Immune Mediators in a Murine Model for Occupational Asthma: Studies with Toluene Diisocyanate

Joanna M. Matheson, Victor J. Johnson, and Michael I. Luster

Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505

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Isocyanate-induced asthma, which is the most common type of occupational asthma, has been difficult to diagnose and control, in part, because the biological mechanisms responsible for the disease and the determinants of exposure are not fully defined. To help address these issues, we recently established a murine model of toluene diisocyanate (TDI) asthma using inhalation exposure paradigms consistent with potential workplace exposure. In order to confirm our hypothesis that TDI-induce asthma, like allergic asthma, is predominantly a Th2 response, the ability of mice that were deficient in CD4 or CD8 cells or specific Th1 and Th2 cytokines to develop TDI asthma was examined. The development of allergic asthma was evaluated by monitoring lungs for the presence of eosinophilia, goblet cell metaplasia, epithelial cell alterations, airway hyperreactivity (AHR), and Th2 and Th1 cytokine expression, as well as serum IgE levels and TDI-specific IgG antibodies. Transgenic CD8 or CD4 knockout (KO) mice exhibited significant reductions in AHR, cytokine expression, serum antibody levels, airway inflammation, and histopathological lesions, although in a number of the endpoints the effects were more attenuated in CD4 KO mice. IFNγ depletion ablated the increase in AHR in TDI-allergic mice, but had only slight to moderate effects on airway histopathology, serum antibody levels, and cytokine expression compared to sensitized/challenged controls. IL-4 and IL-13 deficiency had moderate inhibitory effects, while combined IL-4/IL-13 depletion effectively prevented almost all asthma-associated pathologies. Taken together, these results indicate that TDI asthma, like immune-mediated asthma produced by large-molecular-weight materials, is driven primarily by CD4+ T cells and is dependent upon the expression of Th2 cytokines. However, as with protein-induced asthma models, certain pathologies are influenced by CD8+ T cells and Th1-derived cytokines, such as AHR and cytokine production.

Key Words: isocyanate-induced asthma; toluene diisocyanate; airway hyperreactivity; cytokines; occupational asthma.

Asthma is present in 10–15% of the adult population and is more common in industrialized settings, where it has been estimated that up to 15% of all cases are associated with workplace exposure (Baur et al., 1998). Isocyanates, such as toluene diisocyanate (TDI), are the most common low-molecular-weight class of chemicals responsible for occupational asthma (OA), with over 250,000 workers in the United States exposed every year and 5 to 15% of these developing asthma (Tee et al., 1998). Clinically, diisocyanate asthma displays similar manifestations to allergic asthma induced by high-molecular-weight allergens, suggesting common pathogenic processes, although clinical studies have highlighted several important differences such as a low association with atopy, a low prevalence of specific IgE antibodies, a mixed T-helper (i.e., Th1 and Th2) response, and the presence of high numbers of antigen-specific CD8 T cells (Del Prete et al., 1993; Finotto et al., 1991; Gautrin et al., 2003; Maestrelli et al., 1994; Mapp et al., 1994; Rauf-Héimsoth and Baur, 1998; Tee et al., 1998).

