Modulation of allergic inflammation in mice deficient in TNF receptors

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1Departments of Pathology and Immunology, Genentech, Incorporated, San Francisco CA 94080; 2Department of Molecular Biology, Deltagen, Incorporated, San Carlos, California 94070; and 3Department of Pathobiology, Purdue University, West Lafayette, Indiana 47907

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Rudmann, Daniel G., Mark W. Moore, Jeffery S. Tepper, Melinda C. Aldrich, Juergen W. Pfeiffer, Harm Hogenesch, and Daniel B. Tumas. Modulation of allergic inflammation in mice deficient in TNF receptors. Am J Physiol Lung Cell Mol Physiol 279: L1047–L1057, 2000.—Tumor necrosis factor-α (TNF) is implicated as an important proinflammatory cytokine in asthma. We evaluated mice deficient in TNF receptor 1 (TNFR1) and TNFR2 [TNFR(−/−)] mice in a murine model of allergic inflammation and found that TNFR(−/−) mice had comparable or accentuated responses compared with wild-type [TNFR(+/+) ] mice. The responses were consistent among multiple end points. Airway responsiveness after methacholine challenge and bronchoalveolar lavage (BAL) fluid leukocyte and eosinophil numbers in TNFR(−/−) mice were equivalent or greater than those observed in TNFR(+/+) mice. Likewise, serum and BAL fluid IgE; lung interleukin (IL)-2, IL-4, and IL-5 levels; and lung histological lesion scores were comparable or greater in TNFR(−/−) mice compared with those in TNFR(+/+) mice. TNFR(+/+) mice chronically treated with anti-murine TNF antibody had BAL fluid leukocyte numbers and lung lesion scores comparable to control antibody-treated mice. These results suggest that, by itself, TNF does not have a critical proinflammatory role in the development of allergic inflammation in this mouse model and that the production of other cytokines associated with allergic disease may compensate for the loss of TNF bioactivity in the TNFR(−/−) mouse.

knockout mice; asthma; animal models; T cell subset 2 cytokines

Asthma is a multifactorial disease that has had escalating morbidity and mortality in the last 20 years (45, 52). The mRNA and/or protein levels of several cytokines, including tumor necrosis factor-α (TNF), are increased in the sputum or serum of asthmatic patients, but the exact roles of these cytokines in disease pathogenesis are not well defined (4–6, 11, 29, 34, 39, 53). TNF is generally considered a potent proinflammatory cytokine, and in the lung, TNF is synthesized and stored primarily in mast cells and alveolar macrophages (4, 17, 39). TNF may have multiple proinflammatory roles in the pathogenesis of asthma, including upregulation of endothelial cell integrin receptors; activation of mast cells, macrophages, and eosinophils; promotion of epithelial cytotoxicity; and aggravation of airway hyperreactivity (15, 27, 32, 37, 38, 47, 48).

Acute blockade of TNF bioactivity in sensitized animals can attenuate pulmonary eosinophilic inflammation and/or airway hyperresponsiveness after a single aerosol challenge with antigen (33, 42, 51, 54). These studies have not examined the role of TNF after multiple aerosol challenges nor have they examined the response in sensitized mice after chronic TNF blockade [i.e., antibody-treated mice or TNF receptor (TNFR) knockout mice]. We were interested in studying the role of TNF in a model of pulmonary eosinophilic inflammation that was CD4+ T cell dependent (14, 16). We chose a model that used multiple aerosol challenges to avoid evaluating only the acute effects of altering TNF bioactivity. We used a CD4+ cell-dependent model because CD4+ cells are thought to be important in orchestrating the chronic inflammatory response in asthma, and recently, TNF has been demonstrated to downregulate CD4+ T cell function (2, 7, 8, 49).

Gene-deleted mice. TNFR(−/−) mice were generated by backcrossing TNFR1(−/−) and TNFR2(−/−) mice, the phenotypes of which have been described previously (12, 13, 28, 31, 35, 36, 41, 43). The genotype was confirmed in vitro with TNFR1- and TNFR2-specific DNA probes and oligonucleotide primers in Southern blot and PCR experiments (12). In vivo,

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TNFR(−/−) mice had no clinical signs associated with systemic administration of recombinant murine TNF (Genetech, South San Francisco, CA) given at a 10-fold 100% lethal dose (Rudmann, unpublished data). All TNFR(−/−) mice were on a C57BL/6 (Charles River, Gilroy, CA) and 129/Sv (Taconic, Boston, MA) background, and TNFR(+/+ ) mice were derived from the littermates of TNFR(−/−) mice. In select experiments, responses to ovalbumin (Ova) were also studied in the background strains C57BL/6 and 129/Sv for the TNFR(+/+ ) and TNFR(−/−) mice. All mice were housed in microisolator cages under laminar flow conditions and were 6–10 wk old at the start of the experiments. Sentinel mice were housed in the same room and serologically evaluated on a regular basis for murine viral and bacterial agents. All experiments were approved by Genentech’s Animal Protocol Review Committee.

