American Journal of Pathology, Vol. 157, No. 2, August 2000

Animal Model

TRAF4 Deficiency Leads to Tracheal Malformation with Resulting Alterations in Air Flow to the Lungs

Helena Shiels,*† Xiantang Li,‡
Paul T. Schumacker,§ Emin Maltepe,§¶
Philip A. Padrid,§ Anne Sperling,†§
Craig B. Thompson,*†§¶ and Tullia Lindsten*§

From the Gwen Knapp Center for Lupus and Immunology Research, the Committee on Immunology,† the Department of Pathology,‡ and the Department of Medicine,§ Howard Hughes Medical Institute,¶ University of Chicago, Chicago, Illinois*

TRAF4 is one of six identified members of the family of TNFR-associated factors. While the other family members have been found to play important roles in the development and maintenance of a normal immune system, the importance of TRAF4 has remained unclear. To address this issue, we have generated TRAF4-deficient mice. Despite widespread expression of TRAF4 in the developing embryo, as well as in the adult, lack of TRAF4 expression results in a localized, developmental defect of the upper respiratory tract. TRAF4-deficient mice are born with a constricted upper trachea at the site of the tracheal junction with the larynx. This narrowing of the proximal end of the trachea results in respiratory air flow abnormalities and increases rates of pulmonary inflammation. These data demonstrate that TRAF4 is required to regulate the anastomosis of the upper and lower respiratory systems during development. ***(Am J Pathol 2000, 157:679–688)***

The tumor necrosis factor receptor (TNFR) family is a still expanding family of receptors that are involved in signal transduction pathways primarily during immune and inflammatory responses.[1] Each receptor binds to a ligand from the TNF family, which results in multimerization of receptor monomers and in signaling responses ranging from proliferation and differentiation to apoptosis. The various family members have considerable homology in their extracellular domains due to the presence of cysteine-rich motifs required for ligand binding. With the exception of a certain subset of receptor family members that contain the so-called death domain (DD) motif in their cytoplasmic domain, the cytoplasmic tails of the proteins have a much more limited homology.

Recently, a group of proteins that bind to the cytoplasmic tail of several TNFR family members has been identified. These proteins are named TRAFs (for TNFR-associated factors), and to date six family members have been described. All TRAFs share a ~150-amino acid C-terminal TRAF domain that mediates the interaction between the TRAF and its receptor(s)[2–4] or other proteins, as well as a more loosely conserved region of zinc finger repeats. A similar domain can also be found in the meprin family of mammalian zinc metalloendopeptidases.[5] Among some of the TNF receptors, a PXQX(T/S) motif has been shown to be a common TRAF domain binding site.[6–8] With the exception of TRAF1, all TRAFs contain an N-terminal RING finger, which seems to be required for their downstream signaling potential. Dominant negative (DN) versions of the TRAFs can be generated by removal of the RING finger domain.[9,10] TRAF2, 5, and 6 share an ability to mediate NF-κB and JNK activation through their N-terminal domains.[10–12] TRAFs are also able to bind to non-TNFR family members, as exemplified by TRAF6's ability to associate with the interleukin-1 (IL-1) receptor.[13] To date, published reports have been generated on the phenotype of mice that lack TRAF2,[14] TRAF3,[15] TRAF5,[16] or TRAF6[17] or that overexpress TRAF1[18] or the DN form of TRAF2.[19] The TRAF2-, TRAF3-, or TRAF6-deficient mice that are born appear normal at birth but die at a very young age as a result of severe runting. TRAF5-deficient mice appear outwardly normal.

The immune system of TRAF2-deficient mice is affected, with depletion of lymphocytes in thymus and spleen and an absence of secondary germinal centers, perhaps due to a combination of hypersensitivity to TNF and increased serum TNF levels. B-cell development is impaired, but T cells develop normally. TNF-mediated NF-κB responses are normal, but JNK activation is defi-

Supported in part by grants by the National Institutes of Health.

Accepted for publication April 27, 2000.

Address reprint requests to Dr. Tullia Lindsten, Abramson Family Cancer Research Institute, Department of Pathology and Laboratory Medicine, Room 450, BRB II/III, 421 Curie Boulevard, Philadelphia, PA 19104-6160. E-mail: lindsten@mail.med.upenn.edu.

cient. Interestingly, in TRAF2 DN mice with the transgene being expressed only in lymphocytes, there is an increase in the number of B cells, splenomegaly, and lymphadenopathy. JNK activation, but not NF-κB responses, is affected just as in TRAF2-deficient mice.

In mice lacking TRAF3, despite hypotrophy of spleen and thymus similar to what is seen in TRAF2-deficient animals, the immune system is defective when it comes to T-cell-dependent responses to antigen but otherwise is fairly normal.

TRAF5-deficient mice show defects in CD40- and CD27-mediated signaling. TRAF6-deficient mice have enlarged spleens, despite normal T-cell function and reduced B-cell proliferation in response to CD40 or lipopolysaccharide stimulation. IL-1, CD40, or lipopolysaccharide-mediated NF-κB activation is also disrupted, as is IL-1-mediated JNK activation. In addition, these mice are osteopetrotic, which is most likely due to the recently described association between TRAF6 and the receptor activator of NF-κB (RANK),[20] which is required for osteoclast differentiation and lymph node formation.[21]

TRAF4 was cloned in a differential expression screen, using mRNA from metastatic breast cancer samples.[22,23] The 2-kb gene is located on human chromosome 17, q11-q12,[22] which is a region that also contains the oncogene c-*erbB2*, a gene known to be involved in breast cancer. The predicted molecular weight of TRAF4 is 53 kd. TRAF4 was originally found to be expressed in the nucleus of the breast cancer cells, using a polyclonal rabbit anti-serum against a TRAF-C domain synthetic peptide. In mice, using Northern blot analysis, as well as *in situ* hybridization,[24] it was shown that TRAF4 is expressed at high levels during embryogenesis, peaking at embryonic day 13.5. Specifically, transcripts are present in the developing neuroepithelium, neural crest cell condensations, and the first, second, and third branchial arches. Transcripts are also detected in the neuroepithelium of the nasal fossae, as well as in thymus, salivary glands, and intestine of embryos. In the adult, TRAF4 was among the genes induced in postmitotic and undifferentiated cells of the central nervous system.

