

Critical link between TRAIL and CCL20 for the activation of T_H2 cells and the expression of allergic airway disease

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The role of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in immune responses mediated by T-helper 2 (T_H2) lymphocytes is unknown. Here we characterize the development of allergic airway disease in TRAIL-deficient (*Tnfrsf10*^{-/-}) mice and in mice exposed to short interfering RNA targeting TRAIL. We show that TRAIL is abundantly expressed in the airway epithelium of allergic mice and that inhibition of signaling impairs production of the chemokine CCL20 and homing of myeloid dendritic cells and T cells expressing CCR6 and CD4 to the airways. Attenuated homing limits T_H2 cytokine release, inflammation, airway hyperreactivity and expression of the transcriptional activator STAT6. Activation of STAT6 by interleukin-13 restores airway hyperreactivity in *Tnfrsf10*^{-/-} mice. Recombinant TRAIL induces pathognomic features of asthma and stimulates the production of CCL20 in primary human bronchial epithelium cells. TRAIL is also increased in sputum of asthmatics. The function of TRAIL in the airway epithelium identifies this molecule as a target for the treatment of asthma.

Asthma is a chronic inflammatory disease of the airways that is a considerable health burden in many developed countries¹. Although inflammation has been linked to disease severity and progression²⁻⁴, the early signals that are released from the respiratory epithelium in response to allergen exposure and that promote inflammation and bronchoconstriction are poorly defined. It is these signals that are crucial switches for the induction of downstream disease processes.

Of the immune pathways activated in allergic asthma, CD4⁺ T_H2 cells have a central role in the development of many hallmarks of the disease²⁻⁴. T_H2 cells are activated in an allergen-specific manner by local antigen-presenting cells to release cytokines such as interleukin (IL)-4, IL-5, IL-9 and IL-13, which contribute to the eosinophilia, mast cell influx, mucus hypersecretion, airway remodeling and enhanced bronchial reactivity to spasmogens (airway hyperreactivity, AHR) that characterize this disorder⁵⁻¹². The initiation and maintenance of the T_H2-type response is primarily dependent on the activation of pulmonary dendritic cells (DCs)¹³⁻¹⁶. The local cytokine and chemokine environment that regulates the recruitment of immature DCs to the airway wall and their transport to regional lymph nodes is tightly regulated and dependent, in part, on the activation of airway epithelial cells and the expression of specific receptors on DCs¹⁷⁻²².

TRAIL is a member of the tumor necrosis factor (TNF) superfamily of cytokines^{23,24}, which have essential roles in the regulation of

different types of immune and inflammatory response²⁵. TRAIL is expressed in various cells (human T_H2 cell clones, eosinophils, neutrophils, DCs and some mouse liver natural killer cells) in an activation-dependent manner²⁶⁻³⁰. Studies of its biological functions have been confined mainly to determining its effects on tumor growth and survival, because TRAIL selectively induces apoptosis in tumor cells, leaving most tissues unharmed²⁹⁻³². Some studies, however, have suggested that this molecule is involved both in the differentiation of human T_H-cell clones *in vitro* and in inflammatory diseases^{27,28,33,34}. Notably, TRAIL has been shown to be upregulated in individuals with asthma after allergen provocation²⁶. Nevertheless, the role of TRAIL in the regulation of T_H2 responses and in the pathogenesis of asthma remains to be determined.

We report here that inhibition of TRAIL function in mice with allergic airway disease, either by gene disruption or by specific RNA interference in the lung, inhibits production of the pulmonary chemokine CCL20 (also known as macrophage inflammatory protein-3 α) and recruitment of myeloid DCs (mDCs) and memory CCR6⁺CD4⁺ T cells into the airways, which in turn attenuates T_H2 cytokine release, inflammation and AHR in a manner dependent on the transcriptional activator STAT6. Thus, we have identified TRAIL as an early signal that shows increased expression in the respiratory epithelium in response to allergen exposure and plays an essential part in promoting T_H2 responses and pathogenesis of asthma.

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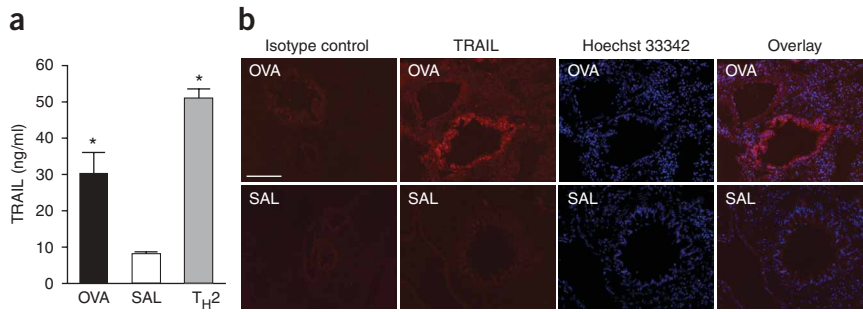


Figure 1 TRAIL is increased in allergic mice and regulated by T_H2 cells. **(a)** Amount of TRAIL in whole-lung lysates from allergic (OVA) mice, mice that were adoptively transferred with ovalbumin-specific T_H2 cells (T_H2), and non-allergic (SAL) mice. Results are mean $\pm$ s.e.m. ($n = 2-3$ mice per group). * $P < 0.05$, OVA versus SAL, and T_H2 versus SAL. **(b)** Pulmonary expression of TRAIL assessed by immunofluorescence staining in representative lung sections from allergic and non-allergic mice. Nuclei were also stained with Hoechst 33342. Scale bar, 50 μ m.

RESULTS

Expression of TRAIL is regulated by T_H2 cells

To identify a potential role for TRAIL in the pathogenesis of allergic airway disease, we first sensitized BALB/c mice with allergen (ovalbumin) and then challenged them with ovalbumin and compared their responses with those of non-allergic, saline-treated mice. Sensitized and challenged (allergic) mice developed a marked increase in total lung TRAIL as compared with non-allergic mice (**Fig. 1a**). Challenge of the airways with ovalbumin without prior sensitization, or conversely sensitization without lung challenge, did not result in an increase in TRAIL in the lung (data not shown).

To determine the direct contribution of T cells to TRAIL production, we transferred ovalbumin-specific T_H2 cells to naive mice and then challenged the mice with ovalbumin (**Fig. 1a**). We have

previously shown that transferred T_H2 cells induce the characteristics of allergic airway disease in response to allergen challenge³⁵. Here we found that transfer of these cells resulted in an increase in TRAIL in the lung (**Fig. 1a**). The cellular source of TRAIL after allergen challenge was primarily the airway epithelium (**Fig. 1b**). Thus, expression of TRAIL in the airway epithelium is upregulated by allergen-specific T_H2 cells.

TRAIL deficiency abolishes AHR and reduces inflammation

Allergic wild-type mice demonstrated AHR with a marked increase in enhanced pause (P_{enh}) and lung resistance, and a concomitant decrease in dynamic compliance and expiratory flow at 50% of tidal volume (EF_{50}) in response to methacholine (**Fig. 2a**; a representative flow curve is shown in **Fig. 2b**). By contrast, lung function