As recently reviewed by Johnson et al. (2004), studies in murine models of occupational asthma using epicutaneous sensitization protocols have supported an immune etiology in diisocyanate-induced asthma. For example, Dearman et al. (1996) demonstrated that topical application of TDI induced the production of specific IgE antibodies with both CD4+ and CD8+ T cells participating as response effectors. Scheerens et al. (1996), using epicutaneous sensitization and intranasal challenge, showed that TDI led to increased airway hyperreactivity (AHR) to carbachol, and the response could be adoptively transferred with lymphocytes from sensitized mice, suggesting an immunological etiology. Herrick et al. (2002), using a similar exposure design with hexamethylene diisocyanate (HDI), detected airway eosinophilia, mucous hypersecretion, and induction of both Th1 and Th2 cytokines. Subsequent studies from this group (Herrick and Bottomly, 2003) showed that CD4+ T cells were critical for airway eosinophilia, while CD8+ T cells were the major effector cells in contact hypersensitivity. Previous studies in our laboratory demonstrated that low-level subchronic and, to a lesser extent, acute high-dose inhalation to TDI effectively sensitized mice, resulting in challenge-induced increases in AHR, airway eosinophilia, mucous hypersecretion, TDI-specific serum antibodies, and elevated pulmonary Th1 and Th2 cytokines (Matheson et al., 2005). Furthermore, in extending observations by...
Experimental animals. All knockout (KO) mice had been bred onto C57BL/6 J backgrounds by the supplier. Female wild-type C57Bl/6 J, CD4 KO (original stock B6.129S2-Cd4tm1Zav), CD8 KO (original stock B6.129S2-Cd8tm1Zav), and IL-4 KO (original stock B6.129P2-Il4tm1Zav) mice were obtained from Jackson Laboratory (Bar Harbor, ME), at approximately 5 to 6 weeks of age. Upon arrival the mice were acclimated for 2 weeks prior to experimentation. Animals were housed in microisolator cages in specific pathogen-free and environmentally controlled conditions at NIOFS facilities in compliance with AAALAC approved guidelines and an approved IACUC protocol (#03-JM-M005). Food and water were provided ad libitum. Groups of C57BL/6 J mice received filtered neutralizing antibodies to IFNγ (200 μg, BD-PharMingen, San Diego, CA), intravenously, 24 h prior to TDI exposure, and intraperitoneally (ip), at week 4 of the exposure and are designated x-IFNγ. Additional groups of wild-type control and IL-4 KO mice were administered 200 μl polyclonal rabbit anti-mouse IL-13 antibodies (representing 140,000 units, Biosource) or an equal volume of nonimmune sera, intravenously, 24 h prior to the study and ip weekly. Mice receiving neutralizing IL-13 antibodies are designated x-IL-13. These concentrations of antibody administered to mice 24 h prior to injection of 0.5 mg/kg LPS (Sigma Chemical Co., St Louis, MO) fully prevented the detection of the specific cytokine in the serum, as assessed by enzyme-linked immunosorbent assay (ELISA) (data not shown). Isotype control antibodies did not influence any of the parameters examined (data not shown).

Experimental design. Mice were exposed by inhalation to 20 ppb of TDI (Mondur TD60; 80:20 molar mixture of 2,4:2,6 isomers provided by Bayer, USA, Pittsburgh, PA) for 6 weeks, 5 days per week, 4 h per day in a 10-l inhalation chamber, with only the heads of the animals extended into the chamber. TDI vapors in the chamber were generated by passing dried air through an impinger that contained 3 ml TDI. A computer interfaced mass flow controller (Model GPC-37, 0–20 LPM, Aalborg Instruments, Orangeburg, NY) regulated the diluent air. Temperature GFC-37, 0–20 LPM, Aalborg Instruments, Orangeburg, NY) regulated the TDI that contained 3 ml TDI. A computer interfaced mass flow controller (Model GPC-37, 0–20 LPM, Aalborg Instruments, Orangeburg, NY) regulated the TDI vapor, free of TDI aerosol. Real-time transmissions never varying more than 0.5 ppb. Challenge by inhalation (1 h, 20 ppb TDI) was performed following a rest period of 14 days during which there was no exposure to TDI. The 6-week exposure period is the time during which sensitization to TDI develops in the current model (Matheson et al., 2005). Therefore mice that were exposed to TDI during this 6-week period followed by challenge are, henceforth, referred to as “sensitized/challenged” groups. Two controls groups were examined, including an air-sensitized/air-challenged and air-sensitized/TDI-challenged treatment group. As both control groups responded similarly, for convenience, only results from the air-sensitized/TDI-challenge control treatment are shown and are, henceforth, referred to as “controls.” Administration of cytokine-specific neutralizing antibodies to the control mice did not influence any of the parameters tested (data not shown). In addition, CD4 KO, CD8 KO, and IL-4 KO mice exposed to air for 6 weeks followed by challenge with TDI were not different from concurrent wild-type mice for the parameters examined (data not shown). Thus, for convenience only wild-type control values are presented.

