

Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand Regulates Hallmark Features of Airways Remodeling in Allergic Airways Disease

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Abstract

Allergic asthma is a complex disease characterized by acute inflammation of the airways that over time leads to the development of significant structural changes termed remodeling. TNF-related apoptosis-inducing ligand (TRAIL) has an important regulatory role in acute allergic airways inflammation through up-regulation of the E3 ubiquitin ligase Midline-1 (MID-1), which limits protein phosphatase 2A (PP2A) activity and downstream dephosphorylation of proinflammatory signaling molecules. The relevance of TRAIL in the development of airways remodeling has yet to be determined. In this study, the lungs of wild-type (WT) BALB/c and *Tnfsf10* knockout (TRAIL^{−/−}) mice were chronically exposed to ovalbumin (OVA) for 12 weeks to induce hallmark features of chronic allergic airways disease, including airways hyperreactivity (AHR), subepithelial collagen deposition, goblet cell hyperplasia, and smooth muscle hypertrophy. TRAIL^{−/−} mice were largely protected from the development of AHR and peribronchial eosinophilia and had reduced levels of mast cells in the airways. This correlated with

lower levels of cytokines, including IL-4, -5, -10, and -13, and with lower levels of proinflammatory chemokines from cultured cells isolated from the draining lymph nodes. TRAIL^{−/−} mice were also protected from the characteristic features of airways remodeling, including peribronchial fibrosis, smooth muscle hypertrophy, and mucus hypersecretion, which correlated with reduced TGF-β1 levels in the lungs. MID-1 expression was reduced in TRAIL^{−/−} mice and up-regulated in allergic WT mice. Raising PP2A activity using 2-amino-4-(4-heptyloxyphenyl)-2-methylbutan-1-ol in allergic WT mice reduced eosinophilia, TGF-β1, and peribronchial fibrosis. This study shows that TRAIL promotes airways remodeling in an OVA-induced model of chronic allergic airways disease. Targeting TRAIL and its downstream proinflammatory signaling pathway involving PP2A may be of therapeutic benefit in reducing the hallmark features of airways remodeling observed in chronic allergic airways inflammation.

Keywords: TNF-related apoptosis-inducing ligand; asthma; remodeling

Allergic asthma is a complex disease, driven by an exaggerated immune response in the airways against what would normally be an innocuous environmental antigen. This aberrant response is traditionally

characterized by T helper (Th)2 T cells and their associated cytokine milieu, which drives the recruitment of eosinophils, activation of mast cells, and overproduction of mucus by epithelial cells. In an acute

asthma attack, the smooth muscle associated with the airways constricts, resulting in reduced airflow, cough, and wheeze. Over time, the inflamed airways undergo significant remodeling, with smooth

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Clinical Relevance

Our data highlight a more complex role of tumor necrosis factor-related apoptosis-inducing ligand in the pathogenesis of fibrosis than previously identified, which requires consideration of the underpinning disease process and severity associated with fibrosis for the future development of novel therapeutic approaches.

muscle hyperplasia and peribronchial fibrosis evident in the lungs of patients with asthma even when symptoms are considered to be well controlled (1). Airway constriction promotes subepithelial collagen deposition and mucus production in the airways of patients with asthma even in the absence of pronounced inflammation, thereby causally relating episodic airways obstruction occurring chronically in subjects with persistent asthma to airways remodeling (2).

The role of Th2 cells in the development of airways remodeling is a consequence of their cytokine expression patterns. IL-5 is essential for the recruitment, differentiation, and activation of eosinophils. Eosinophils are the primary cellular source of TGF- β , linking Th2 cells to one of the primary factors responsible for fibrogenesis. Decreased levels of TGF- β after treatment with antibodies inhibiting IL-5 have been demonstrated to reduce the expression of extracellular matrix proteins in bronchial biopsies from patients with mild asthma (3). IL-13 contributes to the features of airways remodeling with constitutive overexpression in transgenic mice, resulting in subepithelial fibrosis, smooth muscle cell hypertrophy, and goblet cell metaplasia (2). In tetracycline-inducible IL-13 transgenic mice, lung fibrosis persists beyond 4 weeks after the transgene is switched off despite the resolution of eosinophilic airway inflammation and mucus production (4, 5). Eosinophils induce airways hyperreactivity (AHR), release TGF- β , and promote IL-13 production by Th2 cells (6), all of which play a role in the initiation of airways remodeling.

