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TRAIL regulates MID1, TSLP, inflammation and remodelling in experimental eosinophilic oesophagitis

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Abstract

Background—Eosinophilic Oesophagitis (EoE) is an inflammatory disorder of the oesophagus defined by eosinophil infiltration and tissue remodelling with resulting symptoms of oesophageal dysfunction. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) promotes

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Author contribution

L.A.S and A.C performed and designed mouse experiments, analysed data, generated figures and edited the manuscript. M.E.R and J.S performed and supervised studies on healthy subjects and subjects with eosinophilic oesophagitis and performed experiments. S.N, N.J.T and M.M.W provided assistance in interpreting and analysing data. L.H performed experiments and analysed data. J.M. conceptualized, coordinated, designed and supervised mouse experiments, interpreted and analysed data, drafted and edited the manuscript. All authors contributed to data discussion and revised the manuscript.

Competing financial interest

MER is a consultant for Immune Pharmaceuticals, Celsus Therapeutics and Receptor and has an equity interest in each. MER has an royalty interest in reslizumab, a drug under development by Teva Pharmaceuticals. MER is an inventor of EoE related patents owned by Cincinnati Children's Hospital. These activities are not directly related to the content of this manuscript. The other authors declare no competing financial interests.

inflammation by upregulation of the E3 ubiquitin-ligase midline-1 (MID1), which binds to and deactivates the catalytic subunit of protein phosphatase 2 A (PP2Ac) resulting in increased NF- κ B activation.

Objective—To elucidate the role of TRAIL in EoE.

Methods—We used *Aspergillus fumigatus* (*Asp F*) to induce EoE in TRAIL sufficient (wildtype) and deficient ($-/-$) mice and targeted MID1 in the oesophagus with small interfering (si) RNA. We also treated mice with recombinant TSLP and TRAIL.

Results—TRAIL deficiency and MID1 silencing employing siRNA reduced oesophageal eosinophil and mast cell numbers and protected from oesophageal circumference enlargement, muscularis externa thickening and collagen deposition. MID1 expression and NF- κ B activation were reduced in TRAIL $-/-$ mice, while PP2Ac levels were increased compared to wildtype controls. This was associated with reduced expression of CCL24, CCL11, CCL20, IL-5, IL-13, IL-25, TGF- β and TSLP. Treatment with TSLP reconstituted hallmark features of EoE in TRAIL $-/-$ mice and recombinant TRAIL induced oesophageal TSLP expression *in vivo* in the absence of allergen. *Post hoc* analysis of gene array data demonstrated a significant upregulation of TRAIL and MID1 in a cohort of children with EoE as compared to diseased controls.

Conclusion—TRAIL regulates MID1 and TSLP, inflammation, fibrosis, smooth muscle hypertrophy and expression of inflammatory effector chemokines and cytokines in experimental EoE.

Keywords

Eosinophilic Oesophagitis; Eosinophilic Esophagitis; Allergy; Tissue remodelling; Fibrosis; Cytokine; TRAIL; MID1; PP2Ac; NF- κ B; TSLP; IL-25; CCL20; CCL24; CCL11

Introduction

Eosinophilic Oesophagitis (EoE) is characterised by eosinophil-dominant inflammation of the oesophagus that is resistant to proton pump inhibitor therapy. Although an orphan disease, the prevalence of EoE is increasing in the western world [1, 2]. EoE can manifest at any age with a trend towards affecting atopic male children [3-5]. Patients classically present with oesophageal or abdominal pain, vomiting, and dysphagia and young children are at particular risk of significant weight loss as a result of prolonged oesophageal inflammation [6, 7]. EoE sufferers, particularly children, have comorbid atopic disorders such as food allergy or asthma [3, 8, 9].

Dietary exposure to food allergens has been linked to the development of EoE, with dietary modifications shown to be a successful therapeutic approach in many patients [9-11]. Alternatively, topical administration of corticosteroids to the oesophageal mucosa has also been shown to be effective in reducing oesophageal eosinophilia [6, 12, 13]. However, neither dietary interventions nor steroid treatments are universally effective with a subset of patients experiencing persistent EoE symptoms and/or refractory oesophageal eosinophilia. In events where chronic inflammation of the oesophagus is left untreated or patients fail to respond to therapies, remodelling of the oesophagus can lead to oesophageal stricture

formation and worsening food impaction [14, 15]. Clinical guidelines suggest that patients with severe remodelling may receive endoscopic dilation therapy to alleviate symptoms, with most patients responding well to this therapy in the short term [16, 17]. However, up to 75% of patients experience post-dilation complications including pain, bleeding and perforation [18], highlighting the need for an effective pharmacological alternative to treat elimination diet-resistant and steroid-resistant EoE.

