7). Although the expression levels of these genes did not change after exposure to acrolein, the lack of these cytokines in mice deficient in  $\alpha\beta$  T cells may contribute to the cells' significant decrease in macrophage accumulation observed.

Notably, the CCL5 receptor, CCR5, has been implicated in the chronic pulmonary pathology due to cigarette smoke (Ma et al. 2005).

The dominant response observed in mice deficient in  $\gamma\delta$  T cells after repeated acrolein exposure was increased sloughing of epithelial cells into the airways and increased expression of the apoptotic marker, active caspase 3. Consistent with a previous report (King et al. 1999), we observed an accumulation of necrotic epithelial cells in the airways of mice deficient in  $\gamma\delta$  T cells after exposure to acrolein. We have extended these findings by examining the result of repeated exposure and assessing more subtle changes in the epithelium by measuring apoptosis. Our data suggest that mice deficient in  $\gamma\delta$  T cells fail to clear damaged or stressed cells, which attenuates the repair and repopulation of the airways with healthy cells. Global gene-expression analysis of  $\gamma\delta$  T cells also gave us important insights into the cells' function in maintaining the integrity of the epithelial barrier in the airways.

We hypothesize that the response to acrolein exposure in  $\gamma\delta$  T cells can be affected by multiple pathways, including the attenuated recruitment of neutrophils and macrophages involved in clearing necrotic cells as well as the lack of clearance of stressed or apoptotic cells through mechanisms mediated by MHC class I and MHC class I-like molecules. The hypothesis is supported by the increase in the expression of genes associated with neutrophil accumulation (*Cxcl1* and *Cxcl2*) and the changes in expression of genes associated with the recognition of MHC class I molecules on target cells (*Klra2*, *Klra9*, *Mpeg1*). Although not known for their roles in tissue remodeling, genes such as *Cd14* (Jiang et al. 2006) and *Cd44* (Teder et al. 2002), which are up-regulated in  $\gamma\delta$  T cells in the current studies, have also been implicated in the response to pulmonary injury and the resolution of inflammation.

In addition to the potential roles of genes whose expression was significantly changed in  $\gamma\delta$  T cells after acrolein exposure, transcriptome analyses indicated that these cells express significant levels of > 20 genes that may be involved in the recognition and removal of injured tissue (data not shown). These genes included lectin-like receptors (MHC class I molecules) and *Ncr1* (a natural cytotoxicity-triggering receptor). These genes are of particular importance because previous studies in our laboratory have demonstrated that levels of MHC class I molecules are decreased on epithelial cells coincident with stress or injury, accompanied by the induction of stress signals on the surface of epithelial cells (Borchers et al. 2006). These findings, along with the localization of  $\gamma\delta$  T cells within the pulmonary epithelium (Wands et al. 2005), are consistent with the idea that, after acrolein exposure,  $\gamma\delta$  T cells

are critical for efficient removal of stressed or injured cells through the loss of self-recognition and the expression of danger signals (Hayday et al. 2001).

Furthermore, it has been suggested that the fibroblast growth factor *Fgf7*, due to its potent impact on epithelial-cell survival, may account for the protective effect of  $\gamma\delta$  T cells in response to injury or infection. However, we observed no differences in *Fgf7* mRNA levels in the whole lungs of wild-type mice or mice deficient in  $\alpha\beta$  T cells or  $\gamma\delta$  T cells at baseline or after repeated acrolein exposure (data not shown).

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### SUMMARY

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Taken together, these results provide compelling evidence to support the hypotheses that  $\alpha\beta$  T cells are critical for the initial and sustained accumulation of macrophages after repeated acrolein exposure, and  $\gamma\delta$  T cells serve a protective role in the lung, maintaining the integrity of the airway epithelium in response to repeated exposure to acrolein. Although we present direct and indirect evidence of roles for  $\alpha\beta$  and  $\gamma\delta$  T cells, the mechanisms remain to be determined. However, these findings provide a strong rationale for study of the effector functions of these cells in the modulation of lung pathophysiology.

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 ABOUT THE AUTHORS
 

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**Michael Borchers**, Ph.D., the principal investigator of this research, is an assistant professor in Environmental Health and Internal Medicine at the University of Cincinnati Medical Center.

**Scott C. Wesselkamper**, Ph.D., is a research scientist at the University of Cincinnati Medical Center. He has extensive experience in pulmonary toxicology and has conducted many studies of the respiratory health effects of nickel, acrolein, and ozone.

