Role of Tumor Necrosis Factor in Toluene Diisocyanate Asthma

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Nearly 9 million workers are exposed to chemical agents associated with occupational asthma, with isocyanates representing the chemical class most responsible. Isocyanate-induced asthma has been difficult to diagnose and control, in part because the biologic mechanisms responsible for the disease and the determinants of exposure have not been well defined. Isocyanate-induced asthma is characterized by airway inflammation, and we hypothesized that inflammation is a prerequisite of isocyanate-induced asthma, with tumor necrosis factor (TNF)-α being critical to this process. To explore this hypothesis, wild-type mice, athymic mice, TNF-α receptor knockout (TNFR), and anti–TNF-α antibody–treated mice were sensitized by subcutaneous injection (20 μl on Day 1; 5 μl, Days 4 and 11), and challenged 7 d later by inhalation (100 ppb; Days 20, 22, and 24) with toluene diisocyanate (TDI). Airway inflammation, goblet cell metaplasia, epithelial cell damage, and nonspecific airway reactivity to methacholine challenge, measured 24 h following the last challenge, were reduced to baseline levels in TNF-α null mice and athymic mice. TNF-α deficiency also markedly abrogated TDI-induced Th2 cytokines in airway tissues, indicating a role in the development of Th2 responses. Despite abrogation of all indicators of asthma pathology, TNF-α neutralization had no effect on serum IgE levels or IgG-specific TDI antibodies, suggesting the lack of importance of a humoral response in the manifestation of TDI-induced asthma. Instillation studies with fluorescein-conjugated isothiocyanate and TDI suggested that TNF-α deficiency also resulted in a significant reduction in the migration of airway dendritic cells to the draining lymph nodes. Taken together, these results suggest that, unlike protein antigens, TNF-α has multiple and central roles in TDI-induced asthma, influencing both nonspecific inflammatory processes and specific immune events.

Asthma is a chronic respiratory disease characterized by the presence of reversible airway constriction and airway hyperresponsiveness (AHR) to various stimuli. Isocyanates, such as toluene diisocyanate (TDI), are the most common cause of occupational asthma, with over 100,000 workers in the United States exposed every year and 5–10% of these developing asthma (1). Isocyanate-induced asthma has been difficult to diagnose and control, in part, because the biologic mechanisms responsible for the disease and the determinants of exposure have not been well defined, and validated animal models do not yet exist. It has been suggested that multiple mechanisms may be involved in the induction of isocyanate-induced asthma, including immunologic, inflammatory, genetic, pharmacologic, and neurogenic (2–4). In humans, TDI-induced asthma shares many characteristics of atopic asthma manifested by a latency period, persistent airway hyperreactivity to chemical-specific and nonspecific (e.g., methacholine) challenge, and airway inflammation involving the presence of activated T cells, eosinophils, neutrophils, and mast cells. Airway inflammation in TDI asthma is a particular hallmark, being both severe and persistent, evident even months after exposure (4).

Tumor necrosis factor (TNF)-α is a pleiotropic cytokine and a central regulator of inflammatory processes. Like other cytokines, TNF-α confers its signals to target cells through binding to distinct membrane receptors, referred to as p55 or TNFR1 and p75 or TNFR2 (5). Although TNFR1 is the major biologically active receptor, both receptors exert unique activities (5). TNF-α is likely a central mediator of airway inflammation and bronchial hyperresponsiveness in asthma (6). It is measured at high levels in bronchoalveolar lavage fluid (BALF) and can regulate inflammatory cell infiltration, locally enhance vascular permeability, and aid in the release of bronchoactive substances such as histamine (8). Among other functions, TNF-α promotes the migration of dendritic cells, part of a family of professional antigen-presenting cells present in many organs (10). These cells migrate into regional lymph nodes, where they present processed antigen to T cells. In contrast to these airway proinflammatory activities, studies utilizing the ovalbumin murine model of allergic asthma, demonstrated either little significant effect of neutralizing antibodies to TNF-α on AHR and eosinophilia, or comparable responses of TNF-α receptor knockout mice to that observed in wild-type exposed animals (11).

We hypothesized that TNF-α is critical in the development of isocyanate-asthma due to its ability to regulate inflammation and initiate the migration of lung dendritic cells. The role of TNF-α in TDI-induced asthma was demonstrated in TNF-α–deficient mice, produced by either administration of neutralizing antibodies or by deletion of the gene controlling TNF receptors, with the abatement of TDI-

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Abbreviations: airway hyperresponsiveness, AHR; bronchoalveolar lavage fluid, BALF; complementary DNA, cDNA; TNF-α double receptor knockout, DKO; enzyme-linked immunosorbent assay, ELISA; fluorescence-activated cell sorter, FACS; fluorescein-conjugated isothiocyanate, FITC; de-3-phosphate-dehydrogenase, G3PDH; interferon, IFN; interleukin, IL; lymphotactin, Ltn; lymphotoxin β, Ltβ; enhanced pause, Penh; parts per billion, ppb; reverse transcription-polymerase chain reaction, RT-PCR; glyceraldehyde ribonuclease protection assay, RPA; toluene diisocyanate, TDI; tumor necrosis factor, TNF; TNF-α receptor knockout, TNFR ; TNF-α receptor 1 (p55) knockout, TNFR1; TNF-α receptor 1 (p75) knockout, TNFR2.

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induced airway hyperresponsiveness and inflammation, but not specific antibody formation.

Materials and Methods

Experimental Animals

Female wild-type and athymic (nu+/nu+) C57BL/6J mice, ~ 6–8 wk old, were obtained from Jackson Laboratory (Bar Harbor, ME). Upon arrival, the mice were quarantined for 2 wk and acclimated to a 12-h light/dark cycle. Transgenic mice deficient in tumor necrosis factor receptor 1 (TNFR1), receptor 2 (TNFR2), or both receptors (double knockout; DKO), were kindly provided by Immunex Corp. (Seattle, WA) through Dr. Larry Schook (University of Minnesota, St. Paul, MN). All transgenic mice were re-derived (Charles River, Charlestown, MA). Animals were housed in microisolator cages in pathogen-free and environmentally controlled conditions at NIOSH facilities in compliance with AAALAC approved guidelines. Food and water were provided *ad libitum*.

Anti-TNF Antibody Treatment

The preparation of polyclonal rabbit anti-mouse TNF-α antisera and specificity testing were described elsewhere (13). C57BL/6J mice were injected intravenously with 0.2 ml of filtered anti-TNF antisera (representing 250,000 units) or an equal volume of nonimmune sera 24 h before TDI sensitization and 24 h before TDI inhalation challenge. This dose of antiserum effectively inhibits any increase in serum TNF-α following endotoxin administration.