Oesophageal remodelling is thought to result as a consequence of prolonged eosinophilic inflammation promoting collagen deposition and fibrogenesis, oesophageal muscle hypertrophy and angiogenesis [14, 19]. T_H2 cytokine signalling plays a central role in EoE pathogenesis by driving the recruitment and proliferation of eosinophils to the oesophagus [20]. In turn, eosinophil derived proteins including transforming growth factor (TGF)- β have been shown to driving pro-fibrotic SMAD2/3 pathways [14]. IL-13 also plays a key role with activation of the IL-4/IL-13 receptor induces eotaxins (CCL11 and CCL24 in mice and CCL26 in humans) by STAT-6 mediated pathways [21]. However, recent studies have indicated that symptoms and remodelling can persist even when eosinophilia has been corrected [22], suggesting eosinophil independent pathways may also be key drivers of oesophageal remodelling in EoE [7]. Mast cell and basophilic inflammation is also observed clinically and experimentally, with mast cells believed to contribute to a thickened muscularis externa via TGF- β , histamine and tryptase [23]. A major EoE genetic susceptibility locus exists at the *TSLP* gene (5q22) [24] and release of TSLP from oesophageal epithelial cells promotes basophil infiltration [25] and has been demonstrated to induce cellular senescence and fibrosis in asthma models [26]. Upstream regulators of the remodelling and TSLP pathways in EoE have yet to be elucidated, however they may be promising therapeutic targets.

TNF-related apoptosis-inducing ligand (TRAIL) has been increasingly recognised as a proinflammatory cytokine [27-29]. We have shown previously that in allergic airways disease models (AAD) and in patients with asthma, TRAIL is released by structural airway cells in response to allergen stimulation [28] resulting in upregulation of the E3 ubiquitin ligase Midline-1 (MID1) [29]. MID1 in turn monoubiquitinates the $\alpha4$ subunit of protein phosphatase 2A (PP2A), promoting the proteosomal degradation of the catalytic subunit of PP2A (PP2ac) and preventing the A and B subunits forming an active complex [30, 31]. Due to the central role of PP2A in the regulation of inflammatory cascades via dephosphorylation, including the NF- κ B and MAP kinase pathways [32, 33], the inhibition of PP2Ac permits the activation of inflammatory cascades, primarily through T_H2 mediated mechanisms but also through early inflammatory factors such as CCL11, CCL20, IL-25 and 33 [28, 29, 34]. TRAIL-induced upregulation of MID1 has been shown to promote allergic inflammation and airways remodelling in the lung through inhibition of PP2A activity [28, 29, 35].

While EoE and allergic asthma remain distinct disorders, eosinophilic inflammation with subsequent remodelling is common to both diseases. Given the crucial role of TRAIL in the promotion of eosinophilic inflammation and remodelling in AAD, we hypothesised it would contribute to oesophageal inflammation and remodelling in an allergen induced murine model of EoE (e.g. *Aspergillus fumigatus* (*Asp F*) induced).

Methods

RNA sequencing of human biopsies

Patient cohorts and methods for RNA sequencing and analyses have been described previously (Sherrill, *et al.*[36]). In brief, distal oesophageal biopsies from six healthy controls (no EoE diagnoses, 0 eosinophils per high-power field) and 10 patients with active EoE (EoE diagnosis, 163+/-29 eosinophils per high-power field [mean+/-SEM]) were subjected to RNA sequencing. Sequencing reads were aligned against the GRCh37 reference genome using the UCSC gene models. Raw expression data (fragments per kilobase of transcript per million mapped reads [FPKM]) were assessed for statistical significance using a Welch t-test with Benjamini-Hochberg false discovery rate and a threshold of $P < 0.05$ and a 2.0-fold cut-off filter and cluster analysis was performed in GeneSpring® GX (Agilent Technologies Incorporated, Clara, CA). These data were deposited into the Gene Expression Omnibus (GEO) (GSE58640).

