

26 **Abstract**

27 Introduction: Food antigens are common inflammatory triggers in pediatric eosinophilic
28 esophagitis (EoE). TNF-related apoptosis-inducing ligand (TRAIL) promotes eosinophilic
29 inflammation through the upregulation of Midline (MID)-1 and subsequent downregulation
30 of Protein Phosphatase 2A (PP2A) but the role of this pathway in EoE that is experimentally
31 induced by repeated food antigen challenges has not been investigated.

32 Methods: Esophageal mucosal biopsies were collected from children with EoE and controls
33 and assessed for TRAIL and MID-1 protein and mRNA transcript levels. Wild type and
34 TRAIL deficient (*Tnfsf10* ^{-/-}) mice were administered subcutaneous ovalbumin (OVA)
35 followed by oral OVA challenges. In separate experiments OVA challenged mice were
36 intraperitoneally administered salmeterol or dexamethasone.

37 Results: Esophageal biopsies from children with EoE revealed increased levels of TRAIL and
38 MID-1 and reduced PP2A activation as compared to controls. *Tnfsf10*^{-/-} mice with
39 experimental EoE, were largely protected from esophageal fibrosis, eosinophilic
40 inflammation, and the upregulation of TSLP, IL-5, IL-13 and CCL11 when compared with
41 wild type mice. Salmeterol administration to wild type mice with experimental EoE restored
42 PP2A activity and also prevented esophageal eosinophilia, inflammatory cytokine expression
43 and remodeling which was comparable to the treatment effect of dexamethasone.

44 Conclusion: TRAIL and PP2A regulate inflammation and fibrosis in experimental EoE,
45 which can be therapeutically modulated by Salmeterol.

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47 **New and Noteworthy**

48 The data presented in this article demonstrates the essential role of proinflammatory TRAIL
49 signaling in eosinophilic esophagitis. The study characterizes TRAIL, MID-1 and PP2A in
50 esophageal biopsies from EoE patients, and demonstrates that TRAIL deficient mice are
51 protected from a chicken egg ovalbumin induced model of EoE. Finally, modulation of
52 esophageal PP2A activity using Salmeterol is also shown to have therapeutic potential in this
53 model.

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67 Introduction

68 Eosinophilic esophagitis (EoE) is a chronic inflammatory upper gastrointestinal tract (GIT)
69 disorder, characterized by allergy-driven inflammation. Symptoms of EoE may be similar to
70 other upper GIT disorders, presenting clinically with regurgitation, retrosternal or epigastric
71 pain (19, 27, 40), which may be misdiagnosed as gastro-esophageal reflux disease. Untreated
72 or poorly diagnosed EoE patients suffer long-term effects associated with prolonged
73 inflammation, such as remodeling of esophageal structure in the form of strictures and
74 extensive weight loss (24, 35). Although the disease can manifest at any age, the condition is
75 more common in young, atopic males with 75% of patients being male and 80% of them
76 having an additional allergic disorder (13, 30).

77 Eosinophilic inflammation of the esophageal mucosa can be treated through administration of
78 topical (swallowed) corticosteroids; however, the effectiveness of such treatment varies and
79 symptoms may persist despite reduced numbers of esophageal eosinophils (3, 31). Some
80 patients non-responsive to current treatments have worse clinical outcomes (26). Because
81 food allergens are considered an important trigger of EoE in pediatric patients, dietary
82 elimination of common food antigens has increasingly been employed as an alternative to
83 corticosteroid treatment (14, 15, 18). Empirical elimination diets have demonstrated that the
84 most prevalent food antigens associated with EoE are cow's milk, wheat, egg and soy (20),
85 and the success rate of elimination diets for EoE is around 70% in children (18, 21, 32). The
86 need for more effective therapeutics in some patients is highlighted by the rising number of
87 EoE cases, the risk of stricture formation despite current best practice, treatment side effects
88 and poor adherence (1, 5, 25).