Murine Ova model of allergic inflammation. TNFR(−/−), TNFR(+/+ ), and C57BL/6 and/or 129/Sv mice were ear tagged and injected intraperitoneally on day 1 with Dulbecco’s PBS (nonsensitized control groups; HyClone, Logan, UT) or 25 μg OVA (Sigma) in 11 μl of aluminum hydroxide (Alum, Intergen, Purchase, NY) in 0.1 ml of Dulbecco’s PBS (sensitized groups). In a 3-day Ova challenge model, mice received an additional Ova-Alum immunization or PBS injection (nonsensitized groups) on day 8 followed by an aerosol challenge with sterile Ova (10 mg/ml) in PBS with 0.01% Tween 20 (Sigma). After 1 wk, mice were aerosol challenged for 3 consecutive days and necropsied ~18 or 36 h after the last aerosol challenge. In a 7-day model, mice were immunized only on day 1, were Ova aerosol challenged for 7 consecutive days beginning on day 15, and were necropsied ~18 h after the last aerosol challenge.

For each 20-min aerosol challenge, a PARI IS-2 nebulizer (PARI, Richmond, VA) was used at 22 ps. Mice were challenged individually with Ova in a Plexiglas pie chamber (Braintree Scientific, Braintree, MA) that housed 12 mice simultaneously. Under these conditions, the mass mean aerodynamic diameter of the fully saturated particle was 2.27 μm, with a span of 2.04 μm as measured by a Malvern Mastersizer (Malvern, Malvern, UK). The estimated lung dose of Ova per mouse at each daily exposure was 10.4 μg.

The Ova used for aerosolization contained 1 ng endotoxin/ml Ova as determined by the Limulus amebocyte lysate assay (Associates of Cape Cod, Boston, MA). This endotoxin concentration is at least 105 times lower than the concentration necessary to produce lung inflammation as evaluated by bronchoalveolar lavage (BAL) and histology (Rudmann, unpublished data).

Anti-murine TNF-treated mice. Neutralizing anti-murine TNF monoclonal antibody (Mab) was used to abrogate TNF bioactivity in groups of nonsensitized control or Ova-sensitized TNFR(+/+ ) mice. For these experiments, 100 μg of an anti-TNF Mab (46) or 100 μg of control antibody (hamster IgG; Cappel, Durham, NC) were administered to TNFR(+/+ ) mice by intraperitoneal injection every other day starting 2 wk before sensitization and continuing until the end of the study. Control groups of nonsensitized TNFR(+/+ ) mice received anti-TNF Mab or control antibody. The dose of antimurine TNF Mab given has been demonstrated (46) to completely neutralize TNF bioactivity and has a half-life of ~7 days in mice. The anti-TNF Mab has been administered to mice for up to 3 mo with no evidence of a neutralizing antibody response (46).

Airway reactivity measurements. Breathing mechanics during methacholine (MCh) provocation were evaluated 18–24 h after the third or seventh Ova aerosol challenge. Initial experiments were performed with the 7-day challenge protocol. Briefly, these mice were anesthetized, tracheal and esophageal cannulas were placed, and the spontaneously breathing mice were challenged with inhaled MCh. This method was determined to be unacceptable because the sensitized TNFR(−/−) mice were severely impaired (showing dyspnea, mucus accumulation, and gasping) at the time of pulmonary function testing. Other than this observation (see RESULTS), the data from this experiment are not included.

Mice receiving 3 days of Ova challenge were anesthetized (25 mg/kg of pentobarbital sodium and 1.8 g/kg of urethane), the jugular vein was catheterized (PE-10 connected to PE-50 tubing), and a cannula (1.7 cm of PE-160 tubing connected to a 19-gauge tubing adapter) was inserted into the trachea. The resistance of the tracheal cannula was ~0.92 cmH2O·ml−1·s−1. Mice were placed on a heated cradle and ventilated with a four-port manifold. Two of the three ports were used for the inspiratory and expiratory lines of the solenoid-actuated constant-flow ventilator. A third port was connected to a differential pressure transducer for measurement of airway opening pressure. The fourth port was connected to a pneumotachograph made of 100% O2 at a frequency of 170 breaths/min and a tidal volume of 9 ml/g. The cradle was enclosed in a Plexiglas volume-displacement plethysmograph (Century, Philadelphia, PA) for measurement of thoracic flow with a differential pressure transducer (SenSym, Milpitas, CA). The two pressure transducers (airway opening and thoracic flow) were linear in frequency and phase over the normal tidal breathing range. Breathing mechanics (respiratory system resistance and dynamic compliance) were calculated from the digitally converted pressure and flow signals with the use of the covariance method (44). Before baseline measurements, mice were paralyzed with pancuronium (0.2 mg/kg), given a volume history of 22.5 ml/g, and allowed to stabilize for 3 min. Immediately after baseline measurements were obtained, a computer-controlled syringe pump (pump 22, Harvard Apparatus, Boston, MA) delivered 35 μg/kg of intravenous MCh (stock concentration 100 μg/ml) over 10 s. Five additional MCh doses were automatically delivered every 5 min by doubling the flow rate until a final dose of 1,120 μg/kg was achieved. All pulmonary function parameters were continuously monitored with a computerized (Buxco Electronics, Sharon, CT) data acquisition program and were reported at 15-s intervals. An integrated measurement of the MCh response at each dose was obtained by averaging the 15-s logged data over the 5-min period. Log-linear dose-response curves were constructed for each animal, and the provocative concentration (μg MCh/kg body wt) that causes a 20% increase over baseline resistance (P20) was determined by linear interpolation. The data are reported as means ± SE.

BAL and cytology. Approximately 18 h after the last Ova aerosol challenge, mice were anesthetized with halothane and euthanized by exsanguination. The diaphragm was incised, and the trachea was exposed and cut just below the larynx. A polyurethane flexible tube (0.040-cm outer diameter; Braintree Scientific, Braintree, MA) ~4 cm in length and attached to a blunt 23-gauge needle (Sherwood Medical, St. Louis, MO) was placed 6 mm into the trachea, and the lung was lavaged three times with the same bolus of sterile saline. The amount of saline used for BAL was 30 μl/g mouse wt. This bolus filled the lung to ~60% of total lung capacity. BAL fluid return averaged 80% and did not vary by treatment group.

