Tumor necrosis factor (TNF-α) is a potent cytokine with immunomodulatory, proinflammatory, and pathobiologic activities. Although TNF-α is thought to play a role in mediating airway inflammation and airway hyperresponsiveness (AHR), its function is not well defined. TNF-α–deficient mice and mice expressing TNF-α in their lungs because of a TNF-α transgene placed under the control of the surfactant protein (SP)-C promoter (SP-C/TNF-α–transgenic mice) were sensitized to ovalbumin (OVA) and subsequently challenged with OVA via the airways; airway function in response to inhaled methacholine was monitored. In the TNF-α–deficient mice, AHR was significantly increased over that in controls. In contrast, the transgenic mice failed to develop AHR. In addition, sensitized/challenged TNF-α–deficient mice had significantly increased numbers of eosinophils and higher levels of interleukin (IL)-5 and IL-10 in their bronchoalveolar lavage fluid than were found for control mice. However, in SP-C/TNF-α–transgenic mice, both the numbers of eosinophils and levels of IL-5 and IL-10 were significantly lower than in sensitized/challenged transgene-negative mice. γδ T cells have been shown to be activated by TNF-α and to negatively regulate AHR. Depletion of γδ T cells in the TNF-α–transgenic mice in the present study increased AHR, whereas depletion of these cells had no significant effect in TNF-α–deficient mice. These data indicate that TNF-α can negatively modulate airway responsiveness, controlling airway function in allergen-induced AHR through the activation of γδ T cells.

Keywords: TNF-α; γδ T cells; airway hyperresponsiveness; inflammation

Allergic asthma is a complex syndrome associated with airway hyperresponsiveness (AHR) and airway inflammation, and is characterized by an influx of activated eosinophils and T lymphocytes into the airways (1–3). The selective accumulation of these cells at allergic inflammatory sites depends on the interactions between adhesion molecules on the infiltrating cells and endothelial cells, and a number of pivotal cytokines, including interleukin (IL)-4 and IL-5. Tumor necrosis factor (TNF-α) is a multifunctional cytokine with widespread proinflammatory and antineoplastic activities. It was originally identified as the factor induced in mice exposed to bacterial endotoxin that caused the hemorrhagic necrosis of certain tumors (4). TNF-α is now implicated in the pathogenesis of other chronic inflammatory diseases such as rheumatoid arthritis, and is thought to be an important mediator of inflammation in endotoxin-induced shock and ischemia/reperfusion injury (5–7). TNF-α has also been implicated in the pathogenesis of idiopathic pulmonary fibrosis (IPF), and TNF-α messenger RNA (mRNA) and protein have been detected in the lungs of IPF patients. In mouse models, TNF-α depletion protects against pulmonary fibrosis induced by exposure to bleomycin and silica (6–11). Therefore, several lines of evidence indicate that TNF-α may play a significant role in airway inflammation, but its role in asthma remains to be defined.

TNF-α is secreted mainly from monocytes, alveolar macrophages (AM), mast cells, neutrophils, and T lymphocytes, depending on the conditions or stimulus used (12–15). In previous studies, the effects of TNF-α on AHR have been inconsistent. TNF-α was reported to increase responsiveness to electrical field stimulation, albeit to a low level, in nonsensitized human bronchial tissue as compared with control tissue; however, this effect was not dose-dependent, and TNF-α had no effect on sensitized human bronchial tissue (16). In a rat model, TNF-α inhalation induced AHR to 5-hydroxytryptamine, but to a lower degree than observed after administration of lipopolysaccharide (LPS), despite an increase in neutrophil numbers in bronchoalveolar lavage fluid (BALF) (17). On the other hand, the pulmonary pathology of surfactant protein (SP)-C/TNF-α–transgenic mice, whose expression of TNF-α mRNA was limited to the lungs, consisted of a chronic mononuclear cell infiltrate with a predominance of T lymphocytes, and an increase in neutrophils in lavage fluid. This lymphocytic pneumonitis in time led to air space enlargement and emphysematous changes (8, 18). The affected animals also had an increase in γδ T cells in their lungs (19).