In addition to inflammatory immune cells such as Th2 cells, eosinophils, and neutrophils, there is a mounting body of evidence that resident airway epithelial cells orchestrate the immune response in

asthma (7). The epithelial cells of the airway express a plethora of pattern recognition receptors, leaving them well equipped to detect the presence of pathogen-associated molecular patterns within the lung. In response to pattern recognition receptor activation and signaling, airway epithelial cells release chemokines and cytokines, which attract and activate innate and adaptive immune cells. Recent studies have demonstrated that an aberrant

activation of these pathways by inhaled allergen is responsible for activating the local dendritic cells, which coordinate the inflammatory immune response, including Th2 cells and eosinophils, responsible for the host damage and symptoms associated with allergic asthma (8).

We have recently demonstrated a role for epithelial-derived TNF-related apoptosis-inducing ligand (TRAIL)-dependent signaling through the E3

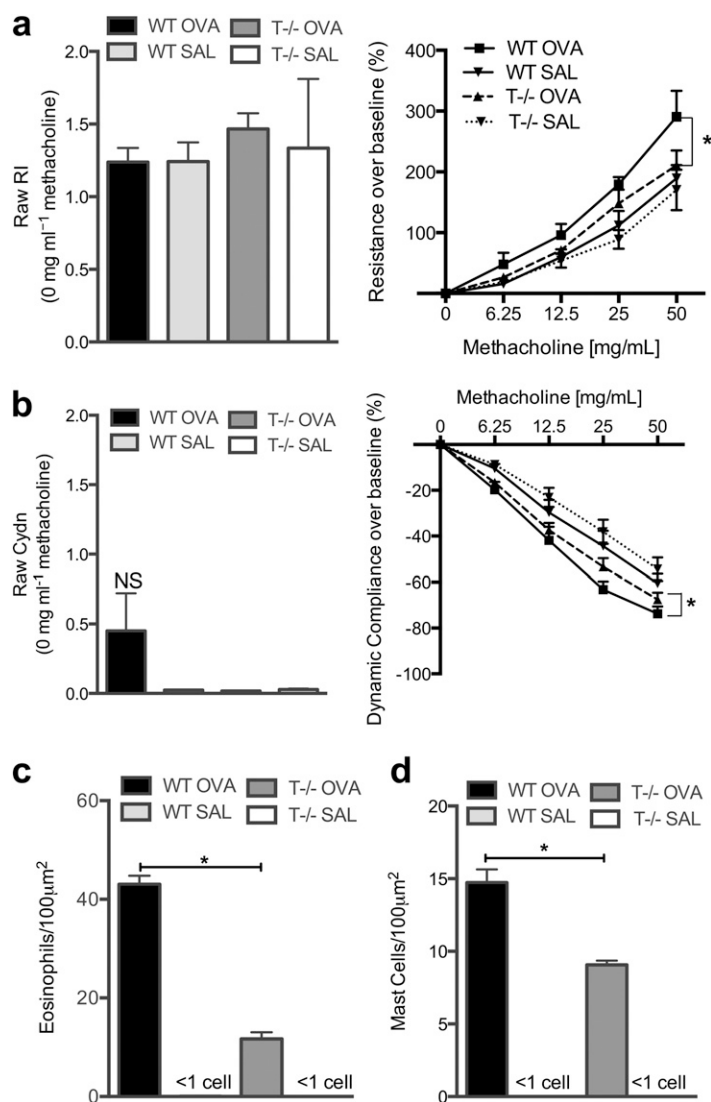


Figure 1. Tumor necrosis factor-related apoptosis-inducing ligand deficient (T-/-) mice were protected from the development of airways inflammation and hyperreactivity. (a) Total lung resistance (RI) at baseline (left) and as a percentage change of baseline measurement in response to inhaled methacholine (right) ($n = 5-6$). (b) Dynamic lung compliance (Cdyn) at baseline (left) and as a percentage change of baseline measurement to inhaled methacholine (right). (c) Number of peribronchial/perivascular eosinophils identified with hematoxylin and eosin staining per 100 μm^2 . (d) Number of mast cells identified by Toluidine blue staining per 100 μm^2 . * $P < 0.05$; $n = 3$ mice per group. OVA, ovalbumin; SAL, saline.

ubiquitin ligase Midline-1 (MID1) in the pathogenesis of allergic airways disease (AAD) (9). The ligase activity of MID1 results in reduced activation of the protein phosphatase 2A (PP2A), which results in elevated proinflammatory NF- κ B, p38, and JNK signaling due to impaired dephosphorylation (9). Although this signaling cascade was demonstrated to be required for acute ovalbumin (OVA)-, house dust mite (HDM)-, and rhinovirus-induced inflammation in mouse models of AAD (9–11), the relevance of this pathway in the development of airways remodeling is unknown. Here we use a model of chronic AAD induced by 12 weeks of exposure to OVA to demonstrate that TRAIL deficiency largely protects against the development of key features of airways remodeling.

