Tumour necrosis factor-related apoptosis-inducing ligand promotes cigarette smoke-induced experimental COPD

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Chronic obstructive pulmonary disease (COPD) is a life-threatening inflammatory respiratory disorder, often induced by cigarette smoke (CS) exposure. The development of effective therapies for COPD is impaired by a lack of understanding of the underlining mechanisms. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a cytokine with inflammatory and apoptotic properties. We interrogated a mouse model of CS-induced experimental COPD and human COPD tissues to identify a novel role for TRAIL in pathogenesis. CS-exposure of wild-type mice increased TRAIL and its receptor mRNA expression and protein levels, as well as the number of TRAIL⁺CD11b⁺ monocytes in the lung. TRAIL and its receptor mRNA was also increased in human COPD. TRAIL-deficient mice had decreased; smoke-induced pulmonary inflammation; pro-inflammatory mediators; emphysema-like alveolar enlargement, and; improved lung function. TRAIL-deficient mice also developed spontaneous airway remodelling characterized by increased epithelial cell thickness and collagen deposition, independent of CS exposure. Importantly, therapeutic neutralization of TRAIL, after the establishment of early-stage experimental COPD, reduced CS-induced pulmonary inflammation, emphysema-like alveolar enlargement and airway remodelling. These data provide further evidence for TRAIL being a pivotal inflammatory cytokine in respiratory diseases, and the first preclinical evidence to suggest that therapeutic agents that target TRAIL may be effective in COPD therapy.
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is the third leading cause of morbidity and death worldwide and imparts a major socioeconomic burden.¹ It is a complex, heterogeneous disease characterized by chronic pulmonary inflammation, airway remodelling and emphysema, that are associated with progressive lung function decline.² It is primarily caused by cigarette smoke (CS) exposure in Western countries but additional factors are also important in other areas.³ Once induced the patient’s condition continues to deteriorate often even after smoking cessation.⁴ Glucocorticoids, anticholinergic agents and long acting muscarinic antagonists are the current mainstay therapies for COPD. However, many patients are refractory to steroid treatment, and these agents do not modify the inducing factors or halt the progression of disease even at high doses.⁵ The lack of effective treatments for COPD is largely due to the poor understanding of the underlying mechanisms of disease pathogenesis.

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), also known as tumour necrosis factor superfamily member 10 (TNFSF10), is a cytokine that induces both inflammation and apoptosis.⁶ TRAIL is expressed by a variety of cells including epithelial cells, monocytes/macrophages, neutrophils, dendritic cells, and T cells.⁷⁻¹¹ Four cell surface receptors have been identified for TRAIL; death receptor 4 (DR4, also known as TRAIL-R1, which is not expressed in mice), DR5 (TRAIL-R2), decoy receptor 1 (DcR1; TRAIL-R3) and DcR2 (TRAIL-R4).¹²⁻¹⁴ Both DR4 and DR5 possess an intracellular death domain that induces apoptosis.¹²,¹³ In contrast, DcR1 and DcR2 lack a functional death domain and, therefore, act as decoy receptors.¹⁴ Emerging evidence from us, and others, implicate TRAIL in chronic lung diseases such as asthma and pulmonary fibrosis.⁶,⁷,⁹,¹⁵,¹⁶ Experimental studies show that
TRAIL promotes allergic airway disease (AAD) by increasing (C-C motif) ligand (CCL)20 production and myeloid dendritic cell (mDC) migration into the lungs resulting in increased inflammation and airway hyperresponsiveness (AHR). TRAIL-dependent signalling pathways are also critical for rhinovirus-induced AAD exacerbations. Furthermore, TRAIL promotes Chlamydia respiratory infection-induced pulmonary inflammation, AHR and emphysema-like alveolar enlargement. It is also implicated in collagen deposition in a mouse model of ovalbumin-induced AAD, but conversely appears to protect against lung injury and fibrosis in some situations in mice. The role of TRAIL in CS-induced inflammation, airway remodelling, emphysema, impaired lung function and the pathogenesis of COPD is unknown.

Here, we investigated the role of TRAIL using our recently established mouse model of chronic CS-induced experimental COPD that recapitulates the critical features of human disease. TRAIL and its receptors were increased in the model and in lung samples from human COPD patients. Mice that were deficient in TRAIL or were treated with an anti-TRAIL neutralizing antibody had reduced disease features, and the mechanisms involved were investigated. This study is the first to characterize the role of TRAIL in COPD pathogenesis and identifies TRAIL as a potential therapeutic target for this disease.
RESULTS

TRAIL mRNA expression and protein levels increase in CS-induced experimental COPD

To investigate whether TRAIL expression is altered during the pathogenesis of COPD, we interrogated our established model of experimental COPD in mice.\textsuperscript{18-23} Chronic CS exposure for eight weeks to induce experimental COPD significantly increased TRAIL mRNA (Figure 1a and b) and protein levels (Figure 1c and d) in the airways and parenchyma, compared to control mice that breathed normal air, assessed by real-time quantitative PCR (qPCR) and enzyme-linked immunosorbent (ELISA) assay. TRAIL expression occurred on small airway epithelial cells and parenchyma-associated inflammatory cells in histology sections detected by immunohistochemistry (Figure 1e). CD11b\textsuperscript{+} monocytes were the predominant TRAIL expressing cells in lung homogenates by flow cytometry (Figure 1f and 1g). Minimal changes were observed in the numbers of TRAIL\textsuperscript{+} neutrophils, γδT cells, natural killer T (NKT) cells or CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. Chronic CS exposure also increased DR5 mRNA expression in the airways (Figure 1h) but not parenchyma (Supplementary Figure S1a). DcR1 mRNA was increased in parenchyma (Figure 1i) but not airways and DcR2 expression was unaltered in both the airways and parenchyma (Supplementary Figure S1b-d).