**Hitesh Deshmukh**, Ph.D., is a postdoctoral fellow at the National Institute of Environmental Health Sciences. He was a graduate student at the University of Cincinnati Medical Center during the project and assisted with several of the technical aspects involved in examining pulmonary pathology in the mice.

**Erin Beckman**, B.S., is a graduate student at the University of South Carolina. She was a research assistant in Dr. Borchers' laboratory throughout the project and assisted in all aspects of the work.

**Mario Medvedovic**, Ph.D., is an associate professor in the Department of Environmental Health at the University of Cincinnati Medical Center. Dr. Medvedovic's expertise is in developing and applying new statistical and computational procedures for the analysis of complex genomic, functional genomic data, and the development and application of unsupervised statistical learning approaches based on the Bayesian infinite mixture model. Dr. Medvedovic contributed to the design and analysis of the microarray studies.

**Maureen Sartor**, Ph.D., is a research associate in the Department of Environmental Health at the University of Cincinnati Medical Center. She has extensive experience in biostatistics, particularly the analysis of microarray data. Dr. Sartor performed the microarray data analysis, including the MAPPFinder classifications.

**George Leikauf**, Ph.D., is a professor of environmental health at the University of Cincinnati Medical Center. His scientific interests include the genetic analysis of acute lung injury in response to ozone, aldehyde, and nickel exposure. Dr. Leikauf served as a consultant for the acrolein exposure during this project.

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 OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH
 

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Borchers MT, Wesselkamper SC, Harris NL, Deshmukh H, Beckman E, Vitucci M, Tichelaar JW, Leikauf GD. 2007. CD8+ T cells contribute to macrophage accumulation and airspace enlargement following repeated irritant exposure. *Exp Mol Pathol* 83:301–310.

Borchers MT, Wesselkamper SC, Eppert BL, Motz GT, Sartor MA, Tomlinson CR, Medvedovic M, Tichelaar JW. 2008. Nonredundant functions of  $\alpha\beta$  and  $\gamma\delta$  T cells in acrolein-induced pulmonary pathology. *Toxicol Sci* 105: 188–199.

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 ABBREVIATIONS AND OTHER TERMS
 

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$\alpha\beta^{-/-}$ mice	mice deficient in $\alpha\beta$ T cells
BAL	bronchoalveolar lavage
COPD	chronic obstructive pulmonary disease
Cy	cyanine (Cy3, Cy5)
ELISA	enzyme-linked immunosorbent assay
$\gamma\delta^{-/-}$ mice	mice deficient in $\gamma\delta$ T cells
GM-CSF	granulocyte–macrophage colony-stimulating factor
GO	Gene Ontology [hierarchy database]
HBSS	Hanks balanced salt solution
IFN	interferon
IL	interleukin
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
mRNA	messenger RNA
NF- $\kappa$ B	nuclear factor- $\kappa$ B
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
qRT-PCR	quantitative real-time PCR
Rage	renal tumor antigen
SEM	standard error of the mean
TCR	T-cell receptor
TLR	Toll-like receptor



Research Report 146, *The Role of T Cells in the Regulation of Acrolein-Induced Pulmonary Inflammation and Epithelial-Cell Pathology*, M.T. Borchers et al.

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## INTRODUCTION

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Acrolein is a reactive aldehyde that injures the airways in humans and other species. It is an important air toxicant, one of a large and diverse group of air pollutants that, with sufficient exposure, are known or suspected to cause adverse human health effects, including illness and death. Even though ambient levels of air toxics are generally low, these compounds are a cause for public health concern because large numbers of people are exposed to them over prolonged periods of time. In the United States, these compounds are not regulated by the National Ambient Air Quality Standards but are subject to other rules set by the U.S. Environmental Protection Agency (as discussed in HEI 2007).

Dr. Michael Borchers, of the University of Cincinnati College of Medicine, submitted an application, "T Cell Sub-Populations Regulate Airway Inflammation and Injury Following Acrolein Exposures," under Request for Applications 03-2, the Walter A. Rosenblith New Investigator Award, which was established to provide support for outstanding investigators beginning an independent research career. Dr. Borchers reasoned that T cells have a key role in acute inflammation and the maintenance of epithelial integrity, but the role of T cells had not been extensively evaluated in response to acrolein exposure. Thus, he proposed to study the role of various subpopulations of T cells in modulating airway cell responses to acrolein exposure. In particular, he proposed to focus on the potentially protective role in the response of  $\gamma\delta$  T cells, a minor subpopulation of T cells that use a two-chain molecule —  $\gamma$  and  $\delta$  — as their antigen-specific T-cell receptor (TCR\*);  $\gamma\delta$  T cells are found

predominantly at mucosal epithelial sites such as the airways and the gastrointestinal tract.