Experimental Design and Tissue Collection

As it has been suggested that cytokine transgenic mice may possess compensatory mechanisms, TNFR knockout mice were compared with mice treated with neutralizing antibodies to TNF-α in selected experiments. Mice were injected subcutaneously with 20 μl of neat TDI (80:20 molar mixture of 2,4:2,6 isomers provided by Bayer, Pittsburgh, PA) on Day 1, followed by 5-μl injections, brought up to 20 μl in olive oil, on Days 4 and 11. Control mice received an equivalent volume of vehicle. Airway challenge (1 h, 100 ppb TDI) was performed on Days 20, 22, and 24 using previously described methods (14). The generation system produces TDI vapor free of TDI aerosol. Real-time monitoring of the chamber atmosphere was performed using an Autostep continuous toxic gas analyzer (Bacharach, Inc, Pittsburgh, PA) with the probe placed 12 cm above the animals.

Twenty-four hours after the last airway challenge, mice were killed and blood, lungs, nares, tracheae, and mediastinal lymph node were collected. Tissues were immersed in 10% neutral buffered formalin, nares were decalcified and the tissues embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin for blinded histopathologic assessment. PAS staining was performed for confirmation of goblet metaplasia. The histopathologic grading system was expressed on a 0–5 scale for each animal, with 0 representing no changes, 1 equal to minimal change, 2 equal to slight/mild changes, 3 equal to moderate changes, 4 equal to moderately/severe changes, and 5 equal to severe changes. Additional groups of mice were used for BALF collection. 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 pooled. A 0.1 ml sample of BALF was used for cytospin preparations. The slides were fixed and stained with Diff-Quik (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. In replicate experiments, the trachea and lungs were collected, frozen in liquid nitrogen, and stored at −80°C for RT-PCR and RPA analysis.

Because TNF-α has been shown to play an important role in the migration of dendritic cells from the skin or gut to the draining lymph nodes, the role of TNF-α in airway dendritic cell migration in this TDI asthma model was examined. To assess pulmonary dendritic cell migration, mice were lightly anesthetized with sodium pentobarbital (50 μl, 30 mg/kg intraperitoneally) and intratracheally instilled with 50 μl of saline-vehicle or FITC-dextran (4,000 MW; Sigma, St. Louis, MO) at doses up to 58 mg/kg. The mediastinal lymph nodes, lungs, and trachea were collected 24 and 48 h after instillation. To assess dendritic cell migration in response to TDI, mice were lightly anesthetized and administered 20 μl of 5% TDI in ethyl acetate:olive oil (1:4) intranasally, divided equally between the two nares. Preliminary dose response studies demonstrated that 5% TDI produces a 5-fold significant increase of CD86+ cells in the airway draining lymph nodes. Mice received neutralizing antibodies to TNF-α 24 h before instillation, and the cervical lymph nodes were collected 24 h after instillation.

Antibody Detection

Total serum IgE was measured using a sandwich ELISA as previously described (15). Briefly, plates were coated with 5 μg/ml of rat monoclonal anti-mouse IgE (PharMingen, San Diego, CA). Serial 2-fold dilutions of test sera, starting at 1:5, were added and incubated with peroxidase-goat anti-mouse IgE (1:1,000; Nordic Immunological Laboratories, Capistrano Beach, CA) and developed with ABTS substrate (2,2′-azino-bis{3-ethylbenzthiazoline-6-sulfonic acid}). 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 as previously described (15). Test sera were added (serial 2-fold dilutions starting at 1:5), to individual wells and were incubated with peroxidase-conjuated, goat anti-mouse antibodies against either IgG (1:400; Sigma), IgE (1:1,000; Nordic Immunological Laboratories), IgG1, or IgG2a (both at 1:400; The Binding Site, Birmingham, UK) and developed with ABTS substrate.

AHR

Airway responsiveness of naïve mice, TDI-exposed mice, or TDI-exposed mice administered TNF-α–neutralizing antibodies was assessed 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. The airway reactivity was expressed as enhanced pause (Penh), which correlates with measurement of airway resistance, impedance, and intrapleural pressure, and is derived from the formula: $Penh = (Te - Tr/Tr) \times Pef/Pif$; where Te = expiration time, Tr = relaxation time, Pef = peak expiratory flow, and Pif = peak inspiratory flow (16). Mice were placed into the plethysmograph and exposed for 5 min to nebulized phosphate-buffered saline (PBS) to establish baseline values. This was followed by increasing concentrations of nebulized methacholine (0–50 mg contained in 1.0 ml of PBS) using an AeroSonic ultrasonic nebulizer (DeVilbiss, Somerset, PA). After each nebulization, recordings were taken for 5 min. The Penh values during each 5-min sequence were averaged and expressed as percentage increase of baseline values following PBS exposure for each methacholine concentration.

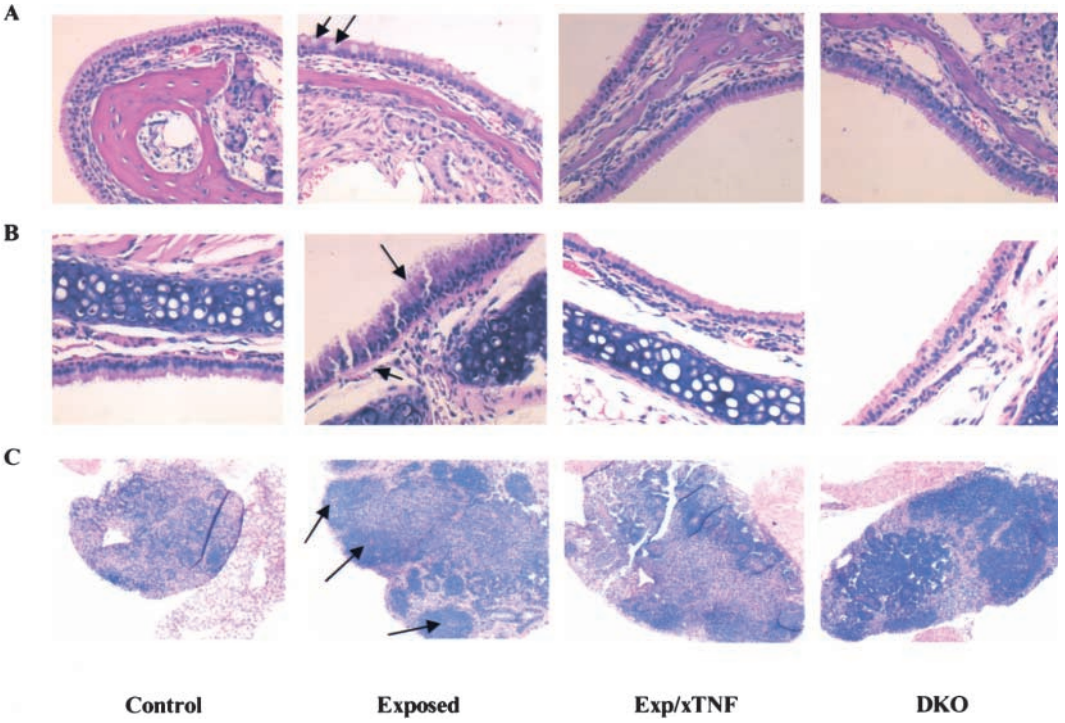

Figure 1. Influence of TNF-α status on TDI-induced pathology. Twenty-four hours after the last TDI inhalation challenge, tissues were fixed, and nares were decalcified, sectioned, and stained with hematoxylin and eosin (H&E). Sections of representative tissues from nares (*A*), tracheae (*B*), and draining lymph nodes (*C*) from control, TDI-exposed, or TDI-exposed receiving neutralizing TNF-α antibodies or TNFR DKO mice are shown. *Original magnification:* ×20–40 (*A*), ×40 (*B*), and ×10 (*C*). *Arrowheads* denote locations of goblet metaplasia, cell piling, inflammatory influx, and follicle formation.