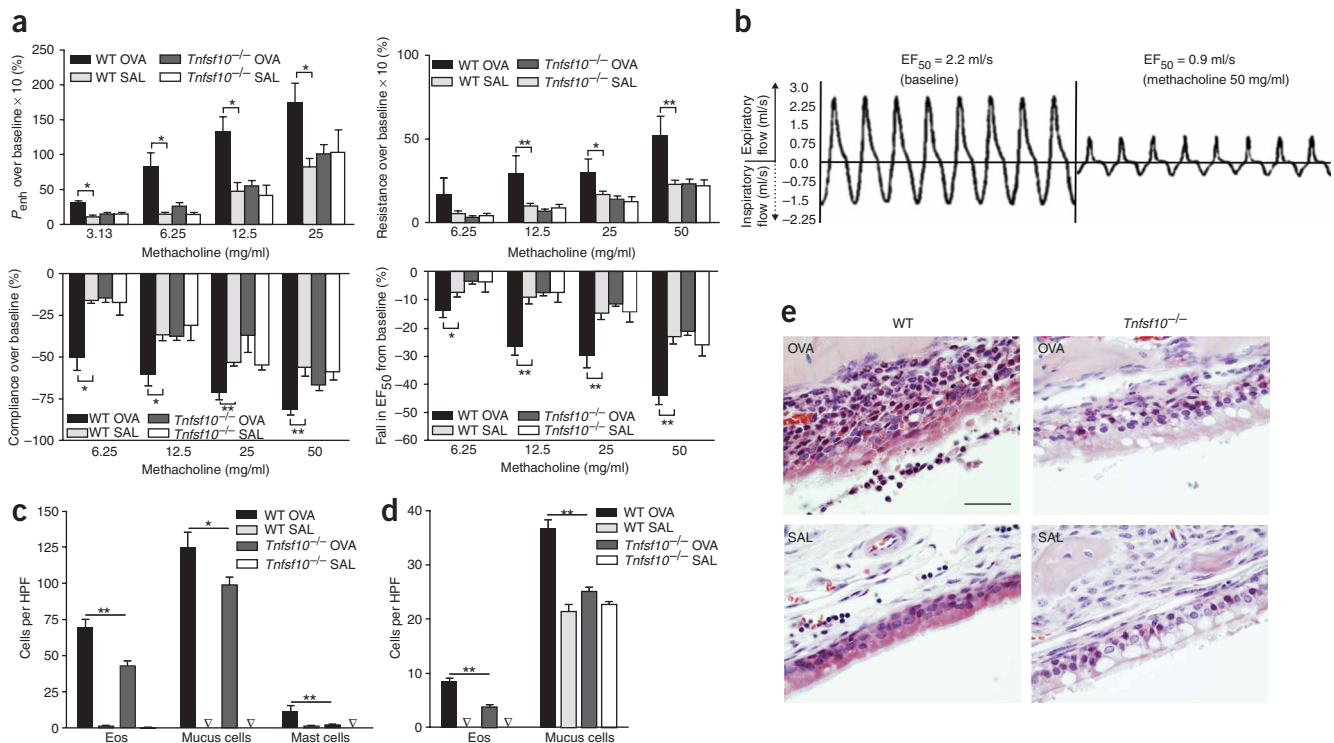


Figure 2 TRAIL deficiency abolishes AHR and reduces airway inflammation. **(a)** P_{enh} , total lung resistance, dynamic compliance and expiratory flow at EF_{50} as a percentage change of baseline measurement (water) in response to inhaled methacholine in allergic (OVA) and non-allergic (SAL) wild-type (WT) and Tnfsf10^{-/-} mice ($n = 6-10$ mice per group). * $P < 0.05$, ** $P < 0.01$. **(b)** Representative flow curve and EF_{50} value at baseline and after inhalation of methacholine in an allergic wild-type mouse. **(c)** Number of peribronchial perivascular eosinophils (original magnification, $\times 1,000$), mast cells ($\times 400$) and mucus-producing cells ($\times 400$) per HPF ($n = 2-3$ mice per group). * $P < 0.05$, ** $P < 0.01$. **(d)** Number of eosinophils ($\times 1,000$) and mucus-producing cells ($\times 400$) in the upper airways (nose) per HPF ($n = 2-3$ mice per group). ** $P < 0.01$. **(e)** Representative sections of the upper airways (nose). Scale bar, 25 μ m. All results shown are mean $\pm$ s.e.m.

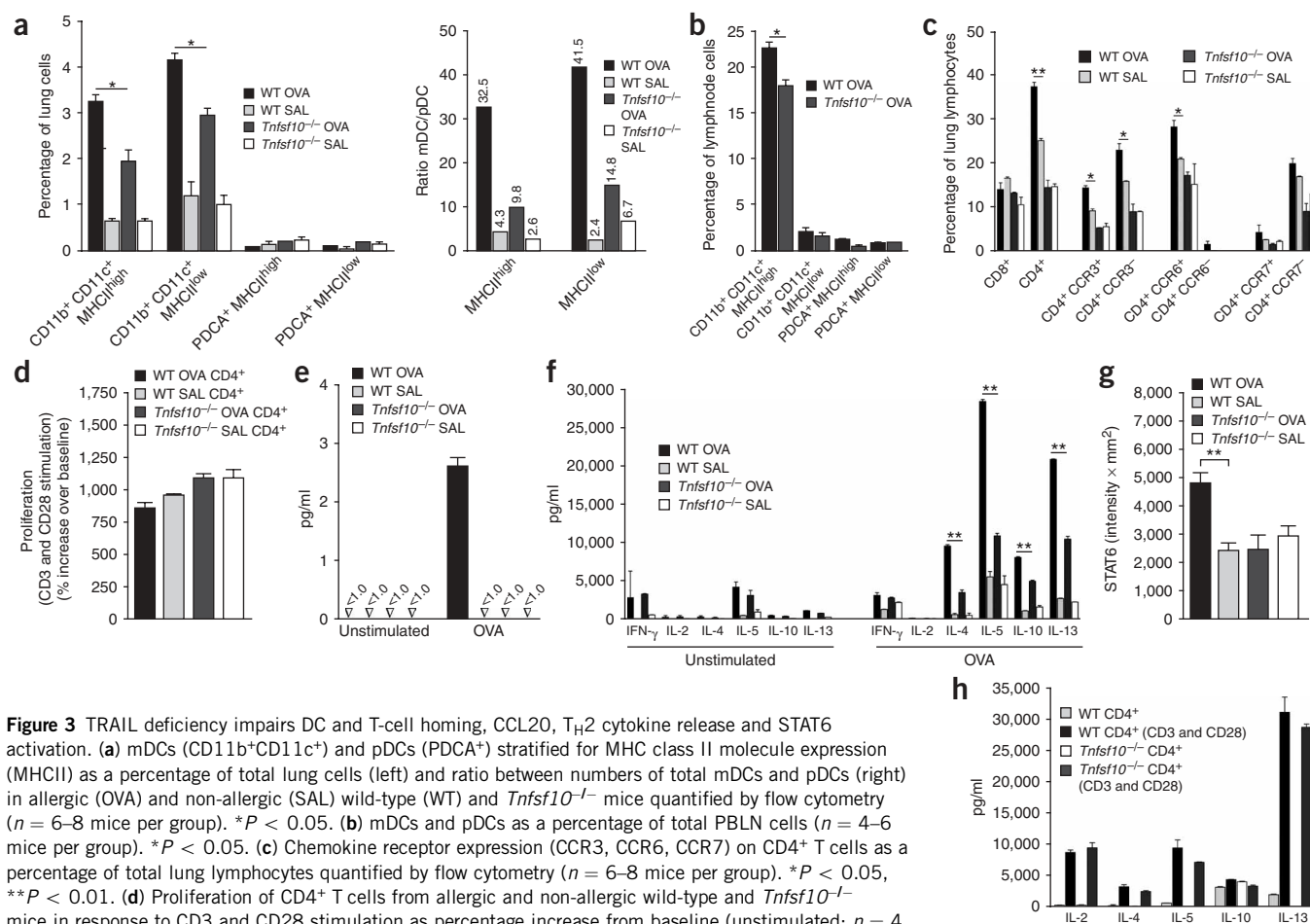


Figure 3 TRAIL deficiency impairs DC and T-cell homing, CCL20, T_H2 cytokine release and STAT6 activation. **(a)** mDCs (CD11b⁺CD11c⁺) and pDCs (PDCA⁺) stratified for MHC class II molecule expression (MHCII) as a percentage of total lung cells (left) and ratio between numbers of total mDCs and pDCs (right) in allergic (OVA) and non-allergic (SAL) wild-type (WT) and *Tnfsf10*^{-/-} mice quantified by flow cytometry ($n = 6-8$ mice per group). $^*P < 0.05$. **(b)** mDCs and pDCs as a percentage of total PBLN cells ($n = 4-6$ mice per group). $^*P < 0.05$. **(c)** Chemokine receptor expression (CCR3, CCR6, CCR7) on CD4⁺ T cells as a percentage of total lung lymphocytes quantified by flow cytometry ($n = 6-8$ mice per group). $^*P < 0.05$, $^{**}P < 0.01$. **(d)** Proliferation of CD4⁺ T cells from allergic and non-allergic wild-type and *Tnfsf10*^{-/-} mice in response to CD3 and CD28 stimulation as percentage increase from baseline (unstimulated; $n = 4$ mice per group). **(e,f)** Ovalbumin-specific CCL20 **(e)** and cytokine **(f)** release from PBLN cells ($n = 5-6$ mice per group). $^{**}P < 0.01$. **(g)** STAT6 levels in nuclear extracts from lung cells ($n = 4$ mice per group). $^{**}P < 0.01$. **(h)** Cytokine release from CD4⁺ T cells isolated from allergic and non-allergic wild-type and *Tnfsf10*^{-/-} mice ($n = 4$ mice per group). All results shown are mean $\pm$ s.e.m.

(P_{enh} , resistance, compliance and EF_{50}) of allergic *Tnfsf10*^{-/-} mice in response to methacholine remained unchanged. Thus, in the absence of TRAIL, AHR in response to pulmonary allergen challenge is completely abrogated.

We also observed a pronounced inflammatory response in allergic wild-type mice that was characterized by an influx of eosinophils and mast cells into the lower airways and mucus hypersecretion (Fig. 2c). These responses were attenuated in allergic *Tnfsf10*^{-/-} mice (Fig. 2c). Inflammation and epithelial shedding were also reduced in the upper airways (nasal mucosa) of allergic *Tnfsf10*^{-/-} as compared with wild-type mice (Fig. 2d,e). These results suggest that TRAIL promotes allergic inflammation of the upper and lower respiratory tract.