BAL fluid samples were centrifuged at 1,000 g at 4°C for 10 min. The supernatants were decanted and immediately frozen at −80°C. The cell pellets were resuspended in 250 μl of PBS with 2% BSA (Sigma), then enumerated with an auto-
nates were centrifuged at 10,000 g for 10 min (Cytospin, Shandon, Pittsburgh, PA). Slides were air-dried, fixed for 1 min in 100% methanol, and stained with Diff-Quik (Baxter Health Care, Miami, FL). At least 200 cells/slide were evaluated to obtain a differential leukocyte count.

**Histopathology.** To evaluate and compare the severity and character of pathological changes in lungs between experimental groups, the left lungs of mice were fixed in 10% neutral buffered Formalin and embedded in paraffin, and 3-μm sections were stained with hematoxylin and eosin or periodic acid-Schiff (PAS). Lung sections were taken along the primary bronchus. The entire section of lung was evaluated blinded by a board-certified veterinary pathologist and scored based on the severity of the inflammation around the airways and blood vessels and in the alveoli and on the extent of airway epithelial cell and mucus-secreting cell hyperplasia/hypertrophy with a scale from 0 (no inflammation or airway changes) to 5 (severe inflammation and airway changes). For evaluation of mucus-secreting cell hyperplasia/hypertrophy, the number and size of mucus-secreting cells were qualitatively evaluated in sections of lung stained with PAS.

**Cytokine and IgE ELISA.** To quantitate and compare cytokine production in lung tissue in mice from different experimental groups, supernatants from lung homogenates were prepared as previously described (33). Briefly, the right lung was placed in 1 ml of sterile chilled PBS with 0.05% Triton X-100 (Baxter Diagnostics, Deerfield, IL) at 800 rpm for 5 min. Supernatants were centrifuged at 10,000 g at 4°C for 5 min. Supernatants were decanted, filtered through a 1.2-μm syringe filter (Whatman, Clifton, NJ), and immediately frozen at −80°C for later use in cytokine quantitation.

Interleukin (IL)-2, IL-4, IL-5, and interferon (IFN)-γ were quantitated in lung homoneguate supernatants and lymphocyte culture supernatants with a sandwich ELISA procedure (1, 21). Briefly, 0.5 μg/ml (for IL-2, Pharmingen, San Diego, CA) or 1.0 μg/ml (for IL-4 and IL-5, Pharmingen; for IFN-γ, Genentech hybridoma 22.1) of anti-murine cytokine MAbs were used to coat high-binding 96-well ELISA plates (Nalge Nunc, Rochester, NY). Plates were coated at 4°C for 12–24 h and blocked for 30–60 min with ELISA buffer (PBS, 0.5% BSA, 2 mM EDTA, and 0.05% Tween 20). Samples were added undiluted or diluted 1:2 to 1:16 to the coated plates in duplicate. The recombinant standard was serially diluted 1:2 in duplicate starting at a concentration of 1,000 pg/ml. All incubation steps were done at room temperature on an orbital shaker. Plates with sample and standard were incubated for 2–4 h. Next, 1 μg/ml of biotinylated anti-murine IL-2, IL-4, or IL-5 (Pharmingen) or rabbit anti-murine IFN-γ (Genentech) MAbs were added, and the plates were incubated for 1–1.5 h. The plates were washed and incubated for 0.5–1 h with 1:3,000 to 1:6,000 dilutions of horseradish peroxidase (HRP)-streptavidin (Zymed, San Francisco, CA) for the IL-2, -4, and -5 assays or HRP-donkey anti-rabbit Ig (Amersham, Arlington Heights, IL) for the IFN-γ assay. HRP development was done for 15 min in the dark with o-phenylenediamine dihydrochloride substrate solution (5 mg of o-phenylenediamine, 12.5 ml of PBS, and 5 μl of 30% H2O2). Plates were read at 490 and 405 nm on a spectrophotometer. The adjusted optical density (OD) was OD490 − OD405 + ODblank. The absolute concentration of the samples was calculated with the standard curve and adjusted OD.

To quantitate lung TNF, the L-M cell bioassay was used with the following modifications (30). Recombinant murine TNFα (Genentech) was used as a standard at a starting concentration of 4 ng/ml, in duplicate, on plated L-M cells. The limit of detection in this assay was 31.25 pg TNF/ml. Included on each plate was a positive control, recombinant murine TNF-β.

The procedures for the total IgG, IgE, and Ova-specific IgE sandwich ELISAs were comparable to those for the cytokine ELISAs except that BAL fluid or serum either undiluted or diluted 1:2 to 1:20 (for BAL fluid) or 1:25 to 1:20 (for serum) in ELISA buffer was used as the sample. For total IgE and total IgG, the capture antibodies were rabbit anti-mouse IgE (Genentech) and goat anti-mouse IgG (Cappel, Durham, NC), respectively (2 μg/ml in PBS), and plates were coated for 24–48 h at 4°C. The standards were murine Igκ chain (PharMingen), or IgG (Cappel), which were diluted serially 1:2 starting with a 100 ng/ml concentration. The detection antibodies, biotinylated FeR1-IgG (Genentech) for IgE and goat anti-mouse IgG-HRP (Boehringer Mannheim, Indianapolicos) for IgG, were diluted 1:1,000 for 0–1 h. HRP-streptavidin and enzyme development steps were identical to those used for the cytokine assays.