Reverse Transcriptase–Polymerase Chain Reaction

Tissues were collected and total cellular RNA was extracted using the Qiagen RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was synthesized as previously described (17). PCR primers for murine G3PDH, interferon (IFN)-γ, interleukin (IL)-4, and IL-5 were purchased from Clontech (Palo Alto, CA), and TNF-α primers were purchased from Stratagene (La Jolla, CA). Samples of cDNA were amplified by polymerase chain reaction (PCR) using a GeneAmp PCR System 9600 DNA Thermal Cycler (Perkin Elmer, Foster City, CA). For each set of primers, dilutions of cDNA were amplified for 20, 23, 25, 28, 30, 35, and 38 cycles to define optimal conditions for linearity and to permit semiquantitative analysis of signal strength as previously described (18). If necessary, the concentrations of cDNA were readjusted to normalize for G3PDH and the PCR repeated. When appropriate, the specificity of the PCR bands was confirmed by restriction site analysis of the amplified cDNA which generated restriction fragments of the expected size (data not shown). Amplified PCR products were separated electrophoretically on 1.5% agarose gel (UltraPure; Sigma), scanned directly with an Eagle Eye

TABLE 1

Summary of pathological changes induced by TD1 exposure in C57BL/6J and TNF receptor knockout mice

Tissue Alterations	Native C57BL	Exposed C57BL*	C57BL Chall	Exposed x-TNF	TNFR1	TNFR2	DKO	Athymic
Nares								
Exudate	0 (0.5)	3 (0.24)	0.5 (0.1)	2 (0)	2 (0.3)	1 (0.18)	1 (0.2)	0.4 (0.3)
Goblet metaplasia	0 (0)	4 (0.65)	1 (0.12)	1.1 (0.3)	4 (0.16)	2 (0.28)	0.4 (0.2)	1.2 (0.2)
Inflammation	0 (0)	2 (0.2)	0 (0)	0.9 (.15)	1.2 (0.43)	1.2 (0.18)	0 (0)	0 (0)
Trachea								
Cilia loss	0 (0)	2 (0)	0.5 (0.3)	0.8 (0.2)	2 (0)	0 (0)†	0 (0)	0 (0)
Goblet metaplasia	0 (0)	1 (0)	0 (0)	1 (0)	1 (0.25)	0.2 (0.2)†	0 (0)	0.4 (0.18)
Cytoplasmic extensions	0 (0)	2 (0.2)	0.7 (0.5)	0.8 (0.2)	0.9 (0.12)	2 (0.7)	0 (0)	0.2 (0.15)
Inflammation	0 (0)	1 (0.2)	0 (0)	0 (0)	0.5 (0.21)	1 (0.75)	0 (0)	0 (0)
Cell piling	0 (0)	2 (0.14)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.2 (0.15)
Lymph Node								
Activation state	0 (0.5)	3 (0.3)	0.5 (0.5)	1 (0.62)	2 (0.21)	1 (0.2)	2 (0.3)	1.2 (0.15)
Germinal cell formation	0 (0)	3 (0.47)	0 (0)	0.8 (0.5)	0.8 (0.3)	0.5 (0.3)	0 (0)	1 (1)

Histopathological changes were assessed 24 hours 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 aniamls.

* Significantly different from control, challenge only, anti-TNF pretreatment group, DKO, and athymic groups for all indices graded.

† Significantly different from TNFR1 group ($P < 0.05$, $n = 6$, ± SEM are presented in parentheses beside each mean value).

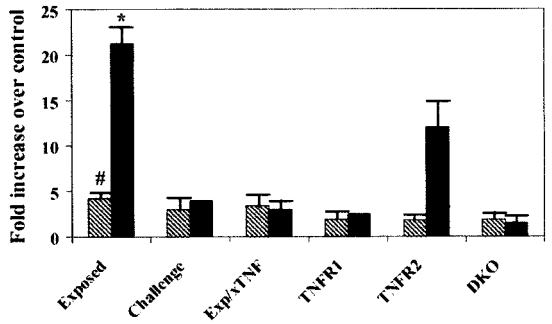

Figure 2. Role of TNFR knockout in TDI-induced bronchoalveolar lavage inflammatory cells. BALF was collected 24 h after the last TDI inhalation challenge. Cytospin preparations were examined for cellular content. Differential cell counts were obtained using light microscopic by evaluation of 300 cells per slide. #Significantly different from control group ($P < 0.05$, $n = 6$, ± SEM). *Significantly different from control, TNFR1, TNFR2, DKO, and anti–TNF-α antibody treatment groups ($P < 0.05$, $n = 6$, ± SEM). *Striped bars*, lymphocytes; *solid bars*, PMNs.

II digital imaging system (Stratagene), and quantified using ImageQuant software (Storm; Molecular Dynamics, Sunnyvale, CA).

RNase Protection Assay

RNA samples were quantified by Rnase protection assay (RPA) using the RiboQuant kit (PharMingen). The dsDNA multiprobe templates mCK-3b and mCK-5 were purchased from PharMingen. ^{32}P-labeled cRNA probes were produced from dsDNA templates using the MAXIscript T7 kit from Ambion (Austin, TX) according to the manufacturer's instructions. Aliquots of total RNA were assayed using the RiboQuant kit (PharMingen). Due to lower levels of RNA, trachea RNA from three mice and lung RNA from three mice were pooled, respectively, such that 20 μg of RNA was utilized for each representative sample in the assay. Samples were electrophoresed on a sequencing gel, and protected fragments were quantified using a phosphor imaging system and ImageQuant software (Storm; Molecular Dynamics).