TRAIL deficiency impairs DC and T_H2 cell homing

To investigate further the role of TRAIL in allergic airway disease, we determined the effect of TRAIL on DC and T-cell numbers in the lung by using a combination of antibodies and flow cytometry (typical cytometry profiles are shown in Supplementary Fig. 1 online). The mean number of total lung cells was not significantly lower in *Tnfsf10*^{-/-} mice than in wild-type mice (allergic mice: 3.7×10^6 versus 4.7×10^6 , respectively, $n = 30$, $P = 0.06$; non-allergic mice: 3.4×10^6 versus 3.7×10^6 , respectively, $n = 30$, $P = 0.21$). In addition, the percentages of myeloid (CD11b⁺CD11c⁺) DCs (mDCs) that promote T_H2 responses and plasmacytoid (PDCA⁺)

DCs (pDCs) that promote immunological tolerance in the lung of non-allergic *Tnfsf10*^{-/-} were comparable to those of wild-type mice, regardless of the expression level of major histocompatibility complex (MHC) class II molecules on the DC surface (Fig. 3a). As expected, allergic wild-type mice had a higher percentage of mDCs but not pDCs in their airways as compared with non-allergic wild-type mice (Fig. 3a). However, both mDCs and the ratio between mDCs and pDCs (Fig. 3a) were markedly lower in the airways of allergic *Tnfsf10*^{-/-} mice than in wild-type mice. The percentage of MHCII^{hi} mDCs, presumably antigen-loaded DCs, was also significantly lower in peribronchial lymph nodes (PBLNs) from allergic *Tnfsf10*^{-/-} mice than in those from wild-type mice (Fig. 3b). In addition, the percentage of CD4⁺ but not CD8⁺ T cells in the airways was significantly lower in the absence of TRAIL (Fig. 3; $P < 0.01$).

Homing of DCs and CD4⁺ T cells in the lung is regulated by chemokine pathways. In particular, the receptors CCR3, CCR6 and CCR7 have been related to the recruitment of both cell types in the context of T_H2 -mediated immune responses. In allergic wild-type mice, more than 90% of the infiltrating CD4⁺ T cells expressed CCR6, and a smaller population expressed CCR3 and CCR7 ($\sim 40\%$ and $\sim 15\%$, respectively; Fig. 3c). Thus, homing of CD4⁺ T cells to the airways independently of CCR3 or CCR7 is possible. The accumulation of CCR6⁺CD4⁺ T cells in the airways was markedly impaired in

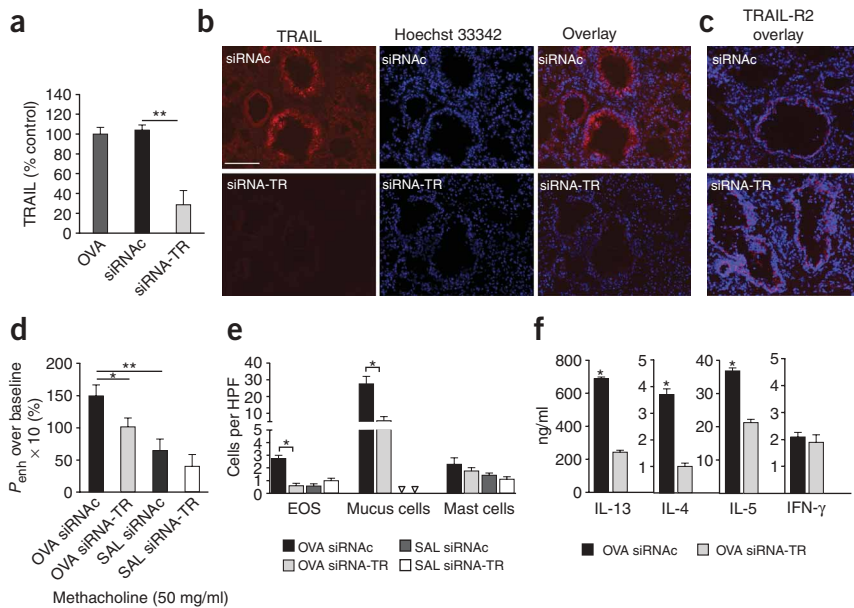


Figure 4 Silencing TRAIL by siRNA abolishes AHR and reduces inflammation and T_H2 cytokine release. **(a)** Amount of lung TRAIL in allergic mice after treatment with siRNA-TR or siRNAc and in allergic (OVA) mice treated with saline only. Results are mean $\pm$ s.e.m. percentages of amount in allergic mice (100%; $n = 3-4$ mice per group). ** $P < 0.01$. **(b,c)** Expression of TRAIL **(b)** and TRAIL-R2 **(c)** assessed by immunofluorescence staining in representative lung sections from allergic wild-type mice treated with siRNA-TR or siRNAc. Nuclei were also stained with Hoechst 33342. Scale bar, 50 μ m. **(d)** P_{enh} as a percentage change of baseline measurement (water) in response to inhaled methacholine in allergic and non-allergic wild-type mice treated with siRNA-TR or siRNAc. Results are mean $\pm$ s.e.m. of maximum response (50 mg/ml of methacholine; $n = 5-6$ mice per group). * $P < 0.05$, ** $P < 0.01$. **(e)** Number of peribronchial eosinophils ($\times 1,000$), mast cells ($\times 400$) and mucus-producing cells ($\times 400$) per HPF. Results are mean $\pm$ s.e.m. ($n = 2-3$ mice per group). * $P < 0.05$. **(f)** Ovalbumin-specific cytokine release from PBLN cells. Results are mean $\pm$ s.e.m. ($n = 5-6$ mice per group). * $P < 0.05$.

allergic *Tnfsf10*^{-/-} mice (**Fig. 3c**); however, TRAIL deficiency did not affect the ability of lung CD4⁺ T cells to proliferate after polyclonal stimulation (**Fig. 3d**). This observation suggests that TRAIL also regulates homing of CCR6⁺CD4⁺ T cells in the airways to promote allergic airway disease.

CCL20, the unique ligand for CCR6, was detected only in PBLNs from allergic wild-type mice (**Fig. 3e**). In addition, release of IL-4, IL-5, IL-10 and IL-13, but not IL-2 or interferon- γ (IFN- γ), from PBLN cells was markedly less in allergic *Tnfsf10*^{-/-} mice than in allergic wild-type mice (**Fig. 3f**) and was correlated with low levels of STAT6 (**Fig. 3g** and **Supplementary Fig. 2** online). However, polyclonal stimulation of equal numbers of CD4⁺ T cells isolated from the PBLNs of allergic wild-type and *Tnfsf10*^{-/-} mice demonstrated comparable amounts of IL-4, IL-5, IL-10 and IL-13 release (**Fig. 3h**). When naive CD4⁺ T cells were cultured under T_H2 -polarizing conditions, no effect of either exogenous TRAIL or loss of endogenous TRAIL on cytokine release was observed (**Supplementary Fig. 3** online). Thus, the reduced amounts of cytokines in the airways of allergic *Tnfsf10*^{-/-} mice are related to activation and homing of T cells, and are not a result of an impaired intrinsic capacity of T_H2 cells to release cytokines in the absence of TRAIL.

Silencing TRAIL abolishes allergic airway disease

We induced loss of TRAIL function specifically in the lung by using synthetic small interfering RNA (siRNA) molecules. First, we verified the specificity of our siRNA duplexes, TRAIL sequence-specific siRNA

(siRNA-TR) and a scrambled control siRNA (siRNAc) *in vitro* (**Supplementary Fig. 4** and **Supplemental Methods** online). siRNA-TR administered directly into the airways of allergic mice reduced TRAIL by 70–90% after 48 h, as compared with siRNAc or saline only (**Fig. 4a**). Immunofluorescence studies confirmed a marked reduction of TRAIL in the airway epithelium of allergic mice treated with siRNA-TR, as compared with siRNAc (**Fig. 4b**). By contrast, the expression of TRAIL receptor 2 (TRAIL-R2) in response to allergen challenge was not affected by siRNA-TR, further demonstrating target specificity (**Fig. 4c**).

To test the therapeutic potential of inhibiting TRAIL in the lung, we used a model of allergic airway disease in which initial sensitization to ovalbumin occurs in the pulmonary compartment and repeated allergen challenge induces a mild allergic inflammatory response. In this model, inflammatory infiltrates are predominantly localized to the airway wall without marked parenchymal inflammation (data not shown and ref. 16), which may reflect human asthma more closely. Treatment of allergic mice with siRNA-TR inhibited the development of AHR (**Fig. 4d**), and reduced eosinophilic inflammation, mucus hypersecretion (**Fig. 4e**) and T_H2 cytokine release by PBLN cells in response to allergen stimulation (**Fig. 4f**). PBLN cells from non-allergic mice treated with siRNA released only small amounts of cytokines (< 5 ng/ml; data not shown). Thus,

directly targeting TRAIL in the airway wall reduces T_H2 responses and allergic airway disease.