Recently, a second TRAF4-specific polyclonal antibody was used to investigate expression patterns in normal embryonic and adult human tissue, as well as in tissue from breast and prostate cancers.[25] Surprisingly, by using this antibody for immunohistochemistry, TRAF4 was found to be primarily localized to the cytoplasm of the cells, and was not expressed in most breast cancer samples. Most epithelial cells in this study, including thymic epithelial cells and lymph node dendritic cells, expressed TRAF4, whereas the thymocytes and lymphocytes did not demonstrate any TRAF4 staining. In addition, Krajewska et al found evidence that TRAF4 is able to associate with the lymphotoxin-β receptor, which has an overlapping expression pattern in the scaffolding tissues of thymus and lymph nodes. A weak interaction with the p75 neural growth factor receptor was also detected. The differences in the expression patterns of TRAF4 in this report as compared to the study using *in situ* hybridization could be explained by posttranscriptional modification of TRAF4 transcripts.[24]

To better understand the role of TRAF4 in development, we have generated TRAF4-deficient mice through gene targeting. The mice are viable and appear outwardly normal. However, about 20% of TRAF4 knock-out mice present with a high-pitched wheezing noise as they breathe. In addition, there is a decrease in weight of the animals, averaging 10% for adult males and 5% for adult females. Pathological analysis demonstrates that lack of TRAF4 leads to failure to properly form the proximal end of the trachea, at the region where the trachea connects to the larynx, leading to various degrees of constriction in all knock-out mice examined. The air flow is altered, as evidenced by a marked decrease in response to a challenge with aerosolized antigen in immunized mice lacking TRAF4. TRAF4-deficient mice also develop spontaneous inflammation of the lungs, correlating with the occurrence of wheezing. The immune system of TRAF4 knock-out mice appears to respond normally to a variety of stimuli. Taken together, the data suggest that the expression of TRAF4 normally seen in branchial arches 2 and 3 during embryogenesis correlates with a required role for TRAF4 during normal laryngotracheal development.

Materials and Methods

Targeting Constructs for the TRAF4 Gene

Polymerase chain reaction amplification of a human cDNA library, using primers derived from the published sequence of human TRAF4, was used to subclone human *TRAF4* cDNA. A 5′ 0.6-kb probe, containing mostly the three zinc finger repeats, was isolated from the cDNA clone and used to screen a phage library (Lambda FIX II; Stratagene Cloning Systems, La Jolla, CA) derived from 129/SvJ mice. The resulting genomic clones were mapped by restriction digest analysis, Southern blotting, and sequencing. A 2.2-kb *Kpn*I-*Xba*I fragment located 3′ of the last exon (exon 7), in combination with either a 7-kb *Sal*I-*Bgl*II fragment containing the first exon (construct 1, Figure 1A) or a 4.1-kb *Sal*I-*Bam*HI fragment containing only sequence 5′ of the gene (construct 2, Figure 1A), was cut out from the same phage preparation (the *Sal*I site is derived from the phage) and subcloned into the pPNT vector.[26] On linearization, the resulting targeting constructs contained the PGK-*neo*^{r} gene in the opposite transcriptional orientation as compared to the *TRAF4* gene, between the two subcloned genomic fragments that were flanked on one side by the *PGK-TK* gene.

Generation of TRAF4-Deficient Mice

The two linearized targeting constructs were used to electroporate R1 ES cells, derived from an F1(129/Sv × 129/SvJ) blastocyst.[27] Two × 10^7 ES cells were used for each electroporation at 270 V, 500 μF, using a Bio-Rad Genepulser (Bio-Rad Laboratories, Hercules, CA), and the cells were plated onto eight 10-cm dishes containing mitomycin C-treated *neo*^{r} mouse embryo fibroblasts.[28] Culture and selection of *neo*^{r}, TK-negative ES cells were

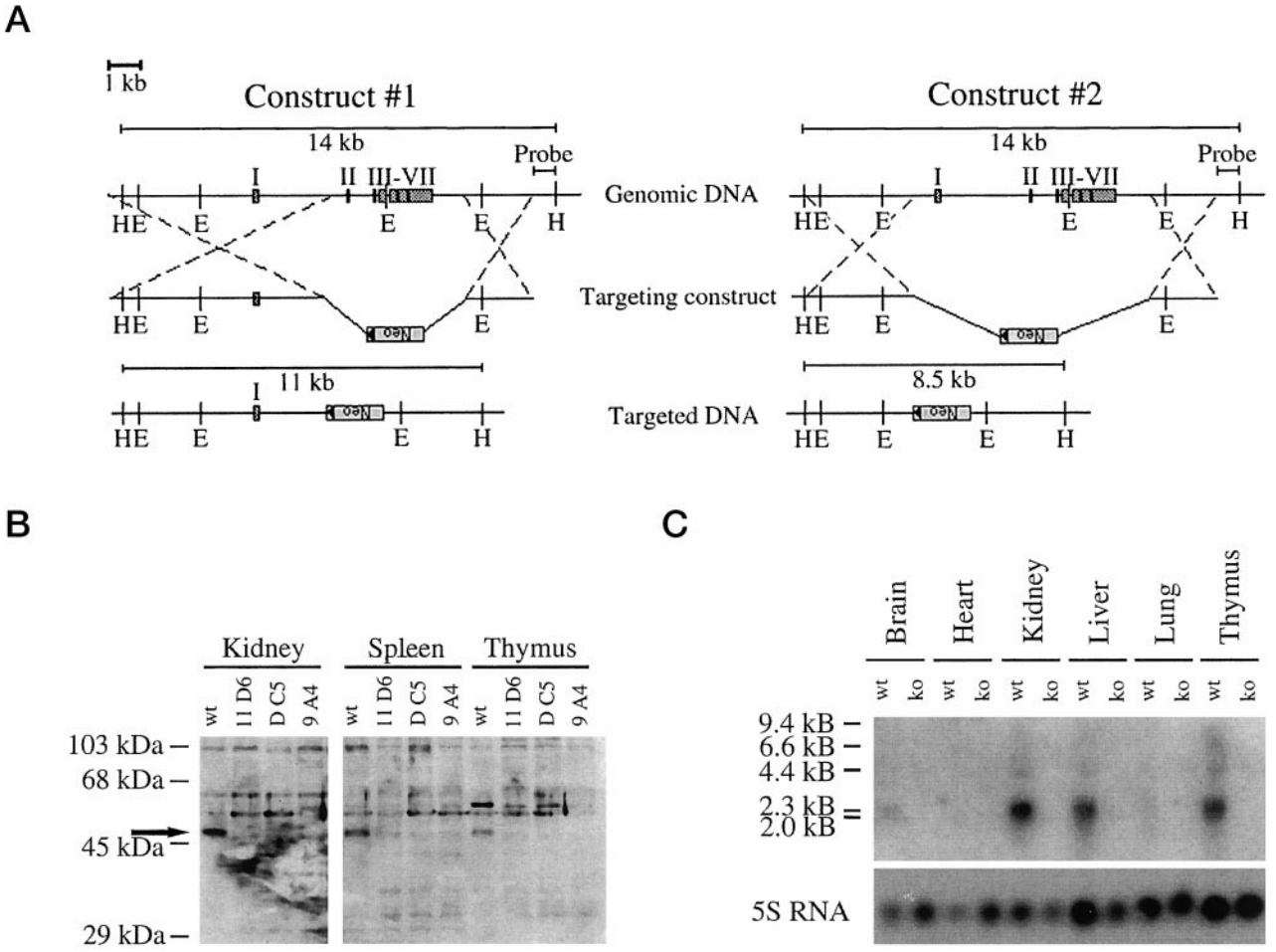


Figure 1. Generation of TRAF4-deficient mice by gene targeting. **A:** Schematic representation of the strategy used to generate ES cell lines with targeted disruptions of the *TRAF4* gene. Constructs 1 and 2 are shown. Exons are marked with roman numerals. The *neo*^{r} gene replaces most or all of the *TRAF4* gene in the targeted loci. E, *Eco*RI; H, *Hin*dIII sites. **B:** Western blot analysis of whole-cell lysates from wild-type or knock-out mice from each of the three ES cell lines (11D6, DC5, and 9A4). Lysates were made from the indicated tissues, and equal amounts of protein were loaded into each lane. The blot was probed with a polyclonal antibody that recognizes N-terminal TRAF4. **C:** Northern Blot analysis of *TRAF4* mRNA expression in wild-type or knock-out mice. Total RNA was extracted, and *TRAF4* transcripts were detected using a probe specific for exons 4–7. (Similar results were obtained by using a probe containing the RING domain.) The relative amounts of RNA were visualized by using an anti-5S RNA probe.