89 TNF-related apoptosis-inducing ligand (TRAIL) has been associated with eosinophilic
90 diseases (34) and inflammatory pathomechanisms *in vivo* (10, 11, 38, 39). In mouse models

91 of allergic airways inflammation, house dust mite and ovalbumin (OVA) have been shown to
92 promote the release of TRAIL from epithelial cells, resulting in the upregulation of the E3
93 ubiquitin ligase Midline (MID)-1 (10, 11). Increased MID-1 expression results in the
94 polyubiquitination of the catalytic subunit of the regulatory enzyme protein phosphatase 2A
95 (PP2A) (10, 11), impeding its central role in the dephosphorylation of p38 mitogen-activated
96 kinases, c-Jun N-terminal kinase and nuclear factor- κ B (NF- κ B) (4, 23, 36). These
97 inflammatory cascades ultimately result in the infiltration of mast cells and eosinophils to the
98 site of inflammation associated with allergic disease (10, 11, 38).

99 The emergence of TRAIL's role in additional pathologies, such as cigarette induced COPD
100 and neonatal respiratory infections, further implicates its role as a key facilitator in multiple
101 inflammatory conditions, affecting adults and children alike (17, 37). In models of allergic
102 airways disease, TRAIL has been shown to promote airways hyperreactivity and remodeling
103 in the form of fibrosis and smooth muscle hyperplasia (10, 11). These effects are associated
104 with elevated levels of T-helper type 2 (TH2) cytokines (IL-5 and 13), eotaxins, transforming
105 growth factor (TGF)- β and epithelial factors including thymal stromal lymphopoietin (TSLP)
106 and CCL20 (10, 11, 38). Inhibition of MID-1 employing siRNA and raising PP2A activation
107 with FTY720 analogues attenuated eosinophilic inflammation and airways remodelling (10,
108 11). Similarly, long acting beta agonists (such as salmeterol) can also increase PP2A activity
109 *in vitro* and *in vivo* studies resulting in amelioration of hallmark features of allergic airways
110 disease (16). Our previous work found that both TRAIL and MID-1 are upregulated in the
111 murine esophageal epithelium upon exposure to *Aspergillus fumigatus* extract and that
112 *Tnfrsf10*^{-/-} mice or mice treated with siRNA silencing MID-1 were protected from EoE like
113 features *in vivo* (12). However, it is unknown if these pathways are utilized in food allergy-
114 driven EoE. As egg antigens are among the most common inducers of EoE in pediatric
115 patients (33), we investigated TRAIL and its downstream signaling pathway in a recently

116 developed OVA-induced mouse model of EoE (38) and compared the hallmark features of
117 EoE between wild type and TRAIL deficient (*Tnfsf10*^{-/-}) mice as well as after salmeterol and
118 dexamethasone treatment.

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Methods

Collection of patient biopsies

Patients presenting to the John Hunter Children's Hospital, Australia, with symptoms consistent with esophageal dysfunction were assessed by a pediatric gastroenterologist and underwent endoscopic esophageal mucosal biopsy (minimum of two per patient) from the distal third of the esophagus. Based on symptoms and eosinophil quantification in biopsies, patients were classified as EoE Active (peak eosinophil count ≥ 15 per high power field), Control (peak eosinophil count < 15 per hpf) and EoE Remission (resolution of symptoms in EoE active patient and a reduction of peak eosinophil count below 15 per hpf). Patients either had gastro-esophageal reflux excluded on esophageal pH metry or were taking proton pump inhibitor therapy at the time of biopsy. Biopsy specimens were then prepared for protein or RNA analysis. Written informed consent was gained from all parents/carers and assent from children (where age-appropriate) prior to sample collection and the study was approved by the Hunter New England Local Health District and The University of Newcastle Human Research Ethics Committees. Patient details involved in qPCR, protein and PP2A assay analysis are shown in Tables 1, 2 and 3.