Flow Cytometry

Lymph nodes were disrupted in fluorescence-activated cell sorter (FACS) buffer (PBS, 0.1% BSA, 0.05% NaN_3), centrifuged, and the cell pellet resuspended to a concentration of 2×10^5 cells/ml. Cells were transferred onto a 96-well, U-bottomed culture plate (200 μl/well), washed, and incubated for 30 min with 10 μl/well of a 1:100 dilution of either phycoerthryin-conjugated rat anti-mouse CD14 monoclonal antibody or CD86 monoclonal antibody (PharMingen). Before staining for CD86, the cells were preincubated with 5 μl/well of blocking antibody (1:50, clone 2.4G2, rat anti-mouse CD16/CD32; PharMingen) or for CD14 FACS buffer. CD86 is present on activated dendritic cells, whereas CD14 is present on murine monocytes and macrophages, but not activated dendritic cells (19). TDI+ cells were detected with rabbit anti-mouse anti-TDI antibody (1:500) (20) followed by FITC-conjugated goat anti-rabbit antibody (1:50; PharMingen). The cell pellets were washed, resuspended in FACS buffer, and 10,000 events assessed on a FACS Calibur (Becton-Dickinson).

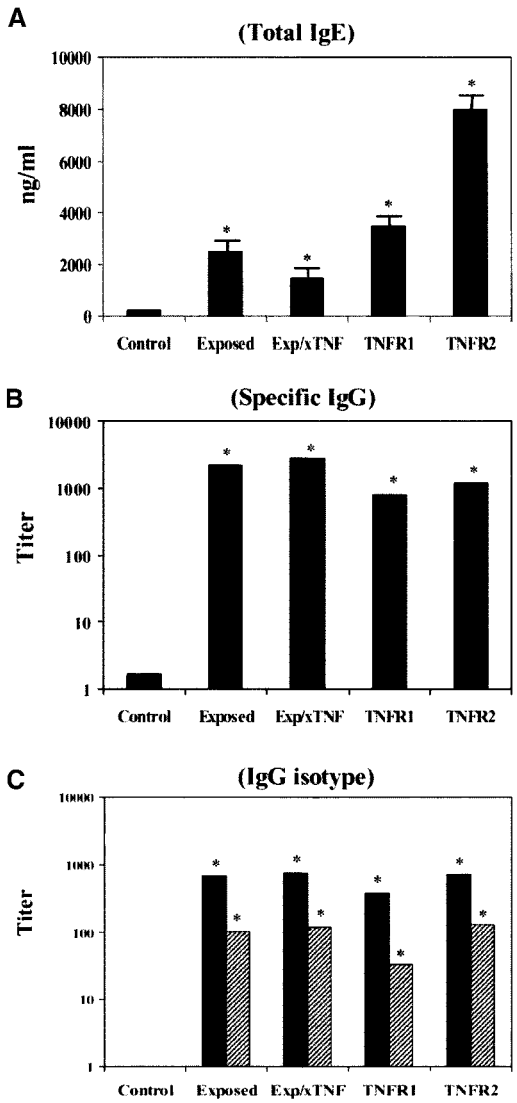

Figure 3. Serum antibody titers to TDI are unaffected by TNF-α status. Sera was collected from control, TDI-exposed, TDI-exposed anti–TNF-α antibody–treated, TDI-exposed TNFR1, TDI-exposed TNFR2, and TDI-exposed DKO mice 24 h after the last inhalation challenge ($n = 6$, ± SEM). Total IgE antibodies (*A*), specific IgG antibodies (*B*), and specific IgG1 (*solid bars*) and IgG2a (*striped bars*) antibodies (*C*). *Significantly different from control group ($P < 0.05$, $n = 6$, ± SEM).

Statistical Analysis

All studies were replicated with representative data shown. For statistical analysis, standard one-way ANOVA followed by Student-Newman-Keuls test was used for multiple group comparisons for all data sets except for the FITC dendritic cell data, for which the Student's two-tailed unpaired *t* test was used to determine the level of difference between the two experimental groups; $P < 0.05$ was considered a statistically significant difference.

Results

TNF-α Neutralization Inhibits TDI-Induced Pathologic Sequelae and Airway Inflammation

Airway inflammation is a characteristic feature of the asthmatic response to TDI and is considered a key manifestation

of underlying bronchial hyperresponsiveness. Although TDI-sensitized and -challenged mice did not reveal appreciable histologic changes in the lungs, both the tracheae and nares exhibited inflammatory responses manifested by neutrophil, lymphocyte, and macrophage infiltration (Figure 1; Table 1). These tissues additionally exhibited degenerative cellular changes including loss of cilia, goblet cell metaplasia, septal exudate, cytoplasmic extensions, and cell piling. When compared with wild-type, sensitized mice, the tracheae and nares of TDI-sensitized mice pretreated with neutralizing antibodies to TNF-α or TNF-α DKO mice displayed reduced inflammation manifested as minimal leukocyte infiltration and degenerative changes (Figures 1A and 1B; Table 1). Moreover, the mediastinal lymph nodes, located near the bronchial bifurcation, were highly activated in TDI-exposed mice, where typically five follicles per field following sensitization were observed compared with zero to two follicles per field in control mice (Figure 1C). The number of follicles were reduced in TNF-α receptor knockout mice and anti–TNF-α antibody–treated mice as reflected by one to two follicles per field (Figure 1, Table 1). Compared with TNFR1 mice, TNFR2 mice were less susceptible to the TDI-induced goblet metaplasia, nare exudate, and loss of cilia (Table 1). The absence of both receptors provided mice almost complete protection to TDI exposure associated with the pathologic sequelae. Thus, both receptors appear to participate in the TDI-induced airway inflammation. To investigate the role of T lymphocytes in TDI-induced asthma, athymic mice were included in the experimental design. As summarized in Table 1, athymic mice demonstrated little, if any, histopathologic sequelae indicative of inflammation or degenerative changes in the nares or trachea when compared with wild-type exposed animals. Challenged only control mice as summarized in Table 1 demonstrated little histopathologic changes, nearly equivalent to naive controls.

Although the lungs of TDI-exposed mice demonstrated only mild histologic changes, the cellular constituents in the BALF indicated the existence of pulmonary inflammation. This was evidenced by a 20-fold increase in neutrophils and 4-fold increase in lymphocytes, although macrophages remained the predominant cell type in the lung, representing over 90% of the cell population in control and sensitized mice (Figure 2). Treatment with neutralizing antibodies to TNF-α resulted in an 80% reduction in the number of neutrophils in BALF, whereas DKO and TNFR1 knockout mice had near control levels of lymphocytes and neutrophils.

Serum Antibody Titers to TDI Are Unaffected by TNF-α Status

Twenty-four hours after the last TDI challenge, blood was collected and serum analyzed for total IgE as well as TDI-specific IgG antibodies (Figure 3). TDI-specific IgG antibodies, including both IgG_1 and IgG_2, are characteristic of TDI asthma (21). Specific IgG antibodies and total IgE were consistently detected and significantly elevated over naive controls (Figures 3A and 3B, respectively). Elevated IgE levels were present in TNF-α–deficient mice, as well as specific IgG antibodies (Figure 3B), including IgG_1 and IgG_2 subclasses (Figure 3C). Although TNFR1-deficient mice displayed lower levels of specific IgG antibodies (a titer of 800 compared with 2,200, 2,700, and 1,200 for exposed, exposed anti-TNF–treated, and TNFR2 mice, respectively), neither TNF-α receptor knockout status nor TNF neutralization affected the specific IgG isotype (Figure 3C). Challenged only control mice responded similarly to naive controls, with no detectable levels of circulating specific antibodies (data not shown). Because the challenge-only group continued to respond similarly to that of naive mice, additional data is not presented.