For the Ova-specific IgE (Ova IgE) and IgG (Ova IgG) ELISAs, the capture reagent was grade V Ova (Sigma) coated at a concentration of 2 μg/ml in PBS on 96-well plates for 12–24 h. In experiments that used a reference standard, the standard was pooled Ova-immunized mouse serum (Genentech). Standard and samples were incubated for 2 h in duplicate. For the Ova IgE ELISA, the samples were diluted 1:10 to 1:640. For the Ova IgG ELISA, the samples were diluted 1:50. The detection antibody was HRP-conjugated goat anti-Ova (Genentech lot 16542-51) and was used at 1:4,000. Enzyme development and OD reading steps for both total IgE and Ova-specific IgE and IgG were identical to those used in the cytokine ELISA protocols. Ova-specific IgE and IgG concentrations were reported in OD (Ova IgG) or in units per milliliter (Ova IgE). When reported in units per milliliter, the OD at the highest concentration of standard, a 1:10 dilution of pooled serum from Ova-immunized mice, was considered 100 U/ml.

**Flow cytometric analysis.** To obtain lung cells for fluorescence-activated cell sorter (FACS) analysis of lymphocytes, lungs from selected mice were digested by a previously described method (25) with modifications. While under anesthesia, mice were exsanguinated from the abdominal aorta. One or both lungs were removed, cut into small pieces, and digested at 37°C for 45 min in 4 mg of collagenase D, 8 U of dispase, and 1 mg of DNase (Boehringer Mannheim) dissolved in 10 ml of RPMI 1640 medium with 0.05 M HEPES and 10% controlled process serum replacement-2 (Sigma). After incubation, lungs were aspirated 20 times through a 30-ml syringe with an 18-gauge needle. The lung digest was filtered through nylon and spun at 1,500 g at 4°C for 5 min. Erythrocytes were eliminated with lysis buffer (0.14 M NH4Cl and 0.017 M Tris, pH 7.2), the lung cell pellet was washed and centrifuged twice, and total lung cells were enumerated on a hemocytometer, excluding those cells that stained with 0.1% trypan blue. Lung cells were stored on ice and used for immunostaining and FACS analysis.

To evaluate the T and B cell populations by FACS in spleen, lung, and tracheobronchial lymph nodes, single-cell suspensions were made as described previously (29).

Cell (1 × 10⁶) suspensions from lung digests of sensitized and control TNFR(+/-) and TNFR(-/-) mice and regional lymph nodes and spleen from sensitized TNFR(+/-) and TNFR(-/-) mice were stained with anti-murine CD4 FITC...
(Calbiochem, La Jolla, CA), CD8 phycoerythrin (Calbiochem), B220 phycoerythrin (Boehringer Mannheim), or the following isotype controls: rat IgG2a FITC, rat IgG FITC, rat IgG2a phycoerythrin, and hamster IgG FITC. Cells were incubated with antibody (10 μg) for 30 min, washed, fixed in paraformaldehyde, and analyzed on a flow cytometer (FACScan) with Cellquest software (Becton Dickinson, San Jose, CA).

Statistical methods. With UNISTAT for Excel software (version 4.5, UNISTAT, London, UK), BAL fluid, flow cytometric, and cytokine ELISA data were compared with a one-way ANOVA and, if significant (P < 0.05), were followed with a protected least significant differences test for between-group comparisons. Pulmonary function measurements from all groups were compared with a one-way ANOVA, and, if significant (P < 0.05), were followed with an uncorrected t-test. Histological lesion scores were compared with Kruskal-Wallis one-way ANOVA and, if significant, were followed with a multiple comparison test (multiple comparisons with t distribution) for between-group comparisons.

RESULTS

Ova-sensitized TNFR(−/−) mice did not have reduced immune or inflammatory responses to aerosol Ova challenge. Asthma is associated with an increased number of leukocytes, especially eosinophils and lymphocytes, in the BAL fluid of patients (3, 9). We quantitated and characterized BAL fluid leukocytes in mice 18 h after 3 or 7 consecutive days of Ova aerosol exposure to determine whether BAL fluid leukocyte numbers would be attenuated in TNFR(−/−) mice. In previous experiments with this model, it was found that the BAL fluid leukocyte response in sensitized mice was maximal 18–24 h after the last aerosol exposure (Tepper, unpublished data). After three aerosol exposures with Ova, sensitized TNFR(−/−) and TNFR(+/+) mice had pulmonary airway inflammation as reflected by marked increases in BAL fluid leukocytes compared with those in control nonsensitized mice (Fig. 1). Eosinophils were the predominant cell population in the BAL fluid; however, lymphocytes were also significantly increased in sensitized mice (Fig. 1). Surprisingly, loss of TNFR function did not significantly affect the BAL fluid leukocyte numbers. Total numbers of BAL fluid total leukocytes, eosinophils, and lymphocytes were, instead, slightly but not significantly higher in TNFR(−/−) compared with TNFR(+/+) mice (Fig. 1).

After seven aerosol exposures with Ova, the pulmonary inflammatory response as measured in the BAL fluid was at least threefold greater in sensitized TNFR(−/−) and TNFR(+/+) mice compared with that in mice challenged for 3 days (Fig. 2). Again, loss of TNFR function did not abrogate the pulmonary inflammatory response as measured by lavage leukocyte numbers. In contrast, the numbers of BAL fluid leukocytes, eosinophils, and lymphocytes were more than twofold higher in TNFR(−/−) mice compared with TNFR(+/+) mice (Fig. 2).

