Exposure and Immunological Determinants in a Murine Model for Toluene Diisocyanate (TDI) Asthma

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Isocyanate-induced asthma, the most commonly reported cause 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 have been difficult to define. Appropriate animal models of isocyanate asthma will be instrumental to further our understanding of this disease. Previous studies have demonstrated that dermal exposure to isocyanates in mice results in systemic sensitization that leads to eosinophilic airways inflammation upon subsequent airway challenge. We hypothesized that inhalation of vapor phase toluene diisocyanate (TDI) will lead to immunologic sensitization in mice and that subsequent challenge will induce pathology and immune system alterations indicative of asthma found in humans. To determine the impact of exposure dose as well as the involvement of immune (allergic) or nonimmune mechanisms, a murine model of TDI asthma was established and characterized following either low-level subchronic or high-dose acute inhalation TDI exposure. C57BL/6 J mice were exposed to TDI by inhalation either subchronically for 6 weeks (20 ppb, 4 h/day, 5 days/week) or by a 2-h acute exposure at 500 ppb. Both groups were challenged 14 days later via inhalation with 20 ppb TDI for 1 h. Mice that underwent the subchronic exposure regimen demonstrated a marked allergic response evidenced by increases in airway inflammation, eosinophilia, goblet cell metaplasia, epithelial cell alterations, airway hyperresponsiveness (AHR), Th1/Th2 cytokine expression in the lung, elevated levels of serum IgE, and TDI-specific IgG antibodies, as well as the ability to transfer these pathologies to naïve mice with lymphocytes or sera from TDI exposed mice. In contrast, mice that received acute TDI exposure demonstrated increased AHR, specific IgG antibodies, and pathology in the lung consistent with asthma, but without the presence of elevated serum IgE, lung eosinophilia, or increased expression of Th1 cytokines. These results describe mouse models for TDI asthma consistent with that found in workers with occupational asthma and indicate that the pulmonary pathology associated with TDI can vary depending upon the exposure paradigm.

Key Words: toluene diisocyanate; isocyanate-induced asthma; lymphocytes.

Asthma occurs 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 (Balmes et al., 2003; Baur et al., 1998). Isocyanates, such as toluene diisocyanate (TDI), are the most commonly reported low-molecular-weight class of chemicals responsible for occupational asthma, with over 250,000 workers in the United States exposed every year and 5 to 15% of these developing asthma (Tee et al., 1998). Although isocyanate-induced asthma has been recognized for over 45 years, the mechanisms responsible are still debated, the determinants of exposure are not well defined, and murine models have only been recently proposed (Herrick et al., 2002; rev. in Johnson et al., 2004; Scheerens et al., 1999). As with high-molecular-weight molecules, such as house dust mite allergens and detergent enzymes, immunologic mechanisms are considered the most likely cause, although inflammatory, pharmacologic, and neurogenic mechanisms have been implicated (Mapp et al., 1994; Rauf-Heimsoth and Baur, 1998; Scheerens et al., 1996). Clinical features of isocyanate-induced asthma can include the onset of the asthmatic symptoms within 1 h following challenge, persistent airway hyperresponsiveness (AHR) to chemical-specific and nonspecific (e.g., methacholine) challenge, and airway inflammation, involving the presence of activated T cells, eosinophils, neutrophils, and mast cells. However, specific IgE antibodies are detected in only 5–30% of individuals with isocyanate-induced asthma (Tee et al., 1998), and a similar percentage of exposed workers have detectable IgG antibodies but no asthmatic symptoms, suggesting that IgG antibodies may represent an indicator of exposure rather than disease (Bernstein et al., 1997). Furthermore, atopy is not considered a risk factor for diisocyanate asthma (Tee et al., 1998). A lack of association with IgE-mediated immunity, however, does not exclude immunological mechanisms, as clinical evidence suggests involvement of CD8+ lymphocytes in occupational asthma induced by exposure to isocyanates (Finotto et al., 1991; Maestrelli et al., 1994). The present studies were conducted to determine the role of the immune system in the asthmatic phenotype induced by TDI and to determine the influence of exposure dose on disease phenotype. To accomplish this goal, we established

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and characterized a mouse model for TDI-induced asthma following exposure conditions relevant to the workplace (i.e., via inhalation route). Mice were exposed to aerosol-free TDI vapor in a nose-only chamber using low-level (20 ppb) subchronic (6 weeks) inhalation or high-dose (500 ppb) acute inhalation, as might occur following an accidental spill. The 20-ppb dose of TDI was selected since this is the Occupational Safety and Health Administration’s (OSHA) current 15-minute Permissible Exposure Limit, expressed as a Ceiling Limit (PEL-C). Workplace concentrations in excess of 20 ppb TDI are still being reported, although less frequently in recent years (Ott et al., 2003).

**Materials and Methods**

**Experimental animals.** Preliminary studies were conducted using several mouse strains including C57BL/6, BALB/c, and B6C3F1 mice. Since the C57BL/6 strain produced the most robust responses under the current exposure conditions (data not shown), the strain was used in the current studies. While BALB/c mice are more commonly employed in studies of respiratory allergy, C57BL/6 mice have been successfully used as an asthma model following exposure to the prototypical protein asthmogen, ovalbumin (Shen et al., 2003; Watanabe et al., 2003). Female wild-type C57BL/6 J and FcεR1 knockout (B6.129-Fcer1g<tm1Rav.N12> mice, deficient in the γ chain of the FcεR1, FcγR1, and FcRIII genes, were obtained from Jackson Laboratory (Bar Harbor, ME), and Taconic (Germantown, NY), respectively, at approximately 5 to 6 weeks of age. Upon arrival the mice were quarantined for 2 weeks and acclimated to a 12-h light/dark cycle. Animals were housed in microisolator cages in pathogen-free and environmentally controlled conditions at NIOSH facilities in compliance with AAALAC approved guidelines and an approved IACUC protocol (03-JM-M-005). Food and water were provided ad libitum.