Tissue collection. Forty-eight hours after airway challenge, mice were sacrificed by CO2 asphyxia, and lungs were collected. Lungs were inflated with 1.0 ml of 10% neutral buffered formalin (NBF), and immersed in 10% NBF for 24 h. The tissues were embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin for histopathological assessment. PAS staining was performed to detect goblet metaplasia, and Chromatique 2R/Mayer’s Hematoxylin staining for eosinophil identification. The histopathological grading system was performed blinded and expressed on a 0–5 scale for each animal, with 0 representing no change, 1 equal to slight, 2 equal to slight/mild changes, 3 equal to moderate changes, 4 equal to moderate/severe changes, and 5 equal to severe changes. Additional groups of mice were utilized for bronchoalveolar lavage fluid (BALF) and blood collection 24 h after challenge. To obtain BALF, mice were anesthetized, exsanguinated, and intubated with a 20-gauge cannula positioned at the tracheal bifurcation. Each mouse lung was lavaged three times with 1.0 ml of sterile HBSS and the fluid pooled. Samples of BALF (105 cells in 0.1-ml volumes) from individual mice were used for cytospin preparations. The slides were fixed and stained with Diff-Quick (VWR, Pittsburgh, PA), and differential cell counts were obtained using light microscopic evaluation of 300 cells/slide. Total cell counts were performed with a hemocytometer. Nonlaved lungs were collected 24 h after challenge, frozen in RNAlater (Qiagen, Valencia, CA) or liquid nitrogen and stored at −80°C for reverse transcription-polymerase chain reaction (RT-PCR) analysis. Tissues frozen in liquid nitrogen were incubated with RNAlaterICE (Ambion, Austin, TX) at −20°C for 24 h prior to RNA isolation.

Antibody detection. Total serum IgE levels were measured using a modified (Matheson et al., 2001) sandwich ELISA (Satoh et al., 1995) employing rat monoclonal anti-mouse IgE (BD-PharMingen) as the capture antibody. Serial two-fold dilutions of test sera, starting at 1:5, were added and incubated with peroxidase-conjugated anti-mouse IgE (1:1000, Nordic Immunological Laboratories, Capistrano Beach, CA) and developed with ABTS substrate (2,2′-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]). TDI-specific IgG antibodies were detected by ELISA using a TDI-mouse serum albumin conjugate, using a previously described procedure (Satoh et al., 1995), kindly provided by Dr. Meryl Karol (University of Pittsburgh PA), and modified in our lab (Matheson et al., 2001).

Eosinophil peroxidase activity (EPO). EPO activity was measured in BALF supernatants of mice according to the method of Bell et al. (1996), with slight modifications. Briefly, 0.1 ml of peroxidase substrate solution consisting of o-phenylenediamine dihydrochloride (OPD), urea hydrogen peroxide, and phosphate-citrate buffer (Sigma Fast Tablets, Sigma, St. Louis, MO) was added to 0.1 ml of the BAL supernatant. The mixture was incubated at 37°C for 30 min before stopping the reaction with 50 μl of 2 N hydrochloric acid. Optical densities were measured at 490 nm (OD490). Nonspecific reactions (always <10%) were corrected by treating duplicate sample sets with the EPO inhibitor, 2 mM 3-aminol-2,4-triazole (Sigma). The results were expressed as OD490 corrected for background and volume of supernatant retrieved.

Airway hyperreactivity (AHR). Airway responsiveness was assessed 24 h following TDI challenge in response to increasing concentrations of methacholine, using a single chamber whole body plethysmograph (Buxco, Troy, NY). A spontaneously breathing mouse was placed into the main chamber of the plethysmograph, and pressure differences between the main chamber and a reference chamber were recorded. AHR was expressed as enhanced pause (Penh), which correlates with measurement of airway resistance, impedance, and intrapleural pressure, and is derived from the formula: Penh = (Te − Tr)/Tr × PeF / Pif; where Te = expiration time, Tr = relaxation time, Pef = peak expiratory flow, and Pif = peak inspiratory flow (Schwarze et al., 1999). Mice were placed into the plethysmograph and exposed for 3 min to nebulized PBS followed by 5 min of data collection to establish baseline values. This was followed by increasing concentrations of nebulized methacholine (0–50 mg contained in 1.0 ml of PBS) for 3 min per dose using an Aerofsonic ultrasonic nebulizer (DeVilbiss,
RESULTS

To help determine the relative contributions of CD4+ and CD8+ T cells to TDI-induced asthma, CD4+ or CD8-deficient mice were subchronically exposed and challenged to TDI and compared to wild-type mice for various asthma-associated pathologies. Wild-type, TDI-sensitized/challenged mice demonstrated a significant increase in nonspecific AHR compared to the control group when exposed to 50 mg/ml concentrations of methacholine (Fig. 1). AHR was significantly reduced in both CD4 and CD8 knockout mice compared to the wild-type sensitized/challenged treatment group. The AHR in CD4 knockout mice, but not CD8 knockout mice, was significantly less \((p < 0.05)\) compared to control mice. Similar trends, although with a less robust responsive, were observed when AHR was measured following administration of the 10 and 25 mg/ml concentrations of methacholine (data not shown).