The HEI Research Committee thought Dr. Borchers' proposed approach to study the role of T cells in responses to acrolein exposure was novel, sound, and likely to produce results useful in understanding the toxicologic mechanism of action of acrolein in the airways. However, the Committee thought it would be valuable for Dr. Borchers to evaluate the role of both  $\gamma\delta$  T cells and  $\alpha\beta$  T cells, the major subpopulation of T cells, which uses a different two-chain molecule as its antigen-specific TCR. In addition, Dr. Borchers had proposed evaluating the effects of acrolein after a 4-week exposure but the Committee thought that evaluating shorter exposures would also provide useful information. After discussions with the Research Committee, Dr. Borchers agreed to investigate the roles of  $\alpha\beta$  and  $\gamma\delta$  T cells and to examine responses after 1, 2, and 4 weeks of exposure. The Research Committee recommended the proposed study for funding.

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## SCIENTIFIC BACKGROUND

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### SOURCES OF ACROLEIN AND EFFECTS OF ACROLEIN EXPOSURE

Like other air toxics, acrolein is emitted by a variety of indoor and outdoor sources but primarily from the incomplete combustion of automotive fuels, tobacco, wood, and cooking oils and fats (ATSDR 2007). Levels of acrolein are usually low in outside air, averaging around 0.12 ppb in rural air and 0.2 ppb in urban air, although levels of acrolein as high as 5.6 ppb have been measured in several large cities (ATSDR 2007).

At relatively low concentrations in controlled human exposures, acrolein is a respiratory irritant: exposure of healthy people to 0.3 ppm acrolein induces eye, nose, and throat irritation and decreased respiratory rate after 10 to 40 minutes (Weber-Tschopp et al. 1977, ATSDR 2007). Accidental human exposures to higher concentrations of acrolein vapors have resulted in symptoms associated with chronic obstructive pulmonary disease (COPD) (Mahut et al. 1993). In vitro studies of human cells exposed to acrolein have found increased levels of expression of mucin genes in airway epithelial cells (Borchers et al. 1999a), consistent with the induction of an inflammatory response.

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Dr. Michael T. Borchers' 3-year study, "T Cell Sub-Populations Regulate Airway Inflammation and Injury Following Acrolein Exposures," began in November 2003. Total expenditures were \$300,052. The draft Investigators' Report from Borchers and colleagues was received for review in July 2007. A revised report, received in January 2008, was accepted for publication in February 2008. During the review process, the HEI Health Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in both the Investigators' Report and in the Review Committee's Critique.

This document has not been reviewed by public or private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views of these parties, and no endorsements by them should be inferred.

\*A list of abbreviations and other terms appears at the end of the Investigators' Report.

Acrolein exposure also damages rodent airways. Acute exposure to acrolein at 1 ppm or less depresses the respiratory rate in mice (Steinhagen et al. 1984) and induces bronchial hyperresponsiveness and airway inflammation in guinea pigs (Leikauf et al. 1989). Exposure of rodents to acrolein at higher concentrations results in changes in airway structure and histologic and biochemical alterations associated with COPD, including airspace enlargement, mucous-cell metaplasia, and increased mucin gene expression (e.g. Costa et al. 1986, Borchers et al. 1998, Borchers et al. 1999b).

### T CELLS AND RESPONSE TO AIR POLLUTANTS

All T cells express one of two different two-chain antigen-specific receptors, either the  $\alpha\beta$  or the  $\gamma\delta$  TCR; thus, expression of the TCR divides T cells into two subpopulations,  $\alpha\beta$  or  $\gamma\delta$  T cells—also referred to as  $\alpha\beta^+$  or  $\gamma\delta^+$  T cells, respectively, to denote expression of the respective genes. Both  $\alpha\beta$  and  $\gamma\delta$  T cells are found in the lung as well as other tissues. The  $\alpha\beta$  T cells are the major subpopulation, predominating in blood and most tissues; with B cells, they play a critical role in the adaptive immune response to antigens when external agents such as microbes enter the body. “Adaptive” signifies that the response improves and changes with a subsequent exposure to the antigen—a memory response is produced. Most  $\alpha\beta$  T cells recognize and respond to the protein components of antigens; to be more specific,  $\alpha\beta$  T cells recognize and respond to peptide fragments of protein antigens that are expressed on the surface of a host cell such as a macrophage; the  $\alpha\beta$  TCR interacts with peptide fragments bound to proteins known as major histocompatibility complex (MHC) molecules that are also expressed on the surface of host cells.