Table 1 Baseline characteristics of subjects with biopsies available for protein measurements

Clinical Features		EoE (n=18)	EoE remission (n=5)	Control (n=25)
Age (Years)	Median (Min, Max)	8.0 (1.0, 13.8)	12.2 (2.6, 13.6)	7.4 (1.5, 16.5)
Sex	Males (%)	13 (72.2)	3 (60)	9 (36)
Peak Eosinophil count	Median (Min, Max)	30 (15, 114)	0 (0, 12)	N/A
Asthma	Total (%)	8 (44.4)	1 (20)	6 (24)
Food Allergy	Total (%)	7 (38.9)	1 (20)	4 (16)

Table 2 Baseline characteristics of subjects with biopsies available for mRNA measurements

Clinical Features		EoE (n=21)	EoE remission	Control
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			(n=10)	(n=29)
Age (Years)	Median (Min, Max)	10.0 (1.0, 13.8)	6.2 (1.3, 13.6)	9.1 (1.8, 16.5)
Sex	Males (%)	17 (81)	6 (60)	12 (41)
Peak Eosinophil count	Median (Min, Max)	34 (20, 82)	0 (0, 5)	N/A
Asthma	Total (%)	7 (33)	2 (20)	11 (38)
Food Allergy	Total (%)	6 (29)	4 (40)	6 (21)

Table 3 Baseline characteristics of subjects with biopsies available for PP2A activity measurements

Clinical Features		EoE (n=15)	EoE remission (n=6)	Control (n=8)
Age (Years)	Median (Min, Max)	9.2 (2.8, 15.0)	10.8 (2.6, 16.1)	11.5 (2.1, 16.1)
Sex	Males (%)	11 (73)	6 (100)	4 (50)
Peak Eosinophil count	Median (Min, Max)	40 (25, 82)	0 (0, 11)	N/A
Asthma	Total (%)	2 (13)	1 (17)	4 (50)
Food Allergy	Total (%)	0 (0)	0 (0)	2 (25)

Ovalbumin mouse model of EoE

Briefly, wild type or *Tnfsf10*^{-/-} BALB/c mice were sensitized to OVA (Sigma-Aldrich, Sydney, NSW) subcutaneously once every three days for 12 days (100µg/50µL) or saline (SAL) only as a negative control (n=5 (SAL wild type), n=8 (OVA wild type), n=5 (SAL *Tnfsf10*^{-/-}), n=7 (OVA *Tnfsf10*^{-/-})). Two days after the final OVA injection, mice were given 100µLs of OVA (50mg/100µL) dissolved with sucralose (Splenda™) or sucralose only as a control, orally via pipette and had their drinking water withheld for one hour. Prior to water re-introduction, OVA treated mice had their water spiked with OVA (1.5g/L). A second oral challenge was administered on day 17, with water withheld again for one hour after treatment as previously described (28). A separate group of wild type mice (n=5-8) were administered salmeterol xinafoate (0.4mg/kg), dexamethasone (2mg/kg) or a combination of both (Sigma-Aldrich, Sydney, NSW) intraperitoneally 24 hours prior to the first OVA challenge on day

13. This dose was then repeated daily until day 17 (n=5 (SAL/SAL), n=8 (OVA/VEH), n=6 (OVA/Salmec), n=8 (OVA/DEX), n=8 (OVA/Salmec/DEX).

Mice were subsequently sacrificed on day 18 via pentobarbitone overdose (Virbac), with esophageal tissue allocated for protein, gene or histological analysis. These animal experiments were approved by the Animal Care and Ethics Committee, The University of Newcastle.

Esophageal Circumference analysis

Excised mouse esophagi were prepared on blotting paper and were sliced longitudinally using a razor. The split esophagus was opened and exposed. Esophageal circumference was determined for each mouse using ImageProPlus (MediaCybernetics) software.

Gene expression analysis

Esophageal tissues were immersed in TRIzol® (Ambion, Life Technologies, Mulgrave, Australia) and RNA was isolated in accordance with the manufacturer's instructions. RNA was then resuspended in nuclease-free water. Samples underwent reverse transcription using BioScript (Bioline, Alexandria, Australia) or SuperScript IV™ (Thermo Fister Scientific, Australia).