Materials and Methods

Chronic Allergy Model

Twelve-week-old wild-type (WT) and *Tnfsf10*^{−/−} BALB/c mice (12) were supplied by Australian Bioresources (Moss Vale, New South Wales, Australia) and, after arrival at the HMRI animal facility, were allowed to settle for 1 week. Mice then received an intraperitoneal immunization on Days 0 and 14 where 200 μ g of OVA (grade v; Sigma-Aldrich, Sydney, Australia) was delivered with 10% alum in 200 μ l of PBS. From Day 28, mice received 50 μ g of OVA (or sterile saline 0.9% for nonallergic control mice) intranasally three times a week for 12 weeks before being killed by pentobarbitone overdose with subsequent sample collection (13). The Animal Care and Ethics

committee at the University of Newcastle approved all experimental procedures.

Histological Preparations

Lungs were inflation fixed at 10 mm H₂O with 10% neutral buffered formalin solution for 24 hours before being transferred to 70% ethanol in accordance with the ATS/ERS guidelines on lung structure assessment (14). Tissue was then embedded in paraffin and stained with hematoxylin and eosin (eosinophils), periodic acid-Schiff (PAS)-positive mucus producing epithelial cells, Masson's Trichrome (Collagen), or toluidine blue (mast cells) or processed further for immunohistochemistry. Lungs across experimental groups were processed as a batch for either the histologic staining or the immunostaining protocol.