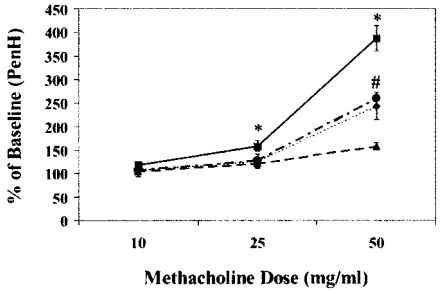

Figure 4. TNF-α modulation of TDI-induced airway hyperreactivity. Mice which received vehicle (*diamonds*), TDI (*squares*), TDI-exposed athymic mice (*circles*) or TDI plus TNF-neutralizing antibodies (*triangles*) were assessed for methacholine reactivity. The change in Penh values from baseline in response to increasing doses of inhaled methacholine was determined 24 h after the last sensitization. *Significantly different from control, athymic, and TNF-α–neutralized groups; #significantly different from TNF-α–neutralized group ($P < 0.05$, $n = 6$, ± SEM)

TNF-α Modulates TDI-Induced AHR

As AHR is also a major characteristic of TDI asthma in humans (4) and animals (21), the effect of TNF-α on increased airway reactivity to methacholine challenge was assessed in animals given neutralizing antibodies to TNF-α before TDI sensitization. Anti–TNF-α antibody prevented hyperreactivity to methacholine, whereas TDI-exposed animals, treated with nonimmune sera, displayed significant hyperreactivity following challenge to 25 or 50 mg/ml concentrations of methacholine when compared with sensitized athymic mice or mice treated with vehicle alone (Figure 4). AHR in naive and athymic mice to 50 mg/ml methacholine was significantly greater than that of the sensitized/anti–TNF-α antibody–treated mice probably reflecting a role for TNF-α in nonspecific AHR.

TNF-α Neutralization Alters Airway Inflammatory Cytokine Expression

To determine the effects of TNF-α on proinflammatory airway cytokine expression, RNA was isolated from the tracheae and lungs of mice 24 h after the last challenge, and the relative levels of IL-4, TNF-α, and IFN-γ mRNA determined by RT-PCR (Figure 5). An elevation in IL-4 and TNF-α, and a reduction in IFN-γ mRNA transcripts, were observed in the tracheae of TDI-exposed mice compared with control animals. TNF-α neutralization minimized the cytokine changes associated with sensitization, as evidenced by lowered expression of IL-4 and TNF-α and increased

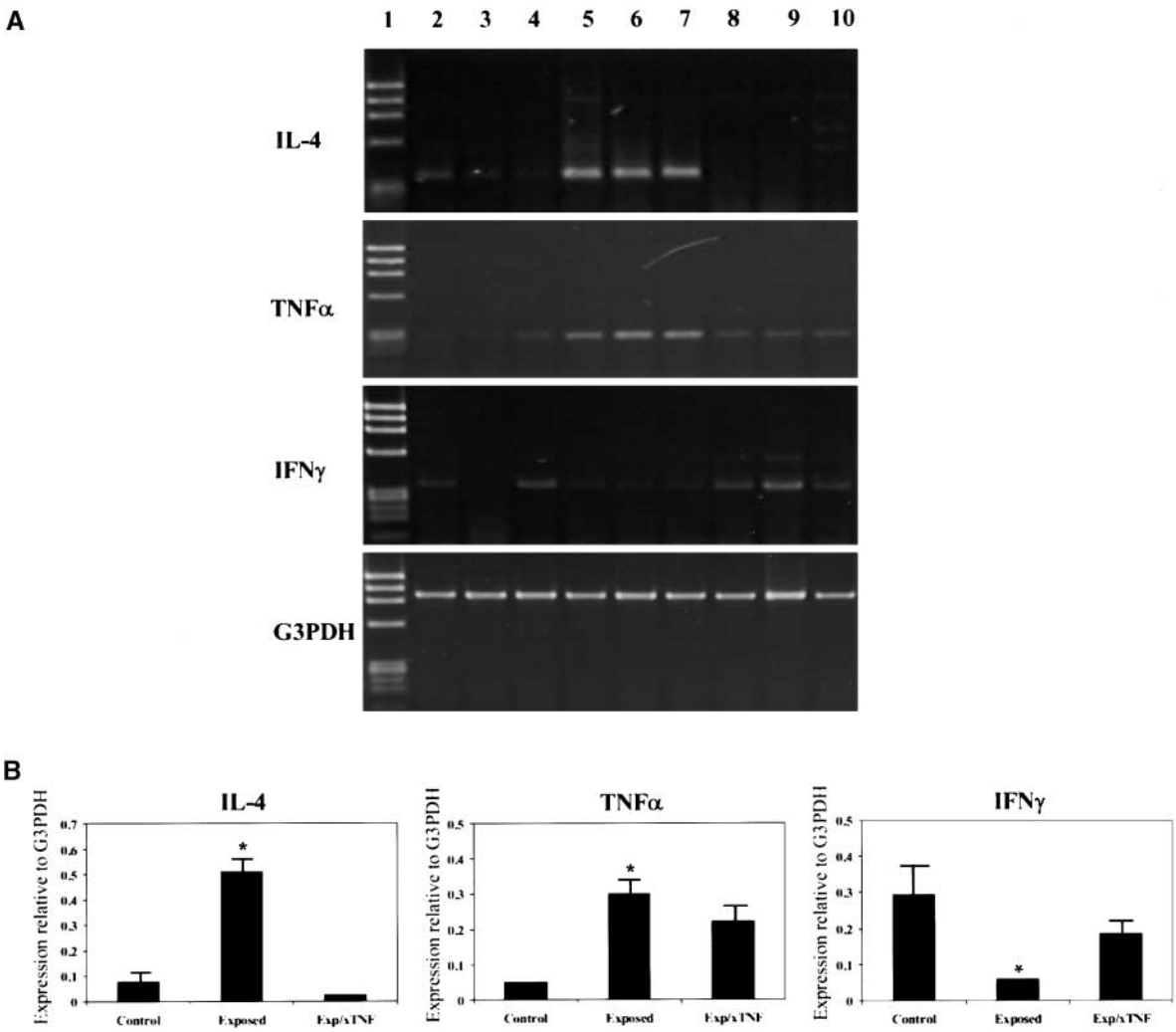

Figure 5. Effect of TNF-α neutralization on inflammatory cytokine expression in TDI-sensitized mice. Twenty-four hours after the last inhalation challenge, RNA was isolated from the tracheae and RT-PCR was performed using IL-4, TNF-α, IFN-γ, and G3PDH-specific primers. (*A*) Ethidium bromide–stained 1.5% agarose gels representative of three experiments and groups of $n = 12$. *Lane 1:* molecular weight markers; *lanes 2–4:* control mice; *lanes 5–7:* TDI-exposed mice; *lanes 8-10:* TDI-exposed anti–TNF-α antibody–treated mice. (*B*) Gels were scanned with a digital analysis system and the PCR products were quantified. Data are expressed relative to G3PDH expression ($P < 0.05$, $n = 12$, ± SEM)