The  $\gamma\delta$  T cells are found predominantly at mucosal epithelial surfaces, such as the skin, airways, and intestinal tract. Compared to  $\alpha\beta$  T cells, less is known about the function of  $\gamma\delta$  T cells, but they have been suggested to play a role in defense at epithelial surfaces, both in response to bacterial antigens and in tissue repair (Born et al. 2000, DeFranco et al. 2007). The  $\gamma\delta$  T cells respond to non-protein antigens and to proteins induced by stress in host cells. Responses of  $\gamma\delta$  T cells occur rapidly after exposure to antigen and it is not clear that re-exposure to antigen changes or improves this response; thus,  $\gamma\delta$  T cells may be considered components of the innate immune system, which also includes cells such as macrophages and neutrophils, or alternatively, one of the minor subpopulations of T cells that has a specialized niche and function (De Franco et al. 2007). Antigen recognition by  $\gamma\delta$  T cells does not involve the recognition of an MHC molecule expressed by a host cell; rather, many antigens are presented to  $\gamma\delta$  T cells by a family of molecules known as CD1.

Few studies have evaluated the involvement of T cells in responses to air pollutants. Some studies on mice, however, have suggested T-cell involvement in the regulation of the inflammatory response after exposure to ozone. For example, protocols that deplete T cells—either by using the immunosuppressive agent cyclosporine (Dziedzic and White 1987, Bleavins et al. 1995) or an antibody to deplete the major subpopulation,  $\alpha\beta$  T cells (Chen et al. 1995)—resulted in increased lung lesion volumes and reaction by acute inflammatory cells. In addition, mice genetically depleted of  $\gamma\delta$  T cells ( $\gamma\delta$ -knockout mice) that were exposed to ozone (1.5 ppm for 8 hours) were unable to recruit inflammatory cells into the airways and had decreased clearance of necrotic epithelial cells in the terminal airways compared to control mice (King et al. 1999). These findings from knockout mice suggest that  $\gamma\delta$  T cells can respond to airway damage and play a role in maintaining epithelial integrity in the response to an air pollutant (in this case, ozone). In the current study, Dr. Borchers and colleagues sought to explore the role of  $\alpha\beta$  T cells and  $\gamma\delta$  T cells in the airway response to another air pollutant, acrolein.

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### STUDY AIMS

The purpose of this study was to examine the role of T cells expressing either  $\alpha\beta$  or  $\gamma\delta$  as their TCR in the response to acrolein exposures. Borchers and colleagues proposed the following two major aims:

1. To determine the role of  $\alpha\beta^+$  and  $\gamma\delta^+$  pulmonary T cells in inflammation and epithelial injury after repeated acrolein exposure, and
2. To examine the gene-expression profiles of purified pulmonary  $\alpha\beta^+$  and  $\gamma\delta^+$  T-cell subpopulations after repeated acrolein exposure of control mice.

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### METHODS

#### MICE AND ACROLEIN EXPOSURE

The investigators used 8–12-week-old wild-type (C57BL/6J) mice and mice genetically deficient either in  $\gamma\delta$  T cells or in  $\alpha\beta$  T cells ( $\gamma\delta$ - or  $\alpha\beta$ -knockout mice). Mice were exposed to 0.5 or 2 ppm acrolein vapor (generated by passing nitrogen over liquid acrolein), or were sham-exposed (to filtered air), for 6 hours per day, 5 days per week, for 1, 2, or 4 weeks.

#### Bronchoalveolar Lavage and Lung-Cell Preparation

Immediately after exposure, mice were anesthetized and bronchoalveolar lavage (BAL) was performed. BAL cells

were examined for total and differential cell numbers. The numbers of epithelial cells detected in BAL fluid were used as a marker of epithelial-cell injury, presumably resulting from necrosis of cells in the proximal airways. Using an enzyme-linked immunosorbent assay (ELISA), BAL supernatant was assayed for levels of mucin (Muc5ac).

After the mice were killed, lung-tissue samples were fixed and prepared for histologic and immunohistochemical analyses. Using histochemical staining, fixed cells were evaluated for expression of activated caspase 3, a marker of apoptosis (programmed cell death). Mucous-cell metaplasia was assessed by performing light microscopy of fixed cells.

