A novel role for the TRAIL signalling pathway in the pathogenesis of Eosinophilic Oesophagitis

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Statement of Originality

I hereby certify that to the best of my knowledge that this thesis is my own written work and contains no material previously published or written by another person except where due references and acknowledgements are made. It contains no material that has been previously submitted by me for the award of any other degree or diploma in any university or other tertiary institution

Leon Sokulsky
Thesis by publication

I hereby certify that this thesis is in the form of three separate papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author, endorsed in writing by the Faculty Assistant Dean (Research Training), attesting to my contribution to any jointly authored papers.
Acknowledgements

It goes without question that completing a doctorate of philosophy is a taxing and challenging experience. However, one of the best things about a PhD candidacy is that you rarely venture forth alone. The work I have presented here in this thesis would not have been possible if it weren’t for the support of the following people.

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*both authors contributed equally
List of Abbreviations:

AAD- Allergic Airways Disease

AHR- Airways Hyperreactivity

Asp F- Aspergillus Fumigatus

BAL- Bronchoalveolar Lavage

CCL11- C-C motif chemokine ligand 11 (Eotaxin-1)

CCL24- C-C motif chemokine ligand 24 (Eotaxin-2)

CCL26- C-C motif chemokine ligand 26 (Eotaxin-3)

DISC- Death Inducing Signalling Complex

EoE- Eosinophilic oesophagitis

FADD- Fas Associated Death Domain

GORD- Gastro-Oesophageal Reflux Disease

IgE- Immunoglobulin E

IKK- I Kappa B

IL- Interleukin

ILC2- Innate Lymphoid Cell Type 2

IL-13Ra- Interleukin IL Receptor α

MAPK- Mitogen-Activated Protein Kinase

MID-1- Midline-1
NFκB- Nuclear Factor Kappa B

OVA- Ovalbumin

PP2A- Protein Phosphatase 2A

PP2Ac- Protein Phosphatase 2A catalytic subunit

PPI- Proton Pump Inhibitor

PPI-REE- Proton Pump Inhibitor Responsive Eosinophilic Oesophagitis

RIP- Receptor Activating Protein

STAT- Signal Transducer and Activator of Transcription

TGF-β- Transforming Growth Factor-β

Th2- T helper 2

TRAIL- TNF-Related Apoptosis Inducing Ligand

TRAIL-/-- TRAIL Deficient

TRAIL-R- TRAIL Receptor

TSLP- Thymic Stromal Lymphopoietin

VCAM-1- Vascular Cellular Adhesion Molecule-1

VEGF-A- Vascular Endothelial Growth Factor-A
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Thesis Abstract

Eosinophilic Oesophagitis (EoE) is an allergen mediated disorder of the oesophagus, associated with eosinophilic infiltration of the oesophageal epithelial layer and remodelling of oesophageal scaffolding. There has been a significant rise in EoE prevalence over the past ten years, however, therapeutic strategies to counter hallmark EoE features have remained relatively unchanged from steroid therapy and dietary restrictions. The apoptotic factor TRAIL has previously been implicated in allergic asthma as a driver for immune cell infiltration, remodelling and airway hyperreactivity. The upregulation of TRAIL through allergen exposure results in the induction of MID-1 and subsequent downregulation of PP2A: a negative regulator of NF-κB and MAP kinase inflammatory pathways. Given the similarities between EoE and asthmatic inflammation, this thesis will explore the role of the TRAIL signalling pathway through the analysis of EoE oesophageal human biopsies and the employment of EoE in vivo models.

In chapter 1, TRAIL and MID-1 expression was found to be elevated in EoE patient biopsies and in vivo modelling of Asp F-driven EoE demonstrated an activation of the TRAIL signalling pathway. TRAIL and MID-1 deficiency resulted in an ablation of EoE hallmark features in vivo, including reduced eosinophil infiltration, fibrosis, eotaxins, Th2 cytokines and TSLP, with TSLP recapitulation found to restore disease properties despite TRAIL deficiency. Chapter 2 further analyses the TRAIL signalling pathway in human EoE, demonstrating a correlation in TRAIL and MID-1 protein and mRNA as well as showing a reduction of PP2A activity in EoE patients. Additionally, chapter 2 demonstrated that TRAIL deficiency ablated ovalbumin driven EoE and that restoring PP2A activity via salmeterol therapy was comparable to corticosteroid treatment in vivo. Finally, in chapter 3, the impact of the Th2 cytokine on the TRAIL signalling pathway was assessed in vivo, where MID-1
silencing was found to ablate eotaxin-1 expression and completely abolish eosinophilia into
the oesophagus. Given the upregulation of MID-1 in TRAIL deficient, but not STAT6
dependent mice, it is likely that MID-1 can operate independently of TRAIL via STAT6 in
IL-13 driven inflammation.

Overall, this thesis has taken multiple approaches in addressing TRAIL’s role in the
perpetuation of EoE hallmark features, through the analysis of human biopsies to the
employment of multiple EoE mouse models. The studies conducted in this thesis have
broadened our understanding of this emerging disorder and highlighted potential therapeutic
strategies to combat this disease.
1. General Introduction

1.1. Introduction into eosinophilic oesophagitis

Eosinophilic oesophagitis (EoE) is characterised by antigen-mediated eosinophilic inflammation of the oesophagus occurring independently of gastro-oesophageal reflux. Individuals presenting with EoE typically display symptoms pertaining to oesophageal dysfunction, notably; dysphagia, food impaction, oesophageal pain, vomiting and in some paediatric patients, a failure to thrive. The infiltration of eosinophils into the upper gastrointestinal tract is not unique to EoE and can also be associated with other oesophageal pathologies, notably gastro-oesophageal reflux disease (GORD) (1-4). While the key differences between EoE and GORD can be simplified down to whether the inflammation is allergen or reflux driven, there are few clinical strategies available to concisely differentiate between these two diseases: patients will often have overlapping symptoms, as heartburn and dysphagia maybe observed in both entities (3). Furthermore, paediatric patients represent a significant diagnostic challenge due to difficulties in expressing symptoms or, conversely, presenting multiple clinical manifestations (5). Without appropriate treatments, inflammation associated with EoE can result in structural remodelling of the oesophagus in the form of oesophageal smooth muscle alterations and fibrosis, worsening EoE associated symptoms (6-8).

As of 2007, it was advocated that EoE diagnosis is given upon the detection of more than 15 eosinophils per high powered field from oesophageal biopsies (9, 10) and eliminating reflux as the cause of inflammation through the administration of acid-suppressive medication (such as proton pump inhibitors (PPIs)) (11, 12). Further complicating diagnosis is the emergence of a sub-group of EoE patients who demonstrate reduced eosinophilia in response to PPI therapy (PPI-REE) (13, 14), with ambient pH testing of the oesophagus required to separate
PPI-responsive EoE from GORD patients (15). Therefore, diagnosis of EoE is highly dependent on a clinocopathological correlation of both symptoms and histological analysis, coupled with an assessment of PPI efficacy and determining if reflux is a contributor to eosinophilic inflammation.

Figure 1: Comparison of a normal oesophageal biopsy (A) with a biopsy extracted from an EoE patient (B). Biopsy A displays a normal squamous mucosa, no alterations in the basal layer or lamina propria, with an absence of inflammatory cells. Biopsy B presents with alterations in the epithelia and basal cell hyperplasia, with a significant influx of eosinophilic infiltration. Modified from (16).

The primary goal of EoE treatment is to achieve EoE remission and prevent remodelling. EoE remission can be achieved through the swallowing of corticosteroids (normally fluticasone or budesonide) or dietary modifications (17-21). Additionally, if patients fall in the category of PPI responsive EoE, clinicians will often encourage PPI therapy if it has demonstrated to effectively resolve EoE symptoms (22), however, the long term effectiveness of this treatment strategy has yet to be examined. It is therefore imperative that an accurate diagnosis of EoE is given as early as possible and a disease management strategy is implemented: a misdiagnosis (or a failure to responded to the previously mentioned treatments) can result in a worsening of the disease condition, notably in the form of extensive remodelling of the proximal and distal oesophagus via collagen deposition and epithelial thickening (23, 24). These patients will often present a ring-like pattern occurring throughout the oesophagus, as observed via endoscopy. In severe cases, this will eventually
progress to form a ‘stricture’, and patients suffering from multiple strictures will often form a ‘feline’ oesophagus, resulting in severe oesophageal pain and an inability to swallow food (25, 26). Further complicating disease management is the idea that eosinophils may be an end result of inflammation rather than causative agents as has been traditionally believed. The poor correlation between absolute numbers of eosinophils, severity of symptoms and risk of progression to serious complications such as stricture formation has contributed to clinical uncertainty about the duration and aggressiveness with which the condition should be treated (15, 27).

Since the publication of the consensus into the recommendation for diagnosing and treating EoE in 2007 (28), scientific publications focused on EoE and its mechanisms have more than doubled in addition to an increased awareness about the disease (29). Despite this, the precise prevalence of EoE is difficult to ascertain due to apparent under-diagnosis, although improved clinical guideline practices have improved the reliability of reporting significantly (30). A systematic review and meta-analysis of EoE epidemiology has ascertained an overall prevalence of 22.7 cases per 100 000 inhabitants a year across the studies (31). These studies were based in either North America (prevalence of 30.7 per 100 000) or Europe (16.1 per 100 000) (31) with the exception of one retrospective study performed in Western Australia in 2006 (8.9 per 100 000 in 2004) (32). Further analysis of these studies identified a sharp increase in the average number of new cases pre and post 2008 (3.7 to 7.2 per 100 000 inhabitants/year) (31), indicative that the disease incidence rate is on the rise.
Figure 2: Diagnosis and treatment strategies for EoE. Diagnosis is dependent on the presence of at least 15 eosinophils per high powered field in oesophageal biopsies post PPI treatment. Treatment strategies involve the administration of topical steroids or specialised diets and the efficacy of such treatments are trialled for 8 weeks. Factors that complicate diagnosis and treatment include the emergence of PPI-REE patients as well as sub-groups of patients that do not respond to mainstream therapies. Modified from (16).
1.2. Pathogenesis and mechanisms involved in EoE

Investigating the pathology of EoE in patients has revealed few potential leads regarding the underlying mechanisms of disease and the factors implicated in its manifestation. The disease has a bias to affect males more than females (9, 33) and is evenly distributed across a variety of ethnic backgrounds (34, 35). There is some evidence to suggest that genetics has an impact on EoE as some sufferers have been shown to have close relatives that also have EoE, however, common environmental exposures within households could also explain this familial trend (33). Although the etiology of the disease is ill-defined, it is common for patients to also have an allergic disorder in addition to EoE, with approximately 75% of EoE patients demonstrating a reaction to common food and aero-allergens (36, 37) and up to 50% having asthma (15).

The primary factor found to be associated with paediatric EoE thus far is diet, where it has been shown that eliminating common allergy related foods like egg, wheat, peanut, cow’s milk or soy from the patients diet can reduce the severity of oesophageal inflammation (19, 38, 39), although there are sub-groups of patients that demonstrate little benefit from these interventions (40). As demonstrated through in vivo models, food allergens such as egg can be used to induce hallmark EoE features in the oesophagus, notably eosinophilic inflammation, fibrosis and angiogenesis after first becoming sensitised to the allergen then subsequently being challenged (41, 42). Using these models, a functional role for thymic stromal lymphopoietin (TSLP), basophils and mast cells were found in food allergen mediated EoE (41, 43) operating independently of Immunoglobulin E (IgE) (41).
Household allergens have also been implicated in EoE, most notably house dust mite, cockroach allergens and mould (23, 45). Combined with the western lifestyle of living in sealed insulated homes, the allergen load in the air that could be inhaled by both children and adults is potentially high. These allergens could then become lodged in the mucosa of the airways, promoting allergic inflammation in sensitised individuals (24). T-helper-2 (Th2) associated cytokines, most notably Interleukin-5 (IL-5), then prime the proliferation and maturation of eosinophils from the bone marrow, promoting their migration to the affected tissue (46). Such cytokines are not only secreted from Th2 cells; it has been revealed that innate lymphoid cells type 2 (ILC2s), a recently described cell type that can regulate and promote inflammation non-adaptively (47), are present in the oesophagus of EoE patients and secrete IL-5 and IL-13 in response to IL-33 and TSLP stimulation (48). As in asthma, these eosinophils are primarily directed to the lungs, however, in EoE there is emerging evidence that they also traffic to the oesophagus (24), though the mechanisms for this distinction remain to be elucidated.