Twenty-four hours after TDI challenge, individual blood samples were collected, and total IgE and TDI-specific IgG, IgG1, and IgG2a serum antibody concentrations were determined (Fig. 2). IgE and IgG1 antibodies are associated with Th2 cytokine response, while IgG2a antibodies are associated with Th1 cytokine response. Serum IgE levels were significantly reduced in CD4 and CD8 knockout mice compared to TDI-sensitized/challenged wild-type mice. Neither TDI-specific IgG nor IgG1 and IgG2a subclass antibodies were detected in CD4 knockout mice (Figs. 2B and 2C). However, IgG antibodies, including both subclasses, were readily detected in CD8 knockout mice at levels similar to those found in TDI-sensitized/challenged wild-type mice.

Lungs, as well as trachea (not shown), of wild-type mice exposed and challenged to TDI exhibited degenerative cellular changes including goblet cell metaplasia, septal exudate, hyaline droplet formation, and epithelial changes (Table 1). Both CD4 and CD8KO mice failed to demonstrate goblet metaplasia and presented consistently less histopathological changes than wild-type mice, particularly with regard to epithelial changes. The lungs of wild-type sensitized/challenged mice also presented significant inflammation manifested by neutrophil, lymphocyte, eosinophil, and macrophage infiltration. Although still present in knockout mice, the presence of these cells was markedly decreased, particularly in CD4 knockout mice.

Changes in the cellular constituents and eosinophil peroxidase activity (EPO) from the BALF following TDI challenge are shown in Figure 3. A significant increase in the number of lymphocytes, neutrophils, and eosinophils occurred following TDI challenge in wild-type sensitized/challenged mice, compared to the control group (Fig. 3A). CD4 knockout mice had minimal lung inflammatory cell infiltration, with numbers comparable to the control group for all cell types (Fig. 3A). CD8 knockout mice also exhibited decreases in inflammatory cell infiltration, but to a lesser degree than CD4 knockout mice (Fig. 3A).

Statistical analysis. All studies were conducted in duplicate or triplicate with representative data shown. For statistical analysis, standard one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) test was used for multiple group comparisons. Student’s two-tailed unpaired \(t\)-test was used to determine the level of difference between two experimental groups, and \(p < 0.05\) was considered a statistically significant difference.
to the changes in eosinophilia observed in the lung, the level of EPO activity was elevated in BALF supernatants from sensitized/challenged wild-type mice and reduced in both CD4 and CD8 knockout mice (Fig. 3B). Phenotypic analysis of BAL cells demonstrated increases in the percent of both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in sensitized/challenged mice with the number of CD8<sup>+</sup>T cells approximately two-fold higher than that of CD4<sup>+</sup>T cells compared to controls (data not shown).

To determine the effects of TDI on the levels of asthma-associated cytokine expression in the airways, RNA was isolated from the lungs of mice 24 h after TDI challenge, and the relative levels of IL-4, IL-5, IFN<sub>γ</sub>, and IL-13 mRNA were determined by real-time PCR (Fig. 4). When compared to the control group, wild-type TDI-sensitized/challenged mice showed three- to five-fold elevations in IL-4, IL-5, IFN<sub>γ</sub>, and IL-13 mRNA transcripts (Figs. 4A–4D). CD4 and CD8 T cell knockout mice demonstrated little, if any, increases in Th1 or Th2 cytokine expression in the lung following subchronic TDI exposure and subsequent challenge.

Cytokines play an integral role in the initiation, propagation, persistence, and resolution of inflammatory and immunological

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**FIG. 2.** Serum IgE levels and TDI-specific IgG antibody titers. Wild-type mice, CD4 knockout mice, or CD8 knockout mice were exposed and challenged to TDI by inhalation as described in Methods, and sera were collected 24 h after TDI challenge. Total IgE levels (A), TDI-specific IgG antibodies (B), and TDI-specific IgG<sub>1</sub> and IgG<sub>2a</sub> antibodies (C) were determined. Significantly different from *wild-type control group or †wild-type sensitized/challenged group (p < 0.05). (n = 5, mean ± SE). ND = none detected.