Gene expression was quantified using either the Eppendorf Realplex (Hamburg, Germany) or the Viia7 (Life Technologies, Mulgrave, Australia) quantitative PCR systems. SYBR Green (Life Technologies, Mulgrave, Australia) was used as the fluorescent marker for dsDNA and standards to target genes were generated to quantify the copy number for each gene. Mouse genes were quantified using CCL11 (F 5-TTCTATTCCTGCTGCTCACGG-3, R 5-AGGGTGCATCTGTTGTTGGTG-3), CCL20 (F 5-CGACTGTTGCCTCTCGTACA-3, R 5-AGGAGGTTACAGCCCTTTT-3) TSLP (F 5-AGGCTACCCTGAACTGAG-3, R 5-

GGAGATTGCA TGAAGGAATACC-3), TGF- β (F 5-TGTGGAAGCTCTACCAGAAATATAGC-3, R 5-GAAAGCCCT GTATTCCGTCTC-3) and β -actin (F 5-GACGGCCAGGTCATCACTATTG-3, R 5-AGGAAGGCTGGAAAAGAGCC-3) primers. Human genes were quantified using TRAIL (F 5-TCGTGATCTTCACAGTGCTCCTG-3, R 5-ACAAGCAATGCCACTTTTGGAGTA-3), MID-1 (F 5-ACAGCTCCCAACCCTCCCACAAT-3, R 5-CGACGTTGGCTTGTCCGGTGAAT-3) and GAPDH (F 5-GAGTCAACGGATTTGGTCGT-3, R 5-TTGATTTTGGAGGGATCTCG-3) primers.

Protein analysis

Esophageal tissue was homogenized in Lysis Buffer consisting of IC#10 diluent (EGTA, EDTA, NP-40 alternative, HEPES and water) and supplemented with Leupeptin (2.5mg/mL), Pepstatin (1.25mg/mL), Aprotinin (1.1mg/mL) and PMSF. Expression for either human or mouse TRAIL, MID-1, IL-5, IL-13, CCL11 and CCL20 protein was determined *via* ELISA (R&D Systems, Minneapolis, Minn or Cusabio, Wuhan, China). Alternatively, PP2A activity was determined by Active PP2A DuoSet IC activity assay (Active Motif). The determined amount of protein or activity was then normalized to tissue weight. The determined amount of protein or activity was then normalized to tissue weight or total protein. In addition to esophageal homogenates, serum collected from blood extracted from euthanized mice was analyzed for IgE specific for OVA using the LEGEND MAX™ Mouse specific OVA IgE ELISA kit (Australian Biosearch).

Histological analysis of oesophageal tissue

Esophageal tissue was fixed in 10% formalin for 24 hours prior to routine processing to paraffin wax, sectioned at 5 μ m and stained with Charbol's chromotrope-hematoxylin to enumerate eosinophils, Toluidine blue to assess mast cells and Masson's Trichrome for collagen quantification.

Eosinophil or mast cell infiltration into the esophagus was determined by counting the number of eosinophils within 1mm^2 of transverse esophageal section. In photographs of Masson's Trichrome stained slides (Olympus, Sydney, Australia) the degree of esophageal fibrosis was determined as the area per micrometer of epithelium ($\mu\text{m}^2/\mu\text{m}$) in each esophagus using Image-Pro-Plus 6 software (MediaCybernetics, Rockville, Md).

Statistical Analysis

Statistical significance was determined between experimental groups using One-Way ANOVA (Holm-Sidak multiple comparison test), Mann-Whitney test or Spearman's Correlation (as appropriate) in Graphpad Prism 6 (La Jolla, CA). Data are presented as mean $\pm$ SEM.