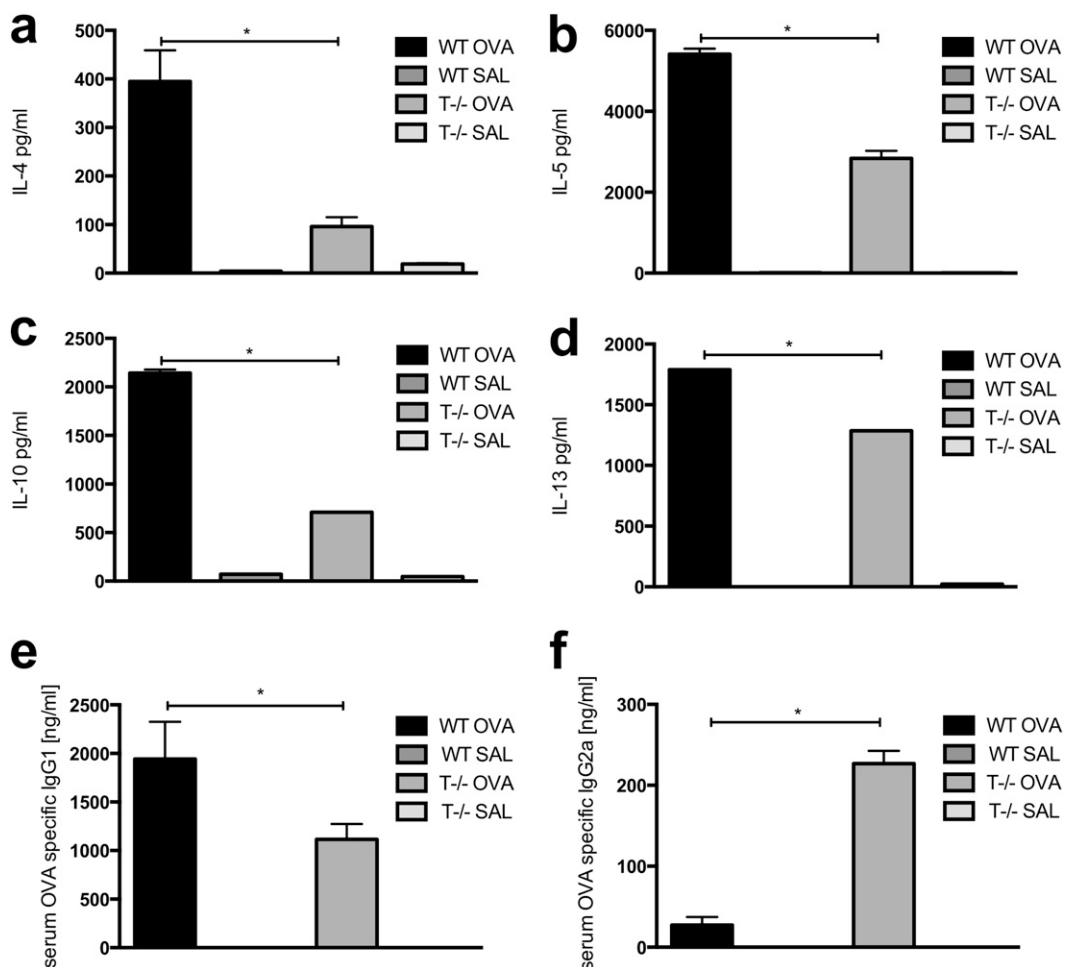


Figure 2. Cytokine expression of IL-4 (a), IL-5 (b), IL-10 (c), and IL-13 (d) detected in supernatant of cells isolated from the draining lymph nodes of the lung, cultured for 6 days in the presence of OVA less background levels from unstimulated cells ($n = 2$ samples from three mice each). (e, f) Serum IgG titers determined by ELISA. * $P < 0.05$; $n = 4$. T-/-, TRAIL-/- mice.

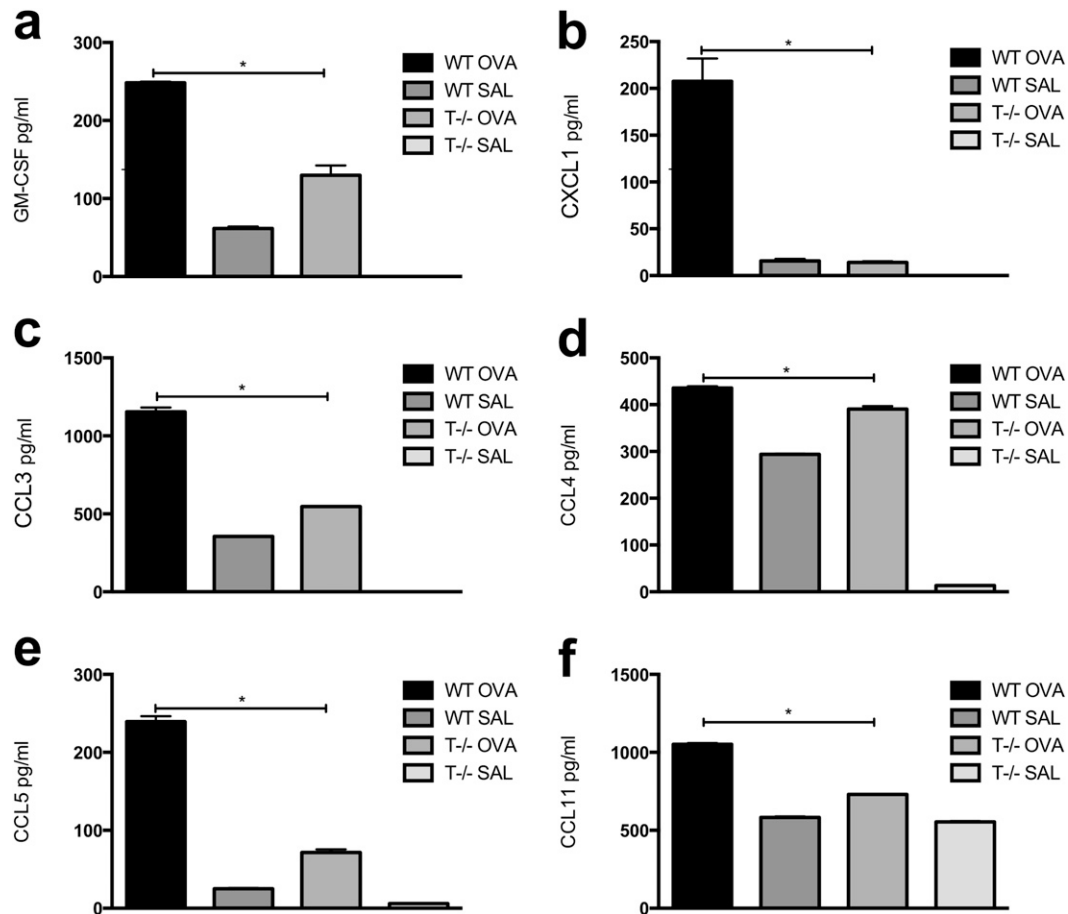


Figure 3. Chemokine expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) (a), chemokine (C-X-C motif) ligand 1 (CXCL1) (b), and chemokine (C-C motif) ligand (CCL)3 (c), CCL4 (d), CCL5 (e), and CCL11 (f) detected in supernatant of cells isolated from the draining lymph nodes of the lung, cultured for 6 days in the presence of OVA less background levels from unstimulated cells. * $P < 0.05$; $n = 2$ samples from three mice per group.