Research into the mechanisms involved in eosinophilic oesophagitis has expanded since the development of a murine EoE models, notably the Aspergillus Fumigatus (Asp F) model of
EoE (23). This model relies on repeated and intense allergen exposure over a period of time, where BALB/c mice are administered nine doses of Asp F extract intranasally over a period of three weeks (23). An interesting point regarding the Asp F model is the requirement that the mice must inhale the allergen to their lungs: histological analysis of anesthetised mice (which inhale most of the Asp F to their lungs) showed a dramatic increase in eosinophilic cells within the epithelium, submucosa and lamina propria of the oesophagus. Non-anesthetised mice that received Asp F do not display any hallmark features of EoE (23), suggesting that direct allergen exposure (via swallowing) is not enough to promote eosinophilic oesophagitis.

In addition to in vivo asthma studies (49), the application of the egg allergen ovalbumin (OVA) in mice has also been used to model EoE. In contrast to the intense allergen insults to the lung and oesophagus as seen through Asp F administration (23), OVA driven EoE models demonstrated how EoE can be induced in the oesophagus without being dependent on lung inflammation. As shown by Noti, et al (2013), EoE pathology was induced in the oesophagus of mice through epicutanous administration of OVA to chemically induced atopic skin lesions, followed by intragastric challenges of OVA (41). Compared to the Asp F model of EoE, this model has arguably greater clinical relevance in regards to EoE given the oral route of the OVA challenge and the lack of dependency on lung inflammation (41). Furthermore, this model mimics the association between atopic disorders (allergic dermatitis in this example) and the presentation of EoE as seen clinically (9, 50), which is relatable to EoE patients that have co-existing atopic disorders.

1.3. Cytokines involved in the pathogenesis of EoE

Key cytokines involved in oesophageal eosinophilia in EoE include epithelial cell derived proteins, such as the Eotaxin family (CCL11, CCL24 and CCL26), TSLP and the Th2 T-cell
derived cytokines (notably IL-5 and IL-13). While Eotaxins, TSLP and IL-5 are involved in eosinophilic migration, IL-5 has a multifactorial role as it is responsible for eosinophil maturation, migration and increased sensitivity to Eotaxins (23, 49), while Eotaxins exclusively promote a baseline homing of eosinophils to the target tissue (23, 51) and TSLP elicits its chemotactic properties through the activation of Th2 responses (52, 53). What is interesting to note about these cytokines are the differences regarding their functions in different organs: IL-5 is primarily a Th2 lung cytokine and has a major role in promoting lung eosinophilia in asthma (49, 54), while Eotaxins do not have a dominate role in the lung and are primarily associated with eosinophil migration in the GIT (23, 51). Indeed, knockout mouse studies have revealed that a lack of CCL11 or IL-5 expression significantly reduces eosinophil migration to the oesophagus in response to Asp F, with the latter ablating eosinophils completely (23).

IL-13 is produced at elevated levels within the asthmatic lung and has been shown to play a key role in eosinophilic recruitment, mucus hypersecretion, airways hyper reactivity and the production of IgE from B cells (55, 56). When investigating IL-13 in the Asp F mouse model, it was found that a genetic deficiency of IL-13 resulted in a partial reduction in oesophageal eosinophilia but no change in blood eosinophilia (57), indicating a local role for IL-13 in the oesophagus for promoting eosinophilia. This was further demonstrated by overexpressing IL-13 in mice using intratracheal administrations of recombinant IL-13 (or using transgenic mice overexpressing IL-13) resulting in an elevated level of eosinophilia in the oesophageal epithelial layer, lamina propria and submucosa but not the blood (57, 58). Further EoE features, such as epithelial cell disruption and hyperplasia, are also present in these mice due to elevated IL-13 levels (58), highlighting an important role for this cytokine in oesophageal remodelling.
While Th2 cytokines play a key role in eosinophilic inflammation, it should be noted that both Th2 cytokines and eotaxins are not the sole promoters of the disease and have previously been shown to be regulated by additional epithelial factors. The epithelium derived cytokine, IL-33, has been shown to be a key factor involved in basal eosinophilic homeostasis and is thus highly associated with Th2 pathology (59). IL-33 signals through its receptor ST2, which has been shown to be expressed on mast cells, eosinophils, basal cells and ILC2s (60). The application of IL-33 in vivo to the oesophageal mucosa was demonstrated to induce a profound Th2 response, which was nullified in IL-13 deficient mutant mice (61). Whilst IL-5 and eotaxins have been demonstrated to facilitate eosinophilia in active EoE, IL-33 appears to have an important role in early development of the disease, as it has been shown to be acutely expressed in Asp F exposed mice before returning to baseline expression at later timepoints (61). This is supported by IL-33’s pleiomorphic nature in the inhibition of T regulatory cells and early induction of IL-13 and eotaxins (61), both which maintain disease phenotype after their induction.

The emergence of TSLP as an essential cytokine involved in EoE was highlighted by genome-wide association studies focused on EoE patient biopsies that exposed the presence of a gain-of-function SNP on the 5q55.1 locus that encompasses TSLP (62). The mechanics of TSLP induced inflammation incorporates the promotion of eosinophilia through CD11c dendritic cells, basophils and ILC2 cells while taking advantage of defective epithelial barrier protection (41, 63, 64), presenting an alternative method of allergen driven inflammation to Th2 cells. As demonstrated in vivo in a model of OVA driven EoE, eosinophilic infiltration into the mouse oesophagus was dependent on the expression of TSLP and basophils in the oesophagus (41). This was further demonstrated in clinical studies, where a basophil/TSLP axis was determined in a cohort of EoE patients (vs control and inactive EoE) (41). Interestingly TSLP may be induced only by particular food antigens, as βLG and casein do
not upregulate TSLP expression \textit{in vitro} despite milk being the most common dietary trigger for EoE (65, 66). This implies that the mechanisms required for EoE development may be more heterogeneous than previously thought and certain allergens maybe more dependent on classical Th2 cell presentation rather than TSLP signalling (67).

\textbf{Figure 4:} EoE is a multifactorial disease that employs a variety of mechanisms to perpetuate its pathogenesis. (A), Food antigen exposure interacts with an impaired epithelial barrier, infiltrating the epithelium. (B), Antigens present in the epithelium layer can interact with dendritic cells and promote the release of Eotaxins and TSLP. This results in the migration of eosinophils that subsequently degranulate, causing epithelial damage. (C), Th2 driven responses, from antigen presenting cells or the release of TSLP. Th2 cells release IL-5, which promotes the maturation and migration of eosinophils to the oesophageal epithelium, and IL-13, resulting in the release of Eotaxin, CAPN14

\textbf{1.4. Oesophageal remodelling in EoE}

Although separate diseases, the remodelling that is observed in the oesophagus in patients with EoE is often compared to remodelling of the airways seen in asthma, in terms of cytokine involvement and associated pathways (68, 69). In asthma and EoE, both Eotaxins and IL-5 contribute to remodelling through the promotion of eosinophils to the target tissue (70). These eosinophils degranulate, releasing remodelling proteins such as Vascular
Endothelial Growth Factor-A (VEGF-A), Transforming Growth Factor β (TGF-β) and IL-8 (68, 70). Th2 cytokines like IL-4 and IL-13, however, can induce the production of remodelling cytokines directly by epithelial cells (71), which is orchestrated through activation the IL-4 Type I and II receptor, where the second arm of the receptor (IL-13Rα1) can accommodate for IL-4 and 13 binding (72, 73). Activation of the IL-4 Type I and II receptor by IL-4 and 13 results in the formation of the transcription factor Signal Transducer and Activator of Transcription (STAT)-6, which in turn activates a variety of genes such as Nuclear Factor Kappa B (NFκB) and the production of CCL26 (74, 75). Activation of the IL-13Rα1 arm will initiate STAT6 and STAT3 signalling, with both transcription factors most likely inducing the expression of the extracellular matrix protein Periostin (76, 77). Periostin, which has been show to play an integral part in eosinophil cell adhesion (77), is important for the induction of fibrosis and collagen cross-linking in diseases such as asthma (78) and could potentially have a similar role in EoE (77). This is supported by data that demonstrates Periostin as the second highest upregulated gene in EoE patients (the first being CCL26) (79), suggesting that these genes, in addition to the STAT3 and 6 pathway, play an integral role in the pathogenesis of EoE.

The IL-13Rα2 arm is an alternate binding site for IL-13, long thought to have a non-active role in cell signalling due to a lack of cell signalling components (80). It has emerged more recently in colitis models of inflammation that the activation of the IL-13Rα2 is necessary for the activation of TGF-β in the colon, which is a potent inducer of fibrosis (81). Human monocytes cultured in vitro that expressed IL-13Rα1 but not IL-13Rα2 are unable to activate the TGF-β promoter in response to IL-13 exposure (82). Similar results were obtained when the cells expressed a mutated version of IL-13Rα2 without a cytoplasmic tail (83) and it had also been found that STAT6 phosphorylation induces IL-13Rα2 expression (84). Although there is a lack of evidence that demonstrates that this pathway is being employed in EoE, it
has been shown in the literature that TGF-β is upregulated in EoE patients, on the mast cells present in the smooth muscle region of the oesophagus (85). The location of these cells suggests that TGF-β can elicit remodelling effects on smooth muscle cells via mast cells (85) in addition to activating fibrosis via fibroblasts in the epithelial region of the oesophagus (86). Further studies into these mechanisms may elucidate if IL-13 is involved in promoting TGF-β expression in these cells and if this is a major driving force of the smooth muscle hyperplasia and fibrosis seen in EoE.

Figure 5: Presentation of EoE remodelling features of the oesophagus as shown by endoscopy. Structural changes of the oesophagus usually present with (A) oesophageal rings, (B) white plaque formations, (C) oesophageal Furrows and (D) stricture formation of the oesophagus. Modified from (16).

The angiogenesis related protein, VEGF-A, has been shown to become significantly upregulated in patients suffering from EoE, suggesting that the oesophagi of these patients are undergoing neovascularization (68). The presence of new blood vessels makes the
oesophagi more accessible to inflammatory cells including eosinophils, which is worsened by a rise in Vascular Cellular Adhesion Molecule-1 (VCAM-1) and IL-8 expression, the latter supporting angiogenesis and epithelial proliferation (68, 87). This increase of eosinophil migration concurrently results in elevated expression of Tumour Necrosis Factor (TNF)-α, as shown in EoE oesophageal biopsies, where it most likely contributes to further eosinophil migration to the oesophagus as it does in the lung (88). At the same time, TNF-α promotes VCAM-1 expression on endothelial cells (89), which results in more immune cell infiltration and ultimately a positive feedback mechanism that result in a worsening prognosis for the patient. The key to these inflammatory mechanisms is NFκB, a transcription factor required for the promotion of inflammatory and remodelling cytokines like TNF-α, VCAM-1 and Th2 cytokines (90). Further evidence of NFκB having a role in EoE is the upregulation of NFκB activity in EoE mucosal biopsies (68), which could be attributed to a downregulation of Inhibitor of NFκB (IkB) that was also observed in this study (68). Although the activation of NFκB remains poorly understood, a recently described inflammatory signalling pathway found to be important in asthmatic inflammation could provide some insight into the inflammation processes underpinning EoE.
Figure 6: Mechanisms involved in eosophageal remodelling in EoE. The secretion of IL-13 from Th2 cells and possibly mast cells results in the release of Eotaxins, promoting eosinophilia. Eosinophils and mast cells release TGF-β, which in combination with Th2 cytokines can promote the fibrosis and changes in the smooth muscle. Modified from (69).