Results

Expression of TRAIL and MID-1 in a pediatric cohort of EoE patients

Esophageal biopsies collected from pediatric EoE patients were assessed for TRAIL and MID-1 mRNA and protein expression and compared to controls. Both TRAIL and MID-1 mRNA expression were increased compared to controls (Fig 1A and B). In addition to gene expression, there was a concomitant increase in both TRAIL and MID-1 protein levels present in EoE patients (Fig 1C and D), as well as a significant reduction of PP2A activity in EoE patients (Fig 1E). There was also a significant correlation between TRAIL and MID-1 for both mRNA and protein (Fig 1F-G).

EoE patients in remission did not demonstrate increased expression of TRAIL and MID-1

Unlike EoE active patients, patients that were in remission did not demonstrate an increase in TRAIL and MID-1 mRNA expression when compared to controls (Fig 2A and B). This was also observed for TRAIL and MID-1 protein expression (Fig 2C and D) in addition to no alterations in PP2A activity (Fig 2E).

OVA-induced EoE increased TRAIL and MID1, which was associated with decreased PP2A activity

OVA sensitization and challenge of wild type mice increased TRAIL protein levels in the esophagus as compared to sham-sensitized controls (Fig 3A). This was associated with an increase in MID-1 protein levels and decreased PP2A activity (Fig 3B-C). Unlike wild type mice, OVA sensitized and challenged *Tnfsf10*^{-/-} mice had no detectable TRAIL protein or induction of MID-1 but had elevated levels of PP2A activity (Fig 3C). TRAIL deficiency did not affect serum IgE levels (Fig 3D).

TRAIL deficiency prevents eosinophilic inflammation of the esophagus

To investigate the impact of TRAIL in OVA driven esophageal inflammation, we enumerated eosinophils and mast cells in the esophagus of mice. Both eosinophils and mast cells were significantly increased in OVA sensitized wild type mice when compared to sham-sensitized controls (Fig 4A-C). However, OVA sensitized *Tnfsf10*^{-/-} mice did not have elevated eosinophilic inflammation (Fig 4B). Mast cell infiltration was not different between both OVA allergic groups (Fig 4C).

TRAIL regulates inflammatory cytokines in OVA-driven EoE

Both qPCR and ELISA were used to determine the inflammatory factors regulated by TRAIL in OVA-induced EoE. In contrast to wild type controls, TSLP, TGF- β and CCL20 mRNA expression were reduced in the esophagi of OVA sensitized *Tnfsf10*^{-/-} mice (Fig 5A-C). CCL11, IL-5 and IL-13 were increased in the esophagi of OVA sensitized wild type mice while levels in *Tnfsf10*^{-/-} remained at non-allergic saline levels (Fig 5D-F).

Esophageal remodeling is TRAIL-dependent

Both esophageal circumference and fibrosis were measured to elucidate the role of TRAIL in OVA induced esophageal remodeling. Compared to wild type mice, *Tnfsf10*^{-/-} mice were protected from increased esophageal circumference after OVA exposure (Fig 6B). In addition, *Tnfsf10*^{-/-} mice were also protected from increased subepithelial fibrosis (Fig 6A, C).

Salmeterol administration restores PP2A activity and reduces EoE hallmark features

To examine the effects of long acting beta agonists (LABAs) on PP2A modulation in the esophagus, OVA sensitized wild type mice were intraperitoneally administered salmeterol,

dexamethasone or a combination of both prior to and during OVA challenge. Administration of salmeterol but not dexamethasone resulted in a restoration of PP2A activity of the esophagus (Fig 7A). Administration of salmeterol, dexamethasone or both resulted in reduced expression of CCL11 mRNA (Fig 7B) as well as protein expression of IL-5 (Fig 7C) in the esophagus of OVA sensitized and challenged mice. While all three treatments reduced CCL20 protein and TSLP mRNA expression (Fig 7D-E), only the combined group was successful in reducing TGF- β expression (Fig 7F).