TRAIL expression increased in human COPD

Given that TRAIL was increased in the parenchyma and small airway epithelial cells of CS-induced experimental COPD, we next sought to determine whether the expression of TRAIL and its receptor mRNA was altered in similar tissues and cells from humans with COPD. TRAIL mRNA expression was assessed in pre-existing
microarray data from COPD patients and non-COPD subjects.\textsuperscript{24–26} TRAIL expression was significantly increased 2.3-fold in airway epithelial brushings from patients with COPD compared to both non-smokers and healthy smokers without COPD (Affymetrix Human Genome U133 Plus 2.0 Array, Accession: GSE5058\textsuperscript{26}, Figure 2a). TRAIL mRNA expression was also significantly increased \textasciitilde4-fold in the parenchyma of COPD patients compared to subjects without COPD (Affymetrix Human Exon 1.0 ST Array, Accession: GSE27597\textsuperscript{24,25}, Figure 2b). The mRNA expression of human TRAIL receptors was also assessed in the same data. The TRAIL receptors DR4 (TNFRSF10A) and DR5 (TNFRSF10B), but not DcR1 (TNFRSF10C) or DcR2 (TNFRSF10D) were increased in epithelial brushings of COPD patients compared to non-smokers and healthy smokers (Figure 2c-f). However, the expression of these receptors was not altered in the parenchyma (Supplementary Figure S2a-d).

\textbf{Pulmonary inflammation is reduced in TRAIL-deficient mice exposed to CS}

Given that TRAIL increased in experimental COPD, we next determined whether this cytokine plays a role in CS-induced pulmonary inflammation. Wild-type (WT) and TRAIL-deficient (\textit{Tnfsf10}^{-/-}) mice were exposed to CS to induce experimental COPD and pulmonary inflammation was assessed in bronchoalveolar lavage (BAL) by staining and differential inflammatory cell enumeration. CS exposure of WT mice significantly increased total leukocytes, macrophages, neutrophils and lymphocytes in BAL compared to normal air-exposed WT controls (Figure 3a-d). In contrast, CS-induced increases in total leukocytes and macrophages were completely inhibited in \textit{Tnfsf10}^{-/-} mice, whereas neutrophils and lymphocytes were elevated compared to normal air-exposed \textit{Tnfsf10}^{-/-} controls. CS-exposed \textit{Tnfsf10}^{-/-} mice also had decreased
total leukocytes and macrophages, but not neutrophils or lymphocytes, compared to CS-exposed WT controls (Figure 3a-d).

Next, we assessed inflammatory cell numbers in the parenchyma by histology. CS exposure of WT mice significantly increased inflammatory cell numbers in the parenchyma compared to normal air-exposed WT controls (Figure 3e). CS exposure of \textit{Tnfsf10}\textsuperscript{-/-} mice also increased inflammatory cell numbers compared to normal air-exposed \textit{Tnfsf10}\textsuperscript{-/-} controls. However, CS-exposed \textit{Tnfsf10}\textsuperscript{-/-} mice had significantly reduced inflammatory cells compared to CS-exposed WT controls.

Since CS-exposed \textit{Tnfsf10}\textsuperscript{-/-} mice had marked reductions in both BAL and parenchymal inflammatory cells, we next determined if there were any differences in the numbers of individual inflammatory cell types in the lung by flow cytometry (Table 1). CS exposure of WT mice increased the numbers of CD11b\textsuperscript{+} monocytes, mDCs and γδT cells in lung compared to normal air-exposed WT controls (Figure 2f-h). CS exposure of \textit{Tnfsf10}\textsuperscript{-/-} mice also increased CD11b\textsuperscript{+} monocytes and mDCs, but not γδT cells compared to normal air-exposed \textit{Tnfsf10}\textsuperscript{-/-} controls. However, CS-exposed \textit{Tnfsf10}\textsuperscript{-/-} mice had reduced CD11b\textsuperscript{+} monocytes, mDCs and γδT cells compared to CS-exposed WT controls. There were no differences in the numbers of alveolar macrophages (AMs), plasmacytoid (p)DCs, NKT cells, CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells between CS-exposed \textit{Tnfsf10}\textsuperscript{-/-} and WT mice (Supplementary Figure S3a-e).

Pro-inflammatory cytokine, chemokine and COPD-related factor mRNA expression are reduced in TRAIL-deficient mice exposed to CS

Given that CS-exposed \textit{Tnfsf10}\textsuperscript{-/-} mice had decreased pulmonary inflammatory cells, we next assessed the mRNA expression of inflammatory cytokines and chemokines and COPD-related factors in the lung. CS exposure of WT mice increased the mRNA
expression of the cytokine tumour necrosis factor-α (TNF-α, Figure 4a), the chemokines CCL2, 3, 7, 12 and 20 (Figure 4b-f) and other COPD-related factors matrix metalloproteinase-12 (MMP-12) and serum amyloid A3 (SAA3, Figure 4g and h) compared to normal air-exposed WT controls. CS exposure of Tnfsf10−/− mice also increased the mRNA expression of these cytokines, chemokines and COPD-related factors compared to normal air-exposed Tnfsf10+/− controls. However, CS-exposed Tnfsf10+/− mice had significantly reduced expression of all of these factors compared to CS-exposed WT mice. We also profiled other factors including interleukin (IL)-33, chemokine (C-X-C) ligand (CXCL)1 and 3, CCL4 and 22 and mucin 5ac (Muc5ac) (Supplementary Figure S4a-f). These were increased by CS but were not different between CS-exposed WT and Tnfsf10+/− mice.

Active NF-κB p65 is reduced in TRAIL-deficient mice exposed to CS

We, and others, have previously shown that TRAIL induces inflammatory responses by mediating NF-κB activity.6,27 In this study, CS exposure of WT mice increased the level of active NF-κB p65 in the lung compared to normal air-exposed WT controls (Figure 4i). In contrast and importantly, active NF-κB p65 did not increase in CS-exposed Tnfsf10+/− mice compared to normal air-exposed Tnfsf10+/− or WT controls. Consequently, active NF-κB p65 was decreased in CS-exposed Tnfsf10+/− compared to CS-exposed WT controls. We also assessed other NF-κB subunits (p50, p52 and RelB) but these were not altered by CS exposure or in the absence of TRAIL (data not shown).

Emphysema-like alveolar enlargement and cell death are reduced and lung function is improved in TRAIL-deficient mice exposed to CS
We previously showed that WT mice with experimental COPD have emphysema-like alveolar enlargement and impaired lung function. Here, we again show that CS exposure of WT mice increased alveolar diameter compared to normal air-exposed WT controls (Figure 5a). CS exposure of Tnfsf10−/− mice also increased alveolar diameter compared to normal air-exposed Tnfsf10−/− controls. However, CS-exposed Tnfsf10−/− mice had significantly reduced alveolar diameter compared to CS-exposed WT controls. Reduced alveolar diameter in CS-exposed Tnfsf10−/− mice was associated with reduced numbers of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)+ cells in the parenchyma, indicating reduced cell death, compared to CS-exposed WT controls (Figure 5b).