IFN-γ expression compared with that observed in the TDI-exposed animals. To extend these observations to additional inflammatory cytokines and chemokines, RPA was performed on pooled mRNA from the tracheae and lungs. Two- to fivefold increases in Rantes, lymphotactin (Ltn), TNF-β, lymphotoxin (LT)-β, and TNF-α occurred in the tracheae of TDI-sensitized mice (Figure 6). The levels of these cytokines in TDI-sensitized mice pretreated with neutralizing anti–TNF-α antisera were similar to control values indicating the importance of TNF-α in their regulation. There were no significant changes observed in the levels of other inflammatory cytokines or chemokines examined in the tracheae among the different treatment groups. No treatment-related changes were observed in the lungs in the expression of any cytokine or chemokine examined (data not shown).

TNF-α Status Inhibits Airway Dendritic Cell Migration

Because TNF-α has been shown to play a key role in the migration of dendritic cells from the skin or gut to the draining lymph nodes (10), the role of TNF-α in airway dendritic cell migration in the TDI asthma model was examined. Following instillation of FITC-coated dextran, cells containing fluorescent particles were observed within the alveolar spaces, interstitium, and paracortex of the mediastinal lymph nodes within 24 h (Figure 7A). FACS analysis showed a dose-dependent increase in FITC-bearing cells in the draining lymph nodes in mice receiving either nonimmune sera or neutralizing antibodies to TNF-α (Figure 7B). However, antibody treatment resulted in a 50% reduction in the total number of FITC+ cells. To identify the FITC-bearing cell populations present in the lymph nodes, the number of cells expressing CD86 (B7–2), a cell surface

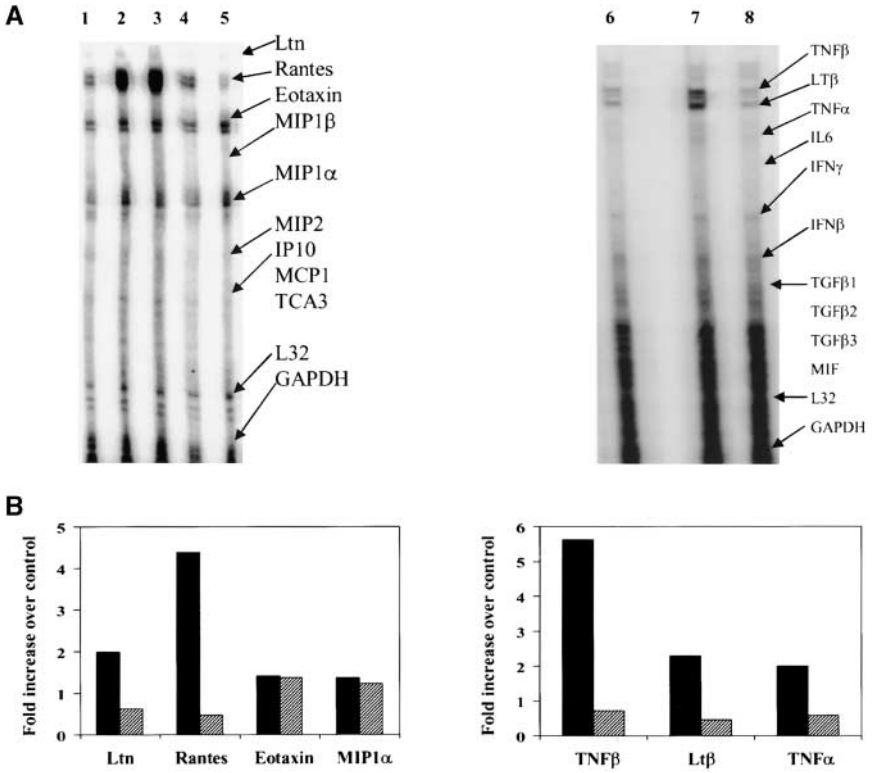

Figure 6. Effect of TNF-α neutralization on TDI-induced cytokine expression in tracheae as determined by ribonuclease protection assay (RPA). Twenty-four hours after the last TDI inhalation challenge, total tracheae RNA was isolated and pooled, and RPA was performed. (*A*) The protected fragments were separated on a polyacrylamide gel, which was dried and exposed to a phosphorimaging screen. *Lane 1:* control mice; *lanes 2–3:* TDI-exposed mice; *lanes 4–5:* TDI-exposed anti–TNF-α antibody–treated mice; *lane 6:* control mice; *lane 7:* TDI-exposed mice; *lane 8:* TDI-exposed anti–TNF-α antibody–treated mice. *Lanes 1–5* were run with the mCK-5 probe and *lanes 6–8* with the mCK-3b probe. (*B*) Bands were quantified using a phosphoimager and data are expressed relative to L32 expression and as fold increase over control values. *Solid bars*, exposed; *striped bars*, TDI-exposed receiving TNF-neutralizing antibodies.

marker that is highly upregulated on activated dendritic cells, and CD14, present on murine macrophages and monocytes, but not on stimulated dendritic cells (19), were enumerated. A 7-fold increase in FITC+/CD86+ cells was demonstrated at the highest dose in concordance with a dose-dependent increase in FITC-bearing cells (Figure 7C), whereas CD14+ cell numbers remained similar to control levels (0.1%) (Figure 7D). TNF-α antibody treatment resulted in a 50% reduction in the total number of FITC+/CD86+ cells, but had no effect on CD14+ cell numbers (Figures 7C and 7D). Consistent with the FITC-dextran studies, intranasal instillation of TDI induced a dose-dependent increase in CD86+ cells in the cervical lymph nodes, with minimal changes in CD14+ cell numbers (data not shown). The increase in CD86+ cells was significantly reduced in TNF-α–deficient mice (Figure 8A). In comparing the TNFR1 and TNFR2 receptor knockout mice, TNFR1 mice had significantly reduced numbers of CD86+ cells in the lymph node, whereas TNFR2 knockout mice had levels equivalent to that of control mice. The number of TDI+ cells detected in the cervical lymph nodes were increased over 3-fold in TDI-exposed mice compared with controls (Figure 8B). Within this TDI+ population, greater than 50% were CD86+ (Figure 8B), whereas less than 3% were TDI+/CD14+ (data not shown). TNF-α antibody treatment reduced the number of cells TDI+ and TDI+/CD86+ to nearly background levels (Figure 8B).