as previously described,[29] with a few modifications as described below. High-glucose liquid Dulbecco's minimum essential medium with L-glutamine (GibcoBRL, Grand Island, NY) was used with the addition of penicillin-streptomycin (GibcoBRL), but without the addition of sodium pyruvate. The fetal bovine serum (HyClone, Logan, Utah) was screened for the optimal growth of undifferentiated ES cells. Selection for targeted ES cells was performed 24 hours after electroporation, using ES cell medium containing 250 μg/ml neomycin and 2 μM gancyclovir. Surviving colonies of ES cells were picked 7 days after electroporation.

The resulting expanded ES cell clones were screened by Southern blot analysis of *Hin*dIII-digested genomic DNA isolated from each clone. The 1-kb *Xba*I-*Hin*dIII probe used to identify homologously recombined ES cell clones is shown in Figure 1A.

ES cells from the selected clones were injected into C57/BL6 blastocysts, and chimeric mice with a large contribution of targeted ES cells could be identified because of their mainly agouti coat color. Largely agouti chimeras were bred to C57/BL6 females, and the resulting F1 progeny were screened by Southern blot analysis of tail DNA to determine their genotype. Mice with germline transmission of a targeted *TRAF4* allele were intercrossed to generate TRAF4-deficient mice.

Western and Northern Blot Analysis

Whole-cell extracts of homogenized tissues from wild-type mice and mice derived from all three of the germline transmitted ES cell lines were made using RIPA buffer (50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.1% Triton X-100, 0.5% deoxycholate, 0.1 mmol/L EGTA, 0.1 mmol/L EDTA) with the addition of protease inhibitors. The protein concentration was determined by colorimetric assay (BCA Protein Assay; Pierce, Rockford, IL), and equal amounts of protein were loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel. The Western blot was probed with a polyclonal antibody raised against an N-terminal peptide from human TRAF4 (Santa Cruz Biotechnology, Santa Cruz, CA).

Total RNA was purified from wild-type and knock-out mice with the use of TRIzol (GibcoBRL). The RNA was equalized as previously described,[30] and the concentration was later determined by spectrophotometric analysis. Approximately 5 μg of total RNA was used for each sample. The samples were loaded on an agarose gel, electrophoresed, and blotted as described.[30] TRAF4 transcripts were detected using a probe specific for exons 4–7. (Similar results were obtained with the use of a probe containing the RING domain.) The relative amounts of RNA were visualized with the use of an end-labeled 5S RNA probe.

Examination of Tracheal Morphology

The mice were sacrificed with a lethal dose of ketamine. The tissues in front of the trachea were removed and the chest was opened, but the mice were otherwise left intact. The outer diameter of each cartilage ring was measured, using a dissection microscope equipped with a scale bar with 0.1-mm increments. The average width of either the first three rings below the cricoid cartilage or the last three cartilage rings before the bifurcation of the trachea was used as a measure of proximal and distal tracheal width, respectively. After the tracheal diameter had been documented, tissue containing the entire airway was removed and fixed. The fixed tissue was sectioned in the coronal plane and stained with hematoxylin-eosin. More detailed histological analysis was performed to determine the circumference, width, and density of the cartilage rings, as well as the occurrence of inflammatory cells in the airway tissue.

Ovalbumin Immunization, Nebulization, and Analysis of Lavage Fluid

The method used for ovalbumin (OVA) immunization and nebulization was as described by Krinzman et al,[31] with some modifications. Specifically, TRAF4-deficient mice, or littermate controls, were left untreated or were immunized intraperitoneally with 10 μg of OVA (grade IV; Sigma, St. Louis, MO) in 20 μl of 5 mg/ml alum in phosphate-buffered saline (PBS). Each experimental group contained three mice. The immunization was repeated 1 week later. Four days after the second immunization, the mice were challenged with aerosolized 6% OVA in sterile PBS, using a nebulizer, for 3 consecutive days for 30 minutes each time. Five days later the mice were sacrificed with a lethal dose of ketamine. Day 5 was chosen to maximize the presence of eosinophils in the bronchoalveolar lavage (BAL) fluid.[32] The airways were rinsed four times with 0.8 ml of sterile PBS, using a syringe with a blunt needle inserted halfway down the trachea, and sealed off with suture thread around the trachea. Similar volumes of BAL fluid were recovered from each mouse. Total white blood cell counts were made directly from the BAL fluid. In addition, samples were centrifuged onto microscope slides with a cytospin (Shandon, Pittsburgh, PA). The cells were stained by standard methods, and the relative contribution of eosinophils *versus* other white blood cell types was determined.

In Situ *Hybridization*

Embryos from timed matings (noon of the day of vaginal plug was considered embryonal day 0.5, E0.5d) were dissected out, fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned. A 0.6-kb probe from the human *TRAF4* cDNA 5′ end was first used on a Northern blot with total mouse RNA to verify specific recognition of mouse *TRAF4* transcript. The probe was subcloned in pBluescript (Stratagene Cloning Systems), linearized on either side of the cloning cassette, and *in vitro* transcribed and α-^{35}S-labeled with T3 (sense) or T7 (antisense) polymerase, depending on the site of linearization. The riboprobes were quantitated and used for *in situ* hybridization, with washing and developing conditions as previously described.[33] The sections were dipped in NTB2 emulsion (Kodak, Rochester, NY), and autoradiography was allowed to proceed for 1 week before the slides were developed and the cell nuclei were counterstained using Hoechst 33258 (Boehringer Mannheim, Mannheim, Germany). Adjacent sections from the same embryo probed with either sense or antisense riboprobe were photographed, using the same exposure time, with a Zeiss Axiophot microscope for epifluorescence and dark-field microscopy.

Results

Gene Targeting and Generation of TRAF4-Deficient Mice

The *TRAF4* gene was disrupted by homologous gene targeting. Three independent ES cell lines derived from two different constructs (Figure 1A) were used to generate TRAF4-deficient mice. Construct I eliminated all but part of the RING finger (exon 1), whereas construct II eliminated the entire gene from the targeted chromosome. Either of the two constructs resulted in loss of both detectable TRAF4 protein (Figure 1B) and TRAF4 mRNA (Figure 1C), and all three lines of the TRAF4$^{-/-}$ mice have the same phenotype, verifying the successful targeting of *TRAF4* in these mice.