Terminal dUTP Nick End Labeling Stain

Colorimetric terminal dUTP nick end labeling (TUNEL) assays were performed on paraffin-embedded sections mounted on microscope slides according to the manufacturer's instructions (Promega, Sydney, Australia). TUNEL-positive cells per 100 μm^2 were enumerated by light microscopy.

Collagen Area Enumeration

The area of peribronchial trichrome staining in a paraffin-embedded lung was outlined and quantified using a light microscope (Olympus, Notting Hill, Australia) attached to an image-analysis system (Image-Pro Plus 6; Media Cybernetics, Silver Spring, MD). Results are expressed as the area of trichrome staining per micrometer length of basement membrane of airways. At least 10 counts were made from each mouse as previously described (15).

Airway Smooth Muscle Staining

Antigen was retrieved in paraffin-embedded lung sections with a 10-minute incubation in a pressure cooker. Sections were stained with monoclonal anti-actin α -smooth muscle Cy3-conjugate antibody (Sigma-Aldrich, Sydney, Australia). The area of Cy3-positive α -smooth muscle actin staining was quantified using a light microscope and image analysis software as with collagen.

Lung Function Testing

AHR was assessed invasively 24 hours after antigen challenge by measurement of total lung resistance and dynamic compliance using a Biosystems XA series RC system (Buxco Research Systems, Harwich Port, MA). Mice were anesthetized with intraperitoneal administration of ketamine/xylazine at 100 and 10 mg/kg, respectively. The trachea was cannulated, and the mice were mechanically ventilated at 170

strokes/min with a 175- μl stroke volume. Mice were then challenged with a baseline dose (PBS) followed by increasing doses of nebulized methacholine (6.25, 12.5, 25, and 50 mg/ml) for 5 minutes while pulmonary resistance and compliance were recorded. Peak values were designated as the maximum response with maximal lung resistance and dynamic compliance expressed as percentage change over baseline (PBS).

Lymph Node Culture

Peribronchial draining lymph nodes were excised, filtered, and cultured in the presence or absence of 200 $\mu\text{g}/\text{ml}$ of OVA (optimal concentration) for 6 days. Cytokine and chemokine concentrations were determined using a Bioplex multiplex kit and read on a Luminex platform according to the manufacturer's instructions (Bio-Rad, Sydney, Australia).