**Exposure.** TDI (80:20 molar mixture of 2,4:2,6 isomers provided by Bayer, USA, Pittsburgh, PA) vapors were generated by passing dried air through an impinger that contained 3 ml TDI. A computer-interfaced mass flow controller (Aalborg Instruments, Orangeburg, NY, model GFC-47, 0–100 LPM) regulated the diluent air. Temperature and relative humidity were monitored by a Vaisala transmitter (Vaisala Inc., Woburn MA, type HP-233) interfacing with the TDI and diluent air controllers in a National Instruments (Austin TX) data acquisition/control system. The generation system produces TDI vapor, free of TDI aerosol. Real-time monitoring of the chamber instrumentation (Austin TX) data acquisition/control system. The generation system was performed blinded and expressed on a 0–5 scale for each animal, with 0 representing no change, 1 minimal, 2 slight/mild, 3 moderate, 4 moderate/severe, and 5 severe. Additional groups of mice were sacrificed 24 h after challenge and utilized for bronchoalveolar lavage fluid (BALF) and blood collection. To obtain BALF, mice were anesthetized with 50 mg/kg of pentobarbital, 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 pooled. BALF recovery was 80 ± 5% for all animals. The BALF samples were centrifuged, and the supernatant frozen at −80°C until enzyme analysis. The cells were resuspended at 105 cells/ml of HBSS, and 0.1 ml was used for cytospin preparations. The slides were fixed and stained with Diff-Quick® (WVR, Pittsburgh, PA), and differential cell counts were obtained using light microscopic evaluation of 300 cells/slide. Total cell counts were performed with a hemocytometer. In replicate experiments, lungs were collected 24 h following challenge, and tissues were frozen in RNAlater® (Qiagen, Valencia, CA) and stored at −80°C for reverse transcription-polymerase chain reaction (RT-PCR) analysis. Tissues frozen in liquid nitrogen were incubated with RNAlater® (Ambion, Austin, TX) at −20°C for 24 h prior to RNA isolation.

**Transfer experiments.** Adoptive and passive transfer experiments were performed to assess the role of specific immunity in the asthma response. For adoptive transfer experiments, single cell suspensions were prepared from groups of mice exposed to TDI for 6 weeks or air sham controls by gently pressing pooled lymph nodes (mesial and auricular) and spleens through a stainless steel screen. The cell suspensions were washed with HBSS (Gibco, Grand Island, New York), the cell number adjusted to 2 × 107 cells/ml, and aliquots layered onto Lympholyte-M (Accurate Chemical, Westbury, NY). After centrifugation at 2,500 rpm, the lymphocyte interface was collected and washed, and 5.0 × 106 cells in 0.5 ml volumes were injected intravenously into naive recipients. B or T cell depletion was conducted by incubating isolated lymphoid cells with either panT or panB Dynabeads® (Dynal Biotech Inc., Lake Success, NY) at a 7:1 cell:bead ratio, according to the manufacturer’s instructions. The respective T and B cell populations were >98% pure, as assessed by FACS analysis on a FACS Calibur® (BD Biosciences, Palo Alto, CA) utilizing anti-CD3 and anti-B220 FITC conjugated monoclonal antibodies (PharMingen, San Diego, CA). The resulting T and B lymphocyte populations were injected intravenously into naive recipients at a concentration of 2.9 × 106 cells and 2.5 × 106 cells, respectively, in 0.5-ml volumes. To measure TDI-specific serum activity, naive mice received an intradimensional injection of 30 μl heat-inactivated (56°C, 4 h) or nonheated pooled serum into the dorsum of the right ear from either TDI sensitized/challenged mice or control mice. Animals were challenged 24 h later with 1% TDI (in acetone:olive oil, 4:1) on the dorsum of the same ear, and the change in ear thickness was compared to the thickness prechallenge. Additional groups of mice received an intravenous injection of 200 μl of either heated or unheated pooled sera from TDI sensitized/challenged or control mice.

Twenty-four hours after intravenous lymphocyte or serum transfer, mice were challenged either by inhalation with 20 ppb TDI for 1 h or by a single application of 25 μl of 1% TDI (in acetone:olive oil, 4:1) onto the dorsum of the right ear, as previously described (Ebino et al., 1999). Respiratory responses including pathology (as outlined above) and airway responsiveness to methacholine (see below) were determined 48 and 24 h following challenge, respectively. The ear challenge response was determined by measuring the change in ear thickness from baseline prechallenge ear thickness 24 h following TDI application. Cell proliferation in the draining lymph node was determined in an additional group of recipient mice using a modification of the local lymph node assay, as originally described by Dearman and Kimber (2000). Twenty-four hours after challenge, the mice were injected intravenously with 200 μl of 3H-thymidine (specific activity 0.1 μCi/ml; Amersham, Piscataway, NJ) and incorporation of 3H-thymidine into DNA in the draining auricular lymph nodes was measured.
Antibody detection. Total serum IgE was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) as previously described (Satoh et al., 1995). Briefly, plates were coated with 5 µg/ml of rat monoclonal anti-mouse IgE (PharMingen). Serial two-fold dilutions of test sera, starting at a 1:5 dilution, were added and incubated with peroxidase-goat 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)]. Total serum IgE concentrations were derived from a standard curve obtained using murine monoclonal anti-DNP IgE (Sigma, St. Louis, MO).

TDI-specific antibodies were detected by ELISA using a TDI-mouse serum albumin conjugate, kindly provided by Dr. Meryl Karol (University of Pittsburgh, Pittsburgh, PA), as previously described (Satoh et al., 1995). Serial two-fold dilutions of test sera, starting at a 1:5 dilution, were added to individual wells and incubated with peroxidase-conjugated, goat anti-mouse antibodies against either total IgG (1:400, Sigma, St. Louis, MO), IgG1, or IgG2a (both at 1:400, The Binding Site, Birmingham, UK) and developed with ABTS substrate. Antibody titers were determined by plotting the serial dilution curve for each sample individually versus the OD for each dilution of that sample. A cutoff OD of 0.2 (average OD of challenge only mouse serum was 0.06 ± 0.005) was used to determine the titer.

Eosinophil peroxidase activity (EPO). Measurement of EPO activity was performed on BALF supernatants 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 BALF supernatant. The mixture was incubated at 37°C for 30 min before stopping the reaction with 50 M of 2 N hydrochloric acid. Optical densities were measured at 490 nm (OD490). Non-specific activity was determined by treating duplicate sample sets with the EPO inhibitor, 3-amino-1,2,4-triazole (2 mM, Sigma), and was always less than 10% of the nontreated samples. Results are expressed as OD490 corrected for background and volume of BALF supernatant retrieved (BALF recovery was 80 ± 5%).