Esophageal inflammation is significantly reduced upon salmeterol treatment

Further investigating salmeterol's effectiveness in alleviating EoE hallmark features, the inflammatory outcomes of treated and non-treated mice were compared by histology (Fig 8A). Mice that were administered salmeterol demonstrated a significant reduction of eosinophils and, unlike *Tnfsf10*^{-/-} mice, a reduction in mast cells (Fig 8B and 8C) in the esophagus comparable to dexamethasone treatment.

Ovalbumin induced remodeling features are significantly reduced by salmeterol therapy

Finally, remodeling features of EoE were assessed salmeterol and dexamethasone treated OVA mice in terms of esophageal enlargement and fibrosis (Fig 9A). A reduction in esophageal circumference was observed in mice that received salmeterol, dexamethasone or both when compared to the vehicle control (Fig 9B). This was also associated with a significant reduction in fibrosis for all three treatment groups (Fig 9C).

Discussion

We have previously identified an essential role for TRAIL and its downstream molecules in an *Aspergillus fumigatus* allergen driven mouse model of EoE. Here, we demonstrate -for the first time- that the levels of mRNA transcript and protein of TRAIL and MID-1 are upregulated in a cohort of EoE patients biopsies compared to controls and patients in disease remission did not demonstrate such an increase. This was correlated with a significant reduction of PP2A activity in the EoE patients with active disease, but not those in those in remission following successful treatment. Thus the levels of TRAIL, MID-1 and PP2A are associated with EoE and its resolution. To further demonstrate the importance of the TRAIL signaling pathway, we employed a model of egg allergen induced EoE by sensitizing and challenging mice to OVA and compared responses in wild type mice to *Tnfsf10*^{-/-} mice. Egg exposure is clinically relevant because some EoE may –at least in part– be mediated by induction of TRAIL expression *via* food allergens including egg.

OVA induced the expression of TRAIL-regulated MID-1 in the esophagus of mice while the activity of PP2A was markedly reduced. This was reversed in *Tnfsf10*^{-/-} mice, with a significant reduction of MID-1 and restoration of PP2A activity observed despite OVA sensitization and exposure. PP2A has an essential role in the dephosphorylation of inflammatory transcription factors (NF- κ B, p38 MAPK) and its downregulation due to MID-1 and α 4 protein interactions are associated with the activation of inflammatory cascades (10, 12). While the polyubiquitination of PP2Ac through MID-1 and α 4 has been established in the context of allergy, other PP2A activity regulatory processes that have been investigated *in vitro*, such as the methylation of the C-terminal or PP2A phosphorylation via tyrosine (7, 8), are yet to be investigated *in vivo*. This highlights the possibility that there could be multiple

regulators of PP2A preventing activity from returning to baseline levels but these require further studies.

While OVA induced eosinophilia was found to be dependent on TRAIL expression, mast cell infiltration and IgE serum levels were not altered by TRAIL in this model. This is different to the *Aspergillus fumigatus* induced EoE where mast cell influx was dependent on TRAIL (12). The different route of sensitization in the models, namely via the epicutaneous route in the OVA model versus oral/lung route in the *Aspergillus fumigatus* model may explain these results but more studies are required to dissect the mechanisms. Both fibrosis and esophageal circumference were increased in egg-induced experimental EoE, with TRAIL $-/-$ mice protected. This could be due to reductions in TRAIL dependent TGF- β expression and TH2 cytokine expression in the esophagus. It is known in OVA-induced allergic airways disease that TH2 lymphocyte homing to the lung is dependent on CCL20 expression, as is dendritic cell infiltration, through the activation of CCR6 (38). Our results are consistent with previous studies that demonstrate the importance of TRAIL signaling for CCL20 expression. Recombinant TRAIL was shown to induce CCL20 expression *in vitro* and its abolition prevented CCL20 expression *in vivo* (12, 38). This outlines one potential inflammatory pathway initiated by TRAIL for the perpetuation of the inflammatory phenotype in EoE.