Using ELISA on whole lung cells, the investigators measured levels of two cytokines, interferon- $\gamma$  (IFN- $\gamma$ ) and granulocyte-macrophage colony-stimulating factor (GM-CSF), that are produced by T cells and activate macrophages.

Lung mononuclear cells were prepared by centrifugation on a Percoll gradient. The  $\alpha\beta^+$  and  $\gamma\delta^+$  T-cell subpopulations from wild-type mice were separated by flow cytometry using appropriate antibodies—that is, using an antibody specific for cells expressing CD3, a T cell-specific surface marker expressed by both  $\alpha\beta^+$  T cells and  $\gamma\delta^+$  T cells, and either the TCR  $\beta$  chain yielding highly purified  $\alpha\beta^+$  T cells) or the TCR  $\delta$  chain (yielding highly purified  $\gamma\delta^+$  T cells).

### Gene-Expression Experiments

**Microarrays** Total cellular RNA was isolated from purified lung  $\alpha\beta^+$  or  $\gamma\delta^+$  T-cell subpopulations from wild-type C57BL/6J mice that had been exposed to either 2 ppm acrolein or filtered air for 1 week. Samples from multiple mice were pooled for the microarray experiments; four separate arrays were performed for experiments with  $\gamma\delta$  T cells, and four arrays for experiments with  $\alpha\beta$  T cells. The microarray contained more than 30,000 70-base-pair probes. Data generated from the microarray experiments were analyzed to identify genes expressed differentially in cells from acrolein-exposed mice (compared to cells from control mice) in either  $\alpha\beta^+$  or  $\gamma\delta^+$  T cells or both.

Borchers and colleagues used the program MAPPFinder to organize the measured changes in gene expression into several discrete biologic pathways; in this way they could determine the types of biologic processes and functions that had been affected in  $\alpha\beta^+$  or  $\gamma\delta^+$  T cells through exposure to acrolein. As a complement to MAPPFinder, they used a similar program, PANTHER database ([www.pantherdb.org](http://www.pantherdb.org)), to obtain complementary information.

**Quantitative Real-Time PCR** To confirm findings for some of the changes in gene expression identified by microarray

experiments, the investigators performed quantitative real-time polymerase-chain reaction (qRT-PCR) on RNA from 15 selected genes.

### Statistical Analysis

For biologic end points, the investigators analyzed data using one-way analysis of variance, with differences between means considered significant when  $P < 0.05$ .

To examine differences in the levels of gene expression between the acrolein-exposure group and the control group, the investigators used log-transformation and local regression to normalize data and applied an empirical Bayesian moderated- $t$  method (Smyth 2004). The investigators considered genes to be differentially expressed when they met the following criteria: both cyanine 3 (Cy3) and cyanine 5 signals were detected, the fold change in expression was  $> 2$ , and the  $P$  value for the differential expression was  $< 0.005$ . Of the transcripts identified as being differentially expressed in at least one of the two T-cell subpopulations examined, expression of a transcript was defined as having changed in both cell subgroups if both  $P$  values were  $< 0.05$ .

## RESULTS

### EFFECTS OF ACROLEIN ON MOUSE AIRWAYS

Critique Table 1 summarizes the responses in the airways—as measured in lung tissue and BAL fluid—of wild-type C57BL/6J mice, mice deficient in  $\alpha\beta$  T cells, and mice deficient in  $\gamma\delta$  T cells, after acrolein exposure for 1, 2, or 4 weeks.

In the airways of wild-type mice and mice deficient in  $\gamma\delta$  T cells, exposures to acrolein increased epithelial-cell sloughing (resulting in increased epithelial-cell numbers in BAL fluid), apoptosis (measured as cells stained with active caspase 3—most of which were found in distal airways and airspaces), and macrophage numbers. The effects appeared to be somewhat more pronounced (having either a higher level of response or an increased response at lower acrolein exposure) in mice deficient in  $\gamma\delta$  T cells than in wild-type mice. By contrast, mice deficient in  $\alpha\beta$  T cells that were exposed to acrolein did not show effects on epithelial-cell injury, apoptosis, or macrophage numbers. All three strains of mice showed similar, but small, increases in the mucus-cell index (reflecting the total amount of mucus in airways and the number of airways affected) and similar increases in levels of the cytokines IFN- $\gamma$  and GM-CSF in the airways.