Airway hyperresponsiveness (AHR). AHR to methacholine challenge was assessed, 24 h following TDI challenge, 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) × Peff / Pif; where Te = expiration time, Tr = relaxation time, Peff = 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 AeroSonic ultrasonic nebulizer (DeVilbiss, Somerset, PA). Recordings were taken for 5 min after each nebulization. Only data from the 50 mg/ml methacholine exposure are presented, as similar but less robust changes were observed between treatment and control groups at the 10 and 25 mg/ml methacholine concentrations (data not shown). The PenH values during each 5-min sequence were averaged and expressed as percentage increase over baseline values following PBS exposure for each methacholine concentration.

Real-time RT-PCR. Tissues were homogenized, and total cellular RNA was extracted using the Qiagen RNeasy kit® (Qiagen, Valencia, CA) according to the manufacturer’s instructions. One microgram of RNA was reverse-transcribed using random hexamers and 60 U of Superscript II (Life Technologies, Grand Island, NY). Real-time PCR primer/probe sets for murine 18S, IFNy, IL-4, IL-5, and TNFα were purchased as predeveloped kits from Applied Biosystems (Foster City, CA). Real-time PCR was performed using Taqman Universal Master mix with Amperase in an iCycler (Bio-Rad, Hercules, CA) for 1 cycle at 50°C for 2 min (degrade carry over using Amperase), and 95°C for 10 min, followed by 60 cycles at 95°C for 15 sec and 60°C for 1 min. The differences in mRNA expression between control and treatment groups were determined by the relative quantitation method developed by Pfaffl (2001) utilizing the threshold cycle (Ct) method and real-time PCR efficiencies of the target gene normalized to the housekeeping gene 18S/RNA.

Statistical analysis. All studies were replicated with representative data shown. For statistical analysis, standard one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls 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. For the analyses of RT-PCR data, the fold change from the mean of the control group was calculated for each individual sample (including individual control samples to assess variability in this group centered around one) prior to ANOVA and SNK.

RESULTS

Mice exposed to 20 ppb TDI by inhalation for 6 weeks and challenged 14 days later demonstrated a marked increase in AHR to methacholine (Fig. 1A). A slight increase in AHR to methacholine occurred in the sensitized-only and challenged-only groups, but was not statistically significant. Mice exposed to an acute high dose (500 ppb) of TDI followed 14 days later with 20-ppb challenge also exhibited significant AHR to methacholine challenge compared to controls (Fig. 1B). No differences in baseline PenH values were observed between treatment groups in the subchronic or acute exposure protocols (data not shown). Furthermore, mice subchronically exposed to TDI show increased PenH values within 2 h following challenge with TDI, indicating TDI-specific airway responsiveness, an important characteristic of asthma (unpublished data). For the remaining studies, the control group will represent mice that received air exposure for 6 weeks (subchronic) or 2 h (acute) followed by TDI challenge (challenge-only).

Twenty-four hours after TDI challenge, blood was collected from control and exposed mice and the serum analyzed for total IgE and TDI-specific IgG antibodies (Fig. 2). Total serum IgE levels in mice that received subchronic TDI exposure were increased by approximately 10-fold compared to control mice, while IgE levels in serum from mice that received an acute exposure to TDI were comparable to controls (Fig. 2A). Total IgG TDI-specific antibodies (Fig. 2B), as well as IgG1 and IgG2a, TDI-specific antibodies (Fig. 2C), were consistently detected and significantly elevated in both the subchronic low-dose and the acute high-dose exposed groups, compared to undetectable levels found in the control group. In addition, while there were equivalent levels of IgG1 and IgG2a antibodies in the acute high-dose group, IgG1-specific antibodies were at least 30-fold higher than IgG2a antibody levels, in subchronically exposed mice. IgG1 and IgG2a antibodies specific for TDI were not detectable in sera of control mice (not shown).

Airway inflammation is a central feature of the asthmatic response to TDI and is considered a key manifestation of underlying bronchial hyperresponsiveness. Mice subjected to the subchronic TDI exposure regimen presented histological changes in the lungs and nars consistent with an inflammatory response, manifested by neutrophil, lymphocyte, eosinophil,
and macrophage infiltration (Table 1). Tissues at these sites exhibited degenerative cellular changes including loss of cilia, goblet cell metaplasia, septal exudate, hyaline droplet formation, and epithelial hyperplasia. Mice exposed by the acute high-dose exposure regimen exhibited similar histopathology as observed in the subchronic exposure, but fewer inflammatory cells, including eosinophils. Control mice revealed minimal histopathological changes that were contained primarily in the nares.

Changes in the cellular constituents and eosinophil peroxidase activity (EPO) from the BALF following TDI challenge are shown in Figure 3. Total cell numbers in the BALF of mice exposed following the subchronic protocol were increased two-fold compared to the control group. Differential analysis showed that large increases in eosinophils and lymphocytes were responsible for the observed increase in cell recruitment. There was also a significant increase in neutrophil infiltration into the lung, although to a much lesser extent...
than other inflammatory cells (Fig. 3A). Macrophages were the predominant cell type in the lung of control mice, representing over 95% of the cells, whereas macrophages decreased to 56% of the total cell population in the subchronically exposed mice following challenge. Mice exposed to the acute high-dose treatment exhibited an 8-fold increase in lymphocyte numbers following challenge, but minimal effects on other inflammatory cells, including eosinophils (Fig. 3B). Corresponding to the increase in eosinophil numbers, EPO activity in BALF supernatants was significantly elevated in subchronically exposed mice after challenge, while no increase in activity was found in the acute high-dose treated animals (Fig. 3C).

Cytokines have been implicated in the recruitment of inflammatory cells to the lung and in the pathogenesis of asthma. To determine the effects of TDI on the relative expression of cytokines in the airway, RNA was isolated from the lungs of mice 24 h after challenge, and the levels of IL-4, IL-5, TNFα, and IFNγ mRNA were determined by real-time PCR (Fig. 4). Compared to the control group, subchronic TDI-exposed mice showed significant elevations in IL-4, IL-5, IFNγ, and TNFα mRNA transcripts following TDI challenge (Fig. 4). In contrast, no increase in expression of IL-4, IL-5, IFNγ or TNFα was observed in the lungs of mice that received acute TDI exposure (Fig. 4).