Mice

Wild Type (WT) and TRAIL deficient (TRAIL^{-/-}) BALB/c mice (male, 8 to 12 weeks of age) were obtained from Australian Bioresources (Moss Vale NSW) under a material transfer agreement with Amgen. All experiments were approved by the Animal Care and Ethics Committee of the University of Newcastle.

Aspergillus fumigatus mouse model of EoE

The *Asp F* mouse model of EoE, described previously by Mishra *et al.*[37], was employed to investigate the role of TRAIL in EoE. Briefly, mice were intranasally challenged with 100µg of *Asp F* extract (Greer, Lenoir, NC) in 50µL of sterile saline three times a week for three weeks under isoflurane anaesthetic. Control mice received 50µL of saline only. Mice were sacrificed for oesophageal samples 24 hours after the final *Asp F* challenge using pentobarbitone sodium (Virbac, Milperra, Australia).

Silencing (si)RNA mediated inhibition of MID1

ON-TARGET siRNAs were purchased from Dharmacon (Millennium Science, Mulgrave, Australia) at a concentration of 50nmols. These siRNAs include an antisense strand sequence of MID1 siRNA (5-AGAGUAAUCUCACCAAUCU-3') and a Nonsense (NONc) strand of siRNA (5'-UGGUUUACAUGUCGACUAA-3') to evaluate any potential off-target effects. Mice were intranasally administered 3.75nmols (in 25µL of sterile saline) of either MID1 or NONc siRNA 24 hours prior to the first *Asp F* challenge. This dose was repeated every second day throughout the course of the model.

Recombinant protein administration

TRAIL^{-/-} mice were administered intranasally 500ng carrier-free recombinant human TSLP (Australian Biosearch, Balcatta, Australia) in 25µL sterile saline or as a control 25µL sterile saline only 24 hours prior to the first *Asp F* challenge and then every second day throughout the course of the model.

In a separate experiment, recombinant human TRAIL (rTRAIL) (Enzo Life Sciences, Farmingdale, NY) was intranasally administered to WT mice (10µg in 25µL of sterile saline) or sterile saline as a control. 24 hours after rTRAIL administration, mice were sacrificed.

Oesophageal circumference measurements

Excised oesophagi were divided into three sections, with the proximal portion allocated for protein analysis and the distal section for histology. The central section was incised longitudinally and flattened on sections of blotting paper. Oesophageal circumference was measured using Image-Pro-Plus 6 software (MediaCybernetics, Rockville, MD) and the oesophageal circumference was determined.

Histological analysis of oesophageal tissue

Distal sections of oesophageal tissue were fixed in 10% formalin for 24 hours before routine processing to paraffin wax, sectioned at 5µm and stained with Charbol's chromotrope-hematoxylin to enumerate eosinophils and Masson's Trichrome for collagen quantification.

Eosinophil infiltration into the oesophagus was determined by counting the number of eosinophils within 1 mm² of transverse oesophageal section. In photographs of Masson's Trichrome stained slides (Olympus, Sydney, Australia) the degree of oesophageal fibrosis was determined as the area per micrometre (µm²/µm) using Image-Pro-Plus6 software. Quantification of the muscularis externa was also determined by measuring the perpendicular width of muscular tissue in each oesophagus.

Immunofluorescent detection of TRAIL

Paraffin-fixed oesophageal slices were blocked with 50% sheep serum (2 hours room temperature) before being incubated overnight (4°C) with either a phycoerythrin-conjugated CD253 (TRAIL) specific antibody (Australian Bioscience, Balcatta, Australia) or anti-human antibody (1:50 dilution in PBS) to act as a control. The oesophagi were then counterstained with DAPI (Sigma-Aldrich, Castle Hill, Australia) and were photographed under UV light exposure via microscopy (Olympus, Sydney, Australia).

Gene analysis of mouse oesophagi

Mouse oesophagi were immersed in RNeasy lysis buffer (Qiagen, Life Technologies Australia, Mulgrave, Australia) before being frozen at -80°C. Total RNA was then isolated using TRIzol® RNA extraction (Invitrogen, Life Technologies Australia, Mulgrave, Australia) and reverse transcribed to cDNA using BioScript (Bioline, Alexandria, Australia).