1.5. The TNF-related apoptosis inducing ligand pathway

TNF-related apoptosis inducing ligand (TRAIL) is recognised as an important molecule involved in cancer and inflammatory disorders. TRAIL, which is transcribed from chromosome 3q26 (91), is a member of the tumour necrosis factor superfamily and is reported to bind to at least five membrane proteins, consisting of death receptor 4 and 5 (also known as TRAIL-R1 and R2), decoy receptor 1 and 2 (TRAIL-R3 and R4) and osteoprotegerin (92). Activation of TRAIL-R1 and R2 results in the recruitment of the Fas-associated death domain (FADD) and procaspase 8, which are then associated into the death inducing signalling complex (DISC) (93). This leads to the conversion of procaspase 8 to caspase 8, which activates caspase 3, 6 and 7 to induce DNA fragmentation, resulting in apoptosis. Alternatively, caspase 8 can activate the Bid/Bax pathway, resulting in the release of cytochrome c and apoptosome formation. Like caspase 8, the apoptosome also mediates DNA fragmentation through caspase 3, 6 and 7.
TRAIL-R3 and TRAIL-R4 have been previously identified as decoy receptors, based on the lack of a death domain on TRAIL-R3 while the death domain on TRAIL-R4 is truncated (94). While it was previously thought that their only role in TRAIL signalling is to protect cells from apoptosis by binding to TRAIL, it has become apparent that TRAIL-R4, in addition to TRAIL-R1 and 2, can promote inflammation through mitogen-activated protein kinase (MAPK) and NFκB (95, 96). TRAIL binding to TRAIL-R4 results in the recruitment of receptor activating protein (RIP) which can then interact with IκB kinases (IKK). This can ultimately result in the downregulation of IKK, leading to an increase of NFκB driven inflammation (97). Furthermore, activation of the MAPK pathway can prevent the activation of caspase 8 (98), preventing the cell from entering an apoptotic state and increase the longevity of the cell.

The inflammatory action of TRAIL is dependent on the presence of ubiquitin ligases, with one notable example being the E3 ubiquitin ligase midline-1 (MID-1) (99). Early studies investigating MID-1 showed that it has an important role in embryonic development, as mutations in the gene can result in midline deformities, such as cleft lip or cleft palate, and has also been linked to the congenital disorder Opitz-GGG syndrome (100). Its specific role in inflammation relates to its interaction with the α4 subunit protein and the c subunit of protein phosphatase 2A (PP2A) (99). Monoubiquitination of the α4 protein via MID-1 will cleave the α4 protein, transforming it from a relatively stable state to a destructive state, where it will cleave the PP2A regulatory subunit (PP2Ac) cumulating in protein degradation (99, 101). Because PP2A is essential for the dephosphorylation of IκB and the inhibition of JNK pathways (102, 103), reduced activation of PP2A due to PP2Ac cleavage will increase NF-κB and JNK mediated inflammation, releasing inflammatory factors, notably Eotaxins, Th2 associated interleukins, as well as TSLP, IL-25 and IL-33.
Figure 7: TRAIL has a dual role as a promotor of apoptosis in tumourgenetic cells and a promotor of inflammation in the diseased state. Activation of TRAIL-R1 and 2 will induce apoptosis in a FADD/Caspase 8 dependent manner, through the activation of cytochrome C employing mitochondrial proteins and activation of caspases 3, 6 and 7. Alternatively, TRAIL may activate TRAIL-R1, 2 and 4 to recruit RIP into the receptor complex, employing the inflammatory TRAIL signalling pathway to activate p38, JNK and NF-κB, resulting in inflammation. Modified from (96).

1.6. TRAIL and its role in allergic inflammation

The link between TRAIL and eosinophilic asthma was established in 2002 via a clinical study involving asthmatic and healthy volunteers, where the asthmatic subjects were found to have a higher concentration of TRAIL in their bronchoalveolar lavage (BAL) fluid compared to healthy controls after an antigen challenge (104). Since then, there has been great interest into the role of TRAIL in allergic inflammation, with Weckmann et al. (105) being the first to demonstrate that TRAIL is regulated by Th2 responses in an in vivo model. Mice deficient in the TRAIL gene (TRAIL-/- mice) and mice administered siRNA against the expression of TRAIL mRNA were protected from eosinophilic inflammation and airways hyperreactivity (AHR) when sensitised and challenged with OVA (105). In addition, mice deficient in the TRAIL gene also demonstrated impairment of T-cell homing, Th2 cytokine release and the activation of STAT6 (105).
Further studies on TRAIL and inflammation reveal that administering recombinant TRAIL protein alone to a non-allergic mouse could promote both AHR and eosinophilia in the lungs (106), and that these processes are heavily reliant on IL-13 (105). While wild type BALB/c mice displayed the eosinophilic phenotype as described previously, mice that were lacking the IL-13 gene did not develop AHR, eosinophilia or Th2 related responses, suggesting that TRAIL is dependent on IL-13 and most likely the STAT6 pathway (105). Furthermore, TRAIL deficient mice that were administered recombinant IL-13 did develop AHR and eosinophilia, highlighting that TRAIL is dependent on IL-13 and the pathology of eosinophilic related disease is orchestrated through these cytokines (99, 105, 106).

The role of TRAIL in inflammatory disease is not limited to allergic asthma, however, as it has been shown that TRAIL has a role in additional lung pathologies. Animal models of cigarette smoke induced chronic obstructive pulmonary disorder (107) and neonatal chlamydial respiratory infection (108) have demonstrated that TRAIL is essential in the activation of NF-κB driven inflammation and long term airway remodelling. This implicates the concept of TRAIL’s role as a general facilitator of inflammation, independent of the patient’s age or type of inflammatory condition. Despite this, research regarding the TRAIL signalling pathway in inflammation has seldom expanded past the lung despite TRAIL’s presence throughout multiple organs and possible implication in various inflammatory conditions.
Figure 8: TRAIL exposure to epithelial cells results in an inflammatory cascade. The upregulation of TRAIL upon allergen exposure facilitates the upregulation of MID-1. MID-1 facilitates the degradation of the catalytic subunit of PP2A, preventing PP2A from dephosphorylating MAPK and IκB. This results in increased p38-MAPK, JNK and NK-κB activity, resulting in the release of inflammatory cytokines and the influx of inflammatory cells. Modified from (99).

1.7. EoE and the TRAIL signalling pathway

It is plausible that TRAIL may play an important role in the perpetuation of the EoE disease phenotype, simply due to the similarities the disorder has with allergic asthma as well as what is known about the behaviour of TRAIL in the diseased state. Firstly, TRAIL has been demonstrated to conduct its inflammatory role through the activation of NF-κB and p38 MAP Kinase pathways (shown both in vitro (90, 109) and in vivo (99, 107)), both of which have been shown to contribute to the upregulation of Eotaxins, Th2 cytokines and remodelling
chemokines in the oesophagus in EoE (68, 110). This is in contrast with what is observed in GORD, which relies on chemical injury to induce epithelial cell damage and trigger NF-κB signalling cascades (111). Furthermore, the requirements for TRAIL release from epithelial cells can be triggered through allergen exposure (99), which can occur frequently in EoE patients through either the diet or, potentially, through inhaled household allergens (112). Finally, it has been previously demonstrated that TRAIL is observed in the blood of EoE patients (113), highlighting the plausibility of TRAILs involvement in EoE pathology.

Although there are similarities shared between asthma and EoE, the differences between the two diseases must be considered before any conclusions are drawn regarding TRAILs potential role in promoting EoE. Such differences include the ciliated epithelium of the lungs compared to the squamous epithelium of the oesophagus, a greater upregulation of epithelial factors such as eotaxin-3 in EoE, and a greater association between atopic disorders (such as allergic dermatitis and food allergy) and EoE compared to asthma. Nevertheless, the TRAIL inflammatory signalling pathway has the potential to be involved in the perpetuation of EoE, both in terms of inflammation and in oesophageal remodelling. Targeting factors associated with the TRAIL signalling pathway could provide an alternative means of therapy for patients that respond poorly to conventional EoE treatments. Additionally, the identification of TRAIL signalling molecules in the oesophagus of EoE patients can provide novel diagnostic techniques for a disease that is both difficult to diagnose and high dependent on early treatment for reduced patient morbidity.
Research Chapter 1: TRAIL regulates MID1, TSLP, inflammation and remodelling in experimental eosinophilic oesophagitis

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By signing below, I confirm that Leon Sokulsky was a shared-primary contributor to the publication entitled ‘TNF-related apoptosis inducing ligand (TRAIL) regulates midline-1, thymic stromal lymphopoietin, inflammation and remodelling in experimental eosinophilic oesophagitis’ and conducted mouse models, collected and processed mouse tissue samples, performed experiments on mouse tissue, analysed data and drafted the manuscript.

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2.1. Abstract

2.1.1. Background: Eosinophilic oesophagitis (EoE) is an inflammatory disorder of the oesophagus defined by eosinophil infiltration and tissue remodelling with resulting symptoms of oesophageal dysfunction. TNF-related apoptosis-inducing ligand (TRAIL) promotes inflammation through upregulation of the E3 ubiquitin-ligase midline-1 (MID1), which binds to and deactivates the catalytic subunit of protein phosphatase 2Ac, resulting in increased nuclear factor kB activation.

2.1.2. Objective: We sought to elucidate the role of TRAIL in EoE.

2.1.3. Methods: We used Aspergillus fumigatus to induce EoE in TRAIL-sufficient (wild-type) and TRAIL-deficient (TRAIL-/-) mice and targeted MID1 in the oesophagus with small interfering RNA. We also treated mice with recombinant thymic stromal lymphopoietin (TSLP) and TRAIL.

2.1.4. Results: TRAIL deficiency and MID1 silencing with small interfering RNA reduced oesophageal eosinophil and mast cell numbers and protected against oesophageal circumference enlargement, muscularis externa thickening, and collagen deposition. MID1 expression and nuclear factor kB activation were reduced in TRAIL-/- mice, whereas protein phosphatase 2Ac levels were increased compared with those observed in wild-type control mice. This was associated with reduced expression of CCL24, CCL11, CCL20, IL-5, IL-13, IL-25, TGFB, 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 significant upregulation of TRAIL and MID1 in a cohort of children with EoE compared with that seen in controls.
2.1.5. Conclusion: TRAIL regulates MID1 and TSLP, inflammation, fibrosis, smooth muscle hypertrophy, and expression of inflammatory effector chemokines and cytokines in experimental EoE.
2.2. 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 (32, 114). EoE can manifest at any age with a trend towards affecting atopic male children (9, 33, 50). 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 (15, 57). EoE sufferers, particularly children, have comorbid atopic disorders such as food allergy or asthma (38, 50, 66).

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 (19, 38, 39). Alternatively, topical administration of corticosteroids to the oesophageal mucosa has also been shown to be effective in reducing oesophageal eosinophilia (15, 20, 21). 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 pain, stricture and impactation (86, 115). 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 (116, 117). However, up to 75% of patients experience post-dilation complications including chest pain, oesophageal pain and bleeding (118), 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 (86, 119). Th2 cytokine signalling plays a central role in EoE pathogenesis by driving the recruitment and proliferation of eosinophils to the oesophagus (46). In turn, eosinophil derived proteins including transforming growth factor (TGF)-β have been shown to driving pro-fibrotic SMAD2/3 pathways (86). 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) through signal transducer and activator of transcription (STAT)-6 mediated pathways (58). However, recent studies have indicated that symptoms and remodelling can persist even when eosinophilia has been corrected (120), suggesting eosinophil-independent pathways may also be key drivers of oesophageal remodelling in patients with EoE (57). 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 (85). A major EoE genetic susceptibility locus exists at the TSLP gene (5q22) (62) and release of TSLP from oesophageal epithelial cells promotes basophil infiltration (41) and has been demonstrated to induce cellular senescence and fibrosis in asthma models (121). 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 recognized as a pro-inflammatory cytokine (99, 104, 105). We have shown previously that in allergic airways disease (AAD) models and in patients with asthma, TRAIL is released by structural airway cells in response to allergen stimulation (105) resulting in upregulation of the E3 ubiquitin ligase Midline-1 (MID1) (99). In turn, MID-1 monoubiquinates the α4 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 (101, 122).
Due to the central role of PP2A in the regulation of inflammatory cascades via dephosphorylation, including the nuclear factor κB (NF-κB) and mitogen-activated protein kinase pathways (102, 103), the inhibition of PP2Ac permits the activation of inflammatory cascades, primarily through Th2 mediated mechanisms but also through early inflammatory factors such as CCL11, CCL20, IL-25 and 33 (99, 105, 123). TRAIL-induced upregulation of MID1 has been shown to promote allergic inflammation and airways remodelling in the lung through inhibition of PP2A activity (99, 105, 106).

Although 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 hypothesized it would contribute to oesophageal inflammation and remodelling in an allergen induced murine model of EoE (e.g. *Aspergillus fumigatus* induced).
2.3. Methods

2.3.1. RNA sequencing of human biopsies

Patient cohorts and methods for RNA sequencing and analyses have been described previously (Sherrill, et al.(124)). 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 Benjamin-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 (GSE58640).