We next assessed the role of TRAIL in impaired lung function. CS exposure of WT mice increased pressure-volume loops (PV-loops) and lung compliance compared to normal air-exposed WT controls (Figure 5c and d). In contrast, CS exposure did not increase PV-loops or lung compliance in Tnfsf10−/− mice compared to normal air-exposed Tnfsf10−/− controls or, importantly, CS-exposed WT controls.

Spontaneous airway remodelling occurs in TRAIL-deficient mice

We previously showed that mice develop small airway remodelling in experimental COPD. Here, we replicate our previous observations and show that CS exposure of WT mice increased small airway epithelial cell area compared to normal air-exposed WT controls (Figure 6a). CS exposure of Tnfsf10−/− mice also increased small airway epithelial cell area compared to normal air-exposed Tnfsf10−/− controls, which was not different to CS-exposed WT smoke controls. Notably, however, small airway epithelial cell area increased spontaneously in normal air-exposed Tnfsf10−/− compared to WT controls.
We then determined whether increased epithelial area was associated with increased small airway epithelial cell numbers. Consistent with expanded small airway epithelial cell area, CS exposure of WT mice increased the number of epithelial cells in the small airways compared to normal air-exposed WT controls (Figure 6b). Epithelial cell numbers in CS-exposed Tnfsf10−/− mice were not different to normal air-exposed Tnfsf10−/− or CS-exposed WT controls. The former observation was because normal air-exposed Tnfsf10−/− controls had increased epithelial cell numbers compared to WT air controls.

We next examined airway fibrosis in terms of collagen deposition around the small airways. CS exposure of WT mice increased collagen deposition compared to normal air-exposed WT controls (Figure 6c). CS exposure of Tnfsf10−/− mice did not alter collagen deposition compared to normal air-exposed Tnfsf10−/− or CS-exposed WT controls. The former observation was because normal air-exposed Tnfsf10−/− mice had increased collagen deposition compared to normal air-exposed WT air controls.

Pulmonary inflammation is suppressed and emphysema-like alveolar enlargement is inhibited in experimental COPD by therapeutic neutralization of TRAIL

We previously showed that the hallmark features of experimental COPD are emerging by Week 6, established by Week 8 and progressively worsen by Week 12 of CS exposure.18 Hence, to assess the therapeutic potential of targeting TRAIL, WT mice were exposed to CS or normal air for 12 weeks and were treated with a neutralizing anti-TRAIL monoclonal antibody or isotype control intraperitoneally (i.p) from Week 7 to Week 12.
We first examined the effect of TRAIL neutralization on pulmonary inflammation in BAL. As expected CS exposure of isotype-treated WT mice increased total leukocytes, macrophages, neutrophils and lymphocytes in BAL compared to isotype-treated normal air-exposed WT controls (Figure 7a-d). CS-exposure of anti-TRAIL-treated mice only partially increased total leukocytes, macrophages, neutrophils and lymphocytes in BAL compared to anti-TRAIL-treated air controls. Furthermore, anti-TRAIL-treatment significantly reduced all inflammatory cells compared to isotype-treated CS-exposed controls, although levels remained increased compared to normal air-exposed controls.

Next, we examined the effect of neutralizing TRAIL on inflammatory cell numbers in the parenchyma. As expected CS exposure of isotype-treated mice increased inflammatory cells in the parenchyma compared to isotype-treated normal air-exposed controls (Figure 7e). CS exposure of anti-TRAIL-treated mice partially increased inflammatory cells compared to anti-TRAIL-treated normal air-exposed controls. Furthermore, anti-TRAIL treatment significantly reduced inflammatory cells compared to isotype-treated CS-exposed controls.

Next, we assessed the effect of neutralizing TRAIL on active NF-κB p65 levels in the lung. Active NF-κB p65 was increased in CS-exposed isotype-treated mice compared to normal air-exposed isotype-treated controls (Figure 7f). In contrast, CS-exposed anti-TRAIL-treated mice completely inhibited the increase in active NF-κB p65 compared to anti-TRAIL-treated normal air-exposed or isotype-treated CS-exposed controls.

We then assessed the effect of neutralizing TRAIL on CS-induced emphysema-like alveolar enlargement. CS exposure of isotype-treated mice increased alveolar diameter compared to isotype-treated normal air-exposed controls (Figure
In contrast, CS-exposed anti-TRAIL-treated mice were completely protected against increased alveolar diameter with no increase compared to anti-TRAIL-treated normal air-exposed or isotype-treated CS-exposed controls. Similarly, CS exposure of isotype-treated mice resulted in increased numbers of TUNEL$^+$ cells in the parenchyma compared normal air-exposed isotype-treated controls (Figure 7h). However, CS-exposed anti-TRAIL-treated mice were protected against increases in TUNEL$^+$ cells in the parenchyma with no increase compared to CS-exposed isotype-treated controls.

**Airway remodelling is suppressed in experimental COPD by therapeutic neutralization of TRAIL**

We then assessed the effect of neutralizing TRAIL on airway remodelling in experimental COPD. As expected CS exposure of isotype-treated mice increased small airway epithelial cell area and number compared to isotype-treated normal air-exposed controls (Figure 8a-b). CS exposure of anti-TRAIL-treated mice only partially increased small airway epithelial cell area and number compared to anti-TRAIL-treated normal air-exposed controls. Furthermore, CS-exposed anti-TRAIL-treated mice had significantly reduced small airway epithelial cell area and number compared to isotype-treated CS-exposed controls. In addition, unlike in normal air-exposed Tnfsf10$^{-/-}$ mice, administration of anti-TRAIL to similarly exposed WT mice did not increase airway epithelial cell area or number compared to isotype-treated controls.

Finally we examined the effect of neutralizing TRAIL on airway fibrosis. As expected CS exposure of isotype-treated mice increased collagen deposition around the small airways compared to isotype-treated normal air-exposed controls (Figure
In contrast, CS-exposed anti-TRAIL-treated mice did not have increased collagen deposition compared to anti-TRAIL-treated normal air-exposed controls. In addition, unlike in normal air-exposed \textit{Tnfsf10}\(-\)/ mice, administration of anti-TRAIL to similarly exposed mice did not increase collagen deposition around the small airways compared to isotype-treated controls.