Gene expression within the oesophagus was determined using RT-qPCR (Eppendorf Realplex, Hamburg, Germany) and SYBR Green (Invitrogen, Life Technologies Australia, Mulgrave, Australia). Primers specific for murine MID1 (Forward: 5'-CACTCGCTGAAGGAAAATGACCA-3, Reverse: 5'-AATCCAAGGCAAAAGTGTCAAA CG-3), CCL11 (F: 5-TTCTATTCCTGCTGCTCACGG-3, R: 5-AGGGTGCATCTGTTGT TGGTG-3), TGF-β (F: 5-TGTGGAAGTCTACCAGAAATATAGC-3, R: 5-GAAAGCCCT GTATTCCGTCTC-3), TSLP (F:5-AGGCTACCCTGAAACTGAG-3, R:5-GGAGATTGCA TGAAGGAATACC-3) CCL20 (F:5-CGACTGTTGCCTCTCGTACA-3,

R: 5-AGGAGGTTACAGCCCTTTT-3) IL-25 (F: 5-ATGTACCAGGCTGTTGCATTCTTG-3) (R: 5-CTAAGCCATGACCCGGGGCC-3) (Sigma-Aldrich, Castle Hill, Australia) and CCL24 (Biomol, VMPS-907, Enzo Life Sciences, Farmingdale, NY) were used to quantify mRNA copy numbers as described previously [38]. Murine β -actin was used as a housekeeper gene and gene expression was determined as mRNA copies of the gene of interest per copy of β -actin (F:5-GACGGCCAGGTCATCACTATTG-3, R:5-AGGAA GGCTGGAAAAGAGCC-3).

Protein quantification in mouse oesophagi

Snap frozen oesophageal samples were weighed prior to being homogenised (Tissue Tearor, BioSpec products) and protein levels for IL-5, IL-13, MID-1 and PP2Ac were determined by ELISA (Ramp;D systems, Minneapolis, MN or Cusabio, Wuhan, China). Results are normalised to oesophageal tissue weight.

NF- κ B activity assay

Active p65 was determined in oesophageal homogenates using a TransAM Transcription Factor assay kit (Active Motif, Carlsbad, CA) in accordance with the manufacturer's instructions. Expression of p65 was normalised to the weight of oesophageal tissue.

Flow cytometry

Mouse oesophagi were homogenised using the GentleMACS™ Dissociator system and the cell suspension was stained with phycoerythrin-conjugated anti-CD4 and FITC-conjugated anti-TCR β chain (BD Bioscience, Sparks, MD). The number of CD4 positive T cells in the oesophagus was determined using flow cytometry (FACSCanto) and the final percentage was multiplied by the total cell count for each group. Data was analysed using FlowJo software (FlowJo, Ashland, OR).

Statistical Analysis

Statistical significance was determined between experimental groups using Student's *t*-tests (Welch *t*-test for human studies) in Graphpad Prism 6 (La Jolla, CA). Data presented as Mean $\pm$ SEM.

Results

The TRAIL signalling axis is altered in EoE

Global RNA sequence analysis demonstrated that the expression of TRAIL and MID1 was significantly upregulated in a cohort of EoE patients in comparison to healthy counterparts (Figure 1A and 1B).

Thus, we investigated the potential role of TRAIL in EoE pathogenesis *in vivo* through the use of the *Asp F* mouse model of allergic EoE. Intranasal *Asp F* exposure resulted in the upregulation of MID1 and downregulation of PP2Ac in the oesophagus (Figure 1C and 1D). We also saw a significant increase in the activity of the p65 subunit of NF- κ B (Figure 1E). This was associated with higher levels of mast cells in the oesophagus and elevated

eosinophils in the oesophagus and blood (Figure 1F to 1H) but not CD4⁺ TCR⁺ cells (Figure 1I).

TRAIL regulates PP2Ac and p65 activity in addition to eosinophil and mast cell infiltration

To determine the significance of TRAIL expression in oesophageal inflammation, we employed mice genetically deficient in TRAIL. TRAIL^{-/-} mice displayed markedly reduced expression of MID-1 in the oesophagus as compared to wildtype mice (Figure 2A). TRAIL^{-/-} mice were protected from PP2Ac downregulation in the oesophagus following *Asp F* challenge (Figure 2B) and also displayed lowered p65 activity (Figure 2C). There was also a marked decrease in oesophageal eosinophils and mast cells (Figure 2D and 2E), in addition to a marked decrease of eosinophils in the blood (Figure 2F).