**TABLE 1**

<table>
<thead>
<tr>
<th>Tissue alteration</th>
<th>Treatment group</th>
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<tbody>
<tr>
<td></td>
<td>Control Wild-type CD4 KO CD8 KO</td>
</tr>
<tr>
<td>Goblet metaplasia</td>
<td>0               1.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt; 0 0</td>
</tr>
<tr>
<td>Epithelial changes&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.8 ± 0.4       2.0 ± 0.4&lt;sup&gt;a&lt;/sup&gt; 0.5 ± 0.2&lt;sup&gt;b&lt;/sup&gt; 0.6 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.5 ± 0.2       2.7 ± 0.1&lt;sup&gt;a&lt;/sup&gt; 2.1 ± 0.2&lt;sup&gt;ab&lt;/sup&gt; 3.2 ± 0.4&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0               1.8 ± 0.4&lt;sup&gt;a&lt;/sup&gt; 0.2 ± 0.2&lt;sup&gt;b&lt;/sup&gt; 0.9 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Eosinophils</td>
<td>0               2.3 ± 0.3&lt;sup&gt;a&lt;/sup&gt; 0.7 ± 0.1&lt;sup&gt;ab&lt;/sup&gt; 1.2 ± 0.2&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Macrophages</td>
<td>0.6 ± 0.1       2.3 ± 0.3&lt;sup&gt;a&lt;/sup&gt; 0.9 ± 0.3&lt;sup&gt;b&lt;/sup&gt; 2.5 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

Note. Histopathological changes were assessed 48 h after the last TDI inhalation challenge. Values are expressed on a 0–5 scale, with 0 representing no changes, 1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately/severe, and 5 = severe. Mean individual severity within a group was calculated by adding severity scores of all animals and dividing by the total number of animals. Each value represents a mean ± SE (n = 5).

<sup>a</sup>Significantly different from wild-type control group (p < 0.05).

<sup>b</sup>Significantly different from wild-type sensitized/challenged group (p < 0.05).

<sup>*</sup>Epithelial changes represent epithelial hyperplasia, epithelial regeneration, and loss of structure.
IL-13 deficiency had no inhibitory effects, IL-4 and IL-4/IL-13 cytokine-deficient mice had little if any serum IgE and significant decreases in TDI-specific IgG antibody levels compared to sensitized/challenged wild-type mice.

A summary of the pulmonary histopathological changes associated with TDI exposure in cytokine-deficient mice is shown in Table 2. Evidence for goblet metaplasia was not observed in any of the TDI-exposed cytokine-deficient animals following challenge, while epithelial cell changes were significantly reduced in IL-4, IL-13, and IFNγ cytokine-deficient mice and absent in IL-4/IL-13 mice when compared to wild-type controls. Similarly, a decrease in the presence of inflammatory cells was generally found in the lungs of cytokine-deficient mice compared to wild-type mice, with IL-4/IL-13 cytokine-deficient mice being similar to controls.

Changes in the cellular constituents and EPO activity in the BALF following TDI challenge are shown in Figure 7. Cellular infiltration was decreased in IL-4, IL-13, and particularly in IL-4/IL-13 cytokine-deficient mice, with eosinophils virtually absent in the latter group (Fig. 7A). Corresponding to the decrease in eosinophil numbers, EPO activity was reduced in all IL-4 and IL-4/IL-13 cytokine-deficient mice. IFNγ deficiency produced a slight, but not statistically significant, decrease in BALF cell numbers compared to sensitized/challenged wild-type mice.

The relative changes in IL-4, IL-5, IFNγ, and IL-13 mRNA expression in cytokine-deficient mice were determined from lung tissue by real-time PCR 24 h following TDI challenge (Fig. 8). The values obtained from analogous cytokine-deficient animals (e.g., IL-4 expression in IL-4 cytokine-deficient mice) are not shown, since the specific protein is either not produced or not functional in these animals despite high mRNA expression. Lung tissue was not available in IL-4/IL-13 double-deficient mice to conduct PCR. When compared to the control group, sensitized/challenged mice had four- to sixfold increase in IL-4, IL-5, IFNγ, and IL-13 mRNA expression (Fig. 8). IL-4 expression was decreased in IL-13, but not in IFNγ cytokine-deficient mice. IL-5 mRNA expression was decreased in all cytokine-deficient mice, particularly IL-4 KO mice. IL-13 deficiency had no inhibitory effects, IL-4 and IL-4/IL-13 cytokine-deficient mice had little if any serum IgE and significant decreases in TDI-specific IgG antibody levels compared to sensitized/challenged wild-type mice.