In addition to CCL20, we also noticed a marked reduction of TSLP, a cytokine previously shown to act downstream of TRAIL in EoE as an inducer of eosinophils, basophils and esophageal remodeling (12, 29). Abolition of TSLP in OVA-induced EoE has previously been demonstrated to ameliorate esophageal remodeling and eosinophilic inflammation *in vivo* and TSLP was found to be upregulated in EoE patient biopsies (29). This confirms that TRAIL is essential for the expression of TSLP in OVA-induced EoE and indicates an important role for TRAIL in the promotion of inflammation through its regulation of TSLP induced inflammation.

The administration of salmeterol in an *in vivo* model of EoE has demonstrated its immunomodulatory properties outside the lung for the first time through the upregulation of PP2A activity. Whilst conventional dexamethasone therapy ameliorated EoE hallmark features without modulating PP2A activity, salmeterol driven restoration successfully reduced eosinophil infiltration and esophageal remodelling while also reducing CCL11, IL-5 and TSLP expression. Interestingly, a significant reduction of mast cells was also observed in salmeterol treated wild type mice in contrast to no change in mast cell inflammation in *Tnfsf10*^{-/-} OVA mice. This is not surprising, however, as LABAs have previously been shown to regulate mast cells in the lung (6, 9). We therefore cannot exclude that salmeterol may have anti-inflammatory properties independent of PP2A.

While no eosinophils are expected to be present in healthy human esophagus, mice esophagi will typically show a small number of eosinophils at baseline (2, 29), which is a critical difference to note when comparing this *in vivo* model to clinical disease. We did not address the impact that TRAIL may have on esophageal morphology during embryonic and postnatal development, which is beyond the scope of this study. We found a baseline protein expression of MID-1 in *Tnfsf10*^{-/-} mice while MID-1 mRNA expression was almost completely abolished (12). This suggests a dissociation between TRAIL and MID-1 that could be explained by post-transcriptional induction of MID-1 translation independent of TRAIL or a lack of specificity of the protein assay.

In summary, our findings highlight the essential role for TRAIL signaling in the promotion of the inflammatory phenotype in experimental EoE. Salmeterol reconstitutes TRAIL-induced reduction in PP2A activity which may –at least in part- explain its anti-inflammatory properties in experimental EoE. Our experimental studies suggest that Salmeterol could be of therapeutic value in EoE if sufficient drug levels can be reached in the esophagus via the oral route without unacceptable side effects.

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Figure Legends

Figure 1: Upregulation of TRAIL and MID-1 mRNA and protein levels in patients diagnosed with eosinophilic esophagitis compared to controls. **A** and **B**, Copy numbers of TRAIL (Fig 1A) and MID-1 (Fig 1B) in patient esophageal biopsies (expressed per copy of GAPDH) as determined by quantitative (q) PCR (EoE active; n=21, control; n=32). Excluded samples were found to be below detection. **C** and **D**, Protein levels of TRAIL (Fig 1C) and MID-1 (Fig 1D) in esophageal biopsies normalized to biopsy weight (EoE active; n=18, control; n=25). **E**, PP2A activity (as a percentage of control levels) in esophageal biopsies (EoE active; n=15, control; n=8) normalized to total protein (Fig 1E). Data expressed as means $\pm$ SEM. **F** and **G**, Correlation of TRAIL and MID-1 expression in both mRNA (Fig 1F, n=51) and protein (Fig 1G, n=48). * P <0.01 and **** P <0.0001.

Figure 2: No observable difference in TRAIL signaling between Control and EoE remission patients. **A**, Copy numbers of TRAIL (Fig 2A) and MID-1 (Fig 2B) in patient esophageal biopsies (expressed per copy of GAPDH) as determined by quantitative (q) PCR (control; n=32, EoE remission n=10). Excluded samples were found to be below detection. **C** and **D**, Protein levels of TRAIL (Fig 2C) and MID-1 (Fig 2D) in esophageal biopsies normalized to biopsy weight (control; n=25, EoE remission; n=5). **E**, PP2A activity (as a percentage of control levels) in esophageal biopsies (control; n=8, EoE remission; n=6) normalized to total protein (Fig 2E). Data expressed as means $\pm$ SEM.