TRAIL induces AHR and inflammation

To test whether TRAIL alone is sufficient for the development of characteristics of asthma, we delivered recombinant protein to the airways of naive wild-type mice. Within 24 h, AHR developed along with mucus hypersecretion and an influx of eosinophils and mast cells (**Fig. 5a,b**). Notably, recombinant TRAIL induced the accumulation of both mDCs that were MHCII^{low} and CD4⁺ T cells in naive mice (**Fig. 5c,d**). We instilled inactivated recombinant TRAIL into the airways as a control but did not observe an effect on AHR or cell influx (data not shown).

Next, PBLN cells were isolated from mice treated with recombinant TRAIL or control vehicle and cultured for 6 d. TRAIL treatment resulted in the release of substantial amounts of IL-13 and IL-5 from PBLN cells (**Fig. 5e**). Delivery of recombinant TRAIL did not result in an increase in nuclear DNA fragmentation, indicative of apoptosis, in pulmonary cells (**Supplementary Fig. 5** online). Thus, recombinant TRAIL promotes the development of AHR, influx of mDCs and T cells, and release of T_H2 -like cytokines in the lungs of naive mice.

TRAIL-mediated airway hyperactivity is dependent on IL-13

We examined the role of IL-13 as an effector molecule downstream of TRAIL, because this molecule (like TRAIL) is known to be an essential regulator of AHR, mucus hypersecretion and influx of inflammatory

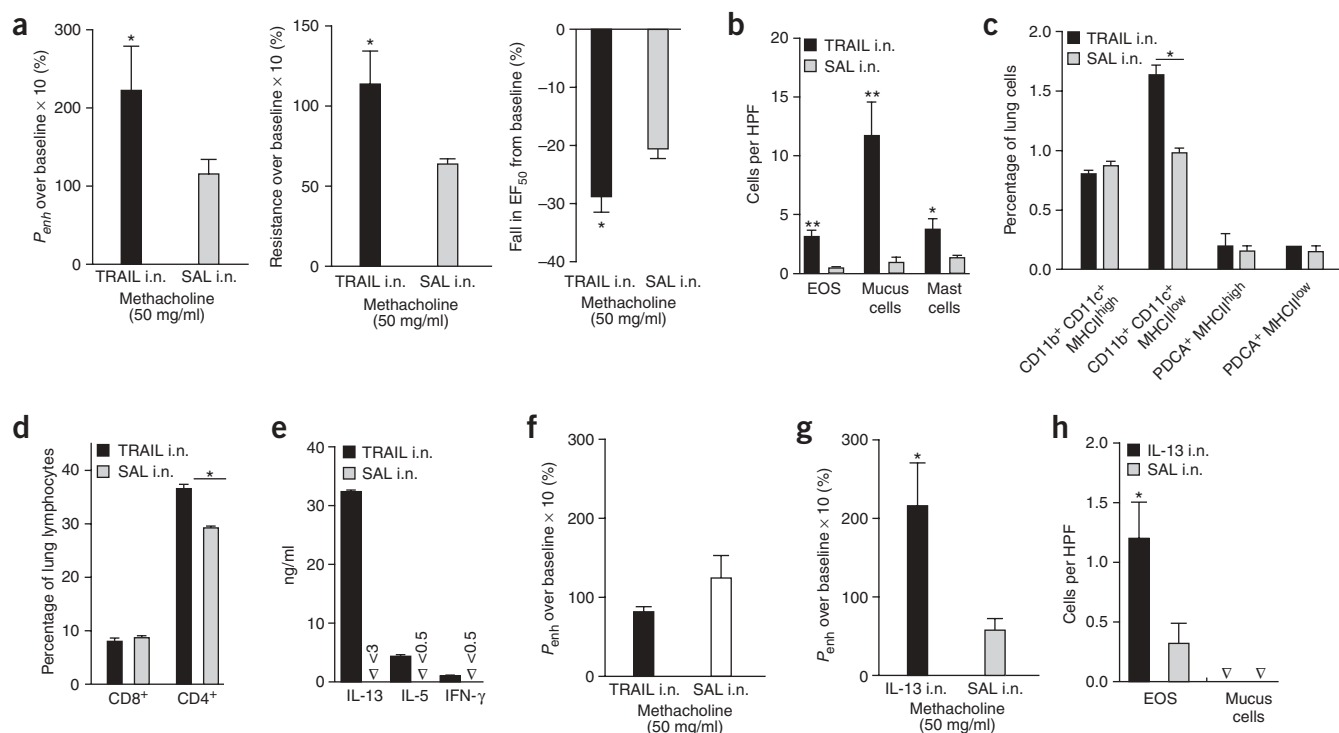


Figure 5 TRAIL induces AHR, inflammation and cytokine release in an IL-13-dependent manner. (a) P_{enh} , total lung resistance and expiratory flow at EF_{50} as a percentage change of baseline measurement (water) in response to inhaled methacholine in naive wild-type mice 24 h after i.n. application of TRAIL or saline (SAL). Results are mean $\pm$ s.e.m. of maximum response (50 mg/ml of methacholine; $n = 5-6$ mice per group). * $P < 0.05$. (b) Number of peribronchial eosinophils ($\times 1,000$), mast cells ($\times 400$) and mucus-producing cells ($\times 400$) per HPF. Results are mean $\pm$ s.e.m. ($n = 2-3$ mice per group). * $P < 0.05$, ** $P < 0.01$. (c) mDCs (CD11b $^{+}$ CD11c $^{+}$) and pDCs (PDCA $^{+}$) stratified for MHC class II molecule expression as a percentage of total lung cells. Results are mean $\pm$ s.e.m. ($n = 4-5$ mice per group). * $P < 0.05$. (d) CD4 $^{+}$ T cells as a percentage of total lung cells gated for lymphocytes. Results are mean $\pm$ s.e.m. ($n = 5-6$ mice per group). * $P < 0.05$. (e) Cytokine release from PBLN cells. Results are mean $\pm$ s.e.m. ($n = 5-6$ mice per group). (f) P_{enh} in naive *Il13* $^{-/-}$ mice 24 h after i.n. application of TRAIL or saline. (g) P_{enh} in naive *Tnfsf10* $^{-/-}$ mice 24 h after i.n. application of IL-13 or saline. In f,g, results are mean $\pm$ s.e.m. of maximum response (50 mg/ml methacholine; $n = 5-6$ mice per group). * $P < 0.05$. (h) Number of peribronchial eosinophils ($\times 1,000$) and mucus-producing cells ($\times 400$) per HPF. Results are mean $\pm$ s.e.m. ($n = 2-3$ mice per group). * $P < 0.05$.

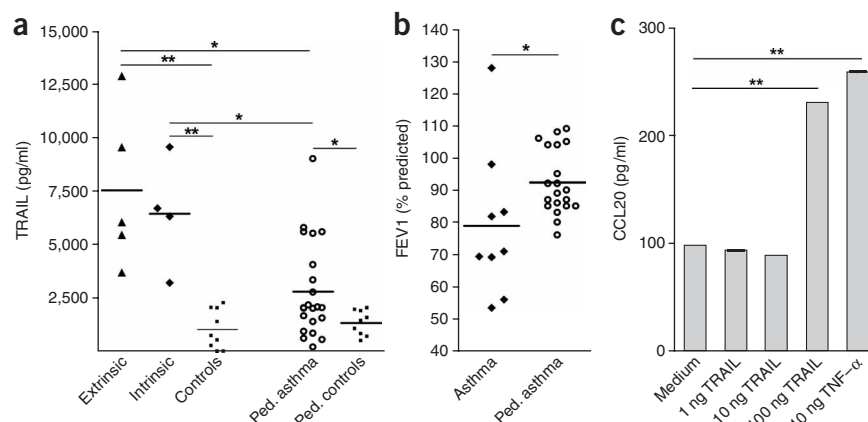
cells. We delivered recombinant TRAIL into the lungs of naive *Il13* $^{-/-}$ mice or conversely IL-13 into the airways of naive *Tnfsf10* $^{-/-}$ mice. In contrast to wild-type mice, *Il13* $^{-/-}$ mice did not develop AHR (Fig. 5f), airway inflammation or T_H2 like responses (Supplementary Fig. 6 online) after exposure to recombinant TRAIL; however, delivery of IL-13 into the airways of *Tnfsf10* $^{-/-}$ mice induced AHR (Fig. 5g) and an increase in the number of airway eosinophils (Fig. 5h).

Therefore, TRAIL regulates AHR and airway inflammation ultimately through IL-13-mediated pathways.

TRAIL is increased in sputum from asthmatics

We analyzed TRAIL in induced sputum samples from individuals with asthma and detected larger amounts in sputum from both allergic and non-allergic adults with asthma and in sputum from children with

Figure 6 TRAIL is increased in sputum of individuals with asthma and induces CCL20 release from bronchial epithelial cells. (a) TRAIL in induced sputum samples determined by ELISA. Results are mean $\pm$ s.e.m. (5 allergic and 4 non-allergic adults with asthma, and 9 adult controls; 21 allergic children with asthma and 9 children without asthma). * $P < 0.05$, ** $P < 0.01$. (b) Forced expiratory volume in the first second as a percentage of the predicted volume (FEV1%). * $P < 0.05$. (c) Release of CCL20 from primary bronchial epithelium cells after stimulation with recombinant TRAIL or recombinant TNF- α . Results are mean $\pm$ s.e.m. ($n = 2$ per group). ** $P < 0.01$.



allergic asthma, as compared with their respective controls (Fig. 6a). Notably, adults with asthma had significantly more sputum TRAIL as compared with children with asthma (Fig. 6a; $P < 0.05$). In addition, a greater impairment of lung function was observed in the former group (Fig. 6b).