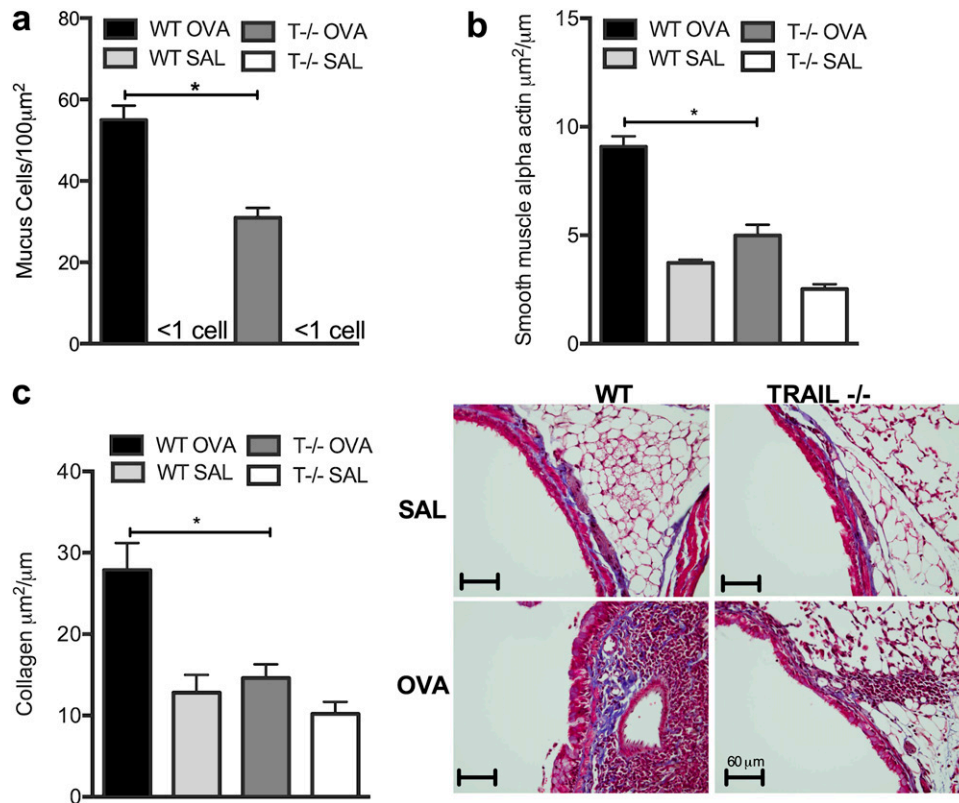


Figure 4. (a) Number of periodic acid-Schiff–positive mucus producing airway epithelial cells per 100 μm^2 . (b) Area of α -smooth muscle actin–positive staining per length of major airway epithelium ($\mu\text{m}^2/\mu\text{m}$). (c) Area of peribronchial fibrosis determined by Masson's trichrome staining per length of major airway epithelium ($\mu\text{m}^2/\mu\text{m}$). Scale bars, 60 μm . * $P < 0.05$; $n = 3$ mice per group.

Serum IgG

Blood was collected from mice immediately after death via cardiac puncture and spun down to isolate the serum. We determined the concentration of OVA-specific IgG1 and IgG2 using ELISA (Pharmingen, Sydney, Australia) as previously described (16).

Quantitative RT-PCR

We performed quantitative RT-PCR with SYBR Green (Invitrogen, Sydney, Australia) on a Realcyler (Eppendorf, Sydney, Australia) with the following cycling conditions: 2 minutes at 50°C followed by 2 minutes at 95°C. Amplification was recorded during 40 cycles of 15 seconds at 95°C followed by 45 seconds at 60°C. We quantified mRNA copy numbers using cDNA standards for all genes of interest. We normalized expression to the housekeeper gene *Hprt*. Primer sequences can be found in Table 1.

Statistical Analysis

Statistical significance was analyzed using Student's *t* test or Mann-Whitney test as appropriate. Comparisons between curves for lung function data was performed by one-

way ANOVA. All statistical analyses shown are comparisons between strain-matched OVA groups unless otherwise stated. Data are expressed as mean $\pm$ SEM.

Results

To investigate the role of TRAIL signaling in airways remodeling, we used a chronic OVA model where, after sensitization, BALB/c mice were challenged with intranasal administration of OVA for 3 months. This model has previously been described to be of sufficient length to allow the development of structural alterations of the airways (15), providing additional outcomes over the acute

mouse models previously used to investigate the role of TRAIL signaling in the pathogenesis of AAD. Here we observed that OVA-sensitized and challenged ("allergic") BALB/c mice with the *Tnfsf10* gene knocked out (TRAIL^{-/-}) were protected from the development of AHR defined as percentage increase in airways resistance to increasing concentrations of methacholine as compared with allergic WT mice and OVA-sensitized, saline-challenged ("nonallergic") mice (Figure 1a). The percentage decrease in dynamic compliance from baseline in response to methacholine was also ameliorated in allergic TRAIL^{-/-} as compared with WT mice (Figure 1b). There was no difference in baseline (without

Table 1. Primer List

Target	Forward	Reverse
Tgfb1	TGTGGAACCTCTACCAGAAATATAGC	GAAAGCCCTGTATTCCGTCTC
Mid1	CACTCGCTGAAGGAAAATGACCA	AATCCAAGGCAAAAAGTGTCAAACG
Hprt1	AGGCCAGACTTTGTTGGATTGAA	CAACTTGCGCTCATCTTAGGCTTT