TNF-α has also been identified as a mediator of early T-cell activation, but may differentially influence the response of γδ and γδ T cells. Greater responsiveness of γδ T cells to TNF-α has been correlated with higher levels of the inducible TNF-α receptor p75 (20). We recently reported that γδ T cells down-regulate AHR through an αβ T-cell-independent mechanism (21). To clarify the significance of TNF-α and the role of γδ T cells in controlling the development of allergen-induced AHR, we examined both TNF-α–deficient mice and SP-C/TNF-α–transgenic mice after sensitization and challenge to ovalbumin (OVA). We monitored airway function in response to inhaled methacholine (MCh), and inflammatory cell infiltration in the airways.

METHODS

Animals

Mice genetically deficient in TNF-α (22, 23) were a gift from Dr. John Hartly of the University of Iowa, Iowa City, IA. These mice were originally derived from intercrosses of 129Sv × C57BL/6F1 mice heterozygous for the mutated 129Sv TNF-α gene, and have been maintained since 1996 as a line of mixed 129B6–gene–background mice homozygous for the mutation. Intercrossed mice not expressing the mutated TNF-α gene served as controls. Mice expressing the TNF-α gene under the control of the SP-C promoter (SP-C/TNF-α–transgenic mice) (8) were a gift from Dr. Yoshitaka Miyazaki of the Department of Clinical Immunology, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan. The transgenic founder mice (C57BL/bxBDA/2 F1) were backcrossed with C57BL/6 mice to generate F1 hybrid transgenic mice, and have been maintained as a heterozygous line by repeated backcrossing since 1995. All transgenic
mice were identified by polymerase chain reaction (PCR) analysis of genomic DNA as previously described (8). Littermate transgene-negative mice were used as controls. The mice were maintained on OVA-free diets.

Antibodies
Monoclonal anti-murine T cell receptor (TCR)-δ antibodies (GL3 and 403A10) were gifts from Drs. Leo LeFrançois (24) and Susumu Tonegawa (25). The antibody is panspecific for TCR-δ. The dose administered was optimized for T cell depletion, and routinely depleted more than 90% of splenic and pulmonary CD3 T cells (21). Monoclonal antibodies (mAbs) were prepared from antibody-secreting hybridoma cell lines. Antibodies were purified on affinity columns and quantified.

Sensitization and Airway Challenge
Each strain of mouse was grouped on the basis of the following treatments (4 mice/group/experiment): (1) airway challenge (×3) with OVA nebulization alone (N group); and (2) intraperitoneal sensitization with OVA and OVA airway challenge (IPN group). Mice were sensitized by intraperitoneal injection of 20 μg of OVA (Grade V; Sigma, St. Louis, MO) emulsified in 2.25 mg of alum (AlumImuject; Pierce, Rockford, IL) in a total volume of 100 μl on Days 0 and 14. Mice were challenged via the airways with OVA (1% in saline) for 20 min on Days 28, 29, and 30 by ultrasonic nebulization (particle size 1 to 5 μm; De Vilbiss, Somerset, PA). Lung resistance (Rl) and dynamic compliance (Cdyn) were assessed 48 h after the last allergen challenge, and the mice were killed to obtain tissues and cells for further assay.

Determination of Airway Responsiveness
Rl and Cdyn were determined as changes in airway function after inhaled MCh challenge (21, 26). After each aerosolized MCh challenge, the data were continuously collected for 1 to 5 min, and maximum values of Rl and minimum values of Cdyn were taken to express changes in these functional parameters.

Cytokine Levels in BALF
After assessment of Rl and Cdyn, lungs were lavaged once via the tracheal tube with Hank’s balanced salt solution (1 ml, 37°C). Cytospin slides (Shandon, Sewickley, PA) were stained with Leukostat (Fisher Diagnostics, Pittsburgh, PA) and cells were differentiated in a blinded fashion by counting at least 300 cells under light microscopy.

Cytokine levels (IL-4, IL-5, IL-10, and interferon IFN-γ) in BALF supernatants were measured with enzyme-linked immunosorbent assays (ELISAs) as described (27), and IL-12 (p70) was also assayed with an ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s recommendations. Cytokine levels were determined by comparison with known standards. The limits of detection were 4 pg/ml.

T Cell Purification and Fluorescence Activated Cell Sorter Analysis
Lung cells were isolated as previously described (28), and were passed through nylon wool columns to yield an enriched T cell preparation containing >90% CD3+ cells as previously described (29).