2.3.2. 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.

2.3.3. Aspergillus fumigatus mouse model of EoE

The *A.fumigatus* mouse model of EoE, described previously by Mishra *et al.*(23), was employed to investigate the role of TRAIL in EoE. Briefly, mice were intranasally challenged with 100 mg of *A.fumigatus* extract (Greer Laboratories, Lenoir, NC) in 50 mL of sterile saline 3 times a week for 3 weeks after administration of isoflurane anaesthetic. Control mice
received 50 mL of saline only. Mice were killed for oesophageal samples 24 hours after the final *A. fumigatus* challenge by using pentobarbital sodium (Virbac, Milperra, Australia).

**2.3.4. 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'-AGAGUAAUCUCACAAUACA-3') and a Nonsense (NONc) strand of siRNA (5'-UGGUUUACUGUCGACUAA-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 *A. fumigatus* challenge. This dose was repeated every second day throughout the course of the model.

**2.3.5. 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 *A. fumigatus* 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.

**2.3.6. 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.

2.3.7. 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.

2.3.8. 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).

2.3.9. Gene analysis of mouse oesophagi

Mouse oesophagi were immersed in RNAlater (Ambion, 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-AATCCAAAGGCAAAAAGTGTCAAA CG-3), CCL11 (F: 5-TTCTATTCCTGCTGCTCACGG-3, R: 5-AGGGTGCACTCTGTGTGT TGGTG-3), TGF-β (F: 5-TGTGGAACCTCTACGAAATATAGC-3, R: 5-GAAAGCCCT GTATTCCGTCTC-3), TSLP (F: 5-AGGCTACCCTGAAAACGTGAG-3, R: 5-GGAGATTGCATGAAGGAATACC-3) CCL20 (F:5-CGACTGTTGCTCCTCTGTACA-3, R: 5-AGGAGTTTCACAGCCCTTTT-3) IL-25 (F: 5-ATGTACCAGGTCCATACATTCTTG-3) (R: 5-CTAAGCCATGACCGGGGACC-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 (125). Murine β-actin (ACTB) was used as a housekeeper gene and gene expression was determined as mRNA copies of the gene of interest per copy of ACTB (F:5-GACGGCCAGGTCTCCTATGG-3, R:5-AGGAA GGCTGGAAAAGAGCC-3).

2.3.10. Protein quantification in mouse oesophagi

Snap frozen oesophageal samples were weighed prior to being homogenized (Tissue Tearor, BioSpec products) and protein levels for IL-5, IL-13, MID-1 and PP2Ac were determined by ELISA (R&D systems, Minneapolis, MN or Cusabio, Wuhan, China). Results are normalized to oesophageal tissue weight.
2.3.11. NF-κB 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 normalized to the weight of oesophageal tissue.

2.3.12. Flow cytometry

Mouse oesophagi were homogenized 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).

2.3.13. 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 +/- SEM.
2.4. Results

2.4.1 The TRAIL signalling axis is altered in the setting of EoE

Global RNA sequence analysis demonstrated that expression of TRAIL and MID1 was significantly upregulated in biopsies from a cohort of patients with EoE in comparison with that seen in healthy counterparts (Fig 1, A and B).

Thus we investigated the potential role of TRAIL in EoE pathogenesis in vivo through use of the *A. fumigatus* mouse model of allergic EoE. Intranasal *A. fumigatus* exposure resulted in upregulation of MID1 and downregulation of PP2Ac in the oesophagus (Fig 1, C and D). We also saw a significant increase in the activity of the p65 subunit of NF-kB (Fig 1, E). This was associated with higher numbers of mast cells in the oesophagus and increased eosinophil counts in the oesophagus and blood (Fig 1, F-H) but not CD4+ T-cell receptor–positive cells (Fig 1, I).

2.4.2 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 used mice genetically deficient in TRAIL. TRAIL−/− mice displayed markedly reduced expression of MID1 in the oesophagus compared with WT mice (Fig 2, A). TRAIL−/− mice were protected from PP2Ac downregulation in the oesophagus after *A. fumigatus* challenge (Fig 2, B) and also displayed decreased p65 activity (Fig 2, C). There was also a marked decrease in oesophageal eosinophil and mast cell counts (Fig 2, D and E) in addition to a marked decrease in eosinophil counts in the blood (Fig 2, F). This was also seen in bronchoalveolar fluid (Fig 2, G).
Figure 1: The TRAIL signalling pathway is active in EoE. (A) and (B), Individual fragments per kilobase of transcript per million mapped reads (FPKM) values of TNFSF10 (TRAIL; Fig 1, A) and MID1 (Fig 1, B) from RNA sequencing performed on oesophageal biopsy specimens from healthy control subjects (NL; n=6) and patients with active EoE (n=10). (C) and (D), Mouse MID1 (Fig 1, C) and PP2Ac (Fig 1, D) protein levels from oesophagi of *A. fumigatus* (Asp F)–treated and saline control (SAL) mice, as assessed by using ELISA. E, Activity assay for the NF-κB subunit p65. (F)-(H), Histologic enumeration of oesophageal eosinophils using congo-red stain (Fig 1, F), mast cell infiltration using toludine blue (Fig 1, G), and eosinophilia in the blood determined by using Giemsa stain (Fig 1, H). (I), oesophageal T cells (TCR1CD4+), as enumerated by means of fluorescence-activated cell sorting. Data are expressed as mean +/- SEMs. *P < .05 and ***P < .005.
Figure 2: TRAIL regulates PP2Ac and p65 activity in addition to eosinophil and mast cell infiltration. (A), MID1 expression was determined by using quantitative PCR. Gene expression was normalized to the copy number of ACTB. (B) and (C), Protein expression of PP2Ac (Fig 2, B) and p65 activity (Fig 2, C). (D), Histologic enumeration of oesophageal eosinophils using congo-red stain. (E), Enumeration of mast cells stained with toludine blue. (F), Eosinophilia in the blood determined by using Giemsa stain. (G), Differential cell count of total cells isolated from mice through bronchoalveolar lavage. BALF, Bronchoalveolar lavage fluid. *P < .05. Asp F, A fumigatus; SAL, saline.
2.4.3. Inflammatory cytokines and chemokines involved in EoE are regulated by TRAIL

To investigate which EoE-related factors are regulated by TRAIL, we performed quantitative RT-PCR on cDNA derived from mouse oesophagi. mRNA expression of the eosinophil chemoattractants CCL11 and CCL24 (Fig 3, A and B) was found to be significantly lower in TRAIL-/- mice in comparison with that seen in WT mice after *A. fumigatus* exposure. This trend was also present for mRNA expression of TGFB and TSLP (Fig 3, C and D). We also determined that IL-5 and IL-13 protein levels were reduced in addition to STAT6 expression (Fig 3, E to G).

2.4.4. Oesophageal remodelling in EoE requires TRAIL expression

The significance of TRAIL expression on oesophageal remodelling with EoE was determined through histologic analysis of mouse oesophageal tissue (Fig 4, A). TRAIL-/- mice were protected from an increase in oesophageal circumference when challenged with *A. fumigatus* (Fig 4, B). TRAIL-/- mice were also protected from increases in muscularis externa thickness and oesophageal fibrosis (Fig 4, C and D) compared with WT mice.

2.4.5. TSLP is sufficient to restore *A. fumigatus* induced remodelling in TRAIL deficient mice and TRAIL induces TSLP

TRAIL-/- mice that received recombinant TSLP throughout the *A. fumigatus* model had restored oesophageal eosinophilia (Fig 5, A and B) compared with TRAIL-/- mice that received only vehicle control in addition to *A. fumigatus*. TSLP-treated mice also displayed increased oesophageal circumference, muscularis externa thickness, and subepithelial collagen deposition (Fig 5, D-F). Non-allergic mice treated with recombinant TRAIL had increased oesophageal expression of TSLP 24 hours after treatment compared with vehicle-treated mice (Fig 5, C). Thus TRAIL regulates TSLP, which is sufficient to induce EoE, at least in this murine experimental system.
Figure 3: Inflammatory and remodelling cytokines involved in EoE are regulated by TRAIL. (A)-(D) and (G), Expression of CCL11 (Fig 3, A), CCL24 (Fig 3, B), TGFB (Fig 3, C), TSLP (Fig 3, D), and STAT6 (Fig 3, G) was determined by using quantitative RT-PCR. Gene expression was normalized to copy numbers of ACTB. (E) and (F), IL-5 (Fig 3, E) and IL-13 (Fig 3, F) protein expression was determined by using ELISA. * \( P < .05 \). *Asp F, \textit{A} fumigatus; \textit{SAL}, saline.
Figure 4: Oesophageal remodelling in EoE is dependent on the expression of TRAIL. (A) Representative images of transverse oesophageal sections at x100 and x200 magnification (stained with Masson trichrome) from WT and TRAIL-/- mice challenged with A. fumigatus or saline control. (B)-(D), Muscularis externa (Fig 4, B), oesophageal circumference (Fig 4, C), and subepithelial collagen deposition (Fig 4, D), as determined with Image-Pro Plus analysis (n=5-6). *P < .05. Asp F, A fumigatus; SAL, saline.
**Figure 5:** TSLP is sufficient to restore Asp F induced remodelling in TRAIL deficient mice. (A) Representative images of transverse oesophageal sections at x100 and x200 magnification (stained with Masson trichrome) from TRAIL−/− mice challenged with *A. fumigatus* and treated with recombinant TSLP or vehicle control. (B), Histologic enumeration of oesophageal eosinophils using congo-red staining. (C), TSLP expression in oesophagi of WT mice after treatment with recombinant TRAIL or saline control determined by using quantitative PCR. Gene expression was normalized to copy numbers of ACTB. (D)-(F), Muscularis externa (Fig 5, D), oesophageal circumference (Fig 5, E), and subepithelial collagen deposition (Fig 5, F), as determined with Image-Pro Plus analysis (n=6-8). *P < .05 and **P < .01. Asp F, *A. fumigatus*; SAL, saline; VEH, vehicle control.
2.4.6. Eosinophilic inflammation and remodelling are dependent on MID1 expression

siRNA-mediated silencing of MID1 expression compared with treatment with nonsense control (NONc) siRNA in the oesophagus (Fig 6, B) reduced oesophageal eosinophilia and protected against increases in oesophageal circumference, muscularis externa thickness, and oesophageal fibrosis (Fig 6) when challenged with *A. fumigatus*. MID1 siRNA–treated mice had corresponding reductions in expression levels of CCL11 and CCL24 (Fig 7, A and B) but did not show any difference in expression of TGFB or TSLP (Fig 7, C and D). Thus MID1 inhibition ameliorates EoE independent of TSLP.