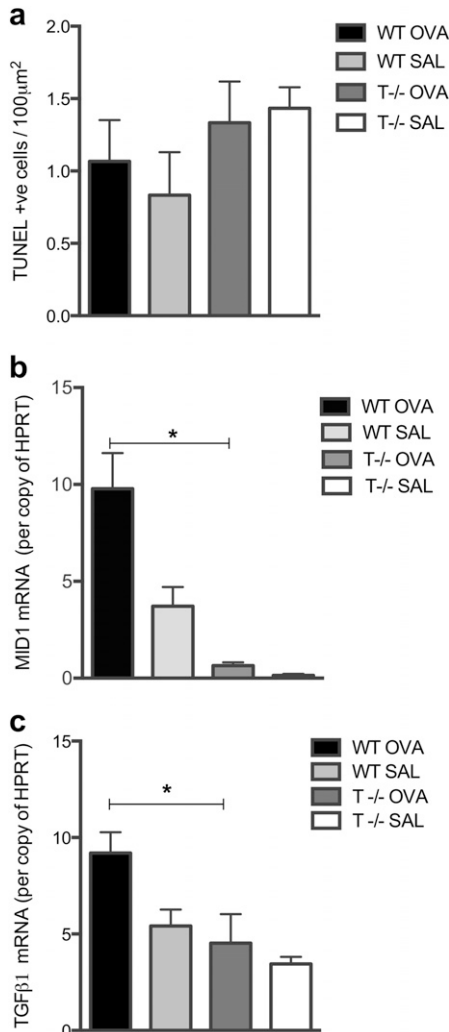


Figure 5. (a) Terminal dUTP nick end labeling (TUNEL)-positive apoptotic cells per 100 μm^2 ($n = 3$). (b, c) Expression of Midline-1 (MID1) and TGF- β 1 normalized to hypoxanthine phosphoribosyltransferase (HPRT) expression in lung tissue as determined by quantitative RT-PCR. * $P < 0.05$; $n = 6$ mice per group.

methacholine challenge) resistance or compliance observed between WT and TRAIL-/- mice (Figures 1a and 1b). Allergic TRAIL-/- mice were also largely protected from eosinophilia in the lungs (Figure 1c) and had significantly fewer mast cells present (Figure 1d). These findings were consistent with those previously reported in acute OVA- and HDM-induced models of AAD (9, 10).

The secretion of IL-4, IL-5, IL-10, and IL-13 by cells isolated from the draining lymph nodes of the lung cavity was reduced in TRAIL-/- mice (Figures 2a–2d).

Serum OVA-specific IgG1 levels were reduced in the TRAIL-/- (Figure 2e), whereas serum OVA-specific IgG2a levels (Figure 2f) were elevated, consistent with a reduced Th2 response to chronic allergen exposure.

Proinflammatory chemokines were also reduced, with allergic TRAIL-/- mice displaying significantly reduced levels of granulocyte-macrophage colony-stimulating factor, chemokine (C-X-C motif) ligand 1, chemokine (C-C motif) ligand (CCL)3, CCL4, CCL5, and CCL11 when compared with WT mice (Figure 3).

These differences in inflammatory signaling networks correlated with reduced structural alterations in the lungs. OVA-challenged TRAIL-/- mice had fewer PAS-positive mucus-expressing epithelial cells than OVA-challenged WT mice (Figure 4a). Allergic TRAIL-/- mice also had reduced smooth muscle hyperplasia (Figure 4b) and collagen deposition (Figure 4c) associated with the major airways as compared with allergic WT mice.

To explore whether TRAIL signaling through apoptotic pathways could partially explain the observed differences between TRAIL-/- and WT mice, lung sections were TUNEL stained. No differences in numbers of TUNEL-positive apoptotic cells were observed between allergic and nonallergic nor TRAIL-/- and WT mice (Figure 5a). The expression of MID-1 and the profibrotic signaling molecule TGF- β 1 was elevated in allergic WT mice but not in TRAIL-/- mice (Figures 5b and 5c). We have previously shown that MID-1 impairs PP2A activity (9, 11), and we therefore treated WT mice with the PP2A-activating drug AAL(s) to reverse MID1 effects *in vivo* throughout the challenge phase. OVA-allergic WT BALB/c mice treated with AAL(s) showed reduced eosinophilia, TGF- β 1 expression, and collagen deposition in the major airways (see Figure E1 in the online supplement).

Discussion

We have previously demonstrated a role for TRAIL and its downstream proinflammatory signaling pathways in the induction of AAD in acute OVA- and HDM-driven mouse models (9, 10). In the acute setting, genetic TRAIL deficiency or

siRNA silencing of TRAIL or downstream signaling (MID-1) was sufficient to inhibit AHR- and Th2-driven airways inflammation, including eosinophilia (9, 10). Here we show for the first time in a chronic model of AAD that key aspects of airways remodeling, including smooth muscle hyperplasia and peribronchial fibrosis, are TRAIL dependent. Allergic TRAIL-/- mice were also protected from AHR, which can most likely be attributed to the lower numbers of peribronchial eosinophils and mast cells when compared with allergic WT mice (Figure 1).

TRAIL-/- mice had markedly reduced production of the classical Th2 cytokines IL-4, -5, and -13 (Figures 2 a–2d). The Th2-dependent serum IgG1 antibody titer (17) was also reduced in the absence of TRAIL (Figure 2e). B cells express the TRAIL-binding death receptor DR5, and recent studies have shown a direct suppressive role of TRAIL on memory B-cell responses in chronic HIV infection involving apoptosis (18). However, other studies showed that human nonmalignant, noninfected B cells are not responsive to TRAIL-induced apoptosis. Thus, our data demonstrating a complete shift in IgG1 versus IgG2a profiles may indicate indirect TRAIL effects on memory B lymphocytes through impairment of Th2 responses rather than direct TRAIL effects through promoting B-cell apoptosis.