For cytofluorographic analysis, mAbs were conjugated with N-hydroxysuccinimidobiotin (Sigma) and/or fluorescein isothiocyanate isomer I on Cellite (Sigma), and were analyzed on an XL2 cytfluorograph (Coulter, Miami, FL). We used streptavidin–phycoerythrin (diluted at 1:100 per 1 × 10⁶ cells; Tago Immunologicals Biosource, Camarillo, CA) for the biotin-conjugated antibodies in order to enhance detection as described (30).

Anti-TCR-δ mAb Depletion of T Cells
T cell depletion was achieved after injection of 200 μg hamster monoclonal anti-TCR-δ IgG antibody (1:1 mixture of GL3 and 403A10) into the tail vein of mice 3 d before the first OVA challenge (21). Sham depletion was done with hamster IgG (Jackson Laboratories, Bar Harbor, ME).

Figure 1. (A) Rl and (B) Cdyn in TNF-α-sufficient mice (B6) and TNF-α-deficient mice. Rl and Cdyn values were obtained in response to increasing concentrations of inhaled MCh as described in MATERIALS. Data represent the mean ± SEM (n = 8 in each group). The TNF-α-deficient mice were hyperresponsive to MCh. *Significant differences (p < 0.05) between TNF-α-sufficient mice subjected to OVA challenge alone (TNF+/+/N) and OVA sensitization and challenge (TNF+/+/IPN). **Significant differences (p < 0.05) between TNF-α-deficient mice subjected to OVA challenge alone (TNF−/−/N) and OVA sensitization and challenge (TNF−/−/IPN). #Significant differences (p < 0.05) between TNF-α-deficient mice (TNF−/−/IPN) and TNF-α-sufficient mice (TNF+/+/IPN).
Measurement of Serum Anti-OVA Antibody and Total Ig Levels

Anti-OVA IgE antibody levels and total IgE were measured with ELISAs as previously described (31). The limits of detection were 100 pg/ml for IgE.

Histologic and Immunohistochemistry Studies

After obtaining BALF, we inflated lungs through the tracheal tube with 2 ml of air and fixed them in 10% formalin. Portions of lung tissue were cut around the main bronchus and embedded in paraffin blocks. Tissue sections 5 μm thick were cut, deparaffinized, stained with hematoxylin and eosin (H&E), and examined under light microscopy. The examiner was masked with regard to the treatment group.

Cells containing major basic protein (MBP) in lung sections were identified by immunohistochemical staining and quantitated as described, using a rabbit antimouse-MBP antibody (provided by Dr. James J. Lee of Mayo Clinic, Scottsdale, AZ) (28).

Statistical Analysis

Values for all measurements were expressed as the mean ± SEM. Student’s two-tailed unpaired t test was used to determine the levels of difference between two experimental groups. Analysis of variance (ANOVA) was used to compare percent changes of Rl and Cdyn in different groups with the same treatment. Significance was set at p < 0.05.

RESULTS

AHR Is Increased in the Absence of TNF-α

We first assessed airway responsiveness to inhaled MCh in TNF-α-deficient mice. Both OVA-sensitized and nonsensitized TNF-α-deficient mice were challenged with an aerosol of OVA on three consecutive days, in parallel with TNF-α-sufficient controls. After OVA sensitization and challenge, TNF-α-deficient mice developed significant increases in RL and decreases in Cdyn in an MCh dose-dependent manner, as compared with mice only challenged with OVA (Figure 1). Mice genetically deficient in TNF-α developed AHR to a (significantly) greater extent than did the control animals. In both naive and nonsensitized mice undergoing airway challenge alone, there were no significant differences in airway responsiveness between the two strains.

To further extend these results and directly compare the results of allergen challenge in sensitized mice, we sensitized...
both TNF-α-sufficient and -deficient mice to OVA (together with alum) on Days 0 and 14, and exposed them to phosphate buffered saline (PBS) or OVA by aerosol challenge on Days 28, 29, and 30. As shown in Table 1, challenge with OVA increased Rl with increasing concentrations of inhaled MCh in a dose-dependent manner (as compared with exposure to PBS) in both TNF-α-sufficient and -deficient mice, and the increases in Rl were significantly greater in the TNF-α-deficient animals.