TRAIL promotes CCL20 release from bronchial epithelial cells

To investigate the direct effect of TRAIL on CCL20 production, we incubated bronchial epithelium cells obtained from individuals with asthma with increasing concentrations of recombinant TRAIL or recombinant TNF- α as a positive control²¹. Recombinant TRAIL induced the release of CCL20 from primary bronchial epithelium tissue (Fig. 6c), suggesting that TRAIL may promote homing of DCs and T_H2 cells in the airways of individuals affected with asthma.

DISCUSSION

We have shown that T_H2-dependent production of TRAIL in the airway epithelium is both necessary and sufficient for the development of characteristics of allergic airway disease. We found that TRAIL promoted the development of T_H2 immune responses through CCL20-mediated recruitment of mDCs and memory CCR6⁺CD4⁺ T cells to the lungs and through activation of STAT6-mediated pathways—all elements known to be crucial for the development of allergic inflammation and AHR^{8–10,13,14,35–41}.

After allergen provocation, TRAIL was rapidly expressed (within 48 h) in the airway epithelium, which is in immediate proximity to regions where mucosal DCs are recruited to capture antigens that are transported across the epithelial tight junction barrier. It is by inhibiting mDC recruitment to the lung that TRAIL seems to exert, in part, suppression of T_H2 responses and allergic inflammation. We found here that inhibition of TRAIL signaling altered the balance between mDCs that promote T_H2 responses and pDCs that maintain tolerance to inhaled antigens in the airways. Specifically, allergic *Tnfsf10*^{−/−} mice had a reduced mDC/pDC ratio (~10:1) in contrast to allergic wild-type mice (~30:1; Fig. 3b). It has been previously shown in culture that pDCs exert a suppressive effect on mDC-induced cytokine production from antigen-activated T_H2 cells¹⁶. When mDCs are cocultured with antigen-specific T cells, the suppressive effect of pDCs is most pronounced at low ratios (for example, an mDC/pDC ratio of 10:1), and at higher ratios the inhibitory effect is almost lost¹⁶. Thus, *Tnfsf10*^{−/−} mice may be more predisposed to develop tolerance, rather than a harmful immune response, to inhaled allergens in the lung, as reflected in the reduced release of T_H2 cytokines without a concomitant T_H1 bias. The role of mDCs in the activation of memory T-cell responses is also supported by the fact that their depletion at the time of allergen challenge abrogates important features of allergic airway disease in mice including airway inflammation, mucus hypersecretion and AHR³⁷. DCs can also affect T-cell responses independently of allergen through co-stimulatory molecules^{37–39}. Such mechanisms may underpin our observations with recombinant TRAIL: when TRAIL was directly instilled into the lungs of naive mice, it promoted (in the absence of allergen) the homing of mDCs to the lung that were low in MHC class II molecule expression, and this homing correlated with the release of T_H2 cytokines.

TRAIL also markedly altered the spatial distribution of CD4⁺ T cells in the lung. Reduced numbers of CD4⁺ T cells were found in the airways of *Tnfsf10*^{−/−} mice, and conversely recombinant TRAIL induced a substantial increase in the number of these cells in the lung. Migration of CD4⁺ T cells into the lung was associated with CCR6 expression on the cell surface, and homing of CCR6⁺CD4⁺ T cells into the airways was markedly impaired in the absence of

TRAIL. Accordingly, production of CCL20, the unique ligand for CCR6, must be tightly regulated by TRAIL, because this chemokine was not detected in the lung of allergic *Tnfsf10*^{−/−} mice. Notably, CCR6 regulates not only the recruitment of allergen-primed memory T cells and but also the homing of DCs^{18–20}. Thus, loss of CCL20 production in allergic *Tnfsf10*^{−/−} mice may explain the reduced numbers of mDCs in the airways after allergen challenge. The essential role of CCL20-CCR6 interactions in the pathogenesis of asthma has also been observed in CCR6-deficient mice, which are protected from the development of crucial features of allergic disease such as eosinophilic inflammation and AHR^{40,41}. The molecules that induce CCL20 release from epithelial cells *in vivo* have not been defined, but *in vitro* studies have suggested that TNF- α and IL-1 β can control expression²¹. We have shown here that TRAIL regulates the release of CCL20 both in primary human bronchial epithelium tissue and in allergic mice to promote antigen-specific T-cell memory responses in the lung.

Although it is established that T_H2-regulated effector pathways such as those mediated by IL-13 and STAT6 crucially regulate disease processes in asthma^{8,9,35,36,42–45}, we have only limited knowledge on the factors that activate these important mechanisms in the airways in response to allergen provocation. We have shown here that inhibition of TRAIL attenuates IL-13 release and abolishes STAT6 activation in the lung. *Il13*^{−/−} mice were fully protected from TRAIL-induced effects such as induction of inflammation, AHR and T_H2 cytokine release, indicating that TRAIL plays a central part in the activation of IL-13- and STAT6-mediated effector functions. By contrast, delivery of IL-13 to the lungs of *Tnfsf10*^{−/−} mice restored the ability of these mice to develop AHR and airways inflammation. Collectively, these data suggest that TRAIL—like IL-13—is essential for the development of pathognomic features of asthma. TRAIL, however, clearly mediates its effects upstream of IL-13, and its mechanism of action is linked to the upregulation of chemokine networks that promote homing of DCs and T_H2 cells in the lung.

Our data indicate that activation of TRAIL in the airway epithelium in response to allergen provocation may be an important initial step that links T_H2 cells to STAT6-dependent processes regulating crucial pathological events associated with asthma. The increase in TRAIL in individuals with asthma and the spatial distribution of TRAIL in the lung identify this molecule as a potential therapeutic target for pharmaceutical intervention against the acute exacerbations and chronicity that are characteristic of this disorder.

METHODS

Mice. We obtained BALB/c mice (aged 6–14 weeks) from the Special Pathogen Free Facility of the University of Freiburg and the University of Newcastle. We crossed BALB/c *Tnfsf10*^{−/−} and *Il13*^{−/−} mice (see Acknowledgments) to the BALB/c background for ten generations. Experiments were approved by the animal ethics committee of the Regierungspraesidium Freiburg, Germany, and the University of Newcastle, Australia. Mice were housed in approved containment facilities.

Induction of allergic airways disease. We sensitized mice by intraperitoneal injection with 50 μ g of ovalbumin in 1 mg of Alhydrogel (CSL), and after 1 week we challenged them with 50 μ g of ovalbumin intranasally (i.n.) for 7 d under light isoflurane anesthesia (Supplementary Fig. 7 online). Nonsensitized mice received 1 mg of Alhydrogel in 0.9% saline and were challenged with sterile saline. Where indicated (Fig. 4), mice were sensitized with 100 μ g of ovalbumin or saline i.n. daily for 2 days, and 10 d later were challenged with 10 μ g of ovalbumin or saline i.n. daily for 7 d. We killed mice 24 h after the last challenge (Supplementary Fig. 7).

T_H2 cell transfer. We generated ovalbumin-specific CD4⁺ T_H2 cells and transferred them intravenously (4×10^6) to naive recipients as described³⁵. Mice received 50 μ g of ovalbumin i.n. on alternate days for 7 d.

AHR measurement. We assessed AHR noninvasively by barometric plethysmography³⁵ and invasively in separate groups of anesthetized mice by measurement of total lung resistance, dynamic compliance and EF₅₀ (Supplementary Methods). Percentage increase over baseline (water) in response to nebulized methacholine was calculated.

siRNA. Target mRNA sequence was downloaded from PubMed (National Center for Biotechnology Information) and applied to the Whitehead siRNA prediction online tool⁴⁶. We ordered siRNAs with no similarities to other sequences from Dharmacon (Option A4, Dharmacon Research). The sequence of the antisense strand of siRNA-TR was 5'-dTdTCUGCGAAGGUUCUACCA GAG-3'. siRNAC was purchased from Dharmacon (nonspecific siRNA VIII) and was chosen to have an equivalent CG content. We started siRNA administration (3.75 nmol) 48 h before pulmonary sensitization and continued it every second day until mice were killed.

Flow cytometry analysis. We stained cells with anti-CCR3-AlexaFluor647, anti-CCR6-AlexaFluor647, anti-CCR7-phycoerythrin (PE), anti-CD4-fluorescein isothiocyanate (FITC), anti-CD8a-PE, anti-CD11b-PerCP, anti-CD11c-PE, biotinylated anti-MHCII conjugated to streptavidin-FITC, respective isotype controls (Pharmingen) and anti-mPDCA-1-PE (Miltenyi). Numbers of positive cells were quantified by flow cytometry (FACS Calibur, Becton Dickinson).