### Table 1

| Tissue alteration | Control | Subchronic | Acute |
|-------------------|---------|------------|-------|
| Nares             |         |            |       |
| Exudate           | 0.2 ± 0.2 | 2.5 ± 0.2 | 2.2 ± 0.6 |
| Goblet metaplasia | 1.2 ± 0.2 | 4.2 ± 0.1 | 4.3 ± 0.2 |
| Inflammation      |         |            |       |
| Lymphocytes       | 0.5 ± 0.2 | 2.2 ± 0.4 | 0.5 ± 0.3 |
| Neutrophils       | 0.8 ± 0.2 | 2.7 ± 0.5 | 1.8 ± 0.6 |
| Eosinophils       | 0.4 ± 0.3 | 2.9 ± 0.5 | 0.7 ± 0.3 |
| Epithelial changes| 0.2 ± 0.2 | 2.1 ± 0.1 | 3.3 ± 0.1 |
| Hyaline droplet   | 0.4 ± 0.3 | 3.1 ± 0.4 | 2.0 ± 0.2 |
| Lung              |         |            |       |
| Goblet metaplasia | 0       | 1.9 ± 0.3 | 2.3 ± 0.7 |
| Inflammation      |         |            |       |
| Lymphocytes       | 0.7 ± 0.3 | 3.3 ± 0.4 | 0.8 ± 0.3 |
| Neutrophils       | 0       | 1.9 ± 0.3 | 0.2 ± 0.2 |
| Eosinophils       | 0       | 3.4 ± 0.3 | 0.2 ± 0.1 |
| Macrophages       | 0       | 2.4 ± 0.3 | 1.7 ± 0.2 |
| Epithelial changes| 0       | 2.4 ± 0.4 | 1.2 ± 0.3 |

**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 added severity scores of all animals and then dividing that by the total number of animals.

**a**Significantly different from control group *p* ≤ 0.05.

**b**Epithelial changes represent epithelial hyperplasia, epithelial regeneration, and loss of structure.

**c**Mean ± SEM (n = 5).
To determine whether specific immunity was involved in the asthmatic response to TDI, adoptive transfer experiments were conducted in which lymphocytes, B cells, or T cells from TDI-exposed mice were transferred into naive recipients. Twenty-four hours following cell transfer, the mice were challenged with 20 ppb TDI, and lung inflammation and airway reactivity were assessed 48 and 24 h later, respectively. Histological examination of lungs from mice that received lymphocytes from subchronic TDI exposed animals showed slight, diffuse infiltration of lymphocytes and eosinophils following TDI challenge, while those receiving lymphocytes for acute TDI exposed group revealed lymphocyte infiltration but no eosinophils (data not shown). No lung inflammation was evident after challenge in transfer mice that received lymphocytes from control animals. Naive mice that received either purified lymphocytes, T cells, or B cells from mice that underwent subchronic exposure also displayed significantly increased responsiveness to methacholine 24 h following TDI challenge, when compared to the control group (Fig. 5A). Recipient mice that received unfractionated lymphocytes from mice in the acute treatment group also showed a significant increase in AHR to methacholine 24 h following TDI challenge (Fig. 5B), although the magnitude of increase over the control group was about half that observed following total cell transfer from subchronic exposure mice. Adoptive transfer experiments with purified B and T cells from mice that received the acute exposure regimen were not conducted.

To help determine whether TDI-specific lymphocytes were present in the transfer experiments, lymphocytes from mice that underwent subchronic TDI exposure were adoptively transferred to naive recipients, and 24 h later the recipients were challenged with 25 μl of 1% TDI on the dorsum of the ear. Ear swelling was determined following an additional 24 h. Mice that received unfractionated lymphocytes, B cells, or T cells produced a significant ear swelling response following TDI ear challenge (Fig. 6A). Cell proliferation in the draining auricular lymph node was also significantly increased in adoptively transferred mice following TDI ear challenge, although the response following transfer of B cells was minimal compared to T cells. This was evidenced by 20-fold, 8-fold, and 2.4-fold increases in ^3H-thymidine uptake in mice receiving total lymphocytes, T lymphocytes, and B lymphocytes, respectively, compared to controls (Fig. 6B). Transfer of lymphocytes from acutely exposed mice was not performed in these experiments.

To help elucidate the role of humoral immunity in TDI-induced asthma, passive transfer experiments were performed in which serum from mice that had been exposed subchronically and challenged with TDI was administered to naive mice (Fig. 7). Histological examination of lungs from mice that received serum from TDI-exposed animals showed minimal diffuse

FIG. 4. Inflammatory cytokine gene expression in the lungs of TDI-exposed mice. Twenty-four hours following challenge, RNA was isolated from lungs and real-time RT-PCR was performed using IL-4, IL-5, IFN-γ, TNFα, or 18s (internal control)-specific primer/probe sets. Cytokine mRNA expression data for subchronic and acute exposure mice are presented as fold change from the respective control group (see Materials and Methods section for details). Open bars represent control group responses, and solid bars represent TDI sensitized/challenged group responses. Significantly different from "control group or "subchronic sensitized/challenged group, (p < 0.05, n = 4, mean ± SEM).
infiltration of lymphocytes and eosinophils 48 h after TDI challenge (data not shown). No lung inflammation was evident after challenge in naive recipient mice that where challenged by TDI inhalation 24 h later. Twenty-four hours following TDI challenge, mice which received vehicle, total lymphocytes, T lymphocytes, or B lymphocytes, as well as a TDI-exposed positive control group (sensitized/challenged) were assessed for methacholine reactivity. The change in PenH values in response to 50 mg/ml of inhaled aerosolized methacholine is expressed as percent change from baseline values (aerosolized saline). The PenH baseline values (0.51 ± 0.07) did not differ between treatment groups. Significantly different from vehicle control group, \( p < 0.05, n = 5, \text{mean} \pm \text{SEM} \). Differences between all transfer groups and the TDI sensitized/challenged group were not significant for both subchronic and acute exposures. (S/C = TDI sensitized/challenged group).

FIG. 5. AHR following adoptive transfer with lymphocytes from TDI-exposed mice. Lymphocytes pooled from the auricular lymph nodes and spleens from TDI-subchronically exposed (A) or acutely exposed mice (B) were injected iv into naive recipient mice that where challenged by TDI inhalation 24 h later. Twenty-four hours following TDI challenge, mice which received vehicle, total lymphocytes, T lymphocytes, or B lymphocytes, as well as a TDI-exposed positive control group (sensitized/challenged) were assessed for methacholine reactivity. The change in PenH values in response to 50 mg/ml of inhaled aerosolized methacholine is expressed as percent change from baseline values (aerosolized saline). The PenH baseline values (0.51 ± 0.07) did not differ between treatment groups. Significantly different from vehicle control group, \( p < 0.05, n = 5, \text{mean} \pm \text{SEM} \). Differences between all transfer groups and the TDI sensitized/challenged group were not significant for both subchronic and acute exposures. (S/C = TDI sensitized/challenged group).