Discussion

We demonstrate that TNF-α, as a central regulator of inflammatory responses, is a major initiator and propagator of airway inflammation and hyperresponsiveness in TDI-induced asthma. Complete ablation of AHR occurred in mice pretreated with neutralizing antibodies to TNF-α, whereas inflammation was significantly reduced in mice either pretreated with neutralizing antibodies to TNF-α or lacking both TNF receptors. Furthermore, TNF-α–deficient mice demonstrated significant reductions in airway dendritic cell migration. By inhibiting TNF-α, the overall effect would be a decrease in antigen presentation, T cell activation, stimulation of chemokine and inflammatory cytokine production, and reduced cellular recruitment (6). Although not demonstrated in asthma models, TNF-α may also contribute to AHR by damaging the pulmonary vascular endothelium and subsequently causing capillary leakage as demonstrated in ischemia/reperfusion studies where anti-TNF antiserum pretreatment significantly decreased pulmonary capillary leakage (25). Other investigators have shown that local infusion of rTNF-α produces a transient increase in airway responsiveness as well as airway inflammation in accompaniment to an increase in pulmonary microvascular permeability (7). In contrast to these airway proinflammatory activities, studies utilizing the ovalbumin murine model of allergic asthma demonstrated either little significant effect of neutralizing antibodies to TNF-α on AHR and eosinophilia, or comparable responses of TNF-α receptor knockout mice to those exhibited by wild-type exposed animals (11).

Airway inflammation and hyperresponsiveness are both hallmarks of TDI asthma (27). Unlike asthma caused by protein antigens, TDI-induced inflammation is observed primarily in the upper airways (21) and results from the ability of isocyanates to react with airway epithelium, creat-

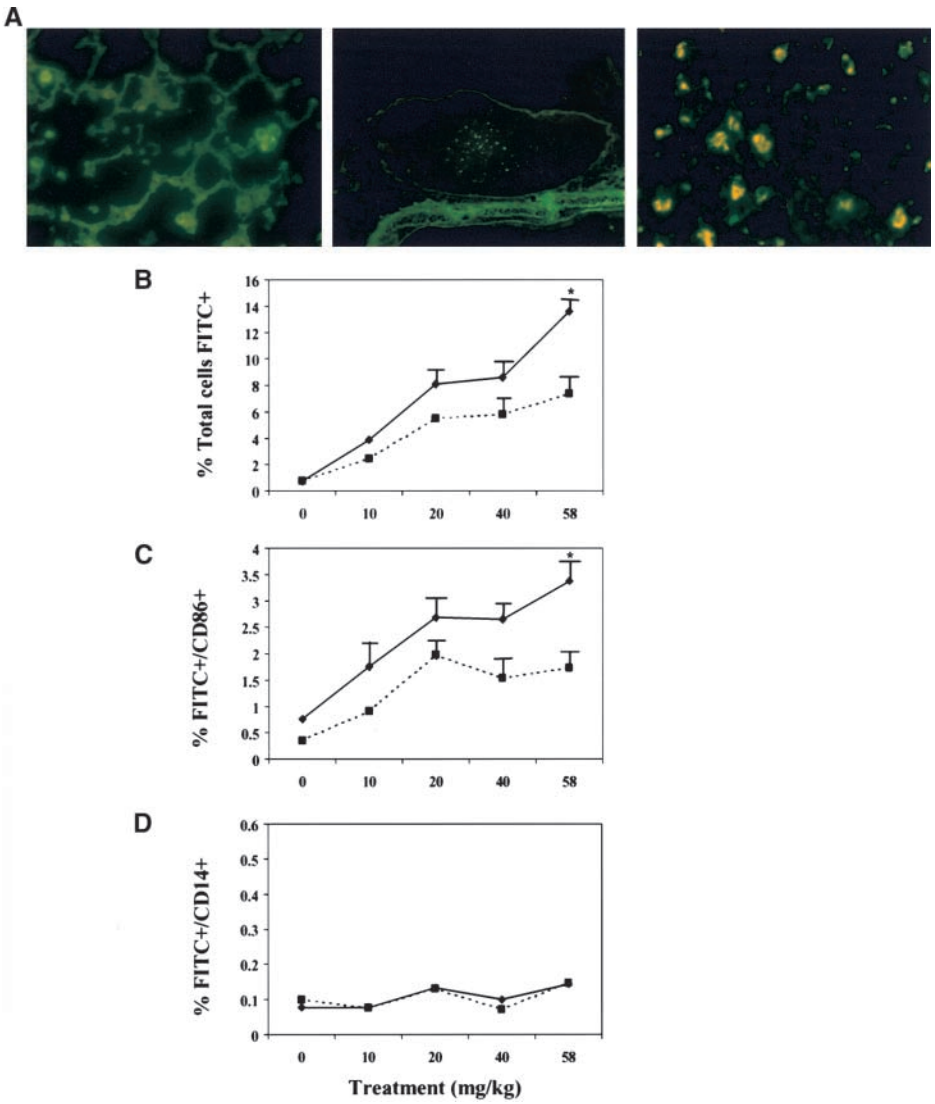

Figure 7. Effect of anti–TNF-α antibody pretreatment on CD86+ cell numbers in draining lymph nodes after instillation with FITC-dextran. C57BL/6J mice were intratracheally instilled with increasing doses of FITC-dextran. Twenty-four hours after instillation, lungs and mediastinal lymph nodes were collected and examined for FITC-bearing cells by fluorescence microscopy (*original magnifications:* ×4 and ×40). Cells containing fluorescent particles were observed within the alveolar spaces, interstitium, and paracortex of the mediastinal lymph nodes within 24 h (*A*). In addition, mediastinal lymph nodes were prepared, immunolabeled, and analyzed by two-color flow cytometry for the presence of FITC-bearing cells (*B*), CD86+ cells (*C*), or CD14+ cells (*D*). *Diamonds*, FITC-exposed mice; *squares*, FITC-exposed mice receiving TNF-neutralizing antibodies.

ing structural abnormalities and the release of biologic mediators (30). It was observed that the upper airway changes, represented by moderately severe goblet metaplasia and mucous secretion in the nares and minimal goblet metaplasia in the trachea, were accompanied by moderate cellular infiltration composed primarily of neutrophils and macrophages. Eosinophilic infiltration, which occurs in humans following TDI exposure, has not been fully demonstrated in this nor other animal models for TDI asthma (31). Corresponding to the histopathologic findings in the upper airways in our studies, BALF contained significant elevations in neutrophil and lymphocyte numbers, although lung tissue damage was not observed.