**DISCUSSION**

In this study, we discovered a previously unrecognized role for TRAIL in CS-induced experimental COPD. TRAIL and its receptors were increased in mice with chronic CS-induced experimental COPD and in human COPD patients. Using a combination of CS-exposure of WT and \textit{Tnfsf10}\(-\)/ mice and a neutralizing antibody, we demonstrate that TRAIL increases pulmonary inflammation and expression of pro-inflammatory mediators, emphysema-like alveolar enlargement and impairs lung function in experimental COPD. Inflammation and alveolar enlargement were associated with TRAIL-induced increases in active NF-κB p65 and apoptosis, respectively. Surprisingly, \textit{Tnfsf10}\(-\)/ mice developed spontaneous airway remodelling characterised by increased epithelial area and collagen deposition. Importantly, therapeutic targeting of TRAIL with a neutralizing monoclonal antibody reduced CS-induced pulmonary inflammation and emphysema-like alveolar enlargement, without inducing airway remodelling. This study advances the emerging knowledge of the roles of TRAIL in inflammatory and respiratory diseases, and its potential for therapeutic targeting.

To investigate the role of TRAIL in the pathogenesis of COPD, we used an established mouse model of experimental COPD.\textsuperscript{18–23} Mice were exposed \textit{via} the nose-only to tightly controlled doses of CS. This protocol induces the development of hallmark
features of human COPD, namely chronic bronchitis (pulmonary inflammation), small airway remodelling, emphysema-like alveolar enlargement and impaired lung function.\textsuperscript{22}

Hence, our model recapitulates key pathological features observed in human disease.

We first showed that chronic CS exposure of WT mice resulted in concomitant increases in TRAIL mRNA and protein levels in the airways and parenchyma. Our examination of lung tissue sections stained for TRAIL identified airway epithelial cells and parenchymal-associated monocytes as sources of TRAIL. We have previously shown that mouse airway epithelial cells express TRAIL following allergen challenge or respiratory infection with \textit{C. murdiamur}.\textsuperscript{6,7,17} Subsequent flow cytometric analysis of lung homogenates from mice with experimental COPD identified CD11b\textsuperscript{+} monocytes as a major source of cell surface-bound TRAIL. Consistent with these observations, we, and others, have shown that TRAIL expressing macrophages are recruited to the lung following respiratory bacterial (e.g. \textit{Chlamydia}, \textit{Streptococcus pneumoniae}) and viral infections (e.g. influenza).\textsuperscript{6,8,10} We also showed that TRAIL receptor expression is elevated in experimental COPD with increases in mRNA levels of DR5 in the airways and DcR1 in the parenchyma. Notably, there were also increases in the mRNA levels of TRAIL and its receptors DR4 and DR5 in human COPD lung tissue and airway epithelial cells.\textsuperscript{24–26} There were some species-specific differences in TRAIL receptor expression with increases in DcR1 in mice and DR4 in humans. Mice do not produce DR4, and so compensatory mechanisms may be present.

\textit{Tnfsf10}\textsuperscript{−/−} mice had reduced CS-induced pulmonary cellular inflammation characterized by reduced influx of total leukocytes and macrophages into the airways and decreased numbers of parenchymal-associated inflammatory cells and CD11b\textsuperscript{+} monocytes, mDCs and γδT cells in the lung. All of these cells have been previously
shown to be increased in experimental models of, or in human COPD.\textsuperscript{28–30} These data are supported by our previous studies that show TRAIL drives pulmonary inflammation.\textsuperscript{6,7,16} Indeed, intranasal administration of recombinant TRAIL to naïve mice increased the numbers of mDCs and CD4\(^+\) T cells in the lung.\textsuperscript{7} Furthermore, silencing of TRAIL using small interfering RNA reduced pulmonary inflammation in a mouse model of acute asthma (ovalbumin-induced AAD).\textsuperscript{7} We also showed that \textit{Tnfsf10}^{-/-} mice had reduced infiltration of CD11b\(^+\) monocytes, mDCs, CD4\(^+\) and CD8\(^+\) T cells into the lung following neonatal \textit{C. muridarum} respiratory infection.\textsuperscript{6}

In support of our inflammatory cell data, \textit{Tnfsf10}^{-/-} mice also had reduced CS-induced mRNA expression of key pro-inflammatory cytokines (TNF-\(\alpha\)), chemokines (CCL2, 3, 7, 12 and 20) and other COPD-related factors (MMP-12 and SAA3) in the lungs. We, and others, have shown increased expression of TNF-\(\alpha\) following CS-exposure in mice and humans, and this cytokine is known to induce the expression of the monocyte chemokines CCL2, 7 and 12 and the neutrophil chemoattractant CCL3.\textsuperscript{18} These chemokines have all been associated with increasing the severity of cellular inflammation and emphysema in COPD.\textsuperscript{31,32} CCL20 was increased by CS and was decreased in \textit{Tnfsf10}^{-/-} mice, which correlated with decreased numbers of mDCs in the lung. We previously showed that inhibition of TRAIL reduced CCL20 and subsequent homing of mDCs to the airways, which was associated with reduced inflammation and AHR in AAD.\textsuperscript{7} We, and others, have also shown that the protease MMP-12 and SAA3, an acute phase protein, are increased in experimental and human COPD.\textsuperscript{18,33,34} To investigate the mechanisms involved in TRAIL-mediated inflammation we assessed the activity of the transcription factor NF-\(\kappa\)B. TRAIL is known to induce NF-\(\kappa\)B activity and promote inflammatory responses.\textsuperscript{6,17,27}

Consistent with these observations, our current study demonstrated that TRAIL-
deficiency or inhibition reduced CS-induced NF-κB p65 activity and this was
associated with reduced pulmonary inflammation. This indicates that in CS-induced
experimental COPD TRAIL induces NF-κB p65 activity causing the transcription of
the mRNA of pro-inflammatory factors that drive inflammation and disease
pathogenesis. We previously showed that TRAIL-deficiency or administration of
neutralizing anti-DcR2 antibody in mice reduced NF-κB activity and pulmonary
inflammation in the lung following neonatal *C. muridarum* respiratory infection.6 We
also recently showed that TRAIL induces the expression of the E3 ubiquitin ligase
midline-1 (MID1) in the airway wall and that siRNA-induced knockdown of MID1
reduces NF-κB activity in AAD and RV infection models.17 Collectively, our current
study advances our understanding of the roles of TRAIL as a pro-inflammatory
mediator by showing that it regulates inflammation in CS-induced experimental
COPD.