The role of antibody in TDI-induced asthma was further explored using FcErIg transgenic mice, which lack the \( \gamma \) chain subunit of the Fc\( \varepsilon \)RI, Fc\( \gamma \)RII, and Fc\( \gamma \)RI receptors and, thus, do not mount functional IgG and IgE immune responses. Transgenic mice were exposed to TDI by subchronic inhalation, and methacholine reactivity was assessed at 24 h following TDI challenge. As shown in Figure 8A, increased AHR in transgenic mice was similar to the controls. Changes in lung cytokine mRNA expression were also examined in FcErIg transgenic mice (Fig. 8B). In contrast to the sensitized/challenged wild-type group, the levels of the asthma-associated cytokines IL-4, IL-5, IFN\( \gamma \) and TNF\( \alpha \) in the subchronically exposed FcErIg transgenic mice were not increased.

FIG. 6. Contact hypersensitivity to TDI following adoptive transfer of lymphocytes from mice subchronically exposed to TDI. Lymphocytes pooled from the auricular lymph nodes and spleens from TDI-exposed mice were injected iv into naive recipient mice. Mice were challenged 24 h later with 1% TDI on the dorsum of the right ear, and after an additional 24 h, contact hypersensitivity responses were measured as a function of challenge-induced increases in ear thickness (A) and \( ^3 \)H-thymidine uptake in the draining auricular lymph nodes (B). Significantly different from vehicle control group or total lymphocyte transfer group, \( p < 0.05, n = 4, \text{mean} \pm \text{SEM} \).
The response to control sera was compared to that of normal mouse sera, and no difference was observed (data not shown). S/C pooled from TDI subchronically exposed mice was injected iv into naive recipient mice. (A) Twenty-four hours later mice were challenged with TDI (20 ppb via inhalation route for 1 h) and 24 h post-inhalation challenge, mice which received control sera, heat-inactivated TDI sera, noninactivated TDI sera, or TDI subchronically sensitized/challenged (positive control) were assessed for methacholine reactivity. The change in PenH values in response to 50 mg/ml of inhaled aerosolized methacholine is expressed as percent change from baseline values (aerosolized saline). The PenH baseline values (0.45 ± 0.04) did not differ between treatment groups. (B) Heat-inactivated or noninactivated pooled serum from TDI subchronically exposed mice was injected intradermally into the dorsum of the right ear of naive recipient mice. Twenty-four hours following transfer, mice were challenged with 1% TDI on the same ear, and responses were measured as a function of challenge-induced increases in ear thickness 24 h post-challenge. Data are presented as percent change from prechallenge ear thickness of the right ear. Significantly different from control serum treated group, noninactivated treated serum group, or subchronically sensitized/challenged group, \( p < 0.05, n = 5, \text{mean} \pm \text{SEM} \). The response to control sera was compared to that of normal mouse sera, and no difference was observed (data not shown). S/C = TDI sensitized/challenged from subchronic exposure.

**FIG. 7.** AHR following passive transfer of TDI immune serum. Sera pooled from TDI subchronically exposed mice was injected iv into naive recipient mice. (A) Twenty-four hours later mice were challenged with TDI (20 ppb via inhalation route for 1 h) and 24 h post-inhalation challenge, mice which received control sera, heat-inactivated TDI sera, noninactivated TDI sera, or TDI subchronically sensitized/challenged (positive control) were assessed for methacholine reactivity. The change in PenH values in response to 50 mg/ml of inhaled aerosolized methacholine is expressed as percent change from baseline values (aerosolized saline). The PenH baseline values (0.42 ± 0.08) did not differ between treatment groups. (B) Heat-inactivated or noninactivated pooled serum from TDI subchronically exposed mice was injected intradermally into the dorsum of the right ear of naive recipient mice. Twenty-four hours following transfer, mice were challenged with 1% TDI on the same ear, and responses were measured as a function of challenge-induced increases in ear thickness 24 h post-challenge. Data are presented as percent change from prechallenge ear thickness of the right ear. Significantly different from control serum treated group, noninactivated treated serum group, or subchronically sensitized/challenged group, \( p < 0.05, n = 5, \text{mean} \pm \text{SEM} \). The response to control sera was compared to that of normal mouse sera, and no difference was observed (data not shown). S/C = TDI sensitized/challenged from subchronic exposure.