Airway morphology studies. Lung tissue and nasal tissue were stained, and cells were identified by morphological criteria and quantified by counting ten high-powered fields (HPFs) in each slide³⁵.

Immunohistological detection. We fixed and incubated snap-frozen lung sections with biotinylated antibody to mouse TRAIL, PE-labeled antibody to TRAIL-R2, or isotype controls. The sections were then washed and incubated with Streptavidin CyChrome (Pharmingen) if appropriate. Nuclei were counterstained with Hoechst 33342 (Sigma). Microscopic analysis was performed with an Axioskop and AxioVision software (Zeiss).

In vivo recombinant TRAIL and IL-13 treatment. Wild-type and *Il13*^{-/-} mice received 10 µg i.n. of functional mouse recombinant TRAIL (Biomol). *Tnfrsf10*^{-/-} mice received 10 µg i.n. of mouse IL-13 (Peprotech).

Nuclear extraction and immunoblotting. We extracted nuclear proteins from lung cells and measured the protein concentration with an NE-PER Nuclear and Cytoplasmic Extraction Reagents and BCA kit (Pierce Biotechnology). Samples were resolved by electrophoresis, transferred onto a nitrocellulose membrane and then blocked. Membranes were incubated with rabbit anti-mouse STAT6 (sc-981; Santa Cruz Biotechnology), washed and then incubated with anti-rabbit IgG-HRP (Sigma). We detected emission with the ChemiDoc XRS system and analyzed it with Quantity One software (Bio-Rad).

Cytokine and chemokine analysis. PBLN and lungs were excised, filtered and cultured in the presence or absence of 200 µg/ml of ovalbumin (optimal concentration) for 6 d. We determined the concentrations of IL-13, human TRAIL (R&D Systems), IL-4, IL-5, IFN-γ (Pharmingen), and mouse and human CCL20 (R&D Systems) in culture supernatants by ELISA. We also quantified cytokines (IL-2, IL-4, IL-5, IL-10 and IL-13) with a five-plex multiplex cytokine analysis kit (Bio-Plex Cytokine Assay, Bio-Rad). Mouse TRAIL was measured by sandwich ELISA using antibodies isolated from the N2B2 clone⁴⁷ in conjunction with a biotinylated antibody to mouse TRAIL (BAF1121, R&D Systems).

Induced sputum. Nonsmoking adults aged 32–74 years ($n = 9$; five males) with a diagnosis of bronchial asthma by American Thoracic Society criteria⁴⁸ and 21 children with allergic asthma aged 7–17 years were recruited. Individuals without respiratory symptoms and without asthma were used as controls (nine adults and nine children). Sputum was induced with hypertonic saline (4.5%) and processed as described⁴⁹. We measured TRAIL in supernatants by ELISA. All studies were approved by the local ethics committee, and written informed consent was obtained.

Primary bronchial epithelial tissue cultures. We obtained primary bronchial epithelial cells from individuals with asthma by bronchoscopy and cultured them as described⁵⁰. After one passage, the cells were seeded onto 12-well trays, cultured until 70% confluency, and incubated with recombinant TNF-α (R&D) or recombinant TRAIL (Biomol) for 24 h. We measured CCL20 in supernatants. The study was approved by the local ethics committee, and written informed consent was obtained.

Statistics. The significance of differences between groups was analyzed by Student's *t*-test or Mann-Whitney test as appropriate.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

M.W. and A.C. performed mouse experiments, analyzed and interpreted data, and generated figures. A.C., J.L.S., M.V.K., P.A.B.W., B.W. and P.G.G. performed and supervised human studies, analyzed and interpreted data, and contributed to manuscript discussion. N.H. performed and analyzed parts of the invasive AHR measurements. M.J.S. and K.I.M. backcrossed and bred mice, contributed to data discussion, and revised the manuscript. H.Y. generated the N2B2 clone and TRAIL 2PK-3 cells, purified TRAIL antibodies, contributed to data discussion, and revised the manuscript. P.S.F. supervised mouse studies, designed mouse experiments, analyzed and interpreted data, and edited the manuscript. J.M. coordinated, designed, supervised and performed mouse and human studies; generated figures; analyzed and interpreted data; and drafted and edited the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/naturemedicine/.

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Online Supplementary

Critical link between TRAIL and CCL20 for the activation of T_H2 cells and the expression of allergic airway disease

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Supplementary Methods

Effect of TRAIL on cytokine release of CD4⁺ T_H2 cells. CD4⁺ T cells from naive WT and *Tnfsf10*^{-/-} mice were isolated from splenocytes with magnetic-activated cell sorting and cultured at 2 × 10⁵ cells/well in complete medium in the presence of anti-CD3 (50 ng/ml; clone 2C11), recombinant murine IL-4 (40 ng/ml), and anti-IFN-γ (80 μg/ml; clone R46A2) for 4 days to generate T_H2 cells. In some experiments, recombinant TRAIL (100ng/ml, optimal dose to elicit CCL20 release in airway epithelial cell cultures) was also added to the cultures ("TRAIL/medium"). T_H2 cells (2 × 10⁵ cells/well) were then restimulated in the presence of anti-CD3 (50 ng/ml) and anti-CD28 (1 μg/ml) in 96-well plates (250 μl/well) for 72 h to determine cytokine release. In some experiments, recombinant TRAIL (100ng/ml) was also added to the cultures during restimulation ("medium/TRAIL"). Cell-free culture supernatants were collected and stored in aliquots at -70°C until analysis by ELISA.

Measurement of AHR. Responsiveness to increasing aerosol concentrations of methacholine was assessed in conscious unrestrained mice by barometric plethysmography and confirmed by invasive measurement of total lung resistance (RI), dynamic compliance (C_{dyn}), and expiratory flow at 50% of tidal volume (EF₅₀) in anesthetized mice (0.2 mg/10g xylazine and ketamine 0.4mg/10g), using apparatus and software supplied by Buxco. The barometric plethysmography yields a dimensionless parameter known as enhanced pause (Penh) that reflects changes in waveform of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expiration. The invasive pulmonary mechanics were measured in response to methacholine in separate groups of mice. Anesthetized animals were tracheotomized and applied to the Buxco restrained animal chamber. Ventilation rate was set at 130 strokes/minute and 175μL stroke volume. The effective

range of peak end expiratory pressure was set between ± 25 cm H₂O, the flow transducer was calibrated to 1ml air injected by a syringe. Animals were given time to acclimate and then exposed to increasing vaporized concentrations of methacholine. Differences in pressure and flow parameters were collected by Buxco's software (Biosystem XA for windows) and analysed according to Buxco's recommended internal algorithm. Percentage increase over baseline (water) of Penh, RI, Cdyn, and EF₅₀ was calculated for each dose.

Fluorescein isothiocyanate (FITC) labeling of siRNA. siRNA was FITC-labeled with Label IT® siRNA Tracker (Mirrus) according to manufactures recommendations for siRNA duplexes.

***In vitro* transfection of cells.** 2PK-3 B lymphoma cells genetically engineered to express murine TRAIL were cultured in complete medium (RPMI 1640, Invitrogen). Twenty-five thousand cells/well were seeded overnight in a 96 well plate (Nunc) and transfected the next day with 300 nM siRNA and a lipid content of 1µl/well oligofectamine (Invitrogen) according to the manufacture's recommendations in the absence of FCS.