**Critique Table 1.** Responses to Acrolein Exposure in Lung Cells and BAL Fluid<sup>a</sup>

Mouse	Mucus Index <sup>b</sup>	BAL Epithelial Cells	Staining with Active Caspase <sup>c</sup>	Macrophages in BAL Fluid	IFN- $\gamma$ and GM-CSF Levels
Wild type	↑ at 2 and 4 weeks	2.0 ppm: ↑ at 1, 2, and 4 weeks 0.5 ppm: =	2.0 ppm: ↑ at 4 weeks	2.0 ppm: ↑ at 1, 2, and 4 weeks 0.5 ppm: ↑ at 2 and 4 weeks	↑ at 2 weeks
$\alpha\beta$ deficient	↑ at 2 and 4 weeks	2.0 and 0.5 ppm: =	2.0 and 0.5 ppm: =	2.0 and 0.5 ppm: =	↑ at 2 weeks
$\gamma\delta$ deficient	↑ at 2 and 4 weeks	2.0 ppm: ↑↑ at 1, 2, and 4 weeks 0.5 ppm: ↑ at 1, 2, and 4 weeks	2.0 ppm: ↑ at 2 weeks 0.5 ppm: ↑↑ at 4 weeks	2.0 ppm: ↑ at 2 weeks 0.5 ppm: ↑ at 2 and 4 weeks	↑ at 2 weeks

<sup>a</sup> = Indicates no change. A single upward arrow indicates a small increase; two upward arrows, a greater increase.

<sup>b</sup> The mucus index reflects the sum of the amount of mucus per airway, as well as the number of airways affected.

<sup>c</sup> Staining with active caspase 3 is a marker of epithelial-cell apoptosis; most cells that were stained were found in distal airways and airspaces.

**EFFECTS OF ACROLEIN EXPOSURE ON GENE-EXPRESSION CHANGES**

**Microarray Experiments**

The investigators reported that the expression of more than 1000 genes was changed in either  $\alpha\beta$  or  $\gamma\delta$  T cells isolated from wild-type C57BL/6J mice after a 1-week exposure to 2 ppm acrolein. The expression of some genes—28% of the total changes in gene expression—was changed in both subpopulations (increased or decreased in both subpopulations, or increased in one, decreased in the other subpopulation). The remaining 72% of the changes in gene expression measured were unique to either the  $\alpha\beta$  or to the  $\gamma\delta$  T-cell subpopulations (either increased or decreased in either  $\alpha\beta$  or  $\gamma\delta$  T cells).

**qRT-PCR Validation of Microarray Data**

To validate the changes in gene expression observed in the microarray experiments, Borchers and colleagues studied 15 genes for qRT-PCR analysis. The genes were selected on the basis of either differential expression (increased or decreased) in  $\alpha\beta^+$  or  $\gamma\delta^+$  T-cell subpopulations or high-level expression in both. Most of the qRT-PCR findings supported the changes in gene expression obtained from the microarray data; however, for some genes, the fold differences in expression differed substantially as measured using the two approaches.

**Candidate Genes and Biologic Pathways Potentially Affected by Exposure to Acrolein**

From the changes in gene expression detected in the microarray experiments, the investigators singled out several

genes in each subpopulation (Tables 1 and 2 of the Investigators' Report) that were considered candidates for further study regarding their responses to acrolein exposure: To explain the major biologic finding associated with  $\alpha\beta$  T-cell deficiency—namely, a lack of macrophage accumulation in the lung—Borchers and colleagues interpreted the transcriptional data from  $\alpha\beta$  T cells after acrolein exposure to suggest that the key changes were in the expression of genes coding for cytokines and chemokines and their receptors, in particular, cytokines and chemokines associated with the activation of T cells and the consequent activation and accumulation of macrophages. These included genes coding for interleukin-3 (IL-3) and for components of the receptors for IFN- $\gamma$ , IL-12, and IL-18.

Similarly, to explain the major biologic finding associated with  $\gamma\delta$  T-cell deficiency—enhanced epithelial-cell injury—the investigators interpreted the transcriptional data to suggest that the key changes in gene expression found in  $\gamma\delta$  T cells only were associated with changes in host-cell recognition, affecting the clearance of damaged host tissue, cellular interactions, and certain cytokines and chemokines and their receptors. These genes included *Klra2*, *Klra9*, *Hmgb1*, *Daf2*, *Cd14*, *Mpeg1*, *Cd28*, *Cd44*, and *Serpine1*.