As in humans, chronic CS exposure causes emphysema-like alveolar
enlargement in experimental COPD.18–23 Here we show that *Tnfsf10*−/− mice are
protected against CS-induced alveolar enlargement. We also show that CS increases
TRAIL+ monocytes and that *Tnfsf10*−/− mice have less TUNEL+ cells, indicating a
reduction in apoptosis, in the parenchyma. Others have shown that TRAIL receptors
are increased in the lungs of patients with emphysema and resected lung explant
cultures from emphysematous patients or A549 cells exposed to CS extract displayed
increased TRAIL-mediated apoptosis.35,36 These data indicate that in CS-induced
experimental COPD TRAIL induces apoptosis of parenchymal cells that leads to
emphysema-like alveolar enlargement.

We then go on to show that the suppression of pulmonary inflammation and
alveolar enlargement in the absence of TRAIL leads to protection against impaired
lung function with the inhibition of increases in lung volumes and compliance. These are important features of human COPD. We previously showed that Tnfsf10−/− mice were protected against neonatal Chlamydia respiratory infection-induced emphysema-like alveolar enlargement and impaired lung function. TRAIL has also been shown to impair lung function in models of AAD.\textsuperscript{7,16,17}

We previously showed that depletion of macrophages with clodronate-loaded liposomes reduced CS-induced airway remodelling, emphysema-like alveolar enlargement and improved lung function in experimental COPD.\textsuperscript{18} Macrophages expressing TRAIL that are recruited to the lung during influenza virus infection induce acute lung injury and alveolar epithelial cell apoptosis.\textsuperscript{8} Collectively, these data indicate that TRAIL induced pulmonary inflammation and that TRAIL+ monocytes may contribute to CS-induced emphysema by promoting apoptosis in the parenchyma, which together reduce lung function.

Surprisingly, naïve Tnfsf10−/− mice developed spontaneous small airway remodelling characterized by increased airway epithelial cell area and numbers and collagen deposition, which was not further increased by CS exposure. In this regard, we previously showed that TRAIL induced airway epithelial thickening and goblet cell metaplasia in neonatal Chlamydia respiratory infection and AAD in mice.\textsuperscript{6} Others have shown that Tnfsf10−/− mice had increased total collagen in the lung in a bleomycin-induced mouse model of pulmonary fibrosis.\textsuperscript{9} Furthermore, recombinant TRAIL treatment inhibited the expression of the collagen-specific molecular chaperone heat shock protein 47 and reduced soluble collagen production in human hepatic stellate cells \textit{in vitro}.\textsuperscript{37} In contrast to these findings, however, Tnfsf10−/− mice had reduced lung collagen in a chronic ovalbumin model of AAD.\textsuperscript{16} This
inconsistency likely results from differences in the pathogenic mechanisms involved in the different disease contexts.

Importantly, anti-TRAIL treatment of established experimental COPD substantially reduced CS-induced pulmonary inflammation, alveolar enlargement, and small airway remodelling. Anti-TRAIL treatment of normal air-exposed WT mice did not induce airway remodelling. This is in contrast to the remodelling that occurred in naive Tnfsf10−/− mice. This suggests that therapeutic neutralization of TRAIL may be effective in suppressing hallmark features of COPD, without causing unwanted airway remodelling that occurs with early-life or lifelong deficiency. Treatment may be further improved with increasing doses or optimizing treatment regimes. The therapeutic benefit in suppressing TRAIL is supported by our previous studies. We showed that TRAIL neutralization protected mice from neonatal Chlamydia respiratory infection-induced pulmonary inflammation and emphysema-like alveolar enlargement.6 Furthermore, inhibition of TRAIL suppressed inflammation and improved lung function in mouse models of AAD.7,17 However, selective inhibition of TRAIL may be contraindicated as a treatment for COPD due to the increased risk of lung cancer and respiratory infections.10 TRAIL is an important inducer of apoptosis of in a variety tumour cells.38 Furthermore, both COPD patients and mice with experimental COPD are more susceptible to bacterial (e.g. S. pneumonias) and viral (e.g. influenza) infections18,20,39 and TRAIL is required for the clearance of such infections.10,40 These potential side effects could be minimized by short-term dosing or with optimized treatment regimes. Furthermore, additional studies may identify specific signalling factors and pathways downstream of TRAIL, which when inhibited do not increase cancer risk or susceptibility to infection. This could be initially assessed in mouse models of cancer and infections.
In summary, our study reveals for the first time that TRAIL has important pathogenic roles in CS-induced experimental COPD. It is pivotal in promoting pulmonary inflammation and NF-κB p65 activation, and emphysema-like alveolar enlargement and apoptosis, which lead to impaired lung function (Supplementary Figure S5). TRAIL also regulates small airway remodelling independent of CS exposure. Importantly, therapeutic neutralization of TRAIL in established experimental COPD reduced hallmark features of the disease. Collectively, our data suggest that therapeutic targeting of TRAIL may be beneficial in COPD.

METHODS

Ethics statement

This study was performed in accordance with the recommendations issued by the National Health and Medical Research Council of Australia. All protocols were approved by the animal ethics committee of The University of Newcastle, Australia.

Experimental COPD

Female, 7-8-week-old, WT or Tnfsf10−/− BALB/c mice were exposed to normal air or CS through the nose only for eight weeks as we have previously described. Some mice were exposed to CS or normal air for 12 weeks and treated with anti-TRAIL neutralizing or isotype control antibodies from Week 7 to 12.