Inflammatory cytokines and chemokines involved in EoE are regulated by TRAIL

To investigate which EoE-related factors are regulated by TRAIL, we performed RT-qPCR on cDNA derived from mouse oesophagi. mRNA expression of the eosinophil chemoattractants CCL11 and CCL24 (Figure 3A and 3B) were found to be significantly lower in TRAIL^{-/-} mice in comparison to wildtype mice following *Asp F* exposure. This trend was also present for the mRNA expression of TGF- β and TSLP (Figure 3C and 3D). We also determined that IL-5 and IL-13 protein was reduced, in addition to STAT6 expression (Figure 3E to 3G).

Oesophageal remodelling in EoE requires TRAIL expression

The significance of TRAIL expression on oesophageal remodelling in EoE was determined through histological analysis of mouse oesophageal tissue (Figure 4A). TRAIL^{-/-} mice were protected from an increase in oesophageal circumference when challenged with *Asp F* (Figure 4B). TRAIL^{-/-} mice were also protected from increases in muscularis externa thickness and oesophageal fibrosis (Figure 4C and 4D) compared to wild type mice.

TSLP is sufficient to restore *Asp F* induced remodelling in TRAIL deficient mice and TRAIL induces TSLP

TRAIL^{-/-} mice which received recombinant TSLP throughout the *Asp F* model had a restored oesophageal eosinophilia (Figure 5B) compared to TRAIL^{-/-} that received only vehicle control in addition to *Asp F*. TSLP-treated mice also displayed increased oesophageal circumference, muscularis externa thickness and subepithelial collagen deposition (Figure 5 D-F). Non-allergic mice treated with recombinant TRAIL had increased oesophageal expression of TSLP 24 hours post treatment compared to vehicle treated mice (Figure 5 C). Thus, TRAIL regulates TSLP which is sufficient to induce EoE, at least in this murine experimental system.

Eosinophilic inflammation and remodelling are dependent on MID1 expression

siRNA-mediated silencing of MID1 expression as compared to treatment with nonsense control (NONc) siRNA in the oesophagus (Figure 6B) reduced oesophageal eosinophilia and protected from increases in oesophageal circumference, muscularis externa thickness and oesophageal fibrosis (Figure 6 A-F) when challenged with *Asp F*. MID1 siRNA-treated mice

had corresponding reductions in expression levels of CCL11 and CCL24 (Figure 7 A,B) but did not show any difference in expression of TGF- β or TSLP (Figure 7 C, D). Thus, MID1 inhibition ameliorates EoE independent of TSLP.

TRAIL expression in the Asp F EoE model

The expression of TRAIL was upregulated 24 hours after the first *Asp F* challenge in epithelial and smooth muscle cells (Figure 8 B, C) which correlated with MID1, CCL20, TSLP and IL-25 expression (Figure 8 D-G). After 3 weeks of allergen challenge (endpoint) no increased expression of TRAIL, CCL20, and IL-25 (Figure 8 H-J) was observed while MID1, TSLP, CCL11, and CCL24 expression persisted (Fig 3 A-D). This suggests a complex temporal and spatial expression pattern of TRAIL and its downstream effector functions.

Discussion

TRAIL has previously been demonstrated to play a key role in the regulation of inflammation in allergic asthma both clinically and in mouse models [28, 29, 39], identifying the possibility that TRAIL signalling may also play an important role in other eosinophilic diseases such as EoE. Gene expression analysis using RNA sequencing demonstrated a significant increase in TRAIL and its downstream pro-inflammatory signalling molecule MID1, in a cohort of EoE patients, implicating a potential role for the cytokine in the pathology of this disease. Notably there was no overlap in TRAIL and MID1 expression levels between healthy children and those with EoE highlighting the need to further explore their potential value as EoE biomarker. Using a mouse model of *Asp F*-induced EoE, we show that mice deficient in TRAIL have significantly less eosinophil and mast cell infiltration into the oesophagus compared to their wild type counterparts. We also demonstrated that muscularis externa hypertrophy, fibrosis and oesophageal circumference is dependent on the expression of TRAIL and that TRAIL is necessary for the upregulation of CCL11, CCL24, TGF- β and TSLP, four key cytokines implicated in the pathogenesis of EoE [21, 23, 25]. TRAIL $^{-/-}$ mice displayed elevated levels of PP2Ac and reduced NF- κ B activity despite *Asp F* exposure.