Using the MAPPFinder program to link the microarray data to a gene database to identify biologic processes affected by acrolein exposure, the investigators found several intracellular pathways that were affected in  $\alpha\beta^+$  and  $\gamma\delta^+$  T cells (Tables 5 and 6 of the Investigators' Report). Some overlap between the subpopulations was noted (e.g., in effects on the “MHC protein complex”), but some differences were also found in the pathways affected in  $\alpha\beta^+$  and  $\gamma\delta^+$  T cells.

On the basis of the most statistically significant findings, organic anion transport and mitochondrial apoptosis were concluded to be affected in  $\alpha\beta^+$  T cells, whereas peptide antigen binding and pathways characterized as defense response, response to biotic stimulus, immune response, and response to stimulus were considered to be affected in  $\gamma\delta^+$  T cells.

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#### HEI REVIEW COMMITTEE EVALUATION OF THE STUDY

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The HEI Review Committee thought that Dr. Borchers and colleagues had successfully designed and conducted a preliminary study in mice to characterize the involvement of two subpopulations of T cells,  $\alpha\beta$  and  $\gamma\delta$  T cells, in the airway response to acrolein exposure via inhalation. Comparisons among the results were facilitated by the investigators' use of strains of mice—wild-type mice and mice genetically deficient in either  $\alpha\beta$  or  $\gamma\delta$  T cells—that were matched with regard to background genes. In addition, the differences in outcomes appeared clear-cut in terms of most airway responses and epithelial pathology.

Thus, this animal study provides useful insights into the host response and pathology associated with acrolein exposure; it also provides useful preliminary descriptions of the possible roles of two groups of T cells during the course of the reparative host response. Overall, the results of the study encourage more-detailed and mechanism-oriented studies to explore the role of T cells in the pathology associated with acrolein exposure.

The Committee agreed with the investigators' general conclusions that T cells appear to play a role in the lungs' responses to acrolein exposure and that the data generally support the view that the  $\alpha\beta$  and  $\gamma\delta$  T-cell subpopulations have different roles in the response to acrolein:  $\alpha\beta$  T cells primarily promote the accumulation of macrophages and  $\gamma\delta$  T cells protect the integrity of airway epithelial cells. The Committee also thought that a separation of the inflammatory response (represented by macrophage accumulation in BAL fluid) from epithelial-cell repair in the airway (represented by increased epithelial cells in BAL fluid) accorded with the interpretation of recent studies of wound healing in the mouse epidermis, in which inflammatory responses could be separated from tissue-repair responses (Martin et al. 2003, Cooper et al. 2005). The Committee also noted that lung toxicants other than acrolein, such as naphthalene, injure airway cells in the absence of inflammation (West et al. 2001).

The Committee noted that caution is needed in the interpretation of the roles of  $\alpha\beta$  and  $\gamma\delta$  T-cell subpopulations in

this and other studies using mice that are genetically deficient in either type of T cells. One reason is that the T-cell deficiency may have affected the development of the animals in ways other than the failure to develop the particular subpopulation of T cells. Thus, other approaches to addressing the roles of specific T-cell subpopulations, such as transiently or locally depleting the cells in an intact animal or transferring purified T-cell subpopulations into deficient animals, should be pursued to confirm or refute the results of the current study.

Another issue to consider with the use of mice completely deficient in  $\gamma\delta$  or  $\alpha\beta$  T cells is that each of these T-cell subpopulations is heterogeneous, containing numerous functionally specialized cell subsets (Born et al. 2000). For example,  $\alpha\beta$  T cells contain natural killer T cells, which have functions and antigen-recognition pathways distinct from those of the classical  $\alpha\beta$  T cells that participate in the adaptive immune system;  $\gamma\delta$  T cells can also be divided into subsets with distinct functions. Thus, mice deficient in all T cells of a given type might display only a net effect of numerous subpopulation deficiencies, some of which may cancel each other out.

Furthermore, as the investigators acknowledge, a genetic deficiency in T cells may result in compensatory changes in the remaining T cells; perhaps of most relevance in this context, Borchers and colleagues report that mice deficient in  $\alpha\beta$  T cells had increased numbers of  $\gamma\delta$  T cells in the pulmonary interstitium. The consequences of such compensatory changes are hard to assess, and assessment was not attempted in the current study. Such changes may result in altered composition of subsets of the  $\gamma\delta$  T-cell subpopulation or a change in functional competence of the  $\gamma\delta$  T cells (Born et al. 2000, Lahn 2000). These considerations do not invalidate the results of the current study but do place important caveats on the interpretation of the results.