The numbers and types of inflammatory cells in the airways of TNF-α-sufficient and -deficient mice were measured in BALF (Figure 2). In TNF-α-sufficient mice, sensitization and challenge with OVA resulted in a marked increase in inflammatory cell numbers as compared with challenge alone. TNF-α-deficient mice showed a similar inflammatory cell response, but the numbers of eosinophils in their BALF were significantly lower than in that of the TNF-α-sufficient mice (Figure 2).

We also examined the inflammatory cell response in lung tissue. In mice that underwent challenge alone, very little inflammatory cell infiltration was detected, whereas intraperitoneal sensitization and subsequent challenge with OVA via the airways increased the number of eosinophils and lymphocytes at these sites (Figure 3). The inflammatory cell response in sensitized and challenged TNF-α-deficient mice was similar to that in sensitized and challenged TNF-α-sufficient animals (Figure 3), including numbers of tissue eosinophils, despite the differences in BALF cell numbers (Figure 4).

**SP-C/TNF-α-Transgenic Mice Fail to Develop AHR**

In view of the heightened airway responses in TNF-α-deficient mice, we investigated airway responsiveness in TNF-α-transgenic mice. Baseline responsiveness in the transgenic mice was not significantly different from that in the transgene-negative or TNF-α-deficient mice, and there were no significant differences between the two strains of mice in airway responsiveness under naive conditions or with challenge alone. Surprisingly, SP-C/TNF-α-transgenic mice failed to develop significant changes in Rl and Cdyn after OVA sensitization and challenge (Figure 5).

The numbers of neutrophils and lymphocytes in the BALF of transgenic mice were significantly increased over those of control mice. However, the numbers of eosinophils in the BALF of transgenic mice were significantly decreased as compared with those in the transgene-negative mice (Figure 6). Histologic sections of the lungs of transgenic mice that underwent challenge alone showed an inflammatory cell infiltration, especially by lymphocytes, within the thickened alveolar septa and under the pleura (Figure 3). Neutrophils were also observed in the same sites. Despite this apparent increase over the background level in the inflammatory infiltrate in the lungs of nonsensitized TNF-α-transgenic mice, there was no AHR. After OVA sensitization and challenge the SP-C/TNF-α-transgenic mice also showed a decrease in eosinophil numbers as compared with the transgene-negative mice (Figure 4).

**Cytokine Levels in BALF in TNF-α-Deficient and Transgenic Mice**

Concentrations of IL-4, IL-5, IL-10, and IFN-γ in BALF supernatants were measured with ELISA. OVA sensitization and challenge significantly enhanced IL-4, IL-5, IL-10, and IFN-γ levels in BALF over the levels in mice that underwent challenge alone in both the TNF-α-sufficient (Figure 7) and TNF-α transgene-negative mice (Figure 8). BALF from OVA-sensitized and -challenged TNF-α-deficient mice contained increased levels of IL-4, IL-5, and IL-10 as compared with the levels in the nonsensitized group. The IL-10 levels in TNF-α-deficient mice were significantly higher than those in the TNF-α-sufficient controls, whereas IFN-γ levels were significantly lower (Figure 7). In contrast, IL-5 and IL-10 lev-
els in the SP-C/TNF-α-transgenic mice were significantly decreased as compared with those of transgene-negative mice, whereas IFN-γ levels were increased; IL-4 levels were similar in the two groups (Figure 8). IL-12 levels were also measured in the BALF, and paralleled those of IFN-γ (Table 2). For each group, sensitization and challenge resulted in an increase in IL-12 levels. The increases in the TNF-α-sufficient mice were significantly greater than in the TNF-α-deficient mice, and the levels in the sensitized and challenged transgenic were the highest, exceeding those in the transgene-negative mice.

Serum Anti-OVA IgE Antibody Levels in TNF-α-Deficient and Transgenic Mice

Anti-OVA IgE and total IgE levels in the sera of sensitized and challenged TNF-α-sufficient mice, TNF-α-deficient mice, transgene-negative mice, and SP-C/TNF-α-transgenic mice were not significantly different from one another (Table 3).