Despite the fact that occupational asthma is often compared with allergic asthma, important differences exist. Patients with allergic asthma are typically atopic, with at least two-thirds producing IgE antibodies to the aeroallergens. Furthermore, asthma prevalence is greater among subjects with high serum IgE levels (4). In contrast, no association of atopics with isocyanate-induced asthma has been found, and the prevalence of isocyanate-asthmatic patients with specific IgE antibodies is reported to be 5–30% (1). In this murine model of isocyanate asthma, specific IgG antibodies in the serum were consistently detected, as were increases in total IgE (21). These responses were not altered in TNF-α receptor knockout mice, nor were they reduced in animals given anti–TNF-α antiserum. Numerous studies of isocyanate-exposed workers indicate that neither specific antibodies nor IgE levels correlate with disease activity, although specific IgG antibodies serve as a marker of exposure (22) and, in animals, antibody titers directly correspond to the exposure concentration (36). The presence of antibodies in TNF-α–deficient mice, in the presence of attenuated lung function and tissue pathology, affirms the dissociation of antibodies and immunoglobulins from pathologic manifestations of isocyanate asthma, including AHR. This is not to preclude that an IgE mechanism may not have other roles in isocyanate asthma. It has been suggested that the lack of linkage between the presence of specific IgE antibodies and human disease is due to phenotyping methods

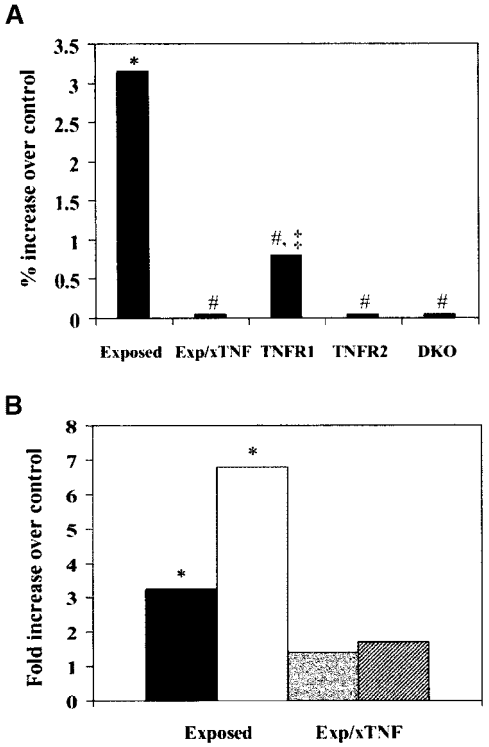

Figure 8. TDI+ and CD86+ cells in the airway draining lymph nodes. Twenty-four hours after intranasal instillation of 5% TDI, cervical lymph nodes were collected from control, TDI-exposed, TDI-exposed anti–TNF-α antibody–treated, TNFR1, TNFR2, or DKO mice. (*A*) Draining lymph node cells were prepared and immunolabeled with anti-CD86/PE and analyzed by flow cytometry (*solid bars*, CD86+). (*B*) Twenty-four hours after intranasal instillation of 5% TDI, cervical lymph nodes were collected from control and TDI-exposed mice. Draining lymph node cells were prepared and immunolabeled with anti-TDI/FITC and anti-CD86/PE and analyzed by two-color flow cytometry for the presence of TDI/CD86 bearing cells (*solid bars*, TDI+; *open bars*, TDI/CD86+; *shaded bars*, TDI/xTNF; *striped bars*, TDI/CD86/TNF). *Significantly different from control group; #significantly different from exposed treatment group; ‡significantly different from TNFR2 group ($P < 0.05$, $n = 6$, ± SEM).

(37), such that usage of polymeric isocyanate conjugates, rather than monomeric isocyanate-human serum albumin test antigens, provides greater assay sensitivity (38). Nonetheless, specific immune mechanisms are involved in TDI asthma. This has been demonstrated in the current studies by the absence of AHR and pathology in athymic TDI-exposed mice in which T cells (21) and putatively Th1 cells (29) were found to be required, and in humans where increased numbers of circulating and BALF CD8+ cells were detected (37) as well as Th1-specific clones identified from the bronchial mucosal tissue (4).

Cytokines play an integral role in the initiation, propagation, and persistence of inflammatory and immunologic processes in asthma (40). Atopic asthma is often associated with Th2-biased responses and can be adoptively transferred with Th2 cells (41). IL-4, a Th2 cytokine, promotes mucus secretion and goblet cell hyperplasia (24). Th1 cytokines, including IFN-γ and IL-2, appear less important in allergic asthma but can induce mediators responsible for airway inflammation (45). Following TDI sensitization, we observed elevated IL-4 expression in the tracheae, which was accompanied by decreased expression of IFN-γ. This would suggest that TDI elicits preferentially a Th2-type cytokine profile. This was observed in C57BL/6 mice, which possess a predominate Th1 background. IL-5 transcripts were not detected in either the tracheae or lungs of the exposed animals, consistent with the absence of eosinophils within the involved tissues.

A hallmark manisfestation of TDI asthma is goblet cell metaplasia within the affected airways. *In vitro* and *in vivo* studies have shown that IL-4 directly induces the differentiation of epithelial cells into goblet cells, which are responsible for mucin secretion (46). The expression of inflammatory cytokines, represented by TNF-α, Rantes, LT-β, and Ltn, was also enhanced in tracheae following TDI exposure. Inflammatory cytokines, such as TNF-α, can be assumed to play multiple roles in occupational asthma. For example, TNF-α can potentiate mucosal inflammation in the airway epithelium and stimulate mucin secretion in airway epithelial cells via the generation of nitric oxide (47). TNF-α also enhances transendothelial migration of Th2 cells, suggesting that inflammatory cytokines are important in Th2 responses (48). In this respect, a crucial event in the inflammatory response is the recruitment and influx of leukocytes into the affected tissues.

Dendritic cells are considered the major antigen-presenting cell in the lung and airway wall (49). Airway dendritic cells are found in secretory epithelium, bronchi, and oro- and nasopharyngeal regions. The migratory properties of dendritic cells are an essential component of their function and the eventual elicitation of specific immunity. This has been aptly demonstrated in dendritic cells of the skin (Langerhan's cells), where TNF-α is critical for signaling their migration to the local draining lymph node (23). Instillation with FITC-coated dextran particles and TDI demonstrated that TNF-α was involved in airway dendritic cell migration and for the most part involved TNFR2 signaling. Despite a marked decrease in dendritic cell migration, a robust antibody response is still observed, with the persistence of TDI-specific antibodies in animals lacking the TNF-α receptor as well as those treated with neutralizing antibodies. This suggests that the lymph nodes are providing sufficient antibody to induce normal antibody responses.

In conclusion, we have found that TNF-α plays a central role in TDI –asthma, as its absence decreases AHR, airway inflammation, and dendritic cell migration. By contrast, TNF-α deficiency had no effect on the induction of specific IgG antibody or total IgE production. Future studies focused on time-related interactions between specific immune responses and inflammatory processes may provide further clarification of underlying mechanisms.

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