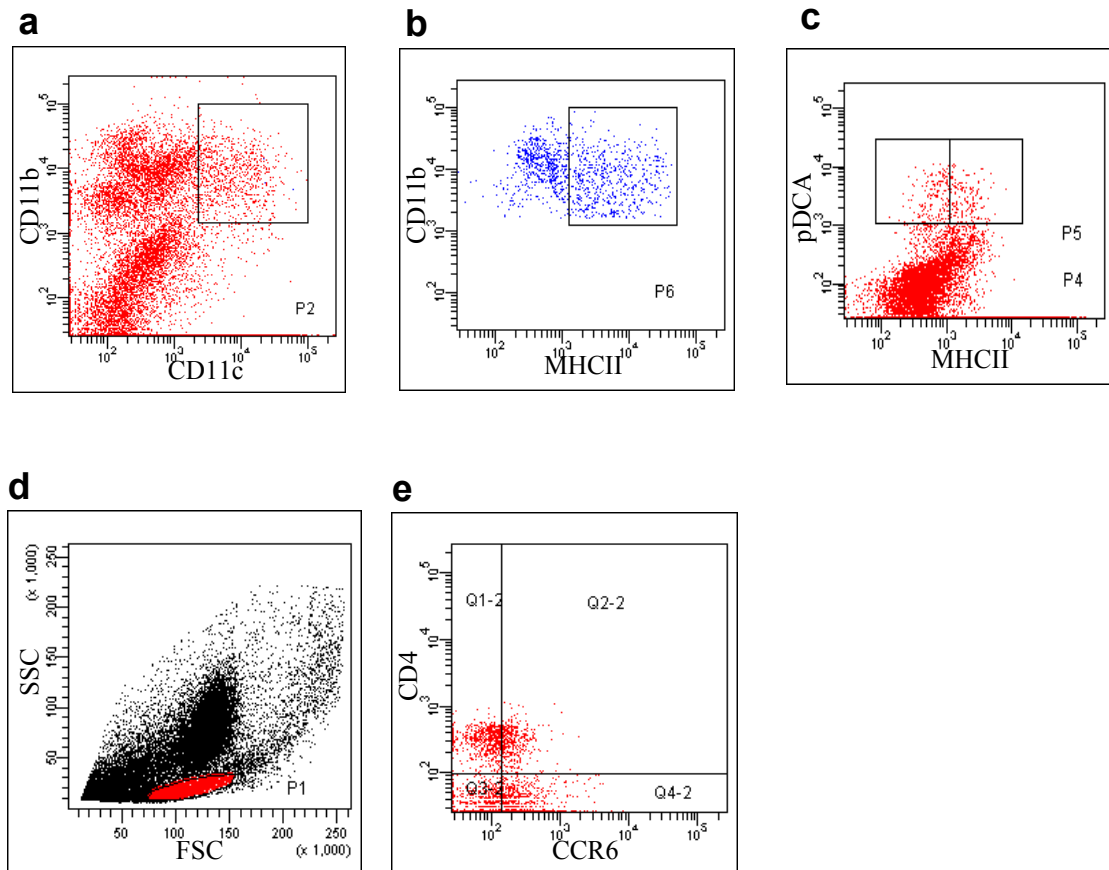
Flow cytometry analysis. 2PK-3 cells were incubated with biotinylated anti-mTRAIL antibody (BAF1121, R&D Systems) at a concentration of 0.25 ng/µL for 60 min, washed, and stained with Streptavidin Cy-Chrome® (0.75µl/250 µl) (BD Biosciences Pharmingen) for 15 min in the dark. Surface expression of mTRAIL was assessed 24, 48, 72, 96 hours after siRNA transfection by flow cytometry (FACS Calibur, BD Biosciences Pharmingen). Analysis (CellQuest, BD Biosciences Pharmingen) was performed by overlapping peaks of mTRAIL-CyChrome marked cells and background ('CyChrome' only marked cells).

Preparation of lung lysates. One hundred mg of tissue was dissolved in 1000 µL PBS and disrupted with D-Stroy-Sticks (Bio-Rad) on ice. Unsolved particles were spun down at 10.000 g for 10 min. and the supernatants were analysed for TRAIL levels immediately. The remaining lung tissue was used for immunohistology.

TUNEL-based apoptosis detection in lung sections. Snap-frozen lung sections (10 micron) were dried and then incubated with 3.7% formaldehyde solution for 10 min. at room temperature. Slides were washed with PBS and processed with an in situ apoptosis detection kit according to the manufactors instructions (TACS™ TdT Kit, R&D Systems). Briefly, after premeabilization with proteinase K endogenous

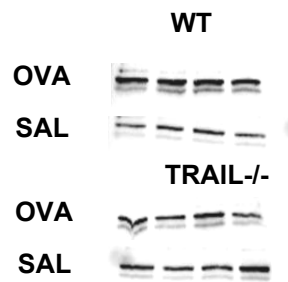
peroxidase was quenched using hydrogen peroxide. Positive control samples were treated with TACS-nuclease after permeabilization to generate DNA breaks in the majority of cells before quenching. Next, samples were incubated with biotinylated nucleotides that were incorporated into the 3'-OH ends of the DNA fragments by Terminal deoxynucleotidyl Transferase (TdT). The biotinylated nucleotides were detected by using streptavidin-horseradish peroxidase conjugate followed by the substrate diaminobenzidine (DAB). Negative controls were processed identically without adding streptavidin-horseradish peroxidase conjugate. Samples were counterstained with methyl green to discriminate apoptotic from necrotic cells.

Supplementary Figure 1



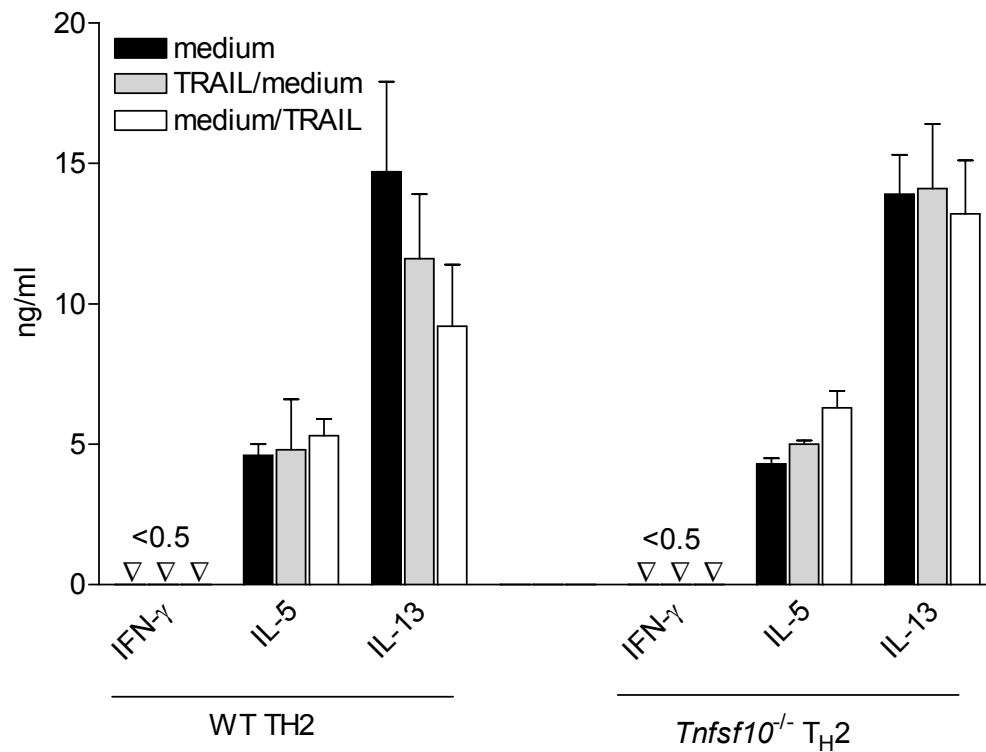
Supplement Fig 1: Phenotypic characterization of DCs and CD4⁺ T cells in lung tissue. **(a)** Lung cells were stained with anti-CD11b PerCP and anti-CD11c PE and respective isotype controls. **(b)** CD11b⁺CD11c⁺ cells (mDCs) were stained with biotinylated anti-MHCII and respective isotype controls. **(c)** Lung cells were stained with anti-mPDCA-1 PE, biotinylated anti-MHCII conjugated with Streptavidin FITC, and respective isotype controls. **(d)** Lung cells gated for lymphocytes. **(e)** Lymphocyte gated region stained with anti-CCR6 Alexa fluor 647 and anti-CD4.

Supplementary Figure 2



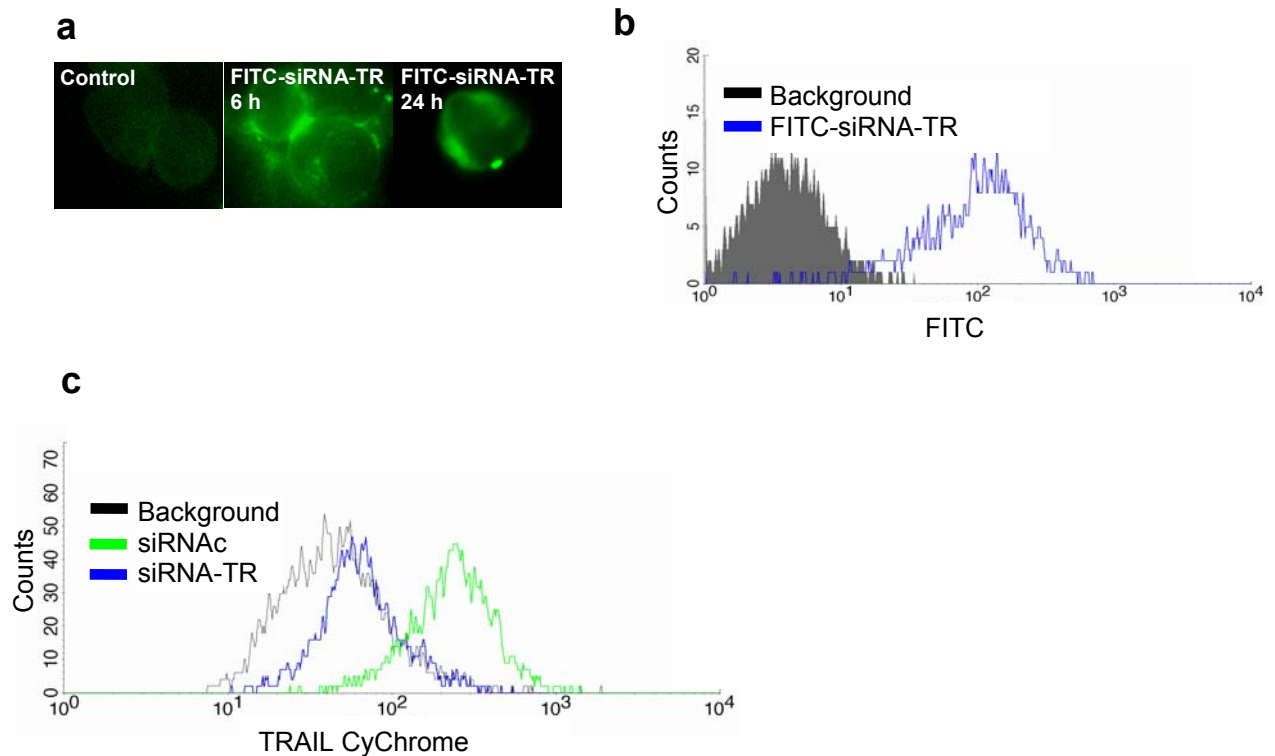
Supplement Fig 2: STAT6 levels in nuclear extracts from whole lungs.