γδ T Cells in SP-C/TNF-α-Transgenic Mice

An increased frequency of γδ T cells has been demonstrated in SP-C/TNF-α-transgenic mice (19). We therefore investigated the effects of monoclonal anti-TCR-δ antibody on the γδ T cell populations in the lungs of OVA-sensitized and -challenged, TNF-α-deficient and transgenic mice. The number of γδ T cells in the lung in TNF-α-deficient mice was significantly lower than in TNF-α-sufficient mice (Figure 9). In contrast, the number of γδ T cells in the transgenic mice was significantly increased as compared with that in littermate transgene-negative mice. Injection of monoclonal anti-TCR-δ antibody significantly suppressed the numbers of γδ T cells in the lung in sensitized and challenged transgenic mice, as well as in TNF-α-sufficient and transgene-negative mice; the low γδ T cell numbers in the TNF-α-deficient mice did not change significantly upon antibody treatment (Figure 9).

Airway Responsiveness in TNF-α-Transgenic Mice after γδ T Cell Depletion

We recently demonstrated that γδ T cells play a role in the regulation of airway responsiveness (21). In view of the increased number of γδ T cells in TNF-α–transgenic mice (19), and the finding that γδ T cells may be activated by TNF-α (20), we examined whether activated γδ T cells might play a role in the failure of TNF-α–transgenic mice to develop AHR. To deplete γδ T cells, we treated mice with monoclonal anti-TCR-δ antibody 3 d before the first challenge with OVA. TNF-α–deficient mice given anti-TCR-δ antibody failed to show any further increase in AHR, although TNF-α–sufficient mice showed some increase, as previously reported (Figures 10A and 10B). The response in SP-C/TNF-α–transgenic mice depleted of γδ T cells was striking, with significant increases in Rt. and decreases in Cdyn that approached those of the transgene negative mice. The transgene-negative mice also showed some in-

Figure 4. Immunohistochemical localization of lung eosinophils. Lung tissue was stained with an antibody to MBP. Eosinophil numbers (per mm²) in the peribronchial, perivascular, and peripheral regions were quantified as described in Methods. TNF-α-deficient mice (TNF−/−N, TNF−/−IPN) showed no significant difference when compared with TNF-α–sufficient mice (TNF+/+/N, TNF+/+/IPN, respectively). *Significant differences (p < 0.05) between OVA-sensitized and -challenged transgene-negative mice (WT/IPN) and OVA-sensitized and -challenged TNF-α–transgenic mice (TNFtg/IPN). Results for each group are expressed as mean ± SEM (n = 8).
increase in AHR after γδ T cell depletion, as predicted by earlier studies (21) (Figures 10C and 10D). This effect on AHR was not correlated with changes in cellular inflammatory response: neither OVA-sensitized and -challenged TNF-α–deficient nor transgenic mice showed any significant differences in the composition of inflammatory cells in their BALF after depletion of γδ T cells (Figure 11).

DISCUSSION

TNF-α is produced in response to numerous stimuli and by a variety of cells (15), and is implicated in the pathogenesis of a number of chronic inflammatory diseases (5–7). The connection between TNF-α and inflammatory responses in patients with asthma has not been clearly defined, nor have the effects of TNF-α on tissue and smooth muscle been consistent (16, 32, 33). In the present study, we examined genetically manipulated mice in an attempt to define the physiologic importance of TNF-α in the development of allergen-induced AHR. Although the TNF-α–deficient mice and the transgenic mice in our study differed in genetic background, such differences are not likely to have played a substantive role in defining the activity of TNF-α in the primary sensitization model used in our study. Direct comparison of controls (challenge alone, Figures 1 and 4) with the TNF-α–deficient or transgenic mice (challenge alone) revealed little difference between the four groups of mice as compared with the differences in airway responsiveness after sensitization and challenge with OVA. The results indicated that TNF-α negatively modulates AHR in the model we used; OVA sensitization and challenge of TNF-α–deficient mice resulted in significant increases in AHR in response to inhaled MCh in a dose-dependent manner as compared with the changes seen in sensitized and challenged control mice. In contrast, SP-C/TNF-α–transgenic mice, which have lung concentrations of TNF-α that are more than 300 times higher than those of transgene-negative mice (34), failed to develop increases in Rl and Cdyn after sensitization and airway challenge.