**DISCUSSION**

These studies describe mouse models for TDI asthma that produce pathologies similar to that which occurs in workers with occupational asthma. The chronic exposure design and exposure level of 20 ppb was used to simulate potential workplace exposures, since levels in excess of 20 ppb are still documented in the workplace (Ott et al., 2003). The Occupational Safety and Health Administration’s (OSHA) current 15-minute Permissible Exposure Limit, expressed as a Ceiling Limit (PEL-C), and the American Conference of Governmental Industrial Hygienists’ (ACGIH) Threshold Limit Value time-weighted average (TLV®TWA) are 20 ppb and 5 ppb, respectively (Cummings and Booth, 2002). In Canada the TLV has been recently reduced from 20 to 5 ppb, although challenge doses as low as 1 ppb can effectively induce an asthmatic response in some individuals with TDI asthma (Lemiere et al., 2002). The acute high-dose (500 ppb) exposure was designed to mimic an accidental spill, such as occurred in 1984 with methyl isocyanate in Bhopal, India (Cullinan et al., 1997). These models should prove useful to investigate mechanisms of sensitization as well as effector responses for isocyanate-induced occupational asthma, because the routes of exposure are more relevant to human exposures than most other animal models.
These data also lend mechanistic understanding to the often-controversial finding in humans, where both IgE and non-IgE etiologies have been suggested. For example, previous clinical studies have shown that specific antibodies do not correlate well with disease activity, although TDI-specific IgG antibodies are often associated with exposure (Bernstein et al., 1997; Lushniak et al., 1998), while in guinea pigs, antibody titers correspond only to exposure concentrations (Karol, 1983). Mice exposed to TDI by inhalation for 6 weeks (20 ppb, 4 h/day, 5 days/week) demonstrated a marked allergic response evidenced by increases in airway inflammation, eosinophilia, goblet cell metaplasia, epithelial cell alterations, airway hyperresponsiveness (AHR), Th1/Th2 cytokine expression in the lung, elevated levels of serum total IgE, and TDI-specific IgG antibodies, as well as the ability to transfer many of these responses to naïve mice with lymphocytes and serum from mice subchronically exposed to TDI. Increased AHR as well as TDI-specific IgG antibodies and asthma-associated lung pathology were also observed in mice following acute high-dose (500 ppb) exposure. Although AHR could be transferred in animals that received acute TDI exposure, mice exposed using the acute exposure design failed to demonstrate elevated serum IgE levels, lung eosinophilia, or elevated Th1/Th2 lung cytokine expression. Thus, unlike that which occurred following subchronic TDI exposure, the pathologies observed following acute exposure lack definitive evidence that Th2 or IgE responses are involved. In this respect, the immunopathogenesis of allergic asthma is usually associated with a Th2 phenotype and can be adoptively transferred with Th2 cells (Cohn et al., 1998; Li et al., 1999; Scheerens et al., 1996). The Th2 cytokines, IL-4 and IL-5 are associated with isotype switching to IgE and eosinophilic responses, both of which are hallmarks of allergic asthma. Although adoptive transfer experiments using cells collected from acute TDI exposed mice resulted in increased AHR in recipients, it has recently been suggested that unrestrained plethysmography, while a sensitive indicator for altered lung function, may not always be a reliable measure of airway responsiveness (Adler et al., 2004) and, without other supporting clinical data, does not indicate that the pathology is of immunological origin. A number of non-IgE mechanisms can be postulated to be responsible for the pulmonary response following acute TDI exposure. For example, the response may involve specific T-cell immunity or IgG1 antibodies. Alternatively, nonspecific immune mechanisms may be evoked, such as the so-called "by-stander" response (Curtsinger et al., 1999). This mechanism would imply that inflammation, induced by TDI acting as an irritant, activates macrophages and neutrophils and the release of mediators such as TNFα and IL-1β, which stimulates T-cell responses independently of MHC-T cell receptor interactions.

The importance of exposure duration and dose in the pathogenic mechanisms that lead to TDI-induced asthma have been addressed previously in animal studies (Scheerens et al., 1999). In these investigations, dermal sensitization for 6 weeks on days 0, 7, 14, 21, 28, and 35, followed by intranasal challenge, resulted in significant respiratory involvement that was not evident following a 2-day sensitization period. Our results, at least using acute exposure, are also consistent with clinical studies which have suggested that isocyanate-induced airway reactivity can result from upper airway irritation without evoking TDI-specific immune mediators (Bernstein, 1982; Leroyer et al., 1998; Luo et al., 1990).

To help clarify the events evoked in TDI-asthma, adoptive and passive transfer experiments were performed. We observed that transfer of lymphocytes from either the acute or subchronic TDI exposed mice to naïve recipients allowed for increased AHR in response to methacholine. Additional adoptive transfer experiments indicated that both T cells and B cells contributed to TDI-asthma following subchronic TDI exposure, as evidenced by both increased AHR and TDI-specific dermal responses in recipient mice of both lymphocyte populations following TDI challenge. These observations are consistent with those of Scheerens et al. (1996), in which TDI asthma was adoptively transferred using lymphocytes from mice epicutaneously sensitized to TDI. A role for antibody was also suggested to be involved in the response following subchronic TDI exposure, as naïve mice administered serum from subchronically exposed mice responded to specific TDI challenge. Reaginic antibodies, particularly those of the IgE class, may be involved, since serum was rendered ineffective by heating and FcεRIg transgenic mice, which lack both IgE and IgG Fc receptors, failed to become sensitized to TDI. It has been suggested that the lack of a strong association between the presence of specific IgE antibodies and human disease in isocyanate-induced asthma may be due to phenotyping methods (Bernstein and Jolly, 1999), as the usage of polymeric isocyanate conjugates, rather than monomeric isocyanate-human serum albumin test antigens, provides greater assay sensitivity (Aul et al., 1999; Park et al., 2001).

Our data are also consistent with the idea that subchronic TDI sensitization involves a mixed Th1/Th2 response. This was evidenced by increased expression of the Th2 cytokines, IL-4 and IL-5, as well as the Th1 cytokine, IFNγ, expression in lungs of mice exposed to TDI using the subchronic paradigm. While allergic asthma is usually considered a Th2-mediated disease, a large body of evidence suggests that Th1 cells participate in the response. For example, cooperation between Th1 and Th2 cells is necessary for a robust eosinophil response, as well as Th2 cell recruitment into the lung in ovalbumin-specific cell transfer experiments (Randolph et al., 1999). In an ovalbumin murine asthma model, significant increases in the Th1 chemokines, such as IP-10, have been observed after challenge, and overexpression of IP-10 augmented AHR, eosinophilia, CD8+ cell numbers, and IL-4 expression while airway hyperresponsiveness in IP-10 knockout mice was ablated (Medoff et al., 2002). Studies in humans have found both CD4+ and IFNγ-secreting CD8+ T cells are present in the airways of asthmatics (Krug et al., 1996; Magnan et al., 2000; van Rijt and Lambrecht, 2001), and that the number of IFNγ producing cells relates to asthma severity, bronchial hyperresponsiveness, and blood eosinophilia (Magnan...
et al., 2000). In this respect, endogenous IFNγ may be necessary for optimal IgE production (Hofstra et al., 1998) and potentiation of IL-13-induced lung inflammation (Ford et al., 2001). Patients with occupational asthma also show both Th1 and Th2 cytokines following in vitro lymphocyte stimulation (Del Prete et al., 1993; Lee et al., 1998; Lummus et al., 1998; Maestrelli et al., 1997; Sumi et al., 2003). Furthermore, Th2 cells comprise only a minor portion of the T-cell population in the airways of TDI-allergic asthmatics, as most cells present a Th1 phenotype (Bernstein and Jolly, 1999). In fact, the majority of T-cell clones derived from bronchial mucosa of patients with isocyanate-induced asthma present a CD8+ phenotype capable of secreting IL-5 (Maestrelli et al., 1994) and IFNγ (Wisnewski et al., 2003).