**DISCUSSION**

These studies indicate that, as with high-molecular-weight asthmogens, isocyanate-induced asthma, a prototypical low-molecular-weight asthmogen, is dependent upon the activation of CD4+ T cells and Th2 cytokines. This was
demonstrated by diminished AHR to methacholine challenge, asthma-associated histopathological changes in the airways, TDI-specific serum antibodies, and Th2 cytokines in CD4 T cell and Th2 cytokine-deficient mice following TDI exposure and challenge. A somewhat less critical, but apparent, role was associated with CD8 T cells and Th1 cytokines. While CD8 knockout mice developed TDI-specific antibodies at levels equivalent to wild type mice, other endpoints, including AHR and histopathology were reduced to levels almost equal to the CD4 knockout mice. Along with other somewhat unique characteristics observed in workers with isocyanate asthma, such as a very low prevalence of specific IgE antibodies and lack of association with atopy (Bernstein et al., 2002; Cartier et al., 1989; Del Prete et al., 1993; Finotto et al., 1991; Maestrelli et al., 1994; Tee et al., 1998; Wisnewski et al., 2003), the numbers of antigen-specific CD8 T lymphocytes found in lung biopsies of patients with diisocyanate asthma are high, exceeding CD4 T cells by approximately four-fold (Del Prete et al., 1993). Although TDI-specific T cells were not measured in the current studies, the percentage of CD8+ cells exceeded those of CD4+ T cells in the BALF of sensitized/challenged mice (data not shown). A role for CD8+ cells has also been demonstrated in the mouse ovalbumin

**FIG. 4.** Th1 and Th2 cytokine mRNA expression. Wild-type mice, CD4 knockout mice or CD8 knockout mice were exposed and challenged to TDI by inhalation as described in Methods, and lung homogenates were collected 24 h following TDI challenge. Total RNA was isolated and real-time PCR was performed using IL-4, IL-5, IFNγ, IL-13, or 18S-specific primer/probe sets. Data are expressed as fold change in cytokine expression from wild-type control mice. Significantly different from wild-type control group or wild-type sensitized/challenged group (p < 0.05). (n = 5, mean ± SE).

**FIG. 5.** Nonspecific AHR airway in mice deficient in IL-4, IL-13, IL-4/IL-13, or IFNγ cytokines. Wild-type mice, IFNγ deficient mice, IL-13 deficient mice, IL-4 knockout mice, or IL-4/IL-13 deficient mice were exposed and challenged to TDI by inhalation, as described in Methods, and assessed for methacholine reactivity 24 h following TDI challenge. Control mice (wild-type) were not exposed to TDI but received TDI challenge. The baseline PenH values for wild-type sensitized/challenged mice were similar to other treatment groups (data not shown). Changes in PenH values to 50 mg/ml inhaled methacholine were determined and are shown as percent change from values obtained in control mice. Significantly different from wild-type control group or wild-type sensitized/challenged group (p < 0.05). (n = 5, mean ± SE).
FIG. 6. Serum IgE levels and TDI-specific IgG antibody titers. Wild-type mice, IFNγ deficient mice, IL-13 deficient mice, IL-4 knockout mice, or IL-4/IL-13 deficient mice were exposed and challenged to TDI by inhalation as described in Methods and sera were collected 24 h after challenge. Total IgE levels (A) and TDI-specific IgG antibodies (B) were determined. Significantly different from *wild-type control group or †wild-type sensitized/challenged group (p < 0.05). (n = 5, mean ± SE). ND = none detected.