Figure 3: Ovalbumin activates the TRAIL signaling pathway *in vivo*. **A**, Protein levels of TRAIL (Fig 3A) and **B**, MID-1 (Fig 3B) determined by ELISA in mouse esophagi normalized to esophageal tissue weight. **C**, PP2A activity (Fig 3C) normalized to esophageal protein weight and saline (SAL) activity. **D**, Quantification of OVA specific IgE levels (ng/ml) in serum determined by ELISA (Fig 3D). Data expressed as means $\pm$ SEM. # P <0.1, * P <0.05, ** P <0.01 and *** P <0.001. n=4-6.

Figure 4: TRAIL deficient mice are protected from ovalbumin induced eosinophilia, but not mast cell infiltration, in the esophagus. **A**, Representative images of transverse mouse esophageal sections stained with chromatin-red (Fig 4A). **B**, Histological enumeration of esophageal eosinophils from stained esophagi (Fig 4B). **C**, Histological enumeration of mast cell infiltration into the esophageal tissue (toluidine blue stain, Fig 4C). Data expressed as means $\pm$ SEM. *** P <0.001 and **** P <0.0001. n=4-8.

Figure 5: Eosinophilic esophagitis associated cytokines induced by ovalbumin are ameliorated in TRAIL deficient mice. **A**, **B** and **C**, TSLP (Fig 5A), TGF- β (Fig 5B) and CCL20 (MIP 2 α , Fig 5C) gene expression determined by qPCR, normalized to β -actin expression. **D**, **E** and **F**, CCL11 (Eotaxin-1, Fig 5C), IL-5 (Fig 5E) and IL-13 (Fig 5F) protein expression in mouse esophagi determined by ELISA and normalized to esophageal weight. Data expressed as means $\pm$ SEM. * P <0.05 and ** P <0.01. n=4-7.

Figure 6: Ovalbumin induced esophageal remodeling is dependent on TRAIL. **A**, Representative images of mouse esophageal sections (transverse) stained with Masson Trichrome (Fig 6A). **B** and **C**,

esophageal circumference (Fig 6B) and subepithelial collagen deposition (Fig 6C) determined using Image-Pro Plus software. Data expressed as means $\pm$ SEM. * P <0.05 and **** P <0.0001. n=4-8.

Figure 7: Salmeterol reduces cytokines and PP2A activity. **A**, PP2A activity (Fig 7A) normalized to esophageal protein weight and saline. **B**, CCL11 expression (Fig 7B) determined by qPCR and normalized to β -actin expression. **C** and **D**, IL-5 (Fig 7C) and CCL20 (Fig 7D) protein expression in mouse esophagi determined by ELISA and normalized to esophageal weight. **E** and **F**, TSLP (Fig 7E) and TGF- β expression (Fig 7F) determined by qPCR and normalized to β -actin expression. Data expressed as means $\pm$ SEM. * P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001. n=3-8

Figure 8: Esophageal inflammation is significantly reduced upon salmeterol comparable to dexamethasone treatment. **A**, Representative images of transverse mouse esophageal sections stained with chromatin-red (Fig 8A). **B** and **C**, Histological enumeration of esophageal eosinophils from Charbol's chromotrope-hematoxylin stained esophagi (Fig 8B) and mast cells from toluidine blue stained esophagi (Fig 8C). Data expressed as means $\pm$ SEM. ** P <0.01 and *** P <0.001. n=4-8

Figure 9: Ovalbumin induced remodeling features are reduced upon salmeterol and dexamethasone administration. **A**, Representative images of transverse mouse esophageal sections stained with Masson's Trichrome (Fig 9A). **B** and **C**, Esophageal circumference (Fig 9B) and esophageal fibrosis (Fig 9C). Data expressed as means $\pm$ SEM. * P <0.05 and ** P <0.01. n=4-8

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