After sensitization and challenge, TNF-α–deficient mice demonstrated increased numbers of eosinophils and higher levels of IL-5 in BALF than did mice that underwent challenge alone, but the levels were lower than in sensitized and challenged TNF-α–sufficient mice. Interestingly, another

Figure 6. Cellular composition of BALF in transgene-negative mice and SP-C/TNF-α–transgenic mice. Groups are the same as in Figure 4. Sensitized and challenged transgene-negative mice show an increase in lung eosinophils, whereas the SP-C/TNF-α–transgenic mice do not. *Significant differences (p < 0.05) between TNF-α transgene-negative mice subjected to OVA challenge alone (WT/N) and OVA sensitization and challenge (WT/IPN). **Significant differences (p < 0.05) between SP-C/TNF-α–transgenic mice subjected to OVA challenge alone (TNFTg/N) and OVA sensitization and challenge (TNFTg/IPN). Statistical differences (p < 0.05) between OVA-sensitized and -challenged littermate transgene-negative mice (WT/IPN) and OVA-sensitized and -challenged transgenic mice (TNFTg/IPN). #Significant differences (p < 0.05) between nonsensitized transgene-negative mice (WT/N) and nonsensitized transgenic mice (TNFTg/N). Results for each group are expressed as mean ± SEM (n = 8).

Figure 7. Cytokine levels in BALF of TNF-α–sufficient mice and TNF-α–deficient mice. (A) IL-4, (B) IL-5, (C) IL-10, and (D) IFN-γ levels in BALF from the groups shown in Figure 1 were measured in supernatants with ELISAs, as described in Methods. TNF-α–deficient mice had equivalent amounts of IL-4 and IL-5 in their lavage fluid, but higher IL-10 and lower IFN-γ after OVA sensitization and challenge. *Significant differences (p < 0.05) between TNF-α–sufficient mice (TNF+/+/IPN) subjected to challenge alone (TNF+/+/N) and to sensitization and challenge. **Significant differences (p < 0.05) between TNF-α–deficient mice subjected to OVA challenge alone (TNF−/−/N) and to OVA sensitization and challenge (TNF−/−/IPN). Statistical differences (p < 0.05) between TNF-α–sufficient mice (TNF+/+/IPN) and TNF-α–deficient mice (TNF−/−/IPN). Results for each group are expressed as mean ± SEM (n = 8).
study found that administration of anti-TNF-α antibody to mice receiving antigen-specific T helper type 1 (Th1) and Th2 cells reduced BALF eosinophil numbers by about 30% (35), indicating only limited control of eosinophil recruitment by TNF-α, in keeping with the results in our TNF-α-deficient mice. In contrast, after sensitization and challenge, the TNF-α transgene-expressing mice in our study exhibited lower levels of IL-5 and eosinophil numbers but increased levels of IFN-γ and IL-12. It is important to note that the present study evaluated the role of TNF-α in a primary sensitization model. Therefore, in the TNF-α–transgenic mice, TNF-α may have influenced initial Th1/Th2 development as a result of the increased release of IL-12 and IFN-γ. IL-10 levels in BALF of the TNF-α–deficient mice were significantly increased, whereas the levels in TNF-α–transgenic mice were decreased as compared with those of sensitized and challenged controls. On one hand, IL-10 may limit eosinophilic inflammation, whereas on the other hand we demonstrated a requirement for IL-10 in the development of AHR (36); mice deficient in IL-10 failed to develop AHR, and only after reconstitution of the IL-10 gene could development of AHR be restored (36). The role of IL-10, however, is complex, with its requirement described both for AHR (36) and for the inhibition of AHR (37, 38).

Some of these differences may be related to the timing and extent of allergen exposure.

TNF-α has been implicated in the recruitment of neutrophils to the lungs under different conditions. The kinetics of neutrophil recruitment to the lung, and its association with development of AHR, are not well delineated. Thomas and colleagues showed that inhaled TNF-α may have influenced initial Th1/Th2 development as a result of the increased release of IL-12 and IFN-γ. IL-10 levels in BALF of the TNF-α–deficient mice were significantly increased, whereas the levels in TNF-α–transgenic mice were decreased as compared with those of sensitized and challenged controls. On one hand, IL-10 may limit eosinophilic inflammation, whereas on the other hand we demonstrated a requirement for IL-10 in the development of AHR (36); mice deficient in IL-10 failed to develop AHR, and only after reconstitution of the IL-10 gene could development of AHR be restored (36). The role of IL-10, however, is complex, with its requirement described both for AHR (36) and for the inhibition of AHR (37, 38).