2.4.7. TRAIL expression in the *A. fumigatus* EoE model

TRAIL expression was upregulated 24 hours after the first *A. fumigatus* challenge in epithelial and smooth muscle cells (Fig 8, B and C), which correlated with MID1, CCL20, TSLP, and IL-25 expression (Fig 8, D-G). After 3 weeks of allergen challenge (end point), no increased expression of TRAIL, CCL20, and IL-25 (Fig 8, H-J) was observed, whereas 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.
Figure 6: Oesophageal remodelling in EoE is dependent on MID1. (A) Representative images of transverse oesophageal sections at x100 and x200 magnification (stained with Masson trichrome) from WT mice challenged with *A. fumigatus* and treated with siRNA targeting MID1, nonsense siRNA, or saline control. (B), oesophageal MID1 expression determined by using quantitative PCR. Gene expression was normalized to copy numbers of ACTB. (C), Histologic enumeration of oesophageal eosinophils using congo-red stain. (D)-(F), Muscularis externa (Fig 6, D), oesophageal circumference (Fig 6, E), and subepithelial collagen deposition (Fig 6, F), as determined with Image-Pro Plus analysis (n=8). *P < .05, **P < .01, ***P < .005, and ****P < .0001. Asp F, *A.fumigatus*; SAL, saline.
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 normalized to copy numbers of β-actin. (n=8) *P<0.05=*, **P<0.01=**. Asp F, A.fumigatus; SAL, saline.
Figure 8: TRAIL and IL-25 expression is highest during establishment of the Asp F EoE model. (A) and (B) Representative immunofluorescence images of oesophageal sections counterstained with 49-6-diamidino-2-phenylindole dihydrochloride and stained with phycoerythrin-conjugated isotype control (Fig 8, A) and phycoerythrin-conjugated anti-TRAIL N2B2 antibody (Fig 8, B). (C)-(J), Expression of TRAIL (Fig 8, C and H), MID1 (Fig 8, D), IL-25 (Fig 8, F and J), CCL20 (Fig 8, E and I), and TSLP (Fig 8, G) was determined by using quantitative PCR after 24 hours (Fig 8, C-G) and 3 weeks (Fig 8, H-J) of A. fumigatus challenges. Gene expression was normalized to copy numbers of ACTB (n=7-9). *P < .05 and **P < .01. Asp F, A. Fumigatus; SAL, saline.
2.5. 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 (92, 99, 105), identifying the possibility that TRAIL signalling might also play an important role in other eosinophilic diseases, such as EoE. Gene expression analysis by using RNA sequencing demonstrated a significant increase in TRAIL and its downstream proinflammatory signalling molecule, MID1, in a cohort of patients with EoE, 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 controls and patients with EoE, highlighting the need to further explore their potential value as EoE biomarkers. Using a mouse model of *A fumigatus*–induced EoE, we showed that mice deficient in TRAIL have significantly less eosinophil and mast cell infiltration into the oesophagus compared with their WT counterparts. We also demonstrated that muscularis externa hypertrophy, fibrosis, and oesophageal circumference are dependent on TRAIL expression and that TRAIL is necessary for the upregulation of CCL11, CCL24, TGFB, and TSLP, 4 key cytokines implicated in the pathogenesis of EoE (41, 58, 85) TRAIL–/- mice displayed increased levels of PP2Ac and reduced NF-kB activity despite *A fumigatus* exposure.

Previous work using *A. fumigatus*–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 (46, 58). Although TRAIL’s role in allergic asthma has previously been identified, for the first time, our study has demonstrated that the production of IL-5 and IL-13 is dependent on TRAIL expression in *A. fumigatus*–induced mouse models of EoE and the subsequent expression of CCL11 and CCL24. This reduction in Th2 cytokine signalling...
might also account for the lack of oesophageal fibrosis, which has been shown to be mediated by eosinophilic release of TGF-β (71, 106).

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 Th2 cytokines are downstream of TRAIL signalling. TSLP has been shown to activate STAT3-mediated remodelling pathways in asthmatic patients and might mediate remodelling independently of eosinophils through this mechanism (121). It is still unknown how TSLP is regulated by TRAIL in EoE, given that no link between TRAIL and TSLP was evident in AAD models (99). However, it has been shown that the TSLP promoter contains several binding sites for the p65 subunit of NF-kB, a transcription factor shown to be TRAIL dependent in AAD (126), which we show is also repressed in the oesophagi of TRAIL-/- mice in the EoE model. It has also been shown that the subsequent expression of TSLP in intestinal epithelial cells is dependent on NF-kB (126). Given NF-kB is upregulated in EoE (68), it is possible that TRAIL is a key regulator of TSLP through p65 activation and contributes to EoE, most likely through basophil activation (41). However, siRNA-mediated silencing of MID1 did not affect TSLP expression while ameliorating the hallmark features of EoE, including CCL11 and CCL24 expression. This observation is in accordance with previous findings showing MID1-independent TSLP expression in AAD (99) and might explain residual eosinophilic inflammation despite MID1 silencing in the EoE and AAD models. Therefore it is possible that TSLP induction might be dissociated from MID1-mediated suppression of p65 activation. Alternatively, more effective MID1 suppression might 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 genome-wide association study demonstrating Calpain14 to be specifically expressed in the oesophagus and dynamically upregulated as a function of disease activity (127). Inhibition of calpains has previously been linked to altered NF-kB activity and TRAIL signalling (128).

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

2.6.1 Acknowledgements

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2.6.2. 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, T.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.

2.6.3 Competing financial interest

MER is a consultant for Immune Pharmaceuticals and has an equity interest. MER has a 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.
3. Research Chapter 2: TRAIL deficiency and PP2A activation with Salmeterol ameliorates egg allergen driven eosinophilic oesophagitis

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This chapter was authored by:

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*both authors contributed equally
Co-author statement

By signing below, I confirm that Leon Sokulsky was a shared-primary contributor to the publication entitled ‘Reversal of inflammatory and remodelling features due to TRAIL deficiency and Salmeterol therapy in egg allergen driven eosinophilic oesophagitis’ and performed gene and protein analysis on human oesophageal tissue, conducted mouse models, collected and processed mouse tissue samples, performed experiments on mouse tissue, analysed data and drafted the manuscript.

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Date 15/10/2016
3.1. Abstract

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

3.1.2 Methods: Oesophageal mucosal biopsies were collected from children with EoE and controls and assessed for TRAIL and MID-1 protein and mRNA transcript levels. Wild type and TRAIL deficient (Tnfsf10 -/-) mice were administered subcutaneous ovalbumin (OVA) followed by oral OVA challenges. In separate experiments OVA challenged mice were intraperitoneally administered salmeterol or dexamethasone.

3.1.3. Results: Oesophageal biopsies from children with EoE revealed increased levels of TRAIL and MID-1 and reduced PP2A activation as compared to controls. Tnfsf10-/- were largely protected from oesophageal fibrosis, eosinophilic inflammation, and the upregulation of TSLP, IL-5, IL-13 and CCL11 when compared with wild type mice. Salmeterol administration to wild type mice with experimental EoE restored PP2A activity and also prevented oesophageal eosinophilia, inflammatory cytokine expression, and remodelling, which was comparable to the treatment effect of dexamethasone.

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

Eosinophilic oesophagitis (EoE) is a chronic inflammatory upper gastrointestinal tract (GIT) disorder, characterised by allergy-driven inflammation. Symptoms of EoE may be similar to other upper GIT disorders, presenting clinically with regurgitation, retrosternal or epigastric pain (15, 33, 57), which may be misdiagnosed as gastro-oesophageal reflux disease. Untreated or poorly diagnosed EoE patients suffer long-term effects associated with prolonged inflammation, such as remodelling of oesophageal structure in the form of strictures and extensive weight loss (129, 130). Although the disease can manifest at any age, the condition is more common in young, atopic males with 75% of patients being male and 80% of them having an additional allergic disorder (9, 50).

Eosinophilic inflammation of the oesophageal mucosa can be treated through administration of topical (swallowed) corticosteroids; however, the effectiveness of such treatment varies and symptoms may persist despite reduced numbers of oesophageal eosinophils (131, 132). Some patients non-responsive to current treatments have worse clinical outcomes (21). Because food allergens are considered an important trigger of EoE in paediatric patients, dietary elimination of common food antigens has increasingly been employed as an alternative to corticosteroid treatment (19, 38, 39). Empirical elimination diets have demonstrated that the most prevalent food antigens associated with EoE are cow’s milk, wheat, egg, and soy (133), and the success rate of elimination diets for EoE is around 70% in children (19, 134, 135). The need for more effective therapeutics in some patients is highlighted by the rising number of EoE cases, the risk of stricture formation despite current best practice, treatment side effects and poor adherence (31, 86, 115).

TNF-related apoptosis-inducing ligand (TRAIL) has been associated with eosinophilic diseases (104) and inflammatory pathomechanisms in vivo (92, 99, 105, 106). In mouse
models of allergic airways inflammation, house dust mite and ovalbumin (OVA) have been shown to promote the release of TRAIL from epithelial cells, resulting in the upregulation of the E3 ubiquitin ligase Midline (MID)-1 (99, 106). Increased MID-1 expression results in the polyubiquination of the catalytic subunit of the regulatory enzyme protein phosphates 2A (PP2Ac) (99, 106), impeding its central role in the dephosphorylation of p38 mitogen-activated kinases, c-Jun N-terminal kinase and nuclear factor-κB (NF-κB) (103, 136, 137). These inflammatory cascades ultimately result in the infiltration of mast cells and eosinophils to the site of inflammation associated with allergic disease (99, 105, 106).

The emergence of TRAIL’s role in additional pathologies, such as cigarette-induced chronic obstructive pulmonary disorder and neonatal respiratory infections, further implicates its role as a key facilitator in multiple inflammatory conditions, affecting adults and children alike (107, 108). In models of allergic airways disease, TRAIL has been shown to promote airways hyperreactivity and remodelling in the form of fibrosis and smooth muscle hyperplasia (99, 106). These effects are associated with elevated levels of T-helper type 2 (Th2) cytokines (IL-5 and 13), eotaxins, transforming growth factor (TGF)-β and epithelial factors including thymal stomic lymphopoiyetin (TSLP) and CCL20 (99, 105, 106). Inhibition of MID-1 employing siRNA and raising PP2A activation with FTY720 analogues attenuated eosinophilic inflammation and airways remodelling (99, 106). Similarly, long acting beta agonists (such as salmeterol) can also increase PP2A activity in vitro and in vivo studies resulting in amelioration of hallmark features of allergic airways disease (138). Our previous work found that both TRAIL and MID-1 are upregulated in the murine oesophageal epithelium upon exposure to Aspergillus fumigatus extract and that Tnfsf10-/- mice or mice treated with siRNA silencing MID-1 were protected from EoE like features in vivo (139). However, it is unknown if these pathways are utilized in food allergy-driven EoE. As egg antigens are among the most common inducers of EoE in paediatric patients (140), we
investigated TRAIL and its downstream signalling pathway in a recently developed OVA-induced mouse model of EoE (105) and compared the hallmark features of EoE between wild type and TRAIL deficient \( (Tnfsf10^{-/-}) \) mice as well as after salmeterol and dexamethasone treatment.

3.3. Methods

3.3.1. Collection of patient biopsies

Patients presenting to the John Hunter Children’s Hospital, Australia, with symptoms consistent with oesophageal dysfunction were assessed by a paediatric gastroenterologist and underwent endoscopic oesophageal mucosal biopsy (minimum of two per patient) from the distal third of the oesophagus. Based on symptoms and eosinophil quantification in biopsies, patients were classified as EoE Active (peak eosinophil count ≥ 15 per high power field \( [hpf] \)), 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-oesophageal reflux excluded on oesophageal 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

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>EoE (n=18)</th>
<th>EoE remission (n=5)</th>
<th>Control (n=25)</th>
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<tr>
<td>Age (Years)</td>
<td>Median (Min, Max)</td>
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<td>12.2 (2.6, 13.6)</td>
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<td>Gender</td>
<td>Males (%)</td>
<td>13 (72.2)</td>
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<tr>
<td>Peak Eosinophil count</td>
<td>Median (Min, Max)</td>
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<td>0 (0, 12)</td>
</tr>
<tr>
<td>Asthma</td>
<td>Total (%)</td>
<td>8 (44.4)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Food Allergy</td>
<td>Total (%)</td>
<td>7 (38.9)</td>
<td>1 (20)</td>
</tr>
</tbody>
</table>
Table 2 Baseline characteristics of subjects with biopsies available for mRNA measurements

<table>
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<th>Control (n=29)</th>
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<td>6.2 (1.3, 13.6)</td>
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<tr>
<td>Gender</td>
<td>Males (%)</td>
<td>17 (81)</td>
<td>6 (60)</td>
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<tr>
<td>Peak Eosinophil count</td>
<td>Median (Min, Max)</td>
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<td>0 (0, 5)</td>
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<tr>
<td>Asthma</td>
<td>Total (%)</td>
<td>7 (33)</td>
<td>2 (20)</td>
</tr>
<tr>
<td>Food Allergy</td>
<td>Total (%)</td>
<td>6 (29)</td>
<td>4 (40)</td>
</tr>
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</table>

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

<table>
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<th>Clinical Features</th>
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<th>EoE remission (n=6)</th>
<th>Control (n=8)</th>
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<td>10.8 (2.6, 16.1)</td>
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<td>Gender</td>
<td>Males (%)</td>
<td>11 (73)</td>
<td>6 (100)</td>
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<tr>
<td>Peak Eosinophil count</td>
<td>Median (Min, Max)</td>
<td>40 (25, 82)</td>
<td>0 (0, 11)</td>
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<tr>
<td>Asthma</td>
<td>Total (%)</td>
<td>2 (13)</td>
<td>1 (17)</td>
</tr>
<tr>
<td>Food Allergy</td>
<td>Total (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

3.3.2. Ovalbumin mouse model of EoE

Briefly, wild type or Tnfsf10/-/- BALB/c mice (n=5-8) 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. Two days after the final OVA injection, mice were given 100μLs of OVA (50mg/100μL) dissolved with sucralse (Splenda™) or sucralse 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 (141). 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.
Mice were subsequently sacrificed on day 18 via pentobarbitone overdose (Virbac), with oesophageal tissue allocated for protein, gene or histological analysis. These animal experiments were approved by the Animal Care and Ethics Committee, The University of Newcastle.