Several inflammatory chemokines were also reduced in the absence of TRAIL signaling, including granulocyte-macrophage colony-stimulating factor, chemokine (C-X-C motif) ligand 1, CCL3, CCL5, and CCL11 (Figure 3). These differences highlight a key role for TRAIL in the perpetuation of the proinflammatory secretory milieu in the chronic OVA model. The expression of CCL5 has been previously demonstrated to be a potent mast cell recruitment factor and activator (19). The cytokine IL-5 and the chemokines CCL11, CCL3, and CCL5 are responsible for driving the recruitment and, in combination with IL-4, the activation of eosinophils in allergic airways (20–23).

Ongoing airways eosinophilia and elevated mast cell activity in the persistent presence of allergen perpetuates the pathological damage and repair processes associated with the development of structural changes within the airways broadly categorized as remodeling (24). Here we

found that, in the absence of TRAIL, with reduced Th2-driven inflammatory signaling and reduced recruitment of eosinophils and mast cells to the airways there were also reductions in the degree of remodeling. Namely, there were significantly fewer PAS-positive mucus-secreting epithelial cells (Figure 4a), reduced peribronchial smooth muscle hyperplasia (Figure 4b), and ameliorated peribronchial fibrosis (Figure 4c). These data may be relevant to patients with asthma because Yurovsky and colleagues showed collagen production by human fibroblasts *in vitro* upon TRAIL exposure at concentrations consistent with those found in human serum (25).

These processes are apparently independent of apoptotic TRAIL signaling because there is no observable difference in TUNEL staining between allergic and nonallergic mice or between WT and TRAIL^{-/-} mice. Thus, a potential role of TRAIL in the regulation of apoptosis in nontransformed cells resident or accumulating in the lung during inflammation is not evident in this model, which is consistent with our previous reports (9, 10) and with those from others reviewed by Fulkerson and colleagues (4). A previous study has demonstrated a role of TRAIL as a prosurvival factor for eosinophils *in vitro* (26), which is consistent with our *in vivo* findings demonstrating reduced levels of eosinophils in TRAIL^{-/-} mice.

A key driver of allergic fibrosis is eosinophil-derived TGF- β 1 (15), the transcription of which was reduced in the airways in the absence of TRAIL. It has previously been demonstrated that TNF- α p55/p75 receptor-deficient mice were protected from remodeling in a chronic OVA model due to reduced recruitment of eosinophils and the ensuing reduction in TGF- β 1 (27). It is likely that the reduction of TGF- β 1 observed in this model is a consequence of the reduced Th2-driven eosinophilic inflammation downstream of TRAIL. MID-1 expression was also markedly reduced in the absence of TRAIL, which is consistent with previous studies using models of acute allergen and rhinovirus exposure (9). MID-1 deactivates PP2A, resulting in impaired dephosphorylation of downstream proinflammatory transcription factors such as NF- κ B, p38, and JNK. Here we show that pharmacological reactivation of PP2A downstream of MID-1 with AAL(s) reduced eosinophilia, TGF- β 1 expression, and collagen deposition in the large airways (Figure E1), identifying a role of the MID-1-PP2A pathway in chronic airways inflammation and TGF- β 1-mediated fibrosis.

TRAIL has the potential to elicit effector functions that are independent of MID-1-PP2A and different effects in a model of interstitial lung fibrosis. Specifically, TRAIL had a protective antifibrotic role in a fulminant bleomycin-induced lung fibrosis

model using susceptible C57BL/6 mice, and its genetic absence was associated with increased neutrophil numbers and reduced neutrophil apoptosis in this model (28). Our data extend these findings by identifying a more diverse role of TRAIL in fibrosis independent of apoptosis by highlighting its profibrotic potential in allergen-induced inflammation models, the pathogenesis of which is Th2-driven eosinophilia, in contrast to fulminant bleomycin-induced lung injury. It would be of great interest to determine the role of TRAIL in other (less fulminant) lung fibrosis models. Together, our data suggest that modulating TRAIL and its proinflammatory downstream signaling pathways have the potential to impair the development of airways remodeling observed in a model of chronic allergen exposure. Our data highlight a more complex role of TRAIL in the pathogenesis of fibrosis than previously identified, which requires consideration of the underpinning disease process and severity associated with fibrosis for the future development of novel therapeutic approaches. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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