### TABLE 2. INTERLEUKIN-12 LEVELS IN BRONCHOALVEOLAR LAVAGE FLUID

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-12 Level (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF+/−/N</td>
<td>ND</td>
</tr>
<tr>
<td>TNF+/−/IPN</td>
<td>19.5 ± 8.6*</td>
</tr>
<tr>
<td>TNF−/−/N</td>
<td>ND</td>
</tr>
<tr>
<td>TNF−/−/IPN</td>
<td>6.9 ± 2.4</td>
</tr>
<tr>
<td>WT+/−/N</td>
<td>ND</td>
</tr>
<tr>
<td>WT+/IPN</td>
<td>24.7 ± 5.7*</td>
</tr>
<tr>
<td>TNFTg+/−/N</td>
<td>ND</td>
</tr>
<tr>
<td>TNFTg/IPN</td>
<td>67.9 ± 12.0*</td>
</tr>
</tbody>
</table>

Definition of abbreviations: IPN = OVA-sensitization and -challenge; N = OVA challenge only; TNF = tumor necrosis factor-α; TNFTg = surfactant protein-C/tumor necrosis factor-α-transgenic; WT = transgene negative (wild-type).

Bronchoalveolar lavage fluid was collected from the same groups as described in the legends to Figures 7 and 8.

* Significant differences (p < 0.05) between TNF-α–sufficient and -deficient mice and transgene-positive and transgene-negative mice. Results for each group are expressed as mean ± SEM (n = 8). ND = none detected (< 4 pg/ml).

### TABLE 3. OVALBUMIN-SPECIFIC IgE AND TOTAL IgE LEVELS IN SERUM

<table>
<thead>
<tr>
<th>OVA-specific IgE levels (EU/ml)</th>
<th>Total IgE levels (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF+/−/N</td>
<td>0</td>
</tr>
<tr>
<td>TNF+/−/IPN</td>
<td>20.73 ± 3.31*</td>
</tr>
<tr>
<td>TNF−/−/N</td>
<td>0</td>
</tr>
<tr>
<td>TNF−/−/IPN</td>
<td>23.9 ± 4.74*</td>
</tr>
<tr>
<td>WT+/−/N</td>
<td>0</td>
</tr>
<tr>
<td>WT+/IPN</td>
<td>25.72 ± 11.58*</td>
</tr>
<tr>
<td>TNFTg+/−/N</td>
<td>0</td>
</tr>
<tr>
<td>TNFTg/IPN</td>
<td>16.1 ± 3.29*</td>
</tr>
</tbody>
</table>

Definition of abbreviations: IPN = OVA-sensitization and -challenge; N = OVA challenge only; TNF = tumor necrosis factor-α; TNFTg = surfactant protein-C/tumor necrosis factor-α-transgenic; WT = transgene negative (wild-type).

Serum titers of OVA-specific IgE antibody and total IgE levels in TNF-α–sufficient mice (B6), TNF-α–deficient mice, transgene-negative mice (WT), and SP-C TNF-α–transgenic mice were determined by ELISA as described in Methods. Neither TNF-α–deficient mice nor transgenic mice showed any significant difference as compared with control mice. Data represent mean ± SEM (n = 8).

* Significant differences (p < 0.05) between mice subjected to OVA challenge alone (TNF+/−/N, TNF−/−/N, WT/N, TNFTg/N, respectively) and OVA-sensitization and challenge (TNF+/−/IPN, TNF−/−/IPN, WT/IPN, TNFTg/IPN, respectively).
Figure 9. Flow-cytometric analysis of γδ T cells in the lung after OVA sensitization and challenge. Numbers of γδ T cells in the lungs of SP-C/TNF-α-transgenic mice were significantly increased after nylon-wool enrichment as compared with those in the lungs of transgene-negative mice. Treatment with monoclonal anti-TCR-δ antibody significantly decreased the numbers of γδ T cells in sensitized and challenged transgenic mice. *Significant differences (p < 0.05) between TNF-α–sufficient mice (TNF+/+/hamsterIgG) and TNF-α–deficient mice (TNF−/−/hamsterIgG). **Significant differences (p < 0.05) between transgene-negative mice (WT/hamsterIgG) and SP-C/TNF-α-transgenic mice (TNFTg+/hamsterIgG). ΔSignificant differences (p < 0.05) between sham-treated, OVA-sensitized and -challenged TNF-α–sufficient mice (TNF+/+/hamsterIgG) and anti-TCR-δ antibody-treated, OVA-sensitized and -challenged TNF-α–deficient mice (TNF−/−/+/anti-TCR-δ). Results for each group are expressed as mean ± SEM (n = 4 to 8).

deficient mice, indicate that although TNF-α can affect neutrophil, eosinophil, and lymphocyte recruitment to the lungs of sensitized and challenged mice, its role is not likely to be an essential one in the accumulation of these cells.