3.3.3. Oesophageal circumference analysis

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

3.3.4. Gene expression analysis

Oesophageal 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 Fisher 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-AGGGTGCACTCGTGTTGGGTG-3), CCL20 (F 5-CGACTGTTGCCTCTCGTACA-3, R 5-AGGAGGTTCACAGCCCTTTT-3) TSLP (F 5-AGGCTACCCTGAAACTGAG-3, R 5-GGAGATTGCA TGAAGGAATACC-3), TGF-β (F 5-TGTGGAACTCTACCAGAAATATAGC-3, R 5-GAAGGCCCT GTATTCCGCTTC-3) and
β-actin (F 5-GACGGCCAGGTCATCACTATTG-3, R 5-AGGAAGGCTGGAAAAAGAGCC-3) primers. Human genes were quantified using TRAIL (F 5-TCGTGATCTTCACAGTGCTCCTG-3, R 5-ACAAGCAATGCCACCTTTGGAGTA-3), MID-1 (F 5-ACAGCTCCCAACCCTCCACAAT-3, R 5-CGACGTTGGCTTGTCCGGTGAAT-3) and GAPDH (F 5-GAGTCAACGGATTTGGTCGT-3, R 5-TTGATTTTGGAGGGATCTCG-3) primers.

3.3.5. Protein analysis

Oesophageal 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 oesophageal homogenates, serum collected from blood extracted from euthanized mice was analysed for IgE specific for OVA using the LEGEND MAX™ Mouse specific OVA IgE ELISA kit (Australian Biosearch).

3.3.6. Histological analysis of oesophageal tissue

Oesophageal tissue was fixed in 10% formalin for 24 hours prior to routine processing to paraffin wax, sectioned at 5μm and stained with Congo red to enumerate eosinophils, Toludine blue to assess mast cells and Masson’s Trichrome for collagen quantification.

Eosinophil or mast cell infiltration into the oesophagus was determined by counting the number of eosinophils within 1mm² 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 of epithelium (μm²/μm) in each oesophagus using Image-Pro-Plus 6 software (MediaCybernetics, Rockville, Md).

3.3.7. 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 +/- SEM.

3.4. Results

3.4.1. Expression of TRAIL and MID-1 in a paediatric cohort of EoE patients

Oesophagal biopsies collected from paediatric 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).
Figure 1: Upregulation of TRAIL and MID-1 mRNA and protein levels in patients diagnosed with eosinophilic oesophagitis compared to controls. A and B, Copy numbers of TRAIL (Fig 1A) and MID-1 (Fig 1B) in patient oesophageal biopsies (expressed per copy of GAPDH) as determined by quantitative (q) PCR. Excluded samples were found to be below detection [EoE active: n=21 (TRAIL), n=20 (MID-1), control: n=29 (TRAIL), n=22 (MID-1)]. C and D, Protein levels of TRAIL (Fig 1C) and MID-1 (Fig 1D) in oesophageal biopsies normalized to biopsy weight (EoE active; n=18, control; n=25). E, PP2A activity (as a percentage of control levels) in oesophageal biopsies (EoE active; n=15, control; n=8) normalized to total protein (Fig 1E). Data expressed as means +/-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.
3.4.2. 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).

![Figure 2: No observable difference in TRAIL signalling between Control and EoE remission patients. A, Copy numbers of TRAIL (Fig 2A) and MID-1 (Fig 2B) in patient oesophageal biopsies (expressed per copy of GAPDH) as determined by quantitative (q) PCR. Excluded samples were found to be below detection [control: n=29 (TRAIL), n=22 (MID-1), EoE remission: n=10 (TRAIL), n=9 (MID-1)]. C and D, Protein levels of TRAIL (Fig 2C) and MID-1 (Fig 2D) in oesophageal biopsies normalized to biopsy weight (control; n=25, EoE remission; n=5). E, PP2A activity (as a percentage of control levels) in oesophageal biopsies (control; n=8, EoE remission; n=6) normalized to total protein (Fig 2E). Data expressed as means +/- SEM.]

Figure 2: No observable difference in TRAIL signalling between Control and EoE remission patients. A, Copy numbers of TRAIL (Fig 2A) and MID-1 (Fig 2B) in patient oesophageal biopsies (expressed per copy of GAPDH) as determined by quantitative (q) PCR. Excluded samples were found to be below detection [control: n=29 (TRAIL), n=22 (MID-1), EoE remission: n=10 (TRAIL), n=9 (MID-1)]. C and D, Protein levels of TRAIL (Fig 2C) and MID-1 (Fig 2D) in oesophageal biopsies normalized to biopsy weight (control; n=25, EoE remission; n=5). E, PP2A activity (as a percentage of control levels) in oesophageal biopsies (control; n=8, EoE remission; n=6) normalized to total protein (Fig 2E). Data expressed as means +/- SEM.
3.4.3. 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 oesophagus 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).

**Figure 3:** Ovalbumin activates the TRAIL signalling pathway in vivo. A, Protein levels of TRAIL (Fig 3A) and B, MID-1 (Fig 3B) determined by ELISA in mouse oesophagi normalized to oesophageal tissue weight. C, PP2A activity (Fig 3C) normalized to oesophageal 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 +/-SEM. #P<0.1, *P<0.05, **P<0.01 and ***P<0.001. n=4-6.
3.4.4. TRAIL deficiency prevents eosinophilic inflammation of the oesophagus

To investigate the impact of TRAIL in OVA driven oesophageal inflammation, we enumerated eosinophils and mast cells in the oesophagus 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).

![Figure 4: TRAIL deficient mice are protected from ovalbumin induced eosinophilia, but not mast cell infiltration, in the oesophagus. A, Representative images of transverse mouse oesophageal sections stained with Congo-red (Fig 4A). B, Histological enumeration of oesophageal eosinophils from stained oesophagi (Fig 4B). C, Histological enumeration of mast cell infiltration into the oesophageal tissue (toluidine blue stain, Fig 4C). Data expressed as means +/-SEM. ***P<0.001 and ****P<0.0001. n=4-8.](image-url)
3.4.5. 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 oesophagi of OVA sensitized Tnfsf10-/- mice (Fig 5A-C). CCL11, IL-5 and IL-13 were increased in the oesophagi of OVA sensitized wild type mice while levels in Tnfsf10-/- remained at non-allergic saline levels (Fig 5D-F).

**Figure 5**: Eosinophilic oesophagitis 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 2α, 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 oesophagi determined by ELISA and normalized to oesophageal weight. Data expressed as means +/-SEM. #P<0.1, *P<0.05 and **P<0.01. n=4-7.
3.4.6. Oesophageal remodelling is TRAIL-dependent

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

Figure 6: Ovalbumin induced oesophageal remodelling is dependent on TRAIL. A, Representative images of mouse oesophageal sections (transverse) stained with Masson Trichrome (Fig 6A). B and C, Oesophageal circumference (Fig 6B) and subepithelial collagen depositation (Fig 6C) determined using Image-Pro Plus software. Data expressed as means +/-SEM. *P<0.05 and ****P<0.0001. n=4-8.
3.4.7. 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 oesophagus, 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 oesophagus (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 oesophagus 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).

Figure 7: Salmeterol reduces cytokines and PP2A activity. A, PP2A activity (Fig 7A) normalized to oesophageal 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 oesophagi determined by ELISA and normalized to oesophageal 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 +/-SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. n=3-8
3.4.8. Oesophageal 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 oesophagus comparable to dexamethasone treatment.

**Figure 8:** Oesophageal inflammation is significantly reduced upon salmeterol comparable to dexamethasone treatment. A and B, Representative images of transverse mouse oesophageal sections stained with Congo-red (Fig 8A) for the histological enumeration of oesophageal eosinophils (Fig 8B) and C mast cells from toludine blue stained oesophagi (Fig 8C). Data expressed as means +/-SEM. **P<0.01 and ***P<0.001. n=4-8
3.4.9. Ovalbumin induced remodelling features are significantly reduced by salmeterol therapy

Finally, remodelling features of EoE were assessed salmeterol and dexamethasone treated OVA mice in terms of oesophageal enlargement and fibrosis (Fig 9A). A reduction in oesophageal 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).

**Figure 9:** Ovalbumin induced remodelling features are reduced upon salmeterol and dexamethasone administration. A, Representative images of transverse mouse oesophageal sections stained with Masson’s Trichrome (Fig 9A). B and C, Oesophageal circumference (Fig 9B) and oesophageal fibrosis (Fig 9C). Data expressed as means +/-SEM. *P<0.05 and **P<0.01. n=4-8
3.5. 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, while 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 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 signalling 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 oesophagus 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-κB, p38 MAPK) and its downregulation due to MID-1 and α4 protein interactions are associated with the activation of inflammatory cascades (99, 139). While the polyubiquination of PP2Ac through MID-1 and α4 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 COOH terminus or PP2A phosphorylation via tyrosine (142, 143), 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 (139). 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 oesophageal 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 oesophagus. 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 (105). Our results are consistent with previous studies that demonstrate the importance of TRAIL signalling for CCL20 expression. Recombinant TRAIL was shown to induce CCL20 expression *in vitro* and its abolition prevented CCL20 expression *in vivo* (105, 139). 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 oesophageal remodelling (41, 139). Abolition of TSLP in OVA-induced EoE has previously been demonstrated to ameliorate oesophageal remodelling and eosinophilic inflammation *in vivo* and TSLP was found to be upregulated in EoE patient biopsies (41). 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 oesophageal 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 (144, 145). 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 oesophagus, mice oesophagi will typically show a small number of eosinophils at baseline (41, 146), 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 oesophageal 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 (139). This suggests 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 signalling 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 oesophagus via the oral route without unacceptable side effects.

3.6. Funding and competing interests

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4. Research Chapter 3: A unique role for IL-13 in Eosinophilic Oesophagitis by inducing eosinophilia through MID-1 and STAT6

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By signing below, I confirm that Leon Sokulsky is the primary contributor to the publication entitled ‘A unique role for IL-13 in Eosinophilic Oesophagitis by inducing eosinophilia through MID-1 and STAT6’ and collected and processed mouse tissue samples, performed experiments on mouse tissue, analysed data and drafted the manuscript.

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4.1. Abstract

4.1.1. Introduction: Eosinophilic Oesophagitis is associated with allergen driven inflammation of the oesophagus, involving the Th2-related cytokine IL-13. Recombinant IL-13 administration to mice induces eosinophilic oesophagitis hallmark features such as increased CCL11 and eosinophil influx. Such inflammatory mechanism have previously been shown to be dependent on the expression of TNF-Related Apoptosis Inducing Ligand (TRAIL) and Midline (MID)-1 which regulated protein phosphatase 2A (PP2A) activity, however the relationship between IL-13 and TRAIL signalling is unknown.

4.1.2. Objective: To investigate the effect of IL-13 administration on TRAIL signalling.

4.1.3. Methods: We administered recombinant IL-13 to wild type (WT) mice, mice deficient in TRAIL (Tnsf10\textsuperscript{-/-}) and STAT6 (STAT6\textsuperscript{-/-}), and targeted mice with small interfering RNA to reduce MID-1 expression.

4.1.4. Results: IL-13 administration to mice increased TRAIL and MID-1 mRNA expression in the oesophagus while reducing PP2A activity. Tnsf10\textsuperscript{-/-}, but not STAT6\textsuperscript{-/-} mice, demonstrated increased MID-1 expression and PP2A activity reduction upon IL-13 challenge, which correlated with eosinophil infiltration of the oesophagus. Silencing MID-1 expression employing siRNA technique completely ablated IL-13 induced eosinophil infiltration of the oesophagus, restored PP2A activity and reduced CCL11 expression.