A prominent component of the histological lesions in sensitized mice that were aerosol challenged with Ova was interstitial (perivascular and peribronchial) leukocyte infiltrates. To determine whether differences in interstitial leukocyte numbers were present in the lung, mice were exsanguinated while under anesthesia, and total and differential leukocyte counts from digested lungs were done on sensitized mice after three Ova aerosol challenges. The number of total and individual leukocytes was two- to threefold higher in lungs of sensitized mice compared with the respective nonsensitized control mice (Fig. 3). However, there were no significant differences in the number of total lung leukocytes or in individual leukocyte populations between TNFR(−/−) and TNFR(+/+) mice (Fig. 3).

Approximately 60% of the interstitial lymphocytes in the lungs of sensitized mice were B220 positive, and the remaining cells were mostly T cell receptor-αβ positive, indicating a B and T cell lineage, respectively.

interstitial leukocyte numbers were present in the lung, mice were exsanguinated while under anesthesia, and total and differential leukocyte counts from digested lungs were done on sensitized mice after three Ova aerosol challenges. The number of total and individual leukocytes was two- to threefold higher in lungs of sensitized mice compared with the respective nonsensitized control mice (Fig. 3). However, there were no significant differences in the number of total lung leukocytes or in individual leukocyte populations between TNFR(−/−) and TNFR(+/+) mice (Fig. 3).
IgE and Ova-specific IgE in sensitized TNFR(−/−) mice. Compared with TNFR(+/+) mice, sensitized TNFR(−/−) mice had increased total leukocyte counts (P = 0.07), eosinophil (P = 0.09), and neutrophil (P < 0.03) counts. There were no differences between interstitial cell counts in TNFR(−/−)S and TNFR(+/+)S mice.

In the tracheobronchial lymph nodes, T cell receptor-αβ-positive cells were the predominant cell population in sensitized mice. IgE- and IgG-positive B220 cells accounted for ~60% of the pooled lymph node and lung lymphocytes. The immunophenotype of lung and regional lymph node lymphocyte subpopulations, as determined by flow cytometry, did not differ between the TNFR(−/−) and TNFR(+/+) mice (Table 1).

The mice used in the above experiments were exsanguinated while under anesthesia; however, the lungs were not perfused with saline before digestion. The predominant peripheral blood leukocyte in the mouse is the lymphocyte, and lymphocytes from the blood remaining in the lung would contribute to the cell population analyzed with FACS. Other leukocyte populations in the peripheral blood (i.e., neutrophils) would also be identified in the interstitial cell differential counts. In effect, interstitial cell differentials or the FACS interstitial lymphocyte immunophenotype may have been influenced by the circulating leukocytes that remained in the lung after exsanguination.

Human asthma is associated with elevated levels of T cell subset 2-type cytokines (2, 4, 5, 11) and IgE (26, 40). Lung concentrations of IL-2, IL-4, and IL-5 in sensitized TNFR(−/−) and TNFR(+/+) mice after 7 Ova aerosol challenges were 2- to 15-fold higher than those in nonsensitized control mice (Table 2). TNFR(−/−) animals did not have lower concentrations of these lung cytokines. Instead, the differences for IL-4 and IL-5 between TNFR(−/−) mice and their control counterparts were approximately twofold greater than the differences between TNFR(+/+) mice and their controls (Table 2). At the time point examined (18 h after the final Ova aerosol exposure), lung homogenate concentrations of TNF and IFN-γ were below the level of detection in sensitized TNFR(−/−) and TNFR(+/+) mice.

There were higher BAL fluid concentrations of IgG and IgE and higher serum concentrations of IgE and Ova-specific IgE in sensitized TNFR(−/−) and TNFR(+/+) mice compared with those in control mice (Table 3). As was the case for the other end points, sensitized TNFR(−/−) mice did not have an attenuated response compared with TNFR(+/+) mice. Lavage fluid IgG was 20-fold higher in sensitized TNFR(+/+) mice and 200-fold higher in sensitized TNFR(−/−) mice compared with that in their respective nonsensitized controls (Table 3). Lavage fluid IgE was ~30-fold higher in sensitized TNFR(+/+) mice and 300-fold higher in sensitized TNFR(−/−) mice compared with that in their respective nonsensitized controls (Table 3).

Perivascular and peribronchial inflammation and airway mucus cell hyperplasia are characteristic findings in biopsies from human asthma patients (3). We performed blind evaluations on sections of lung from mice to determine the extent of airway, perivascular, and interstitial inflammation; airway epithelial hypertrophy; and airway mucus plugging. A total lesion score (0 = no lesions to 5 = severe changes) was derived from each section. Histological changes in sensitized TNFR(+/+) and TNFR(−/−) mice included peribronchiolar, peribronchial, and perivascular lymphocytic and eosinophilic inflammation, airway mucus cell hypertrophy, and alveolar eosinophils and histiocytes (Fig. 4). PAS-stained lung sections confirmed that the extent of hypertrophy and hyperplasia of the mucus-secreting cells corresponded to the lesion severity scores (data not shown). Whereas the histological characteristics of the lesions were comparable between sensitized TNFR(−/−) and TNFR(+/+) mice, lesions were equal or more severe in TNFR(−/−) mice than in TNFR(+/+) mice challenged with Ova for 3 or 7 days (Fig. 5).