Isolation of RNA and protein

Total RNA was extracted from whole lung tissue and blunt-dissected airway and parenchyma as described previously. Briefly, the trachea and lungs were excised, and lung parenchyma was carefully separated from the airways with sterile forceps.
Whole lungs, airways and parenchyma were then snap frozen and stored at -80°C. Tissues were thawed and homogenized in 500μL of sterile Dulbecco’s phosphate-buffered saline (Life Technologies, Mulgrave, Victoria, Australia) using a Tissue-Tearor stick homogenizer (BioSpec Products, Bartesville, OK) on ice. Tissue homogenates were then split equally (250μL) for RNA and protein extraction. Total RNA was extracted using TRIzol (Invitrogen, Mount Waverly, Victoria, Australia) according to manufacturer’s instructions and stored at -80°C. For protein extraction, tissue homogenates were mixed with equal portions of sterile Dulbecco’s phosphate-buffered saline (Life Technologies) supplemented with PhosSTOP phosphatase and Complete ULTRA protease inhibitors cocktails (Roche Diagnostics, Mannheim, Germany). Tissue homogenates were then centrifuged at 8,000xg for 10 mins at 4°C. Supernatants were collected and stored at -20°C for assessment by ELISA assay.

qPCR

Total RNA from whole lungs, airway and parenchyma (1,000ng) were reversed transcribed using Bioscript (Bioline, Alexandria, New South Wales, Australia) and random hexamer primers (Invitrogen).\(^\text{6,19,41–44}\) The mRNA expression of TRAIL, DR5, DcR1, TNF-α, CCL2, 3, 7, 12 and 20, MMP-12, SAA3, DcR2, IL-33, CXCL1, CXCL3, CCL4, CCL7 and Muc5ac were determined by real-time quantitative PCR (qPCR, ABIPrism7000, Applied Biosystems, Scoresby, Victoria, Australia) and expressed as relative abundance to the reference gene hypoxanthine-guanine phosphoribosyltransferase.\(^\text{6,19,41–44}\) Custom designed primers were used (Supplementary Table 1).

ELISA
TRAIL protein levels in airway and parenchyma were quantified with mouse TRAIL/TNFSF10 DuoSet ELISA kits (R&D Systems, Gymea, New South Wales, Australia) with normalization to total protein determined using the BCA Protein Assay Kit (PIERCE, Scorsby, Victoria, Australia) as per manufacturer’s instructions.

**NF-κB assays**

The active NF-κB p65 subunit was measured using the TransAM NF-κB family transcription factor assay kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions.6,17

**Immunohistochemistry**

Lungs were perfused, inflated, formalin-fixed, paraffin-embedded and sectioned (4-6μm). Longitudinal sections of the left lung were incubated with primary antibody (anti-TRAIL, Abcam, Melbourne, Victoria, Australia) overnight at 4°C and followed by anti-rabbit horseradish peroxidase-conjugated secondary antibody (R&D Systems) as per manufacturer’s instructions. 3,3’-Diaminobenzidine chromogen-substrate buffer (DAKO, North Sydney, New South Wales, Australia) was applied to sections and incubated. Sections were counterstained with hematoxylin, mounted and analyzed with a BX51 microscope (Olympus, Tokyo, Shinjuku, Japan) and Image-Pro Plus software (Media Cybernetics, Rockville, MD).

**Flow cytometry**

Numbers of CD11b+ monocytes, neutrophils, mDCs, AMs, pDCs, NKT cells, γδT cells, CD4+ T and CD8+ T cells in lung homogenates were determined based on surface marker expression using flow cytometry (Table 1).6,42,45,46 Flow cytometric
analysis was performed using a FACSriaIII with FACSDiva software (BD Biosciences, North Ryde, Australia). Flow cytometry antibodies were from Biolegend (Karrinyup, Western Australia, Australia) (Supplementary Table 2). OneComp compensation beads (eBioscience) were used to set up assays.

Analysis of differential gene expression
Differential gene expression analysis of published datasets (accession numbers GSE5058 and GSE27597)24–26 was performed with the Array Studio software (Omicsoft Corporation, Research Triangle Park, NC, USA) applying a general linear model adjusting for age and gender and the Benjamini–Hochberg method for p-value adjustment.

Airway and parenchymal inflammation
Airway inflammation was assessed by differential enumeration of inflammatory cells in BAL.18,44,45,47,48 Longitudinal sections of lung were stained with periodic acid-Schiff (PAS) and parenchymal inflammation was assessed by enumerating the numbers of inflammatory cells in 20 randomized, high-powered fields.18

Alveolar enlargement
Lungs were perfused, inflated, fixed, paraffin-embedded and sectioned (4-6μm). Longitudinal sections of the left single-lobe lung were stained with hematoxylin and eosin to assess alveolar diameter using the mean linear intercept technique.6,18–21,43

TUNEL assay
Longitudinal sections of the left single-lobe lung were stained with TUNEL assay kits (Promega, Sydney, New South Wales, Australia) according to manufacturer’s instructions. Apoptosis in lung parenchyma was assessed by enumerating the numbers of TUNEL+ cells in 20 randomized, high-powered fields.

**Lung function**

Lung compliance was assessed by quasi-static PV-loops from oscillation manoeuvres (Flexivent [SCIREQ, Montreal, Québec, Canada]) as the volume of air that entered the lungs when the airway pressure was increased from 2 to 30 cmH2O by the ventilator (PVs-P Flexivent manoeuvre). Three inflations were performed and averaged per mouse.

**Airway remodelling**

Longitudinal sections of the left single-lobe lung were stained with PAS or Masson’s Trichrome. Airway epithelial area (μm²) and cell (nuclei) number, and collagen deposition area (μm²) was assessed in a minimum of four small airways (basement membrane perimeter <1,000μm) per section. Data were normalized to basement membrane perimeter (μm) and quantified using ImageJ software (Version 1.49h, NIH).

**TRAIL neutralization**

Mice were treated with 12.5 mg/kg body weight of neutralizing anti-TRAIL monoclonal antibody (clone N2B2) or rat IgG2a isotype control (clone 2A3, BioXCell, West Lebanon, NH) from Week 7 to 12 (six weeks), by i.p injections three times per week.
Data are presented as means ± standard error of the mean (s.e.m.) and are representative of two independent experiments consisting of 5-6 mice in each group (total of 10-12 mice per group). Statistical significance was determined with two-tailed Mann-Whitney test or by one-way analysis of variance with Bonferroni post-test using GraphPad Prism Software version 6 (San Diego, CA).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

ACKNOWLEDGEMENTS
This work was supported by grants and fellowships from the National Health and Medical Research Council of Australia, Rebecca L. Cooper Medical Research Foundation and a Gladys Brawn Fellowship. TRAIL-deficient mice were obtained from Amgen (Amgen Inc, Seattle, WA, USA). APC-conjugated αGalCer tetramers were obtained from Prof. Dale Godfrey, University of Melbourne, Australia. We thank Kristy Wheeldon for technical assistance.