4.1.5. Conclusion: IL-13 driven inflammation of the oesophagus induces eosinophilia and CCL11 expression in a MID1 and STAT6 dependent manner. This study highlights a novel mechanism employed by IL-13 to perpetuate eosinophilic inflammation via the induction of MID1.
4.2. Introduction

Eosinophilic inflammation of the oesophagus is associated with several inflammatory pathologies, including gastro-oesophageal reflux disease, parasitic infections and eosinophilic oesophagitis (EoE). In the case of the latter disorder, EoE is a recently defined allergic phenomena associated with food antigens and its tendency to present itself primarily in young, atopic males (9, 15, 33). As a potential cause of dysphagia and food impaction, the only treatments currently available to counter its morbidity are dietary restrictions and steroid therapy (17-19, 147). With a rising prevalence in western societies (31), there is a greater need to investigate specific molecular pathways in order to improve the quality of life of patients who do not respond to mainstream therapies.

The inflammation in EoE is associated with T helper 2 (Th2) driven eosinophilia, via the cytokines Interleukin (IL)-5 and IL-13 (49, 56). Both cytokines have been found to be elevated in patients with EoE in addition to in vivo studies modelling EoE. IL-5 is believed to play a core role in eosinophilic trafficking to the oesophagus while IL-13, acts primarily as a regulator of the allergic phenotype by promoting an array of epithelial remodelling (e.g. Periostin) and inflammatory (CCL11) cytokines (23, 57, 58, 77). Indeed, administrating recombinant (r) IL-13 intratracheally to mice demonstrated that rIL-13 alone was enough to induce the hallmark features of EoE (58). IL-13 driven EoE was demonstrated to be dependent on STAT6, IL-5 and CCL11 using knockout mice, indicative of its upstream position (58). Despite this, other EoE related inflammatory pathways have yet to be investigated in regards to IL-13 signalling.

Previous studies on TRAIL signalling in murine models of EoE have shown that inflammation and remodelling of oesophageal tissue is dependent on the expression of TNF-Related Apoptosis Inducing Ligand (TRAIL) and its downstream molecules Midline (MID)-1
We showed previously that TRAIL knockdown prevented allergen-induced increases in IL-5 and IL-13, correlating with a reduction of eosinophils and remodelling (139, 148). TRAIL and MID-1 are known to crucially augment allergen-induced inflammation (92, 96, 99, 105), but their relevance in specifically promoting IL-13-induced effector functions is unknown. In this study, we administered rIL-13 to mice that are deficient in TRAIL (Tnfsf10−/−) and compare their inflammatory responses to mice that were STAT6 deficient (STAT6−/−). We also silenced MID-1 employing small interfering RNAs (siRNAs) to wild type (WT) mice in order to further dissect its role in IL-13-induced eosinophilic inflammation of the oesophagus.

4.3. Methods

4.3.1. Mouse models of inflammation

Specific pathogen free Wild Type (WT), Tnfsf10−/− and STAT6−/− mice were obtained from Australian BioResources (Moss Vale, Australia). All animal experiments were approved by the Animal Care and Ethics Committee of the University of Newcastle.

To induce IL-13 driven inflammation of the oesophagus, WT, Tnfsf10−/− and STAT6−/− mice were intranasally challenged with 15μg of rIL-13 (35μl of saline) under isoflurane anaesthetics.

The role of MID-1 in IL-13 driven inflammation was assessed in mice through the administration of siRNA targeting MID-1 (seq-5-AGAGUAAUCUCACCAAUCC-3) or control siRNA (seq-5-UGGUUUAACUGUCGACUAA-3) (3.75nmol in nuclease free water) intranasally 24 hours prior to rIL-13 administration. Mice were sacrificed 24 hours after the rIL-13 challenge via pentobarbitone overdose (Virbac,) and the oesophagus was collected for protein, histological and gene analysis.
4.3.2. Histological analysis of oesophageal eosinophilia

Oesophageal tissues were collected using methods described previously (139) and were stained for eosinophils (Congo-red). Eosinophil infiltration into the oesophagus was determined by counting the number of eosinophils within 1mm\(^2\) of transverse oesophageal section. Images were taken using the Aperio Slide Scanner (Leica Biosystems).

4.3.3. RNA extraction and gene expression of oesophageal tissue

Oesophageal samples isolated from mice were immersed in TRIzol® (Ambion, Life Technologies, Mulgrave, Australia) prior to being homogenized (Tissue Tearor®). RNA was then extracted using the manufactures instructions and resuspended in nucleus free water. RNA was then reverse transcribed to cDNA using BioScript (Bioline, Alexandria, Australia) in accordance to the manufacturer’s instructions. All genes (shown below) were quantified using SYBR® green (Life Technologies, Mulgrave, Australia) using the Eppendorf Realplex (Hamburg, Germany) and the following primers: β-actin (F 5-GACGGCCAGGTCATCACT ATTG-3, R 5-AGGAAGGCTGGAAAAGAGCC-3), TRAIL (F 5-CCCTGCTTTGCAGGT TA AGAG-3, R 5-GGCCTAAGGTCTTTCCATCC-3), MID-1 (F 5-CACTCGCTGAAGGAAA ATGACCA-3, R 5-AATCCAAGGCAAAAAGTGCAAAACG-3), CCL11 (F 5-TTCTATTTC CTGCTGCTCACGG-3, R 5-AGGGGTGACATCTGTTGTGTTGTG-3), Periostin (F 5-CCGAG AGCCAGTCATTTAAA-3, R 5-TGCAAGGTCTCTCCTGTTTTC-3) and STAT6 (F 5-CTGGG AGTTCCTCGGTGTTGTTTTC-3, R 5-CTGTGGCAGAAAGTAGGGCGAC-3).

4.3.4. Protein quantification and PP2A activity assay

Snap frozen 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. PP2A activity was determined by
Active PP2A DuoSet IC activity assay (Active Motif). PP2A activity was normalised to SAL controls. TRAIL protein expression was quantified through ELISA (R&D Systems).

### 4.3.5. Statistical Analysis

Statistical significance was determined between experimental groups using Student’s *t*-tests or one way ANOVA (Holm-Sidak) in Graphpad Prism 6 (La Jolla, CA) as appropriate. Data are presented as mean +/- SEM.

### 4.4. Results

#### 4.4.1. MID-1 mRNA is induced by IL-13 in WT and Tnfsf10<sup>−/−</sup> mice, but not in STAT6<sup>−/−</sup> mice

Intranasal administration of rIL-13 to WT mice resulted in an upregulation of TRAIL (Fig 1A) and MID-1 mRNA expression (Fig 1B) in the oesophagus. This occurred in correlation with a restoration in PP2A activity (Fig 1C). In line with previous studies, the expression of TRAIL was found to be undetectable in both SAL and rIL-13 administered Tnfsf10<sup>−/−</sup> mice (Fig 1A). In Tnfsf10<sup>−/−</sup> mice, rIL-13 induced a small, but significant increase of MID-1 mRNA expression (Fig 1B) and a reduction in PP2A activity (Fig 1C). In contrast, STAT6<sup>−/−</sup> mice demonstrated baseline expression of TRAIL but no observable differences in MID-1 expression upon IL-13 exposure (Fig 1D & E). PP2A activity, interestingly, was upregulated upon rIL-13 administration in the oesophagus of STAT6<sup>−/−</sup> mice (Fig 1F). Thus IL-13 induces MID-1 in a STAT6-dependent manner and TRAIL deficiency limits IL-13-induced effects on MID-1 expression.
### 4.4.2. TRAIL deficiency attenuates IL-13 driven eosinophilia in the oesophagus

Congo-red stained oesophageal transverse sections were analysed using light microscopy and the number of eosinophils (per mm²) was determined in WT, *Tnfsf10*⁻/⁻ and STAT6⁻/⁻ mice upon IL-13 exposure. While IL-13 administration resulted in a marked increase in eosinophil infiltration in WT mice (Fig 2A), this was significantly attenuated in *Tnfsf10*⁻/⁻ mice (Fig 2B) while IL-13-induced oesophageal eosinophilia was completely abolished in STAT6⁻/⁻ mice (Fig 2C).

**Figure 1:** TRAIL, MID-1 expression and PP2A activity in the oesophagus of WT, *Tnfsf10*⁻/⁻ and STAT6 mice upon rIL-13 administration. A and B, TRAIL (Fig 1A) and MID-1 (Fig 1B) mRNA expression in mouse oesophagi determined by qPCR, normalised to β-actin. C, PP2A activity (Fig 1C) measured in mouse oesophagi normalised to total protein and respective SAL control (%). D and E, TRAIL (Fig 1D) and MID-1 (Fig 1E) mRNA expression in mouse oesophagi determined by qPCR, normalised to β-actin. F, PP2A activity (Fig 1F) measured in mouse oesophagi normalised to total protein and respective SAL control (%). Data expressed as +/- SEM (n=4-6). #=P<0.1, ##=P<0.05 (t-test two tailed), *=P<0.05, **=P<0.01, ***=P<0.001
Figure 2: IL-13 induced oesophageal eosinophilia is partially reduced in Tnfsf10<sup>-/-</sup> mice and ablated in STAT6<sup>-/-</sup> mice. A, B and C, Average eosinophil counts in Congo-red stained traverse oesophageal sections determined via light microscopy per mm<sup>2</sup> (Fig 2A-C). D, Transverse Congo-red stained oesophageal pictographs (A= WT SAL, B= WT IL-13, C= Tnfsf10<sup>-/-</sup> SAL, D= TRAIL IL-13, E= STAT6<sup>-/-</sup> SAL, F= STAT6<sup>-/-</sup> IL-13) (Fig D). Data expressed as +/- SEM (n=3-6). **=P<0.01, ***=P<0.001
4.4.3. IL-13 upregulates CCL11 and periostin in the oesophagus of WT and \textit{Tnfsf10}/- mice, but not in STAT6/-

Both WT and \textit{Tnfsf10}/- mice had a significantly increased CCL11 mRNA expression after IL-13 administration (Fig 3A & B), which was not observed in STAT6/-/ mice (Fig 3C). Similarly periostin expression was significantly upregulated in IL-13 administered WT and \textit{Tnfsf10}/- (Fig 3D & E) but not STAT6/-/ mice (Fig 3F).

\textbf{Figure 3:} TRAIL deficiency does not reduce IL-13 induced CCL11 and periostin mRNA expression in the oesophagus unlike STAT6/-/ mice. A-F, CCL11 (Fig 3A-C) and periostin (Fig 3D-F) mRNA expression in mouse oesophagi determined by qPCR, normalised to \(\beta\)-actin. Data expressed as +/- SEM (n=5-6). *=\(P<0.05\)
4.4.4. MID-1 siRNA administration restored PP2A activity, but not TRAIL or STAT6 expression after oesophageal IL-13 exposure

We investigated the effect of directly targeting of MID-1 mRNA by employing siRNA to the oesophagus. Administration of siRNA designed to selectively inhibit MID-1 had no effect on TRAIL expression (Fig 4A), through MID-1 expression was significantly reduced compared to the nonsense siRNA (NONc) control (Fig 4B). This correlated with a restoration of PP2A activity (Fig 4B) (Fig 4C). STAT6 expression was not altered upon MID-1 inhibition (Fig 4D).

![Graphs A, B, C, D showing data](image-url)

**Figure 4:** Silencing MID-1 prior to IL-13 administration restores PP2A activity in the oesophagus but does not alter TRAIL and STAT6 expression. A, TRAIL protein expression (pg/mg) determined by ELISA normalised to oesophageal tissue weight (Fig 4A). MID-1 mRNA expression in mouse oesophagi determined by qPCR, normalised to β-actin (Fig 4B). C, PP2A activity (Fig 4C) measured in mouse oesophagi normalised to oesophageal tissue weight and respective SAL control (%). D, STAT6 mRNA expression in mouse oesophagi determined by qPCR, normalised to β-actin (Fig 4D). Data expressed as +/- SEM (n=4-8). #=P<0.1, *=P<0.05, **=P<0.01
4.4.5. IL-13 driven eosinophilia to the oesophagus and CCL11 expression is ablated in MID-1 silenced mice

Direct targeting of MID-1 using siRNA resulted in complete abrogation of eosinophilic inflammation compared to the NONc control (Fig 5A & D). There was also a significant reduction of CCL11 mRNA expression in the oesophagus (Fig 5B), however no changes in peristin expression were observed (Fig 5C).