The lungs of the SP-C/TNF-α–transgenic mice used in the current study showed a mononuclear cell alveolitis with lymphocytic infiltration that at 2 mo of age was more prominent in the interlobular septa around the extraalveolar small vessels and under the pleura. Macrophages numbers appeared to be increased and neutrophils could be seen within the infiltrates. In older mice (6 mo of age), the alveolar spaces became enlarged and the inflammatory infiltrate appeared to decrease (8). TNF-α mRNA, which was overexpressed in type II alveolar epithelial cells, was identified only in the lungs, and was not detected in other tissues (8). Using these transgenic mice, we showed that beyond the numbers of neutrophils in the BALF of naive mice, OVA sensitization and challenge further increased the numbers of neutrophils by up to 50%. Despite this influx of neutrophils, lung resistance to inhaled MCh remained lower than in sensitized and challenged control mice. Thus, expression of the TNF-α transgene is associated with negative regulation of airway responsiveness. Because of the finding of decreased airway reactivity in the presence of increased numbers of neutrophils in these mice, it also seems unlikely that neutrophils directly contribute to AHR after sensitization and challenge with OVA, a finding that we previously reported (42).

We recently reported that mice genetically deficient in γδ T cells and γδ T cell depleted (after treatment with monoclonal anti-TCR-γ antibody) mice have altered airway function. The studies in which we made these findings further showed that γδ T cells downregulate AHR through an αβ T cell–independent mechanism and without changes in inflammatory cell accumulation (21). This mechanism may coexist with immunoregulatory effects of γδ T cells on αβ T cell–dependent pathways of AHR (21). Several studies have identified interactions between TNF-α and γδ T cells; not only was early activation of γδ T cells found to be largely dependent on TNF-α, but γδ T cells themselves can produce TNF-α (20, 43, 44). Furthermore, Nakama and coworkers demonstrated an increased frequency of γδ T cells in SP-C/TNF-α–transgenic mice (19), which we confirmed in the present study. In light of these findings, we investigated the effect of γδ depletion in TNF-α–transgenic mice. A marked increase in airway responsiveness to MCh was detected after allergen sensitization and airway challenge in SP-C/TNF-α–transgenic mice depleted of γδ T cells.
which was not seen in sham-treated mice. The changes in airway function were similar to those observed in transgene-negative mice. As seen previously with γδ T cell depletion (21), the effects on AHR were independent of changes in inflammatory cell response. γδ T cell depletion did result in increased AHR in both TNF-α-sufficient and transgene-negative mice, in keeping with previous results (21), but the changes in both groups were much smaller than in TNF-α-transgenic mice. Only the TNF-α-deficient mice failed to show any change in airway function after γδ T cell depletion, a finding in keeping with the very low numbers of γδ T cells in these mice. Therefore, γδ T cells seem to play an important role in the mechanism of suppressing airway responsiveness in SP-C/TNF-α-transgenic mice.

In summary, the findings reported here support complex but important contributions of TNF-α to the overall regulation of allergic inflammatory responses in the lung and to the development of altered airway function, in part through interactions with γδ T cells. The additional finding that increased levels of TNF-α were associated with decreased levels of IL-10—an important factor in the development of AHR (36)—reveals that suppression of IL-10 may be another mechanism by which TNF-α controls airway responsiveness. The possibility that these two mechanisms (increased/activated γδ T cells and IL-10 suppression) are linked is currently being explored.

Acknowledgment: The authors appreciate the advice of Dr. Rebecca O’Brien throughout these studies. We are grateful to Ms. Diana Nabighian for preparation of the manuscript and Ms. Lynn Cunningham for her help in preparing the tissue slides.

References