TRAF4-deficient mice were born at the expected Mendelian ratio. The mice bred as well as their wild-type littermates. On gross examination of the knock-out mice, two findings were evident. First, the mice were smaller overall than their littermates. Figure 2 shows the weight at 7 weeks of 66 knock-out mice and 89 wild-type mice grouped by sex. There is a statistically significant reduction in the average weight of mice lacking TRAF4. Males have undergone a 10% decrease in weight and females a 5% decrease at 7 weeks of age (Figure 2). The weight reduction is not due to loss of any specific tissue, inasmuch as overall gross and histopathological examination revealed no significant differences between TRAF4 wild-type and knock-out mice. Second, a certain percentage (approximately 20%) of the mice at any one time dis-

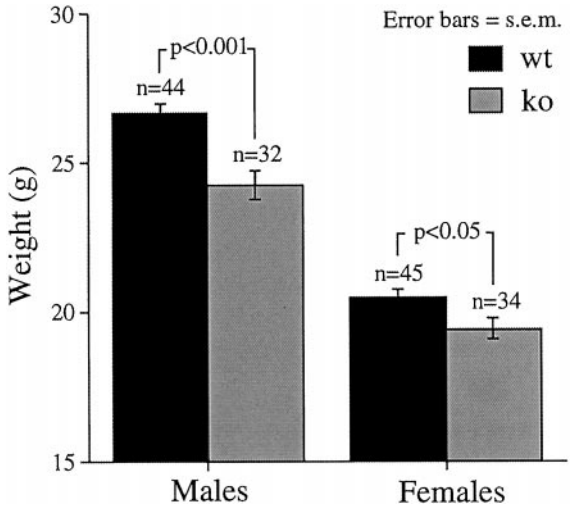


Figure 2. Average total body weight of TRAF4 wild-type and knock-out mice. The mice were placed on a Boston trip balance. Weight was recorded to within an accuracy of 0.1 g. The weights of male and female mice at 7 weeks of age are shown. The mean and SEM can be seen, as well as the calculated *P* values for each data set. The number of animals in each group is denoted by *n*.

played inspiratory stridor, as evidenced by a high-pitched wheezing noise. Mice as young as 2 weeks of age could be heard making the noise. Affected mice could have severe stridor at one time, but within weeks could be asymptomatic and make no or very little noise.

Histological Examination of the Airways of TRAF4-Deficient Mice

To understand the underlying defect(s) of TRAF4-deficient mice, a more detailed analysis of the airways was performed. A total of nine TRAF4-deficient mice and six littermate controls were subjected to analysis. Tissue samples were delivered to the pathologist in a blinded fashion to prevent introduction of investigator bias. On studying the trachea, it became clear that all mice lacking TRAF4 had a narrowing of the proximal part of the trachea, involving the first few cartilage rings immediately below the larynx. When the external diameter of the upper trachea was measured, an average 18% reduction was found in TRAF4-deficient mice as compared to littermate control mice, resulting in an estimated decrease of the cross-sectional area of about 32%. There was no difference in the width of the cartilage rings themselves, and the U-shaped form of the rings remained intact. However, a ventrally pointed appearance was often evident. Lack of TRAF4 expression had no effect on the width of the trachea below this region. The results are summarized in Figure 3A. The location of the tracheal constriction just below the larynx in TRAF4 knock-out mice correlates with the observed inspiratory stridor, because extrathoracic tracheal stenosis would be expected to give rise to inspiratory stridor, whereas intrathoracic tracheal stenosis would result in expiratory stridor.

Figure 3B shows an example of the tracheal defect seen on dissection of a TRAF4-deficient mouse as compared to a wild-type mouse. Fixed tissue containing larynx, trachea, and lungs of another set of mice are shown in Figure 3C. There was no evidence of cartilaginous defects in the trachea of the affected animals, as the tracheal cartilage appearance in stained tissue sections from TRAF4-deficient mice was indistinguishable from that in their wild-type littermates (data not shown). Measuring the length of various bones in the mice, as well as X-ray imaging, also revealed no genotype-dependent differences (data not shown), arguing against a generalized defect in tissues of cartilaginous origin.

In some of the TRAF4 knock-out mice, inflammation of the lungs could be seen. Figure 3D shows a section of lung tissue obtained from one of the affected mice, with arrows pointing to areas of leukocyte infiltration. In several experiments a total of nine wheezing mice were subjected to histological analysis, and six were found to have inflammation of the lungs, whereas none of the asymptomatic mice autopsied displayed evidence of airway inflammation.

Altered Air Flow to the Lungs of TRAF4-Deficient Mice

To investigate the possibility that the tracheal abnormality could result in altered air flow to the lungs, perhaps explaining the susceptibility to lung inflammation and reduced size of mice lacking TRAF4, the response to an aerosolized challenge was examined. TRAF4-deficient mice or littermate controls were left untreated or were immunized intraperitoneally with OVA antigen in adjuvant. The immunization was repeated 1 week later, and after 4 days the mice were challenged with aerosolized OVA in a nebulizer on three consecutive days. Five days later, the mice were sacrificed, and the number of eosinophils and other white blood cells in BAL fluid from the lungs was determined. The TRAF4-deficient mice had a significantly lower number of infiltrating cells in the BAL fluid as compared to immunized control littermates (Figure 4A). However, the BAL fluid samples from the two groups of immunized mice contained similar ratios of eosinophils (Figure 4B), suggesting that although greatly diminished, the response to the aerosolized challenge was normal. The decrease in the response was not due to a generalized T-cell defect, inasmuch as peripheral T-cells isolated from TRAF4$^{-/-}$ animals and wild-type littermates responded equally well to antigen rechallenge in proliferation assays, as measured by [^{3}H]thymidine uptake and cytokine production measured in enzyme-linked immunosorbent assays (data not shown). Taken together, these results suggest that the tracheal stenosis reduced the amount of aerosolized antigen that reached the lungs.

Expression of TRAF4 during Embryogenesis

In situ hybridization was used to study the expression of TRAF4 during embryogenesis. Widespread expression was seen using an antisense riboprobe (Figure 5A), and the specificity of the staining was confirmed by using the corresponding sense riboprobe (Figure 5B). High expression of TRAF4 can be seen in the first, second, and