A protective role for  $\gamma\delta$  T cells on the integrity of lung epithelial cells has also been proposed, on the basis of the comparison of responses of wild-type mice and mice deficient in  $\gamma\delta$  T cells after exposure to ozone (King et al. 1999). Thus, both the current study and the ozone study suggest that  $\gamma\delta$  T cells play a protective role in the airways in response to air toxicants. King and colleagues (1999) interpreted the findings of their ozone study to suggest that exposure to ozone caused damage or stress to epithelial cells in the airways and that these damaged cells were recognized by  $\gamma\delta$  T cells. Although this possibility was not examined in the current study, it is a plausible explanation for the involvement of  $\gamma\delta$  T cells in the response to acrolein.

How  $\alpha\beta$  T cells might play a role in the response to acrolein was also not evaluated in the study by Borchers and colleagues. However, a response involving  $\alpha\beta$  T cells

is plausible because acrolein is highly reactive and is likely to alter the structure of some host proteins. Peptides derived from these altered host molecules would then be presented to  $\alpha\beta$  T cells by antigen-presenting cells (such as dendritic cells or macrophages).

The Committee thought that Dr. Borchers' microarray experiments to assess changes in gene expression in  $\alpha\beta$  and  $\gamma\delta$  T cells purified from wild-type mice after a 1-week exposure to 2 ppm acrolein pointed to potentially useful directions for future research. The Committee agreed that exposure to acrolein up-regulated the expression of many genes and down-regulated the expression of many others and that the two T-cell subpopulations showed some differences in the pattern of gene-expression changes. The Committee thought the investigators' interpretations of the candidate genes and biologic pathways implicated in the response of  $\alpha\beta$  and  $\gamma\delta$  T cells to acrolein exposure were interesting. However, because the expression of so many genes was affected by acrolein and the changes in expression of many of the genes selected by Borchers and colleagues were small, the Committee considered the interpretations to be speculative, requiring further experiments for confirmation or refutation. Nonetheless, the Committee thought that the approaches and results of the current study suggest more-detailed and mechanistic studies to explore the role of T cells in the pathology associated with acrolein exposure.

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## SUMMARY AND CONCLUSIONS

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Dr. Borchers and colleagues successfully designed and conducted a preliminary descriptive study in mice genetically depleted of one or other key subpopulations of T cells—those using either  $\alpha\beta$  or  $\gamma\delta$  as their antigen-specific receptor—to study the airway response to acrolein exposure via inhalation. The study provides useful insights into the host response and pathology associated with acrolein exposure; it also provides useful preliminary descriptions of the roles of the two groups of T cells during the course of the host response.

These findings suggest that T cells play a role in the lungs' responses to acrolein exposure, and the data generally support the view that  $\alpha\beta$  and  $\gamma\delta$  T-cell subpopulations have different roles in the response to acrolein:  $\alpha\beta$  T cells primarily promote the accumulation of macrophages and  $\gamma\delta$  T cells protect the integrity of airway epithelial cells.

In its independent review of the study, the HEI Review Committee generally agreed with the investigators' interpretations of the biologic responses to acrolein exposure and thought that the results showing dissociation between inflammation (attributable to macrophages and their products) and epithelial damage were interesting and compatible with recent data showing injury responses in the absence of inflammation. However, the Committee noted caution in interpreting results of any study based on use of mice that are entirely genetically deficient in  $\alpha\beta$  or  $\gamma\delta$  T cells; of particular concern are uncertainties about the possibilities of unexamined developmental changes in these animals and compensatory increases in the remaining subpopulations of T cells. In addition,  $\alpha\beta$  and  $\gamma\delta$  T cells are heterogeneous, each containing numerous functionally specialized subsets; thus, mice deficient in all T cells of a given type might display a net effect of deficiencies of numerous subsets, some of which may cancel each other out.

The Committee also thought that the studies by Borchers and colleagues of changes in gene expression in purified  $\alpha\beta$  and  $\gamma\delta$  T cells from intact control mice exposed to 2 ppm acrolein for 1 week pointed to potentially useful directions for future research. The Committee agreed that several genes in each subpopulation were up-regulated, and several were down-regulated, by exposure to acrolein; moreover, some genes were expressed differently in the two T-cell subpopulations. However, the Committee found the investigators' interpretations of differences in expression of key genes and involvement of different biologic pathways in  $\alpha\beta$  and  $\gamma\delta$  T cells were interesting but speculative, requiring further experiments for confirmation or refutation. Nonetheless, the Committee concluded that the approaches and results of Borchers and colleagues will encourage more-mechanistic studies to explore the role of T cells in the pathology associated with exposure to acrolein.

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