DISCLOSURE
The authors declared no conflict of interest.

REFERENCES


Figure 1. TRAIL and TRAIL receptor levels increase in cigarette smoke (CS)-induced experimental COPD. Wild-type BALB/c mice were exposed to CS or normal air for eight weeks. TRAIL mRNA levels in blunt-dissected (a) airway and (b) parenchyma expressed as relative abundance to normal air-exposed controls. TRAIL protein levels in (c) airway and (d) parenchyma. (e) Immunohistochemistry for TRAIL protein in whole lung sections (arrowheads indicate TRAIL+ epithelial cells in the small airways or inflammatory cells in the parenchyma). (f) Cell surface expression of TRAIL on inflammatory cell subsets in whole lung homogenates determined by flow cytometry. (g) Representative flow cytometry plots of TRAIL expression on CD45+F4/80+ CD11c−CD11b+ cells in lung homogenates. (h) Death receptor 5 (DR5) and (i) decoy receptor 1 (DcR1) mRNA levels in airways or parenchyma. Data (n = 5-6) presented as means ± s.e.m. are representative of two independent experiments. *p<0.05; **p<0.01; ****p<0.0001 compared to normal air-exposed controls.

Figure 2. TRAIL and TRAIL receptor mRNA levels increase in human COPD. Parenchymal cores or airway epithelial cells were collected from human COPD patients. TRAIL mRNA expression was assessed by microarray profiling in (a) airway epithelial brushings from patients with GOLD stage I or II disease compared to non-smokers (NS) and healthy smokers without COPD (Smoker) and (b) parenchyma from non-COPD controls or patients with severe COPD. mRNA expression of TRAIL receptors (c) death receptor (DR)4, (d) DR5, (e) decoy receptor (DcR)1 and (f) DcR2 in airway epithelium from patients with early-stage, GOLDI and GOLDII stage COPD compared to NS and healthy smokers without COPD. The
numbers in the figures represent the false discovery rate (FDR). *FDR versus NS, #FDR versus smokers.

**Figure 3.** Pulmonary inflammation is reduced in TRAIL-deficient mice exposed to cigarette smoke (CS). Wild type (WT) or TRAIL-deficient (*Tnfsf10* /−) mice were exposed to CS or normal air for eight weeks. (**a**) Total leukocytes, (**b**) macrophages, (**c**) neutrophils, and (**d**) lymphocytes were enumerated in hematoxylin and eosin stained bronchoalveolar lavage (BAL). (**e**) The numbers of parenchymal inflammatory cells (arrowheads) were enumerated in periodic acid-Schiff-stained lung sections. (**f**) CD11b+ monocytes, (**g**) myeloid dendritic cells (mDCs) and (**h**) γδT cells were determined in single cell suspension of whole lung homogenates by flow cytometry. Data (n = 5-6) presented as means ± s.e.m. are representative of two independent experiments. ns; not significant. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 compared to normal air-exposed WT or *Tnfsf10* /− controls. #p<0.05; ##p<0.01; ###p<0.001 compared to CS-exposed WT controls.

**Figure 4.** Pro-inflammatory cytokine, chemokine and COPD-related factor mRNA expression are reduced and NF-κB p65 activity is inhibited in TRAIL-deficient mice exposed to cigarette smoke (CS). Wild type (WT) or TRAIL-deficient (*Tnfsf10* /−) mice were exposed to CS or normal air for eight weeks. (**a**) Tumour necrosis factor-α (TNF-α), (**b**) chemokine (C-C motif) ligand (CCL)2, (**c**) CCL3, (**d**) CCL7, (**e**) CCL12, (**f**) CCL20, (**g**) matrix metalloproteinase-12 (MMP-12) and (**h**) serum amyloid A3 (SAA3) mRNA expression was determined in whole lung homogenates by qPCR. (**i**) NF-κB p65 activity in whole lung homogenates. mRNA data are presented as relative abundance compared to normal air-exposed WT controls. Data
(n = 5-6) presented as means ± s.e.m. are representative of two independent experiments. *p<0.05, **p<0.01; ***p<0.001; ****p<0.0001 compared to normal air-exposed WT or Tnfsf10−/− controls. #p<0.05; ##p<0.01; ###p<0.001; ####p<0.0001 compared to CS-exposed WT controls.

Figure 5. Emphysema-like alveolar enlargement is reduced, apoptosis inhibited and lung function is preserved in TRAIL-deficient mice exposed to cigarette smoke (CS). Wild type (WT) or TRAIL-deficient (Tnfsf10−/−) mice were exposed to CS or normal air for eight weeks. (a) Alveolar diameter (μm) was determined in hematoxylin and eosin-stained lung sections using the mean linear intercept technique. (b) The numbers of TUNEL+ cells (arrowheads) enumerated in whole lung sections. Lung function was assessed in terms of (c) pressure-volume loops and (d) lung compliance at 30cmH2O using forced oscillation techniques. Data (n = 5-6) presented as means ± s.e.m. are representative of two independent experiments. *p<0.05; **p<0.01; ****p<0.0001 compared to normal air-exposed WT or Tnfsf10−/− controls. ##p<0.01; ####p<0.0001 compared to CS-exposed WT controls.

Figure 6. Spontaneous airway remodelling occurs in TRAIL-deficient mice. Wild type (WT) or TRAIL-deficient (Tnfsf10−/−) mice were exposed to cigarette smoke or normal air for eight weeks. (a) Small airway epithelial thickness in terms of epithelial cell area (μm²) per basement membrane (BM) perimeter (μm) was determined in periodic acid-Schiff (PAS)-stained whole lung sections. (b) The number of epithelial cells in PAS-stained lung sections was assessed by enumerating the number of nuclei per 100μm of BM perimeter. (c) Area of collagen deposition (μm²) per BM perimeter (μm) was determined in Masson’s Trichrome-stained lung sections. Data (n = 5-6)
presented as means ± s.e.m. are representative of two independent experiments of 5-6 mice in each group. *p<0.05; ****p<0.0001 compared to normal air-exposed WT or Tnfsf10−/− controls. **p<0.01; ***p<0.001; ****p<0.0001 compared to normal air-exposed WT controls.