Supplementary Figure 3



Supplement Fig 3: Effect of endogenous and exogenous TRAIL on cytokine release in CD4⁺ cells. CD4⁺ T cells from naive WT and *Tnfsf10*^{-/-} mice were cultured under T_H2 polarising conditions in the presence (“TRAIL/medium”) or absence of TRAIL (100ng/ml). T_H2 cells were then restimulated in the presence of anti-CD3 and anti-CD28 to determine cytokine release. In some experiments, recombinant TRAIL (100ng/ml) was also added to the cultures during restimulation (“medium/TRAIL”). Results are mean $\pm$ s.e.m. (n=4 mice per group)

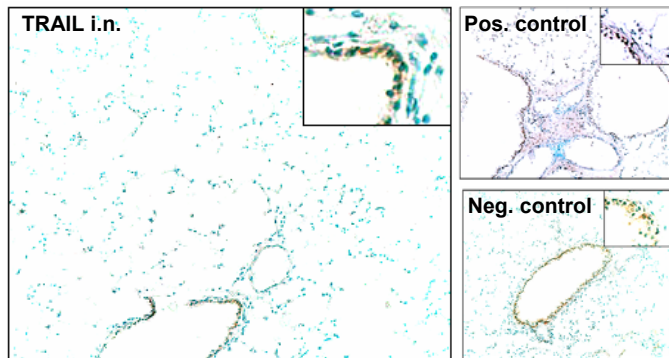
Supplementary Figure 4



Supplement Fig 4: Validation of in-vitro siRNA targeting TRAIL. 2PK-3

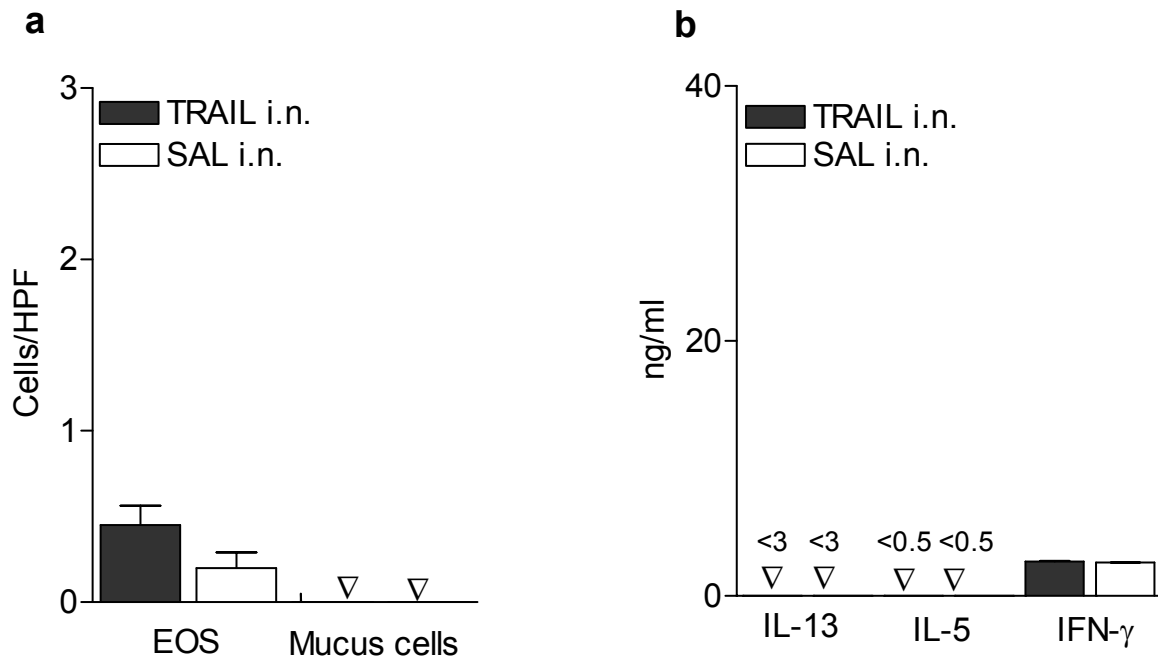
lymphoma cells genetically engineered to overexpress mouse TRAIL were transfected with FITC-labeled TRAIL-specific siRNA (siRNA-TR). **(a)** 6 h after transfection siRNA localised in the cell membrane and 24 h after transfection in the cytoplasm. **(b)** 2PK-3 cells transfected with FITC-labeled siRNA-TR quantified by flow cytometry 24 h after transfection. **(c)** TRAIL surface staining in TRAIL overexpressing 2PK-3 cells transfected with scrambled, non-sense siRNA (siRNA-TR) or siRNA-TR quantified by flow cytometry 48h after transfection. Only siRNA-TR silences TRAIL as determined by lack of TRAIL surface staining.

Supplementary Figure 5



Supplement Fig 5: TUNEL-based apoptosis detection. Representative lung sections of TRAIL treated (TRAIL i.n.) mice. Dark nuclear staining is indicative of DNA fragmentation (pos. control).

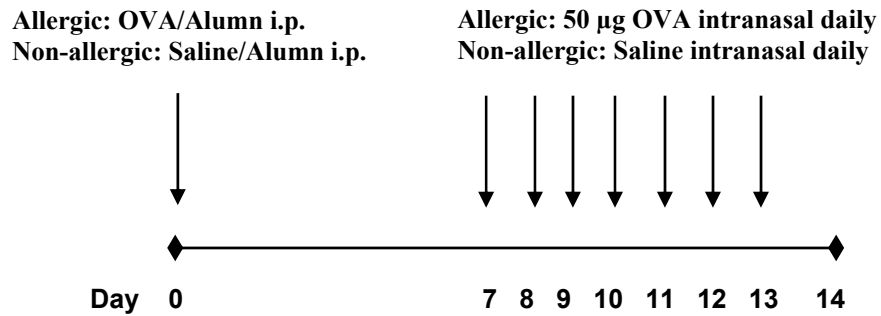
Supplementary Figure 6



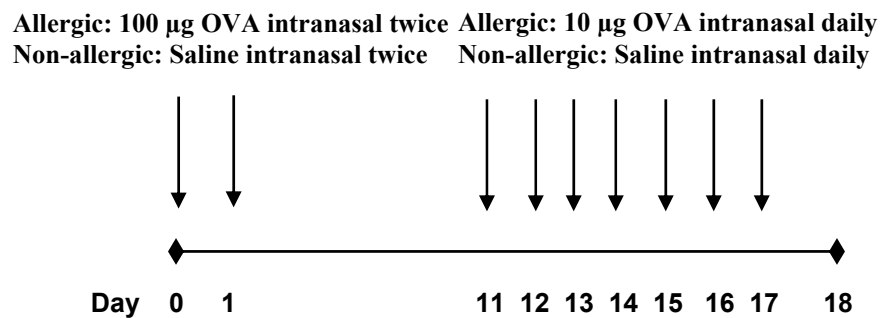
Supplement Fig 6: Induction of inflammation and cytokine release by TRAIL is IL-13 dependent. Airway inflammation and cytokine release in naïve *IL-13*^{-/-} mice 24 h after i.n. application of TRAIL as compared to saline (SAL). **(a)** Number of peribronchial eosinophils (x1000) and mucus producing cells (x400) per high-power fields (HPFs). Results are mean $\pm$ s.e.m. (n=2-3 mice per group). **(b)** Cytokine release from cells isolated from peribronchial lymphnodes. Results are mean $\pm$ s.e.m. (n=5-6 mice per group)

Supplementary Figure 7

a



b



Supplement Fig 7: Protocol diagrams for the induction of allergic airway disease.

(a) Intraperitoneal (i.p.) ovalbumin (OVA) sensitisation followed by pulmonary OVA challenges. (b) Pulmonary OVA sensitisation followed by OVA challenges