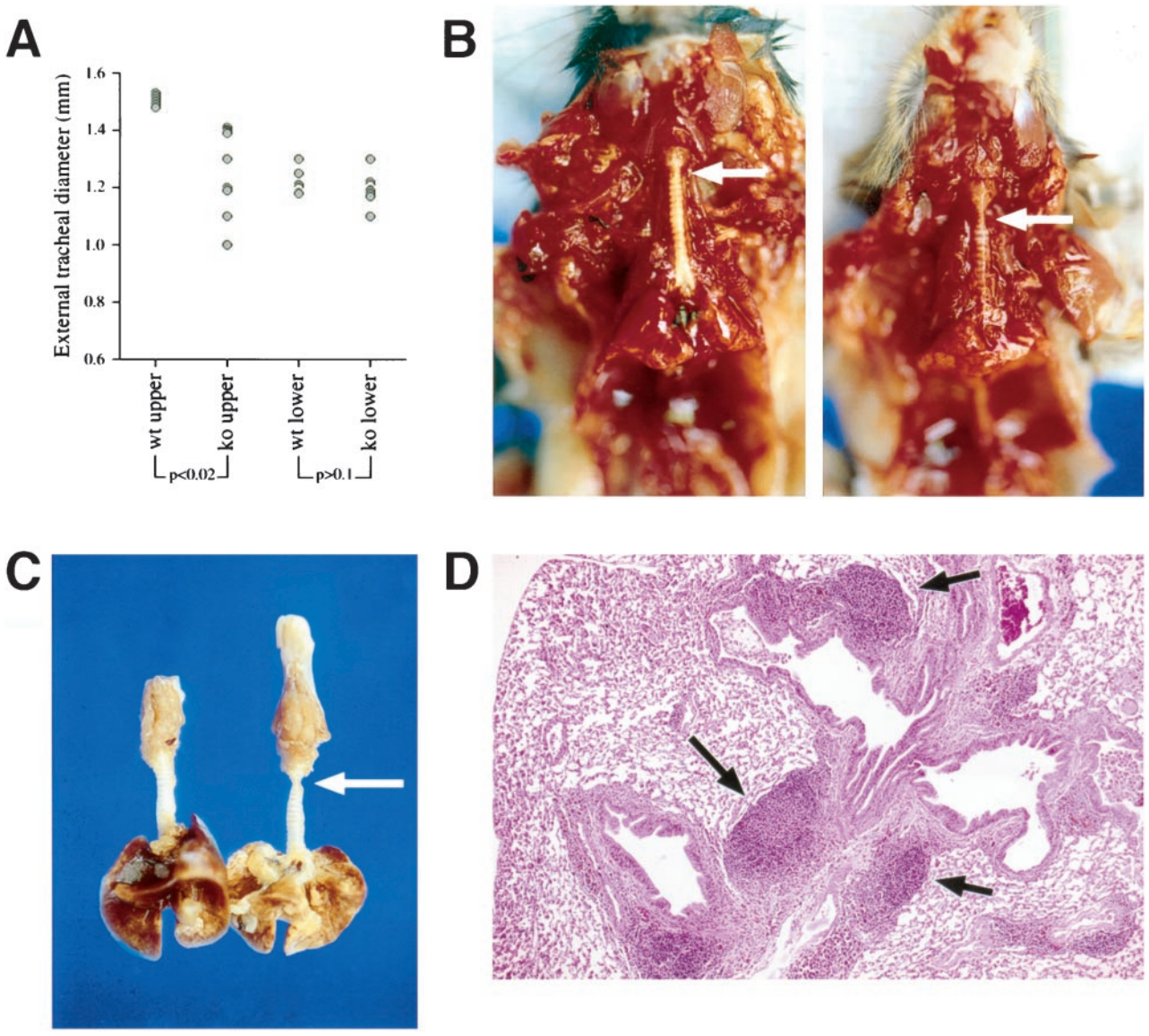


Figure 3. Morphology of the airways in TRAF4 wild-type or knock-out mice, showing the deformed upper trachea of TRAF4-deficient mice. **A:** The diameter of the upper trachea in TRAF4 wild-type or knock-out mice. Each symbol represents the tracheal diameter of an individual mouse. Measurements were performed while the airways were still attached to surrounding tissue, as seen in **B**, and were recorded to within an accuracy of 0.1 mm. The *P* values for each data set are shown. **B:** Representative examples of a wild-type (**left**) and a knock-out (**right**) mouse. Mice were sacrificed and dissected to reveal the airways while they were still intact inside the animals. The **arrows** indicate the presence or absence of tracheal constriction. **C:** Larynx, trachea, and lungs removed from a wild-type (**left**) and a knock-out (**right**) mouse. The point of constriction in the knock-out mouse is indicated by an **arrow**. **D:** An example of inflammation seen in lungs from a "wheezing" mouse. A section of lung tissue was stained with hematoxylin-eosin. Foci of what appear to be lymphocytes are indicated with **arrows**.

third branchial arches during day 10.5 of embryogenesis, according to the antisense probe (Figure 5C) as compared to the sense control probe (Figure 5D). These branchial arches are prominent at this stage of development and later give rise to bones of the jaw and ear, as well as the hyoid bone and upper pharyngeal structures. The laryngeal cartilages, including the thyroid and cricoid cartilages, originate from the subsequent branchial arches.[34] During later stages of development, at embryonal day 12.5 or 13.5, the region where the laryngotracheal tube is establishing a connection with the pharynx[35,36] shows no higher level of expression than the surrounding tissue (data not shown). TRAF4 transcripts can still be found at relatively high levels in the epithelium of the trachea at this later time, as well as in most other epithelia analyzed. TRAF4 is also highly expressed in the developing cerebral cortex as well as in vertebral bodies (Figure 5A and data not shown). Based on the pathology of TRAF4-deficient mice, it appears that the absence of TRAF4 expression results in an inability to properly form the laryngotracheal junction. This correlates with the high expression of TRAF4 seen in this region during early stages of development (Figure 5C). However, there is no discrete point of TRAF4 expression that can explain the unique defect in TRAF4-deficient mice. Despite widespread expression of TRAF4, especially in epithelial and

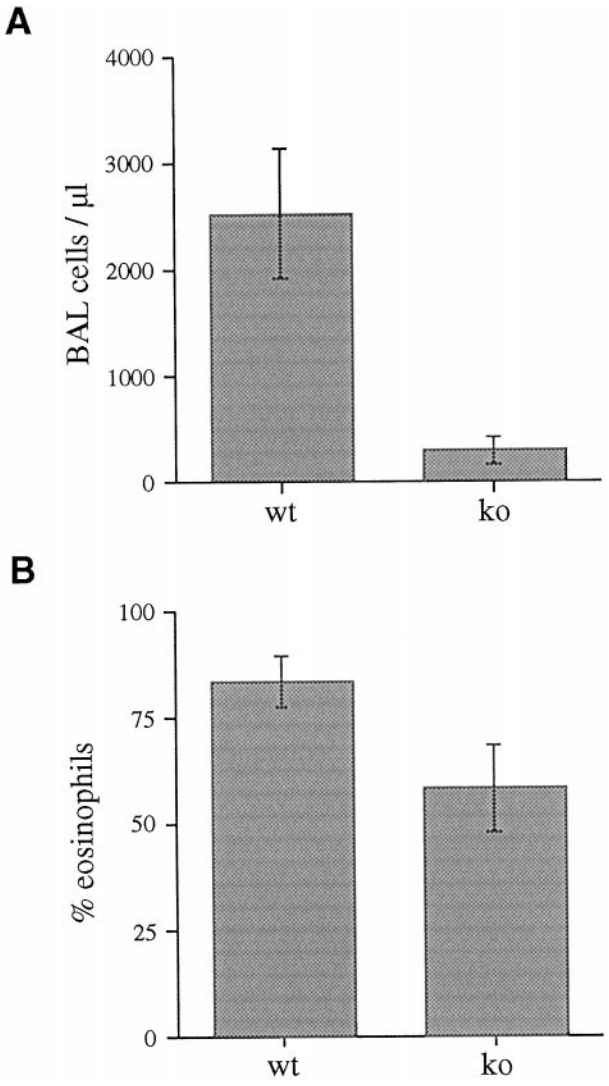


Figure 4. Response to aerosolized challenge with OVA antigen. Mice were immunized twice with OVA and then rechallenged with aerosolized OVA. Each experimental group contained three mice. The mice were then sacrificed, and the influx of inflammatory cells to the airways triggered by the rechallenge was determined. One representative experiment out of two performed is shown. **A:** The concentration of white blood cells in bronchoalveolar lavage (BAL) fluid from wild-type or knock-out mice on rechallenge with aerosolized OVA. **B:** The percentage of eosinophils in BAL fluid from wild-type or knock-out mice.

certain neuronal tissues, it appears that the tracheal junction is the only region where development is critically dependent on TRAF4 expression.