TABLE 2
Summary of Histopathological Changes in the Lung Induced by TDI Exposure

<table>
<thead>
<tr>
<th>Tissue alteration</th>
<th>Control</th>
<th>Wild-type</th>
<th>IL-4 KO</th>
<th>x-IFNγ</th>
<th>x-IL-13</th>
<th>IL-4 KO/x-IL-13</th>
</tr>
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<tbody>
<tr>
<td>Goblet metaplasia</td>
<td>0</td>
<td>1.5 ± 0.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Epithelial changes*</td>
<td>0</td>
<td>2.1 ± 0.4</td>
<td>1.5 ± 0.2⁹</td>
<td>0.9 ± 0.3⁹</td>
<td>0.8 ± 0.5⁹</td>
<td>0</td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.8 ± 0.3</td>
<td>2.0 ± 0.1⁹</td>
<td>1.3 ± 0.3⁹</td>
<td>1.3 ± 0.3⁹</td>
<td>0.3 ± 0.1⁹</td>
<td>0</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0</td>
<td>1.8 ± 0.4</td>
<td>0</td>
<td>0</td>
<td>0.6 ± 0.3⁹</td>
<td>0</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>2.3 ± 0.3</td>
<td>0.8 ± 0.2⁹</td>
<td>1.3 ± 0.3⁹</td>
<td>0.2 ± 0.1⁹</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Macrophages</td>
<td>2.3 ± 0.3</td>
<td>2.0 ± 0.2⁹</td>
<td>2.0 ± 0.4⁺</td>
<td>0.9 ± 0.5⁹</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Note. Histopathological changes were assessed 48 h after the last TDI inhalation challenge. Values are expressed on a 0–5 scale, with 0 representing no changes, 1 = minimal, 2 = slight/mild, 3 = moderate, 4 = moderately/severe, and 5 = severe. Mean individual severity within a group was calculated by adding severity scores of all animals and dividing by the total number of animals. Each value represents a mean ± SE (n = 5).

*Significantly different from *wild-type control group (p < 0.05).
†Significantly different from †wild-type sensitized/challenged group (p < 0.05).
*Epithelial changes represent epithelial hyperplasia, epithelial regeneration, and loss of structure.
asthma model, with the disappearance of IL-5, eosinophilia, and AHR upon antibody depletion of CD8+ T cells (Gonzalo, 1996; Hamelmann et al., 1996; Schwarze et al., 1999; Zhang et al., 1996). One study suggested that these results could be due to antibody-mediated CD8+ cell depletion, which may also eliminate γδ-T cells and CD8+ dendritic cells (van Rijt and Lambrecht, 2001). A more likely explanation, however, is that CD8+ cells help regulate Th2 and inflammatory cytokines. In this respect, CD4+ –CD8+ T-cell interactions have been demonstrated in an in vitro model with CD8+ T cells regulating antigen-triggered lymphokine release from CD4+ cells with the active participation of antigen presenting cells (Gill and Lafferty, 1989). A more likely explanation, however, is that CD8+ cells help regulate Th2 and inflammatory cytokines. In this respect, CD4+ T cells and CD8+ dendritic cells (van Rijt and Lambrecht, 2001). A more likely explanation, however, is that CD8+ cells help regulate Th2 and inflammatory cytokines. In this respect, CD4+ –CD8+ T-cell interactions have been demonstrated in an in vitro model with CD8+ T cells regulating antigen-triggered lymphokine release from CD4+ cells with the active participation of antigen presenting cells (Gill and Lafferty, 1989).

In an ovalbumin ablation and restoration model, adoptive transfer of antigen-specific CD8+ T cells at sensitization, but not transfer at post-sensitization, restored AHR and tissue eosinophilia (Hamelmann et al., 1996). In our studies, either CD4 or CD8 deficiency provided significant decrements in the expression of lung eosinophilia and EPO activity. This is in contrast to adoptive transfer experiments where ovalbumin-specific CD4+ T cells were sufficient for the induction of eotaxin production (Mattes et al., 2001) and to findings by Herrick et al. (2003) that showed no eosinophil decrease in CD8 knockout mice. The potentiating role that CD8+ T cells play in the development of eosinophilia may well be through regulation of IL-5 production and inflammation (Stock et al., 2004).