**Ova-sensitized TNFR(−/−) mice did not have reduced airway hyperreactivity compared with Ova-sensitized TNFR(+/+) mice.** We next determined whether differences in airway hyperreactivity would correspond to inflammatory and immune responses in TNFR(−/−) and TNFR(+/+) mice. In the first experiment, we attempted to measure baseline breathing mechanics and airway hyperreactivity in spontaneously breathing mice.

### Table 1. Immunophenotype of lung and tracheobronchial lymph node mononuclear cells in Ova-sensitized TNFR(+/+) and TNFR(−/−) mice

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<tr>
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<th>Lung</th>
<th>Lymph node</th>
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<tr>
<td>TNFR(−/−)</td>
<td>5.9±3.0</td>
<td>5.4±3.1</td>
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<tr>
<td>TNFR(+/+)</td>
<td>12.0±0.7</td>
<td>6.0±0.03</td>
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<tr>
<td>TCR-αβ</td>
<td>4.2±2.8</td>
<td>4.8±1.4</td>
</tr>
<tr>
<td>TCR-γδ</td>
<td>0.9±0.9</td>
<td>0.07±0.01</td>
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<tr>
<td>CD4</td>
<td>1.6±0.7</td>
<td>2.0±0.6</td>
</tr>
<tr>
<td>CD8</td>
<td>1.4±0.9</td>
<td>2.8±1.2</td>
</tr>
<tr>
<td>B220</td>
<td>7.4±3.3</td>
<td>3.4±1.7</td>
</tr>
<tr>
<td>IgM</td>
<td>5.7±2.1</td>
<td>5.6±3.4</td>
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<tr>
<td>IgG1</td>
<td>3.5±1.2</td>
<td>2.3±0.9</td>
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<tr>
<td>IgE</td>
<td>5.1±1.7</td>
<td>5.5±2.9</td>
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Values are means ± SE in no. of antibody-positive cells (in millions); n = 4 mice/group. Ova, ovalbumin; TNFR, tumor necrosis factor (TNF) receptor; TNFR(+/+), wild type; TNFR(−/−), TNFR deficient; TCR, T cell receptor. Lung and lymph node samples were pooled for IgM, IgG1, and IgE staining. See METHODS for flow cytometric procedure.
Table 2. IL-2, IL-4, IL-5, IFN-γ, and TNF-α levels in lung homogenates from Ova-sensitized and NS mice

<table>
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<tr>
<th></th>
<th>TNFR(−/−)</th>
<th>TNFR(+/+)</th>
<th>TNFR(−/−)NS</th>
<th>TNFR(+/+)NS</th>
<th>P Value</th>
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<tr>
<td>IL-4</td>
<td>2.81 ± 0.60</td>
<td>0.82 ± 0.52</td>
<td>0.19 ± 0.05</td>
<td>0.11 ± 0.02</td>
<td>0.01</td>
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<tr>
<td>IL-5</td>
<td>0.76 ± 0.14</td>
<td>0.42 ± 0.04</td>
<td>0.27 ± 0.02</td>
<td>0.29 ± 0.007</td>
<td>0.003</td>
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<tr>
<td>IL-2</td>
<td>1.93 ± 0.77</td>
<td>0.76 ± 0.18</td>
<td>0.39 ± 0.08</td>
<td>0.19 ± 0.07</td>
<td>0.007</td>
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<tr>
<td>TNF-α</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
<td>NA</td>
<td></td>
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<tr>
<td>IFN-γ</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
<td>BD</td>
<td>NA</td>
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Values are means ± SE in ng; n = 5–6 mice/group for TNFR(−/−) and TNFR(+/+) and 3 mice/group for TNFR(−/−) nonsensitized (NS) and TNFR(+/+)NS. IL, interleukin; IFN-γ, interferon-γ; BD, below detection; NA, not applicable. IL was measured in right lung homogenates in 1 ml of PBS with 0.05% Triton as described in METHODS. Overall group ANOVA P values for IL-4, IL-5, and IL-2 were <0.02. Reported P values are for comparisons between TNFR(−/−) and TNFR(+/+) using Fisher’s protected least significant difference (PLSD) test.

Table 3. Serum and BALF antibody responses in Ova-sensitized and NS mice

<table>
<thead>
<tr>
<th></th>
<th>TNFR(−/−)</th>
<th>TNFR(+/+)</th>
<th>TNFR(−/−)NS</th>
<th>TNFR(+/+)NS</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALF</td>
<td>Serum</td>
<td>BALF</td>
<td>Serum</td>
<td>BALF</td>
<td>Serum</td>
</tr>
<tr>
<td>Total IgG</td>
<td>372.4 ± 179.6</td>
<td>ND</td>
<td>116.8 ± 44.1</td>
<td>ND</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Total IgE</td>
<td>669.0 ± 283.6</td>
<td>703 ± 170.5</td>
<td>30.7 ± 16.9</td>
<td>288.0 ± 83.4</td>
<td>2.3 ± 1.3</td>
</tr>
<tr>
<td>OVA IgG1</td>
<td>ND</td>
<td>1.8 ± 0.2</td>
<td>ND</td>
<td>1.6 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>OVA IgE</td>
<td>ND</td>
<td>7.2 ± 2.8</td>
<td>ND</td>
<td>3.7 ± 1.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE in ng for total IgG and IgE, optical density (OD) for OVA IgG1, and U/ml of OVA IgE in BAL or serum using procedures outlined in METHODS; n = 5–6 mice/group for TNFR(−/−) and TNFR(+/+) and 3 mice/group for TNFR(−/−)NS and TNFR(+/+)NS. BALF, bronchoalveolar lavage fluid. ND, not done. Overall group ANOVA P values for total IgE and IgG data were <0.05. Reported P values are for comparisons between TNFR(−/−) and TNFR(+/+) with Fisher’s PLSD test.
DISCUSSION