Discussion

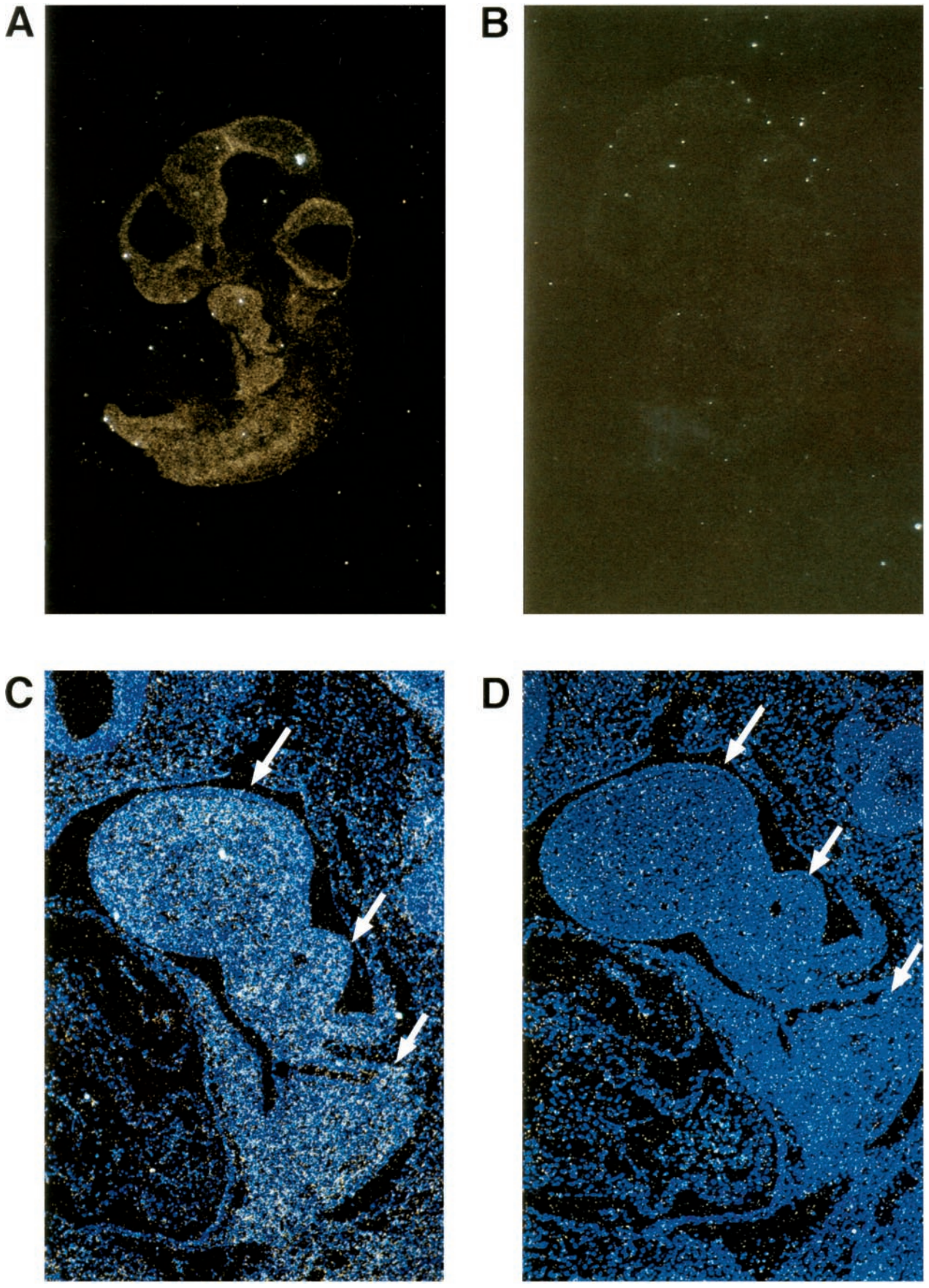
The TRAF family has been under extensive investigation since its initial discovery, and the function of most of the family members has been well established. However, the role of TRAF4 has remained elusive. To gain further insight into the *in vivo* function of TRAF4, we have generated TRAF4-deficient mice. Despite widespread expression of TRAF4 during development and in the adult, lack of TRAF4 results in a specific defect, localized to a small region of the upper trachea. There is no evidence of generalized cartilage or bone formation abnormalities, either in the trachea or in other areas of cartilaginous origin. The deformation of the trachea results in perturbed air flow to the lungs, as manifested by a reduced inflammatory response to challenge with aerosolized antigen. In addition, there is a spontaneous incidence of airway inflammation among TRAF4 knock-out mice.

Laryngotracheal Development

The embryological development of the larynx, trachea, and lungs has been studied mostly through the analysis of serial sections from various stages of development and from wax-plate reconstructions using these sections. These studies established that the larynx and trachea develop as an outpocketing from the ventral side of the foregut (the laryngotracheal groove) (see, for example, ref 34). Lung buds are formed at the caudal end, and the trachea grows by extending downward in length.[37] The formation of the cranial end of the laryngotracheal tube poses a problem, because initially this end is still connected to the esophagus at the point of entry into the pharynx. The formation of a mature larynx is necessary for separating the two structures. In the last couple of decades, several groups have generated detailed insights into how this process occurs in normal human and mouse embryos.[38,39] The mature larynx consists of an infraglottic and a supraglottic cavity. The more cranial supraglottic cavity forms by initial closure of what is termed the sagittal cleft, followed by transverse development of a coronal cleft. This process requires fusion of the two epithelial layers of the sagittal cleft to form the epithelial lamina, which separate the infraglottic cavity from the coronal cleft.[39] Eventually, proliferation and rearrangement of the cells in the epithelial lamina allows for establishment of communication between the infraglottic cavity at the cranial end of the trachea and the pharynx, and eventually for development of a complete supraglottic cavity.[39]

The cartilaginous structures found in adult airways are first seen as clusters of precursor cells that later develop into fully formed cartilage. The origin of the various cartilages can be traced back to the branchial arches that are present before the laryngotracheal groove first forms. The first three branchial arches give rise to structures above the larynx, whereas the laryngeal cartilages originate from the remaining fourth and fifth branchial arches.[34]

Our data suggest a role for TRAF4 in the establishment of a junction between the trachea and the pharynx at the caudal end of the larynx. The crichoid cartilage is unaffected in TRAF4-deficient mice, but the diameter of the proximal trachea is reduced immediately below the crichoid cartilage. As described above, several complex events occur during laryngotracheal development. TRAF4 could play a role in proliferation and/or reorganization of the involved cells. TRAF4 could also be required in normal innervation of this region of the trachea. The transitional area between the laryngopharynx and esophagus seems to be even more densely innervated than the


A
B
C
D

nasal cavities.[39] It is interesting to note that the laryngeal nerve branches stop at the level of the fourth tracheal cartilage, which is precisely the extent of the tracheal defect seen in TRAF4 knock-out mice.