Allergic asthma, which is associated with IgE antibodies and eosinophilia, is considered a Th2 predominant response and can be adoptively transferred with Th2 cells in experimental animal models (Cohn et al., 1998; Li et al., 1999). In this respect, animal studies have demonstrated both the requirement and interdependence for Th2 cytokines in many manifestations of allergic asthma (Foster et al., 1996; Grunig et al., 1998; Hamelmann et al., 1997; Kumar et al., 2002; Webb et al., 2000; Wills-Karp et al., 1998). Our studies also indicate that Th2 cytokines play a primary role in TDI-induced asthma. For example, IL-4 depletion, although only moderately inhibiting AHR, markedly attenuated total serum IgE levels,

**FIG. 8.** Th1 and Th2 cytokine mRNA expression. Wild-type mice, IFNγ deficient mice, IL-13 deficient mice, IL-4 knockout mice, or IL-4/IL-13 deficient mice were exposed and challenged to TDI by inhalation as described in Methods, and whole lung homogenates were collected 24 h following challenge. Total RNA was isolated and real-time PCR was performed using IL-4, IL-5, IFNγ, IL-13, or 18S-specific primer/probe sets. Data are expressed as fold changes in cytokine expression from control mice. Significantly different from *wild-type control group or **wild-type sensitized/challenged group (p < 0.05). (n=4, mean ± SE).
as well as TDI-specific antibody levels and histopathological changes in the lung, including airway remodeling. IL-4 promotes mucus secretion and goblet cell hyperplasia, in addition to the immunoglobulin switch to IgE (Gelfand, 1998; Grunewald et al., 1998; Pauwels et al., 1997). IL-13 deficiency had moderate attenuating effects on most endpoints measured, except in the case of goblet metaplasia, which was markedly attenuated. Combined IL-4/IL-13 deficiency had a profound effect, as almost all endpoints were essentially abrogated. IL-4 and IL-13 have many overlapping functions as a result of sharing a common receptor subunit, IL-4Ra. IL-13 also has distinct functions and, like IL-4, is important in the development of AHR and mucus production (Hershey, 2003).

Some unique capabilities of IL-13 include its ability to suppress NF-kB, as well as to promote eosinophil recruitment through an IL-5 and eotaxin-dependent mechanism (Mattes et al., 2002; Pope et al., 2001) and its dominant role in airway remodeling (Elias et al., 2003).

Our data also demonstrate dissociation between AHR and tissue manifestations of TDI-asthma, since IFNγ depletion diminished AHR to TDI, while only slightly affecting antibody and inflammatory responses. Consistent with these observations, IFNγ expression in the lungs was observed following TDI sensitization. While Th1 cytokines are normally considered to negatively influence allergic responses, increasing data indicate that there is a cooperative interaction between Th1 and Th2 cytokines in the pathogenesis of asthma. For example, it was demonstrated that cooperation between Th1 and Th2 cells is necessary for a robust eosinophil inflammatory response and pulmonary recruitment of antigen-specific T cells (Randolph et al., 1999). In an ovalbumin-mediated asthma model, significant increases in Th1 chemokines, such as IP-10, were observed, and overexpression of IP-10 augmented AHR, eosinophilia, CD8+ cell numbers, and IL-4 expression (Medoff et al., 2002). Adoptive-transfer of ovalbumin-specific Th1 cells not only failed to reverse Th2-mediated airway inflammation, but also caused severe airway inflammation (Hansen et al., 1999) and enhanced AHR (Takaoka et al., 2001). Studies in humans have suggested that the number of IFNγ-producing cells found in asthmatic lungs correlate with asthma severity, bronchial hyperresponsiveness, and blood eosinophilia (Krug et al., 1996; Magnan et al., 2000; van Rijt and Lambrecht, 2001) and endogenous IFNγ potentiates IL-13 induced lung inflammation (Ford et al., 2001; Hofstra et al., 1998).

In conclusion, these studies indicate that occupational asthma, induced by low-molecular-weight chemicals, represented in these studies by TDI, evokes similar immune mechanisms as allergic asthma caused by large-molecular-weight antigens. Activated CD4+ T cells play a predominant role in the pathogenesis of TDI-induced asthma. Furthermore, it would appear that Th2 cytokines are decisive in the initial phase of occupational asthma, in the priming and development of Th2 cells, and in the permeation of eosinophils into the airway lumen. However, as has been suggested previously for allergic asthma (Akdis et al., 1999; Hershey, 2003; Jung et al., 1996; Webb et al., 2000), a cooperative interaction with CD8+ T cells and Th1 cytokines in the pathogenesis of asthma lesions clearly exists. This was particularly evident with IFNγ and the development of AHR and the reduction of AHR, inflammation, and Th1/Th2 cytokine production in CD8 knockout mice.

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