**Figure 7.** Pulmonary inflammation is suppressed and emphysema-like alveolar enlargement inhibited in experimental COPD by therapeutic neutralization of TRAIL. Wild-type mice were exposed to cigarette smoke or normal air for twelve weeks and treated with neutralizing anti-TRAIL monoclonal antibody or isotype control, intraperitoneally three times per week, from Week 7 to 12. (a) Total leukocytes, (b) macrophages, (c) neutrophils and (d) lymphocytes were enumerated in hematoxylin and eosin-stained bronchoalveolar lavage (BAL). (e) The numbers of parenchymal inflammatory cells (arrowheads) were determined in periodic acid-Schiff-stained lung sections. (f) Alveolar diameter (μm) was determined in hematoxylin and eosin-stained lung sections using the mean linear intercept technique. (g) The numbers of TUNEL+ cells (arrowheads) enumerated in whole lung sections. (h) NF-κB p65 activity in whole lung homogenates. Data (n = 5-6) presented as means ± s.e.m. are representative of two independent experiments of 5-6 mice in each group. **p<0.01; ***p<0.001; ****p<0.0001 compared to isotype-treated or anti-TRAIL-treated normal air-exposed controls. #p<0.05; ##p<0.01; ###p<0.001; ####p<0.0001 compared to isotype-treated CS-exposed controls.

**Figure 8.** Airway remodelling is reduced in experimental COPD by therapeutic neutralization of TRAIL. Wild-type mice were exposed to cigarette smoke (CS) or normal air for twelve weeks and treated with neutralizing anti-TRAIL monoclonal or
isotype control antibodies, intraperitoneally three times per week, from weeks 7-12.

(a) Small airway epithelial thickness in terms of epithelial cell area (μm²) per basement membrane (BM) perimeter (μm) was determined in periodic acid-Schiff (PAS)-stained lung sections. (b) The number of epithelial cells in PAS-stained lung sections was assessed by enumerating the number of nuclei per 100μm BM perimeter. (c) Area of collagen deposition (μm²) per BM perimeter (μm) in Masson’s Trichrome-stained lung sections. Data (n = 5-6) presented as means ± s.e.m. are representative of two independent experiments of 5-6 mice in each group. **p<0.01; ****p<0.0001 compared to anti-TRAIL or isotype normal air-exposed controls. #p<0.05; ###p<0.001 compared to isotype-treated CS-exposed controls.
Table 1. Surface antigens used to characterize mouse lung cell subsets by flow cytometry

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<td>CD8$^+$ T cell</td>
<td>CD45$^+$ CD3$^+$ CD4$^−$ CD8$^+$ γδTCR$^−$ TRAIL$^{+/−}$</td>
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mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; NKT cell, natural killer T cell.
SUPPLEMENTARY INFORMATION

Tumour necrosis factor-related apoptosis-inducing ligand promotes cigarette smoke-induced experimental COPD

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*Authors contributed equally and are co-first author.

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**Figure S1.** TRAIL receptor mRNA expression in CS-induced experimental COPD. BALB/c mice were exposed to CS or normal air for eight weeks. (a) Death receptor 5 (DR5), (b) decoy receptor 1 (DcR1) and (c–d) DcR2 mRNA expression in blunt-dissected airway or parenchyma. Data (n = 5-6) presented as means ± s.e.m. are representative of two independent experiments. *p<0.05; **p<0.01 compared to normal air-exposed controls.

**Figure S2.** TRAIL receptor mRNA levels in the parenchyma in human COPD. Parenchymal cores were collected from human COPD patients. mRNA expression of TRAIL receptors (a) death receptor (DR)4, (b) DR5, (c) decoy receptor (DcR)1 and (d) DcR2 in parenchyma from non-COPD controls or patients with severe COPD.

**Figure S3.** Inflammatory cell subsets in the lung are altered in experimental COPD. Wild type (WT) or TRAIL-deficient (Tnfsf10⁻/⁻) mice were exposed to CS or normal air for eight weeks. (a) Alveolar macrophages (AMs), (b) plasmacytoid dendritic cells (pDCs), (c) natural killer T (NKT) cells, (d) CD4⁺ and (e) CD8⁺ T cells were enumerated per 10⁶ CD45⁺ cells in single cell suspensions of whole lung homogenates by flow cytometry. Data (n = 5-6) presented as means ± s.e.m. are representative of two independent experiments. *p<0.05; ***p<0.001; ****p<0.0001 compared to normal air-exposed WT or Tnfsf10⁻/⁻ controls. ϕp<0.05 compared to normal air-exposed WT controls.

**Figure S4.** Pro-inflammatory cytokine, chemokine and COPD-related factor mRNA expression induced by CS. Wild type (WT) or TRAIL-deficient (Tnfsf10⁻/⁻) mice were exposed to CS or normal air for eight weeks. (a) Interleukin (IL)-33, (b) chemokine
Haw, Starkey et al.,

(C-X-C motif) ligand 1 (CXCL1), (e) CXCL3, (d) chemokine (C-C motif) ligand 4 (CCL4), (e) CCL22 and (f) mucin 5ac (Muc5ac) mRNA expression was determined in whole lung homogenates by qPCR. Data (n = 5-6) presented as means ± s.e.m. are representative of two independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to anti-TRAIL- or isotype-treated normal air-exposed controls.

Figure S5. Schematic representation of proposed mechanisms of how TRAIL contributes to CS-induced experimental COPD. Chronic CS exposure increases TRAIL production by small airway epithelial cells and promotes the accumulation of TRAIL+CD11b+ monocytes in the lungs. TRAIL increases NF-κB activity resulting in increased transcription of pro-inflammatory genes that promote pulmonary inflammation. TRAIL also binds to its receptors to induce apoptosis of alveolar epithelial cells causing emphysema-like alveolar enlargement and impaired lung function. Collectively, these TRAIL dependent effects promote the development of COPD.
### Supplementary Table 1. Custom-designed primers used in qPCR analysis

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HPRT, hypoxanthine–guanine phosphoribosyltransferase
**Supplementary Table 2.** Antibodies used in flow cytometry analysis

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