In conclusion, a mouse model is described that demonstrates low-level subchronic TDI inhalation induces pathology, consistent with allergic asthma, manifested by airway inflammation, lung eosinophilia, increased AHR, asthma associated histopathology, Th cytokine expression, elevated serum IgE, and TDI-specific antibodies. Asthmatic symptoms also occur following high-dose, acute exposure, but the response is less robust, failing to demonstrate eosinophilia, elevated serum IgE levels, or Th cytokines. Evidence is also presented that, like allergic asthma, TDI asthma following subchronic exposure, while associated with a Th2 response involving IgE antibodies, also involves Th1 responses. Establishing the relative contribution of Th1 and Th2 mediators is addressed in a subsequent manuscript.

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REFERENCES

Adler, A., Cieslewicz, G., and Irvin, C. G. (2004). Unrestrained plethysmography is an unreliable measure of airway responsiveness in BALB/c and C57BL/6 mice. J. Appl. Physiol. 97, 286–292.

Aul, D. J., Bhaumik, A., Kennedy, A. L., Brown, W. E., Lesage, J., and Malo, J.-L. (1999). Specific IgG response to monomeric and polymeric diphenylmethane diisocyanate conjugates in subjects with respiratory reactions to isocyanates. J. Allergy Clin. Immunol. 103, 749–755.

Balmes, J., Becklake, M., Blanc, P., Henneberger, P., Kreiss, K., Mapp, C., Milton, D., Schwartz, D., Toren, K., and Viegi, G. (2003). Occupational contribution to the burden of airway disease. Am. J. Respir. Crit. Care Med. 167, 787–797.

Baur, X., Chen, Z., and Allmers, H. (1998). Can a threshold limit value for natural rubber latex airborne allergens be defined? J. Allergy Clin. Immunol. 101, 24–27.

Bell, S. J., Metzger, W. J., Welch, C. A., and Gilmour, M. I. (1996). A role for Th2 T-memory cells in early airway obstruction. Cell. Immunol. 170, 185–194.

Bernstein, D. I., and Jolly, A. (1999). Current diagnostic methods for diisocyanate induced occupational asthma. Am. J. Ind. Med. 36, 459–468.

Bernstein, I. L. (1982). Isocyanate-induced pulmonary diseases: A current perspective. J. Allergy Clin. Immunol. 70, 24–31.

Bernstein, J. A., Munson, J., Lummus, Z. L., Balakrishnan, K., and Leikau, G. (1997). T-cell receptor V[beta] gene segment expression in diisocyanate-induced occupational asthma. J. Allergy Clin. Immunol. 99, 245–250.

Cohn, L., Tepper, J. S., and Bottomly, K. (1998). IL-4-independent induction of airway hyperresponsiveness by Th2, but not Th1, cells. J. Immunol. 161, 3813–3816.

Cullinan, P., Acquilla, S., and Dhara, V. R. (1997). Respiratory morbidity 10 years after the Union Carbide gas leak at Bhopal: A cross sectional survey. The International Medical Commission on Bhopal, BMJ 314, 338–342.

Cummings, B. J., and Booth, K. S. (2002). Industrial hygiene sampling for airborne TDI in six flexible slabstock foam manufacturing facilities in the United States: A comparison of the short-term and long-term sampling data. Appl. Occup. Environ. Hyg. 17, 863–871.

Curtsinger, J. M., Schmidt, C. S., Mondino, A., Lins, D. C., Kedl, R. M., Jenkins, M. K., and Mescher, M. F. (1999). Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. J. Immunol. 162, 3256–3262.

Dearman, R. J., and Kimber, I. (2000). Role of CD4(+) T helper 2-type cells in cutaneous inflammatory responses induced by fluorescein isothiocyanate. Immunology 101, 442–451.

Del Prete, G. F., De Calri, M., D’Elios, M. M., Ricci, M., Fabbri, L., and Romagnani, S. (1993). Allergen exposure induces the activation of allergen-specific Th2 cells in the airway mucosa of patients with allergic respiratory disorders. Eur. J. Immunol. 23, 1445–1449.

Ebino, K., Lemus, R., and Karol, M. H. (1999). The importance of the diluent for airway transport of tolune diisocyanate following intranasal dosing of mice. Inhal. Toxicol. 11, 171–185.

Finotto, S., Fabbri, L. M., Rado, V., Mapp, C. E., and Maestrelli, P. (1991). Increase in numbers of CD8 positive lymphocytes and eosinophils in peripheral blood of subjects with late asthmatic reactions induced by toluene diisocyanate. Br. J. Ind. Med. 48, 116–121.

Ford, J. G., Rennick, D., Donaldson, D. D., Venkayya, R., McArthur, C., Hansell, E., Kurup, V. P., Warnock, M., and Grunig, G. (2001). IL-13 and IFN-gamma: Interactions in lung inflammation. J. Immunol. 167, 1769–1777.

Herrick, C. A., Xu, L., Wisnewski, A. V., Das, J., Redlich, C. A., and Bottomly, K. (2002). A novel mouse model of diisocyanate-induced asthma showing allergic-type inflammation in the lung after inhaled antigen challenge. J. Allergy Clin. Immunol. 109, 873–878.

Hofstra, C. L., Van Ark, I., Hofman, G., Nijkamp, F. P., Jardieu, P. M., and Holgate, S. T., Frew, A. J., and Howarth, P. H. (1996). Cytokine secretion patterns of T cells responding to haptenized-human serum albumin in toluene diisocyanate (TDI)-induced asthma patients. J. Allergy Clin. Immunol. 97, 245–250.

Karol, M. H. (1983). Concentration-dependent immunologic response to toluene diisocyanate. Toxicol. Appl. Pharmacol. 68, 229–241.

Krug, N., Madden, J., Redington, A. E., Lackie, P., Djukanovic, R., Schauer, U., Holgate, S. T., Frew, A. J., and Howarth, P. H. (1996). T-cell cytokine profile evaluated at the single cell level in BAL and blood in allergic asthma. Am. J. Respir. Cell Mol. Biol. 17, 319–326.

Lee, M., Park, S., Park, H. S., and Youn, J. K. (1998). Differential effects of endogenous and exogenous interferon-gamma on immunoglobulin E, cellular infiltration, and airway responsiveness in a murine model of allergic asthma. Am. J. Respir. Crit. Care Med. 157, 1769–1777.
Leroyer, C., Perfetti, L., Cartier, A., and Malo, J. L. (1998). Can reactive airways dysfunction syndrome (RADS) transform into occupational asthma due to “sensitisation” to isocyanates? Thorax 53, 152–153.