Previous work utilising *Asp F*-induced EoE models found that allergen induced EoE is IL-5 driven, with a supportive role from IL-13 and its subsequent activation of STAT6-dependent cytokines [20, 21]. While TRAIL's role in allergic asthma has previously been identified, our study has demonstrated, for the first time, that the production of both IL-5 and IL-13 is dependent on TRAIL expression in *Asp F* induced mouse models of EoE and subsequently the expression of CCL11, CCL24. This reduction in T_H2 cytokine signalling may also account for the lack of oesophageal fibrosis, which has been shown to be mediated by eosinophilic release of TGF- β [35, 40].

We also found that TSLP is induced by TRAIL in the absence of allergy and dependent on TRAIL expression in experimental EoE, suggesting that TSLP and T_H2 cytokines are downstream of TRAIL signalling. TSLP has been shown to activate STAT3-mediated remodelling pathways in asthma and could possibly mediate remodelling independently of eosinophils via this mechanism [26]. It is still unknown how TSLP is regulated by TRAIL in

EoE, given that no link between TRAIL and TSLP was evident in models of AAD [29]. It has been shown, however, that the TSLP promoter contains several binding sites for the p65 subunit of NF- κ B; a transcription factor shown to be TRAIL dependent in AAD [41] which we show is also repressed in the oesophagus of TRAIL $^{-/-}$ in the EoE model. It has also been shown that the subsequent expression of TSLP in intestinal epithelia cells is dependent on NF- κ B [41]. Given NF- κ B is upregulated in EoE patients [42], it is possible that TRAIL is a key regulator of TSLP through p65 activation and contributes to EoE most likely through basophil activation [25]. However siRNA-mediated silencing of MID1 did not affect TSLP expression while ameliorating hallmark features of EoE including CCL11 and CCL24 expression. This observation is in accordance with previous findings showing MID1-independent TSLP expression in allergic airways disease [29] and may explain residual eosinophilic inflammation despite MID1 silencing in the EoE and allergic airways disease models. It is therefore possible that TSLP induction may be dissociated from MID1-mediated suppression of p65 activation. Alternatively more effective MID1 suppression may be required for TSLP inhibition and cannot be achieved by siRNA-mediated MID1 targeting. This would be supported by our observation that TRAIL deficiency results in MID1 expression levels well below the levels observed in non-allergic TRAIL sufficient mice and MID1 siRNA treated allergic mice. In any case we have demonstrated an important role for inflammatory TRAIL signalling in the oesophagus during EoE and highlighted its role in oesophageal remodelling in mouse models through regulation of TSLP and MID1. TRAIL has recently been indirectly linked to EoE with a GWAS study demonstrating Calpain14 to be specifically expressed in the oesophagus and dynamically upregulated as a function of disease activity [43]. Inhibition of calpains has previously been linked to altered NF- κ B activity and TRAIL signalling [44].

Together these findings identify TRAIL upstream of MID1 and TSLP as a significant disease pathway in EoE that may cross regulate with emerging oesophageal-specific allergic responses highlighting its potential as a disease target and biomarker.

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Abbreviations

EoE	Eosinophilic Oesophagitis
TRAIL	TNF- α -related apoptosis inducing ligand
MID1	Midline-1
PP2Ac	Protein phosphatase 2Ac

TGF-β	Transforming growth factor β
TSLP	Thymic stromal lymphopoietin
NF-κB	Nuclear factor kappa B
Asp F	Aspergillus fumigatus

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Key Messages

- TRAIL deficiency and MID1 silencing reduced oesophageal eosinophilic inflammation and abolished hallmark features of oesophageal remodelling in experimental EoE
- TRAIL regulates MID1, PP2Ac, NF- κ B, TSLP, CCL11, CCL20, CCL24, IL-5, IL-13, IL-25, and TGF- β in experimental EoE
- TRAIL and MID1 are upregulated in eosinophilic oesophagitis

Figure 1. The TRAIL signalling pathway is active in a model of EoE

Individual FPKM values of (A) TNFSF10 (TRAIL) and (B) MID-1 from RNA sequencing performed on oesophageal biopsies from healthy controls (NL; n=6) and patients with active EoE (EoE; n=10). Mouse MID-1 (C) and PP2Ac (D) protein levels from oesophagi of *Asp F* treated mice and saline controls as assessed by ELISA. (E) Activity assay for the NF- κ B subunit p65. (F) Histological enumeration of oesophageal eosinophils using chromatin stain (G) mast cell infiltration using Toluidine Blue and (H) eosinophilia in the blood determined by Giemsa stain. Oesophageal T cells (TCR+ CD4+) as enumerated by FACS (I). Data expressed as Mean $\pm$ SEM, $p < 0.05 = *$, $p < 0.005 = ***$