TNF has been hypothesized to be an important protagonist in the pathogenesis of asthma (6, 11, 29, 34, 53). For this reason, we used mice unresponsive to TNF [TNFR(−/−)] or modified TNF bioactivity in mice by chronic treatment with anti-TNF MAb in order to better understand the role of TNF in allergic inflammation. We predicted that Ova-sensitized TNFR(−/−) or TNFR(+/+) mice treated with anti-TNF MAb would have reduced immune and inflammatory responses to aerosolized Ova. However, Ova-sensitized and challenged TNFR(−/−) mice had similar or exaggerated airway hyperreactivity and immune and inflammatory responses to aerosolized Ova compared with responses in sensitized TNFR(+/+) mice. Ova-sensitized and challenged TNFR(+/+) mice treated with anti-TNF MAb also had comparable immune and inflammatory responses to aerosolized Ova compared with those in TNFR(+/+) mice treated with the control antibody.

Fig. 4. Histological lesions in lungs of mice examined 18 h after 7 days of Ova aerosol challenge. NS control mice had no histological changes (A). Histological lesions in TNFR(+/+) (B) and TNFR(−/−) (C) mice were characterized by airway mucus (B, arrow), airway epithelial hypertrophy (C, arrow), accumulation of peribronchial and perivascular eosinophils and lymphocytes, and intra-alveolar macrophages and eosinophils. Original magnification of hematoxylin- and eosin-stained sections, ×200. Bars, 100 μm.

Fig. 5. Histological lesion scores after 3 or 7 days of Ova aerosol challenge. Lungs were graded for extent of perivascular/peribronchial inflammation, airway epithelial hypertrophy, and alveolar leukocytic infiltrates (0, no inflammation or airway changes; 5, severe inflammation and airway changes). In both the 3- and 7-day experiments, lesion scores were greater in TNFR(−/−)S mice than in TNFR(+/+)S mice.

Fig. 6. Airway hyperreactivity in TNFR(+/+)S, TNFR(−/−)S, TNFR(+/+)NS, and TNFR(−/−)NS mice (n = 4/group) after 3 days of Ova aerosol challenge. The provocative concentration that causes a 20% increase over baseline resistance (PC_{120}) was significantly decreased in TNFR(−/−)S mice compared with that in TNFR(+/+)S mice, *P < 0.001.
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Fig. 7. Histological lesion scores in NS mice (a-TNFNS and IgGNS; n = 3/group) and TNFR(+/-)S mice (n = 5–6/group) given IgG or anti-TNF antibody (a-TNF). Lungs were graded for extent of perivascular/peribronchiolar inflammation, airway epithelial hypertrophy, and alveolar leukocytic infiltrates. There were no differences between the lesion scores in TNFR(+/-)S mice treated with anti-TNF antibody or IgG.

These data demonstrate that the elimination of TNF bioactivity alone is not sufficient to abrogate the murine inflammatory response to aerosolized Ova. There are several possible explanations for the lack of protection afforded to the TNFR(-/-) mice and anti-TNF MAb-treated TNFR(+/-) mice in the Ova model of allergic inflammation. An unlikely explanation is that TNF may not be involved in the pathogenesis of allergic inflammation in this model. Although TNF was below detectable levels 18 h after 3 days of aerosol challenge (Table 2), TNF has been measured at earlier time points after Ova challenge in the BAL fluid and serum of animals (38, 51, 54). Also, both a TNF neutralizing MAb and a TNFR1 fusion protein, TNFR1-IgG, have been demonstrated (42, 54) to attenuate immune responses in a single-challenge Ova model of allergic inflammation.

What is more likely is that the importance of TNF is different during specific phases of allergic inflammation. TNF is elevated in Ova-sensitized mice for only 1 h after Ova aerosol challenge (38). In the experiments in which neutralization of TNF afforded some benefit to animals sensitized to Ova, the animals were treated with a neutralizing TNF MAb or TNFR1-IgG before a single aerosol challenge with Ova (42, 54). These groups did not examine the benefit or lack of benefit of neutralizing TNF after additional Ova challenges. Except for the present investigation, only one study (19) has evaluated the effect of TNF neutralization after multiple aerosol challenges with Ova, and TNF neutralization did not have a significant effect on the murine immune response or airway hyperresponsiveness. Our data from 3-day and 7-day Ova challenge experiments, combined with those from published single-day Ova challenge experiments (19, 42, 54), suggest that neutralizing TNF only transiently abrogates the murine immune response and airway hyperresponsiveness in models of allergic inflammation.

The immune system has a redundant system of effector molecules. In allergic inflammation, it is possible that other proinflammatory cytokines are more important than TNF after repeated antigen challenges with Ova. We demonstrated increased levels of IL-2, IL-4, and IL-5 in the lung homogenates from Ova-sensitized and -challenged TNFR(+/-) and TNFR(-/-) mice compared with those from control mice (Table 2). Additionally, we showed that TNFR(-/-) mice had higher lung IL-2, IL-4, and IL-5 concentrations than their TNFR(+/-) counterparts. Mice deficient in TNFRs may have an altered immunological feedback mechanism(s), resulting in an accentuated Th2-type response in Ova-induced allergic inflammation. In a model of endotoxemia, TNFR(-/-) mice have markedly increased levels of circulating TNF because of the loss of normal negative feedback from TNFR signaling (Rudmann, unpublished observations).