Li, Y. P., Atkins, C. M., Sweatt, J. D., and Reid, M. B. (1999). Mitochondria mediate tumor necrosis factor-alpha/NF-kappaB signaling in skeletal muscle myotubes. Antioxid. Redox Signal. 1, 97–104.

Lumnus, Z. L., Alam, R., Bernstein, J. A., and Bernstein, D. I. (1998). Diisocyanate antigen-enhanced production of monocyte chemotaxiant protein-1, IL-8, and tumor necrosis factor-[alpha] by peripheral mononuclear cells of workers with occupational asthma. J. Allergy Clin. Immunol. 102, 265–274.

Luo, J. C., Nelsen, K. G., and Fischbein, A. (1990). Persistent reactive airway dysfunction syndrome after exposure to toluene diisocyanate. Br. J. Ind. Med. 47, 239–241.

Lushniak, B. D., Reh, C. M., Bernstein, D. I., and Gallagher, J. S. (1998). Indirect exposure assessment of 4,4’-diphenylmethane diisocyanate (MDI) exposure by evaluation of specific humoral immune responses to MDI conjugated to human serum albumin. Am. J. Ind. Med. 33, 471–477.

Maestrelli, P., Del Prete, G. F., De Carli, M., D’Elios, M. M., Saetta, M., Di Stefano, A., Mapp, C. E., Romagnani, S., and Fabbrini, L. M. (1994). CD8 T-cell clones producing interleukin-5 and interferon-gamma in bronchial mucosa of patients with asthma induced by toluene diisocyanate. Scand. J. Work. Environ. Health 20, 376–381.

Maestrelli, P., Occari, P., Turato, G., Papiris, S. A., Di Stefano, A., Mapp, C. E., Milani, G. F., Fabbri, L. M., and Saetta, M. (1997). Expression of interleukin (IL)-4 and IL-5 proteins in asthma induced by toluene diisocyanate (TDI). Clin. Exp. Allergy 27, 1292–1298.

Magnan, A. O., Mely, L. G., Camilla, C. A., Badier, M. M., Montero-Julian, F. A., Guillot, C. M., Casano, B. B., Prato, S. J., Fert, V., Bongrand, P., et al. (2000). Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma. Increased IFN-gamma-producing CD8(+) T cells in asthma. Am. J. Respir. Crit. Care Med. 161, 1790–1796.

Mapp, C. E., Saetta, M., Maestrelli, P., Stefano, A. D., Chitano, P., Boschetto, P., Ciaccia, A., and Fabbri, L. M. (1994). Mechanisms and pathology of occupational asthma. Eur. Respir. J. 7, 544–554.

Medoff, B. D., Sauty, A., Tager, A. M., MacLean, J. A., Smith, R. N., Mathew, A., Dufour, J. H., and Luster, A. D. (2002). IFN-gamma-inducible protein 10 (CXCL10) contributes to airway hyperreactivity and airway inflammation in a mouse model of asthma. J. Immunol. 168, 5278–5286.

Ott, M. G., Diller, W. F., and Jolly, A. T. (2003). Respiratory effects of toluene diisocyanate in the workplace: A discussion of exposure-response relationships. Crit. Rev. Toxicol. 33, 1–59.

Park, H. S., Kim, H. Y., Lee, S. K., Kim, S. S., and Nahm, D. H. (2001). Diverse profiles of specific IgE response to toluene diisocyanate (TDI)-human serum albumin conjugate in TDI-induced asthma patients. J. Korean Med. Sci. 16, 57–61.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Randolph, D. A., Stephens, R., Carruthers, C. J., and Chaplin, D. D. (1999). Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. J. Clin. Invest. 104, 1021–1029.

Rauff-Heimsoth, M., and Baur, X. (1998). Pathomechanisms and pathophysiology of isocyanate-induced diseases—summary of present knowledge. Am. J. Ind. Med. 34, 137–143.

Satoh, T., Kramarik, J. A., Tollerud, D. J., and Karol, M. H. (1995). A murine model for assessing the respiratory hypersensitivity potential of chemical allergens. Toxicol. Lett. 78, 57–66.

Scheeren, H., Buckley, T. L., Muis, T. L., Garssen, J., Dormans, J., Nijkamp, F. P., and Van Loveren, H. (1999). Long-term topical exposure to toluene diisocyanate in mice leads to antibody production and in vivo airway hyperresponsiveness three hours after intranasal challenge. Am. J. Respir. Crit. Care Med. 159, 1074–1080.

Scheeren, H., Buckley, T. L., Muis, T., Van Loveren, H., and Nijkamp, F. P. (1996). The involvement of sensory neuropeptides in toluene diisocyanate-induced tracheal hyperreactivity in the mouse airways. Br. J. Pharmacol. 119, 1665–1671.

Schwarze, J., Cieslewicz, G., Hamelmann, E., Joetham, A., Shultz, L. D., Lamers, M. C., and Gelfand, E. W. (1999). IL-5 and eosinophils are essential for the development of airway hyperresponsiveness following acute respiratory syncytial virus infection. J. Immunol. 162, 3004–3008.

Shen, H. H., Oechkar, S. I., McGarry, M. P., Crosby, J. R., Hines, E. M., Borchers, M. T., Wang, H., Biechelle, T. L., O’Neill, K. R., Ansay, T. L., et al. (2003). A Causative Relationship Exists Between Eosinophils and the Development of Allergic Pulmonary Pathologies in the Mouse. J. Immunol. 170, 3296–3305.

Sumi, Y., Kyi, M., Miyazaki, Y., Ohtani, Y., Miyake, S., and Yoshizawa, Y. (2003). Cytokine mRNA Expression in Isocyanate-Induced Hypersensitivity Pneumonitis. Respiration 70, 284–291.

Tee, R. D., Cullinan, P., Welch, J., Burge, P. S., and Newman-Taylor, A. J. (1998). Specific IgE to isocyanates: A useful diagnostic role in occupational asthma. J. Allergy Clin. Immunol. 101, 709–715.

van Rijt, L. S., and Lambrecht, B. N. (2001). Role of dendritic cells and Th2 cells in the development of asthma. Crit. Rev. Toxicol. 31, 256–272.

Watanabe, J., Miyazaki, Y., Zimmerman, G. A., Albertine, K. H., and Schwartz, J. (1995). Toluene diisocyanate antigen-enhanced production of monocyte chemoattractant protein-1, IL-8, and tumor necrosis factor-[alpha] by peripheral mononuclear cells of workers with occupational asthma. J. Allergy Clin. Immunol. 102, 265–274.

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