TRAF4 Expression during Development

We and others[24] have used *in situ* hybridization to investigate the expression of TRAF4 during development. For our studies, we focused on the time when the trachea is connecting via the larynx to the pharynx. Despite strong overall expression of TRAF4 in the branchial arches at day 10.5, no localized expression patterns were observed in the region containing the larynx and upper trachea at embryonic day 12.5, which is the time when the connection is starting to form.[35,36] E13.5d embryos also were studied, with the same result. TRAF4 is highly expressed in the epithelium of the trachea, but also in other epithelia. We have therefore not been able to implicate a specific cell type in the tracheal deformation of TRAF4-deficient mice based on the embryonic expression pattern of TRAF4.

Wheezing and Pulmonary Inflammation in TRAF4-Deficient Mice

The TRAF4-deficient animals were housed in a barrier facility and were not exposed to any known murine pathogens, but were not kept under sterile conditions. Airborne particles are deposited in the lungs in a size-dependent manner, and failure to clear them can lead to induction of an inflammatory response. Because of the tracheal constriction of TRAF4 knock-out mice, the air flow would be presumed to have a greater element of turbulence. The high-pitched breathing noise heard in some of the TRAF4-deficient mice supports this, because vortices in the trachea are likely to be the cause of this noise.[40] The increased turbulence could result in alterations in the deposition of certain airborne particles, pathogens included, depending on their size.[41,42] This could contribute to the inflammation observed on autopsy in some mice and the variation in wheezing incidence found. An additional possible explanation for the spontaneous occurrence of airway inflammation is that the mucociliary clearance of larger particles is deficient in these mice, because of tracheal constriction. The reduced weight of TRAF4 knock-out mice may be indicative of the increased effort of respiration in these mice. The energy required for inspiration is dependent on the total airway resistance, and the uppermost part of the airways (including the trachea) is normally responsible for 20–25% of this resistance.[43]

To investigate whether the tracheal constriction resulted in abnormal air flow to the lungs, the response to aerosolized antigen in immunized mice was determined. Although TRAF4-deficient mice showed a qualitatively normal response, as evidenced by the percentage of eosinophils in the BAL fluid, the total number of white blood cells in the BAL fluid was greatly reduced. These results are consistent with the possibility that the decreased response to OVA in TRAF4 knock-out mice is due to a reduction in the amount of aerosol deposited in the lungs because of abnormal air flow. This further supports the notion of an increase in turbulent air flow as a result of tracheal deformation. Turbulence would result in a greater extent of aerosols being deposited in the oropharynx and upper airways, where they would normally be cleared much more rapidly (about 20-fold faster) than aerosols entering the lungs.[41] Even if the tracheobronchial clearance rate is decreased in TRAF4-deficient mice, this presumably would not compensate for the overall reduction in deposited OVA antigen. Although there is spontaneous occurrence of airway inflammation in TRAF4 knock-out mice, the inflammation seen in response to aerosolized antigen is decreased, presumably as a result of differences in deposition patterns of the immunogenic challenge.

TRAF4 Is a Unique Member of the TRAF Family

Despite the importance of other TRAFs in the development and maintenance of a normal immune system, the loss of TRAF4 appears to have no immunological significance. This, as well as the novel role of TRAF4 in tracheal development described here, suggests that TRAF4 plays a unique role, distinct from those of the other TRAF family members. This is consistent with TRAF4 having the most limited sequence homology to the other TRAF family members, and with TRAF4 having yet to be shown to associate with a TNFR family member to a significant extent. The subcellular localization of TRAF4 is also different because, unlike other TRAFs, TRAF4 has been reported to localize to both the nucleus and the cytoplasm.[24,25]

By generating mice deficient in TRAF4 expression, we have shown that TRAF4 plays an unexpected role in tracheal formation. The TRAF4-deficient mice do not share any of the defects described for the three previously generated TRAF family knock-out mice, which is consistent with the notion that TRAF4 is a functionally distinct member of the family.

Acknowledgments

Drs. Andras Nagy, Reka Nagy, and Wanda Abramow-Newerly are gratefully acknowledged for providing the RI ES cell line. We thank Drs. Maria-Luisa Alegre and Celeste Simon for advice and support, and Susan Kerns for expert editorial assistance.

Figure 5. *In situ* hybridization analysis of TRAF4 expression in murine E10.5d embryos. The same embryo was sagittally sectioned, and adjacent sections were used for hybridization with antisense or sense riboprobes. The white silver grains represent probe binding, as detected by dark-field microscopy, whereas the blue staining seen in **C** and **D** is nuclear counterstain. **A:** TRAF4-specific staining, using an antisense TRAF4 riboprobe. **B:** Nonspecific staining, using a sense control TRAF4 riboprobe. **C:** Magnification (×10) of the section in **A**, showing specific staining. The first three branchial arches are indicated with **arrows**. **D:** Magnification (×10) of the section in **B**, showing nonspecific staining.