Figure 2. TRAIL regulates PP2Ac and p65 activity in addition to eosinophil and mast cell infiltration

(A) Expression of MID-1 was determined by qPCR. Gene expression normalised to copy number of β -actin. (B) Protein expression of PP2Ac and (C) p65 activity in Asp F oesophagi (D) Histological enumeration of oesophageal eosinophils using chromatin stain (E) enumeration of mast cells stained with Toluidine Blue, (F) eosinophilia in the blood determined by Giemsa stain (G) Differential cell count of total cells isolated from mice via bronchoalveolar lavage. $p < 0.05 = *$

Figure 3. Inflammatory and remodelling cytokines involved in EoE are regulated by TRAIL
 Expression of (A) CCL11 (B) CCL24 (C) TGF-β (D) TSLP and (G) STAT6 was determined by RT-qPCR. Gene expression was normalised to copy numbers of β-actin. (E) IL-5 and (F) IL-13 protein expression was determined using ELSIA. p<0.05=*

Figure 4. Oesophageal remodelling in EoE is dependent on the expression of TRAIL

(A) Representative images of transverse oesophagi sections at 100x and 200x magnification (stained with Masson's Trichrome), from wildtype (WT) and TRAIL deficient (TRAIL^{-/-}) mice challenged with *Asp For* saline control. (B) muscularis externa, (C) Oesophageal circumference and (D) sub epithelial collagen deposition as determined with ImageProPlus analysis (n=5 to 6). p<0.05=*

Figure 5. TSLP is sufficient to restore Asp F induced remodelling in TRAIL deficient mice
 (A) Representative images of transverse oesophagi sections at 100x and 200x magnification (stained with Masson's Trichrome), from TRAIL deficient (TRAIL^{-/-}) mice challenged with *Asp F* and treated with recombinant TSLP or vehicle control. (B) Histological enumeration of oesophageal eosinophils using chromatin stain. (C) Expression of TSLP in oesophagi of WT mice following treatment with recombinant TRAIL or saline control determined by qPCR. Gene expression was normalised to copy numbers of β -actin. (D) muscularis externa, (E) Oesophageal circumference and (F) sub epithelial collagen deposition as determined with ImageProPlus analysis (n= 6-8). $p < 0.05 = *$ $p < 0.01 = **$

Figure 6. Oesophageal remodelling in EoE is dependent on MID1

(A) Representative images of transverse oesophagi sections at 100x and 200x magnification (stained with Masson's Trichrome), from wildtype (WT) mice challenged with *Asp F* and treated with siRNA targeting MID1, nonsense siRNA or saline control. (B) Oesophageal MID1 expression determined by qPCR. Gene expression was normalised to copy numbers of β -actin. (C) Histological enumeration of oesophageal eosinophils using chromatin stain (D) muscularis externa, (E) Oesophageal circumference and (F) sub epithelial collagen deposition as determined with ImageProPlus analysis (n=8). $p < 0.05 = *$ $p < 0.01 = **$ $p < 0.005 = ***$ $p < 0.0001 = ****$

Figure 7. MID1 regulates a subset of TRAIL regulated inflammatory cytokines in EoE
 Expression of (A) CCL11 (B) CCL24 (C) TGF-β (D) TSLP was determined by RT-qPCR.
 Gene expression was normalised to copy numbers of β-actin. (n=8) p<0.05=* p<0.01=**

Figure 8. TRAIL and IL-25 expression is highest during establishment of the Asp F EoE model
Representative immunofluorescence images of oesophageal sections counter stained with DAPI and stained with PE conjugated isotype control (A) and PE conjugated anti-TRAIL N2B2 antibody (B). Expression of TRAIL (C,H) MID1 (D) IL-25 (F, J) CCL20 (E, I) and TSLP (G) was determined by qPCR following 24hrs and 3 weeks of *Asp F* challenges. Gene expression was normalised to copy numbers of β-actin (n=7-9) p<0.05 = *, p<0.01 = **.