Numerous studies (2, 4, 5, 11) have suggested that IL-4 and IL-5 are important in the pathogenesis of allergic inflammation. In a recent study (19) that examined the effects of antibody neutralization of IFN-γ, IL-4, or TNF in Ova-sensitized mice challenged for 8 consecutive days with aerosolized Ova, only neutralizing IFN-γ attenuated airway hyperreactivity and eosinophil influx into the BAL fluid. Taken together, these data suggest that cytokines other than TNF may have an important role in the allergic response to repeated antigen challenge. These types of experiments demonstrate the time-dependent, redundant, and interactive nature of the cytokine response in allergic disease.

TNF may also have roles other than proinflammatory ones in allergic inflammation. We demonstrated that Ova-sensitized TNFR(-/-) mice that were aerosol challenged with Ova had increased BAL fluid eosinophils, lymphocytes, lung cytokines, IgG, and IgE compared with levels in TNFR(+/-) mice. Investigations have demonstrated that chronic TNF administration downregulates murine T cell responses, peritoneal macrophage 1a expression, and accessory cell function (7, 8, 18, 48, 50). TNF decreases T cell receptor signaling, thereby downregulating the function of both Th1 and Th2 subtypes of T lymphocytes (7). Neutralizing anti-TNF starting 2 wk before sensitization (see METHODS) and IgG antibody or IgG had significantly increased levels of total leukocytes, eosinophils, and lymphocytes (P < 0.01) compared with a-TNFNS and IgGNS mice (n = 3/group). There were no significant differences in BALF leukocyte numbers between IgG-treated and TNF antibody-treated mice.
TNF by treatment with anti-TNF MAb reverses this effect on murine T cells and, in fact, can result in exaggerated T cell function (7, 8). TNF-mediated down-regulation of T cell function may explain why recombinant murine TNF treatment reduces the severity of inflammation in murine models of systemic lupus erythematosus, type 1 diabetes, and delayed-type hypersensitivity (18, 22–24). T cells are likely important in human asthma (2, 9, 49), and murine Ova models of allergic inflammation have been shown to be CD4+ T cell dependent (14, 16). It is possible that the down-regulatory effects of TNF on T cells are an important aspect of the murine response to Ova, especially after repeated Ova aerosol challenges. Absence of the down-regulatory effects of TNF on T cells may have negated the attenuation afforded by losing the proinflammatory effects of TNF in TNFR(−/−) mice or TNFR(+/+) mice treated with anti-TNF. The elevated inflammatory and immune response in TNFR(−/−) mice in this model may also be a result of the loss of TNF-mediated T cell regulation.

It has been demonstrated that airway hyperreactivity in mice and the response of mice in allergy models is influenced by genetic background (10, 20). We tested whether the TNFR(−/−) (C57BL/6 × 129/Sv) and TNFR(+/+) (C57BL/6 × 129/Sv) background strains 129/Sv and C57BL/6 responded differently in the Ova model of allergic inflammation. We found that when aerosol challenged, the Ova-sensitized 129/Sv mice had an approximately twofold greater number of BAL fluid leukocytes and elevated histological lesion scores (data not shown) than identically Ova-sensitized C57BL/6 mice and TNFR(+/+) mice. In separate experiments, BAL fluid total leukocyte, eosinophil, lymphocyte, and macrophage numbers were ~2.6-, 2.8-, 1.7-, and 1.6-fold greater, respectively, in 129/Sv mice compared with those in TNFR(+/+) mice. 129/Sv mice also had greater airway hyperreactivity to MCh challenge than TNFR(+/+) mice (data not shown). Levels of lung IL-4, lung IL-5, BAL fluid IgE, and serum IgE were not significantly different between the strains. The response differences in the background strains demonstrated the importance of using control mice [TNFR(+/+)] derived from littermates of TNFR(−/−) mice as was done in all of the allergy experiments described in this report.

To our knowledge, there have been no published reports describing the immune response to Ova in TNFR(−/−) mice or TNFR(+/+) mice chronically treated with anti-TNF antibodies. Neutralizing TNF with MAbs or TNFR1 fusion proteins in mice or other animal species has been shown to lessen the severity of allergic inflammation (42, 54); however, in those studies, TNF neutralization was done after Ova sensitization and before a single aerosol challenge with Ova. We propose that these models selectively evaluated the early effects of TNF in allergic inflammation. Our data demonstrate that with repeated antigen challenges, neutralization of TNF does not abrogate the mouse inflammatory response to Ova. This is the case whether TNF is chronically neutralized with anti-TNF MAb or whether TNF signaling is blocked by genetic deletion of TNFR1 and TNFR2. These data may suggest that persistent blockade of TNF bioactivity may not provide any clinical benefit in allergic inflammatory diseases such as asthma in humans. Furthermore, our data demonstrate that chronic loss of TNFR signaling could result in an exaggerated T cell-dependent murine response to Ova. In the development of asthma, repeated long-term, low-dose antigen exposure in humans is likely the rule rather than the exception. Whether this exposure is sufficient to stimulate chronic TNF production and whether these low levels of TNF downregulate potential problematic T cell responses in the pathogenesis of asthma requires further investigation.

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REFERENCES


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