References

1. Baker SJ, Reddy EP: Transducers of life and death: TNF receptor superfamily and associated proteins. Oncogene 1996, 12:1–9
2. Cheng G, Cleary AM, Ye ZS, Hong DI, Lederman S, Baltimore D: Involvement of CRAF1, a relative of TRAF, in CD40 signaling. Science 1995, 267:1494–1498
3. Hu HM, O'Rourke K, Boguski MS, Dixit VM: A novel RING finger protein interacts with the cytoplasmic domain of CD40. J Biol Chem 1994, 269:30069–30072
4. Rothe M, Wong SC, Henzel WJ, Goeddel DV: A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. Cell 1994, 78:681–692
5. Uren AG, Vaux DL: TRAF proteins and meprins share a conserved domain. Trends Biochem Sci 1996, 21:244–245
6. Hsu H, Solovyev I, Colombero A, Elliott R, Kelley M, Boyle WJ: ATAR, a novel tumor necrosis factor receptor family member, signals through TRAF2 and TRAF5. J Biol Chem 1997, 272:13471–13474
7. Miller WE, Cheshire JL, Raab-Traub N: Interaction of tumor necrosis factor receptor-associated factor signaling proteins with the latent membrane protein 1 PXQXT motif is essential for induction of epidermal growth factor receptor expression. Mol Cell Biol 1998, 18:2835–2844
8. Mizushima S, Fujita M, Ishida T, Azuma S, Kato K, Hirai M, Otsuka M, Yamamoto T, Inoue J: Cloning and characterization of a cDNA encoding the human homolog of tumor necrosis factor receptor-associated factor 5 (TRAF5). Gene 1998, 207:135–140
9. Lee SY, Kandala G, Liou ML, Liou HC, Choi Y: CD30/TNF receptor-associated factor interaction: NF-kappa B activation and binding specificity. Proc Natl Acad Sci USA 1996, 93:9699–9703
10. Takeuchi M, Rothe M, Goeddel DV: Anatomy of TRAF2. Distinct domains for nuclear factor-kappaB activation and association with tumor necrosis factor signaling proteins. J Biol Chem 1996, 271: 19935–199942
11. Ishida T, Mizushima S, Azuma S, Kobayashi N, Tojo T, Suzuki K, Aizawa S, Watanabe T, Mosialos G, Kieff E, Yamamoto T, Inoue J: Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from amino-terminal domain of the CD40 cytoplasmic region. J Biol Chem 1996, 271: 28745–28748
12. Nakano H, Oshima H, Chung W, Williams-Abbott L, Ware CF, Yagita H, Okumura K: TRAF5, an activator of NF-kappaB and putative signal transducer for the lymphotoxin-beta receptor. J Biol Chem 1996, 271:14661–14664
13. Cao Z, Xiong J, Takeuchi M, Kurama T, Goeddel DV: TRAF6 is a signal transducer for interleukin-1. Nature 1996, 383:443–446
14. Yeh WC, Shahinian A, Speiser D, Kraunus J, Billia F, Wakeham A, de la Pompa JL, Ferrick D, Hum B, Iscove N, Ohashi P, Rothe M, Goeddel DV, Mak TW: Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. Immunity 1997, 7:715–725
15. Xu Y, Cheng G, Baltimore D: Targeted disruption of TRAF3 leads to postnatal lethality and defective T-dependent immune responses. Immunity 1996, 5:407–415
16. Nakano H, Sakon S, Koseki H, Takemori T, Tada K, Matsumoto M, Munechika E, Sakai T, Shirasawa T, Akiba H, Kobata T, Santee SM, Ware CF, Rennert PD, Taniguchi M, Yagita H, Okumura K: Targeted disruption of Traf5 gene causes defects in CD40- and CD27-mediated lymphocyte activation. Proc Natl Acad Sci USA 1999, 17:9803–9808
17. Lomaga MA, Yeh WC, Sarosi I, Duncan GS, Furlonger C, Ho A, Morony S, Capparelli C, Van G, Kaufman S, van der Heiden A, Itie A, Wakeham A, Khoo W, Sasaki T, Cao Z, Penninger JM, Paige CJ, Lacey DL, Dunstan CR, Boyle WJ, Goeddel DV, Mak TW: TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev 1999, 13:1015–1024
18. Speiser DE, Lee SY, Wong B, Arron J, Santana A, Kong YY, Ohashi PS, Choi Y: A regulatory role for TRAF1 in antigen-induced apoptosis of T cells. J Exp Med 1997, 185:1777–1783
19. Lee SY, Reichlin A, Santana A, Sokol KA, Nussenzweig MC, Choi Y: TRAF2 is essential for JNK but not NF-kappaB activation and regulates lymphocyte proliferation and survival. Immunity 1997, 7:703–713
20. Galibert L, Tometsko ME, Anderson DM, Cosman D, Dougall WC: The involvement of multiple tumor necrosis factor receptor (TNFR)-associated factors in the signaling mechanisms of receptor activator of NF-kappaB, a member of the TNFR superfamily. J Biol Chem 1998, 273:34120–34127
21. Dougall WC, Glaccum M, Charrier K, Rohrbach K, Brasel K, DeSmedt T, Daro E, Smith J, Tometsko ME, Maliszewski CR, Armstrong A, Shen V, Bain S, Cosman D, Anderson D, Morrissey PJ, Peschon JJ, Schuh J: RANK is essential for osteoclast and lymph node development. Genes Dev 1999, 13:2412–2424
22. Tomasetto C, Regnier C, Moog-Lutz C, Mattei MG, Chenard MP, Lidereau R, Basset P, Rio MC: Identification of four novel human genes amplified and overexpressed in breast carcinoma and localized to the q11–q21.3 region of chromosome 17. Genomics 1995, 28:367–376
23. Regnier CH, Tomasetto C, Moog-Lutz C, Chenard MP, Wendling C, Basset P, Rio MC: Presence of a new conserved domain in CART1, a novel member of the tumor necrosis factor receptor-associated protein family, which is expressed in breast carcinoma. J Biol Chem 1995, 270:25715–25721
24. Masson R, Regnier CH, Chenard MP, Wendling C, Mattei MG, Tomasetto C, Rio MC: Tumor necrosis factor receptor associated factor 4 (TRAF4) expression pattern during mouse development. Mech Dev 1998, 71:187–191
25. Krajewska M, Krajewski S, Zapata JM, Van Arsdale T, Gascoyne RD, Berern K, McFadden D, Shabaik A, Hugh J, Reynolds A, Clevenger CV, Reed JC: TRAF4 expression in epithelial progenitor cells: Analysis in normal adult, fetal, and tumor tissues. Am J Pathol 1998, 152:1549–1561
26. Tybulewicz VL, Crawford CE, Jackson PK, Bronson RT, Mulligan RC: Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. Cell 1991, 65:1153–1163
27. Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC: Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci USA 1993, 90:8424–8428
28. Askew GR, Doetschman T, Lingrel JB: Site-directed point mutations in embryonic stem cells: a gene-targeting tag-and-exchange strategy. Mol Cell Biol 1993, 13:4115–4124
29. Joyner AL: Gene Targeting: A Practical Approach. New York, Oxford University Press, 1993
30. June CH, Ledbetter JA, Gillespie MM, Lindsten T, Thompson CB: T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. Mol Cell Biol 1987, 7:4472–4481
31. Krinzman SJ, De Sanctis GT, Cernadas M, Kobzik L, Listman JA, Christiani DC, Perkins DL, Finn PW: T cell activation in a murine model of asthma. Am J Physiol 1996, 271:L476–L483
32. Zuany-Amorim C, Haile S, Leduc D, Dumarey C, Huerre M, Vargaftig BB, Pretolani M: Interleukin-10 inhibits antigen-induced cellular recruitment into the airways of sensitized mice: J Clin Invest 1995, 95:2644–2651
33. Wilkinson DG, Nieto MA: Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. Methods Enzymol 1993, 225:361–373
34. Arey LB: Developmental Anatomy: A Textbook and Laboratory Manual of Embryology. Philadelphia, Saunders, 1974
35. Kaufman MH: The Atlas of Mouse Development. London, San Diego, Academic Press, 1995
36. Theiler K: The House Mouse: Atlas of Embryonic Development. New York, Springer-Verlag, 1989
37. Muller F, O'Rahilly R: Somitic-vertebral correlation and vertebral levels in the human embryo. Am J Anat 1986, 177:3–19
38. Henick DH: Three-dimensional analysis of murine laryngeal development. Ann Otol Rhinol Laryngol Suppl 1993, 159:3–24
39. Muller F, O'Rahilly R, Tucker JA: The human larynx at the end of the embryonic period proper. 2. The laryngeal cavity and the innervation of its lining. Ann Otol Rhinol Laryngol 1985, 94:607–617
40. Mori M, Ono M, Hisada T, Kino H, Iguchi M, Nagata T, Koike S, Sugimoto T: Relationship between forced expiratory flow and tracheal sounds. Possible effect of vortices on flow. Respiration 1988, 54:78–88
41. Bouhuys A: Airway Dynamics: Physiology and Pharmacology. Springfield, Thomas, 1970
42. Morrow PE: Physics of airborne particles and their deposition in the lung. Ann NY Acad Sci 1980, 353:71–80
43. Levitzky MG: Pulmonary Physiology. New York, McGraw-Hill, 1991