**Figure 5:** Ablation of IL-13 induced eosinophilia and CCL11, but not peristin, in MID-1 siRNA treated mice. A, Average eosinophil counts in Congo-red stained traverse oesophageal sections determined via light microscopy per mm² (Fig 5A). B and C, CCL11 (Fig 5B) and peristin (Fig 5C) mRNA expression in mouse oesophagi determined by qPCR, normalised to β-actin. D, Transverse congo-red stained oesophageal pictographs (A= SAL, B= NONc siRNA/IL-13, C= MID-1 siRNA/IL-13) (Fig D). Data expressed as +/- SEM (n=4-6). *=P<0.01, ****=P<0.0001
4.5. Discussion

IL-13 alone has been shown to induce experimental EoE in animal models and upregulate the expression of key EoE associated cytokines in vitro. In in vivo allergen models, IL-13 has been shown to be dependent on the expression of TRAIL and MID-1 (139, 148). However, it is unclear if IL-13 has any influence on TRAIL and MID-1. Here, we show that intranasal administration of IL-13 to mice that are deficient in TRAIL experienced a significant reduction of eosinophilic inflammation without effects on CCL11 expression. Silencing MID-1, however, completely ablated eosinophilic migration to the oesophagus comparable to the levels observed in IL-13 challenged STAT6−/− mice. MID-1 silencing also reduced CCL11 expression, but not periostin expression. Thus MID-1 and STAT6 are essential for IL-13-induced CCL11 expression and eosinophilic inflammation, while periostin expression is mediated by a MID-1 independent pathway upon IL-13 exposure. Furthermore TRAIL deficiency limits, but does not completely abrogate IL-13-induced eosinophilic inflammation.

In contrast, we have shown previously that IL-13 is required for TRAIL-induced eosinophilic inflammation of the lung (105). These results identify MID-1 as a novel and integral component of the IL-13-mediated STAT6-dependent pro-inflammatory effector pathway.

Upregulation of MID1 following rIL-13 exposure was reduced in Tnfsf10−/− mice but abolished in STAT6−/− mice. This suggests that IL-13 induced MID1 expression in a TRAIL-dependent and TRAIL-independent manner, both of which however are dependent on STAT6. MID-1 silencing successfully ablated both CCL11 expression and eosinophilia after rIL-13 administration, thus it can be concluded that IL-13 induces eosinophilic inflammation through MID-1 signalling. Both IL-13 and STAT6 have been shown to be dependent on TRAIL and MID-1 expression in allergen driven EoE (139), hence what this finding infers is that IL-13 is not merely an effector pathway of TRAIL induced inflammation but is also a
modulator of MID-1 mediated inflammation in a positive feedback loop. Although this pathway has only been explored in EoE, there is the potential that IL-13/MID-1 interactions are influencing additional TRAIL-related pathologies. These include allergic asthma and early-life neonatal chlamydial infections, both of which have been shown to be dependent on MID-1 (99, 149) and IL-13 (56, 150) signalling.

**Figure 6**: IL-13 upregulation of STAT6 results in the upregulation of Periostin, TRAIL and MID-1, which results in inflammation: This figure is an interpretation of the results shown in this study. STAT6 is essential for IL-13 induced TRAIL and MID-1 expression, however MID-1 can be partially induced in the absence of TRAIL. Upregulated MID-1 expression reduces PP2A activity, which results in the upregulation of CCL11 expression and subsequently eosinophilia. As TRAIL and MID-1 are required for IL-13 expression in allergen induced EoE (139, 148), it is possible that TRAIL induced IL-13 employs a positive feedback mechanism through STAT6 and MID-1 to perpetuate inflammation.
Previous studies showed that intratracheal rIL-13 administration was a critical driver of Th2 driven inflammation of the oesophagus dependent on CCL11 and STAT6 (58), which is augmented by TRAIL (139, 148). Our studies extend these findings and highlight MID-1 as a key mediator of IL-13 mediated effector functions. Subjects with non-functional MID-1 (x-linked Opitz G/BBB syndrome) have midline defects that may include facial anomalies, laryngo-tracheo-oesophageal defects, genitourinary abnormalities and developmental delay, presumably due to a disturbance of neural crest cell migration during early embryonic development. A physiological role of MID-1 beyond embryonic development has yet to be identified, because subjects with MID-1 mutations are phenotypically normal except for displaying congenital anomalies. Thus MID-1 may be a promising therapeutic target.

Although we have demonstrated a novel pathway involved in IL-13 signalling, the study is not without limitations. Similar to issues regarding TRAIL and MID-1 signalling, this study was unable to identify the mechanisms by which IL-13 upregulates MID1 except for showing complete STAT6 and partial TRAIL dependency. In addition, investigating remodelling aspects of IL-13 driven EoE (muscularis externa, eosinophilic circumference, fibrosis) was not possible here as the short term models used do not allow sufficient time for the development oesophageal remodelling. Finally, the role of other Th2 cytokines like IL-4 or IL-5 on members of the TRAIL signalling pathway were not investigated in this study, although it should be noted that administration of these recombinant proteins to mice failed to induce experimental EoE in previous studies (58).

Overall, we propose that IL-13, signalling through the E3 ubiquitin ligase, MID-1, modulates PP2A activity in a STAT6 dependent manner. This work further highlights the mechanisms employed by IL-13 that can manipulate the activation of the TRAIL signalling pathway in the pathogenesis of EoE which may have broader implications in other TRAIL-related pathologies.
4.6. Acknowledgements and competing interests

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5. General Discussion

EoE is characterised by allergen driven inflammation of the oesophagus, associated with eosinophil and mast cell infiltration and structural remodelling of the oesophageal tissue (68, 86, 151). The presence of eosinophils within the oesophageal epithelium and subsequent remodelling are associated with the oesophageal dysfunction observed in EoE patients in the form of oesophageal pain, dysphagia and failure to thrive, although clinical outcomes are poorly correlated with eosinophil numbers (15, 152-154). A majority of patients will find relief with interventions such as dietary modification or corticosteroid treatment (155), however sub-groups of patients remain refractory and develop severe remodelling of the oesophagus, permanently impacting on the patients quality of life. Underdiagnosis remains a significant limitation in improving patient clinical outcomes due to similarities in disease presentation between EoE and GORD (156, 157). The investigation of inflammatory and remodelling pathways is therefore imperative to combat these poor outcomes associated with EoE in regards to discovering novel therapeutic targets and/or diagnostic markers. In these studies, it is revealed that the inflammatory branch of the TRAIL signalling pathway, which was originally defined in the context of asthma, has an essential role in the perpetuation of hallmark EoE features due to its upstream regulation of crucial signalling pathways.

5.1 TRAIL’s role in inflammation and remodelling in EoE

In allergic asthma, TRAIL was demonstrated to promote inflammation through the upregulation of the E3 ubiquitin ligase, MID-1, which in turn facilitates the activity of NF-κB, p38-MAPK and JNK through the polyubiquitination of the catalytic subunit of PP2A (99, 106, 138). Activation of this pathway has been linked to the release of both inflammatory (CCL11, CCL24) and remodelling cytokines (TGF-β, VEGF), which recruit eosinophils, dendritic and Th2 cells to the airways while perpetuating structural changes to the lung
airways (99, 105). When aspects of the TRAIL signalling pathway were investigated in Chapters 1 and 2, patients with EoE were found to have an increased expression of TRAIL and MID-1 in oesophageal biopsies, both in regards to mRNA and protein, coupled with a reduction in PP2A activity. When analysing the TRAIL signalling pathway in in vivo models, Chapters 1, 2 and 3 demonstrated that MID-1 and transcription factors were induced and PP2A activity was reduced in aeroallergen, food allergen or IL-13 driven EoE. Interestingly, Chapter 1 demonstrated that TRAIL was induced in the early stages of allergen sensitisation, implying a complex temporal role for the cytokine in the induction of MID-1 in the oesophagus.

Inhibition of TRAIL-associated factors, through the use of knockout mice (compared to wild type littermates) and siRNAs, demonstrated a reduction in immune cell infiltration of the oesophagus, as were remodelling features such as oesophageal enlargement and fibrosis. This was correlated with decreased IL-5, IL-13, TGF-β, CCL11, CCL24, CCL20 and TSLP. In addition to eosinophils, mast cells were also shown to be dependent on TRAIL expression in Asp F-EoE, and a likely inducer of deep tissue remodelling of the oesophagus through the release of TGF-β (85). Chapter 1 further elucidates the importance of the cytokine TSLP as a downstream mediator of TRAIL driven inflammation, as remodelling and inflammatory features were restored in Asp F/TRAIL deficient mice upon artificial TSLP recapitulation while Chapter 3 focuses on the impact of the Th2 cytokine, IL-13, on TRAIL signalling.

5.2 Food allergens and the impact on TRAIL signalling

Food allergens are considered to be the primary drivers of the EoE phenotype in humans, as a significant portion of EoE patients will show an improvement in symptoms after dietary modifications (15, 18, 133). Previous studies have demonstrated that food allergens (ovalbumin) can be used to induce EoE symptoms in mice, typically through allergen
sensitisation and intragastric challenge (41, 42). As such, these models may provide greater translational power over inhaled allergen driven EoE models given the relevance of food-allergen in EoE patients (most commonly associated with milk or egg) (158). While TRAIL was investigated in great detail in Chapter 1 in a model of EoE induced by *Asp F*, Chapter 2 addressed TRAIL’s role in an egg OVA induced model of EoE that was reliant on systemic sensitisation in an effort to mirror the profile of human disease.

In regards to TRAIL and MID-1 expression, the *Asp F* model only demonstrated an upregulation of these factors 24 hours after a single dose of *Asp F*: the expression of TRAIL and MID-1 significantly decreased to baseline levels at the conclusion of the full EoE model. In contrast, the OVA model demonstrated an elevation of TRAIL and MID-1 expression at the conclusion of the experiment, which in part could be due to short duration of the challenge phase (five days). As both models demonstrated an amelioration of EoE hallmark features in TRAIL deficient mice, this highlights a complex temporal and spatial expression pattern of TRAIL (and its downstream mediator MID-1) in its upregulation of downstream effector cytokines irrespective of allergen sensitisation and challenge.

Another noticeable difference between both models was that TRAIL deficiency had no impact on mast cell infiltration into the muscularis region of the oesophagus, while the expression of mast cells was ameliorated in *Asp F* TRAIL knockout mice. This result was unexpected given the interplay that exists between mast cells and eosinophils through both soluble and membrane activated mechanisms (159). While it is not exactly clear why this difference exists, one possibility is that lung allergen driven *Asp F* model induces an intense Th2/Eosinophilic response that is TRAIL dependent, whereby eosinophils recruit mast cells to the oesophagus through the release of stem cell factor (147, 160). Administering OVA directly to the oesophagus post Th2 activation, however, induces milder EoE features that recruit mast cells independent of eosinophils and TRAIL, correlating more closely with IgE
levels. Thus, these discrepancies observed between both models highlights the caution required when modelling a human disorder *in vivo*, as it can now be revealed that mast cells are not dependent on TRAIL signalling in OVA driven EoE.

### 5.3 Th2 influence on the TRAIL signalling pathway

The Th2 cytokines IL-5 and IL-13 are highly associated with EoE pathology. Previous *in vivo* modelling of EoE has demonstrated that overexpression of both Th2 cytokines induce eosinophilia of the oesophagus, with IL-13 responsible for the induction of pro-inflammatory and remodelling cytokines like CCL11 and periostin (56, 57, 77), with IL-5 being responsible for eosinophil maturation and recruitment to the oesophagus (49). IL-13 has also been shown to be upregulated significantly in EoE patients and induces the expression of CCL26 in oesophageal epithelial cells (161, 162). Therapeutically inhibiting either IL-5 or 13 by employing monoclonal antibodies has been demonstrated to improve clinical outcomes and reduce blood eosinophilia in EoE patients (163, 164).

Because hallmark features of EoE can be induced by IL-13 alone, Chapter 3 investigated the potential that the TRAIL signalling pathway could be involved in the inflammation observed in these models. As shown in rIL-13 administered mice, however, TRAIL deficiency did not completely ablate eosinophilia and CCL11 expression as did STAT6 deficiency when compared to their wild type littermates. IL-13 did significantly induce TRAIL expression, in addition to its downstream inflammatory molecule MID-1 in wild type and TRAIL deficient mice, however not in STAT6 deficient mice. Silencing MID-1 with siRNA technology, in contrast, resulted in a complete ablation of eosinophils and CCL11, in addition to PP2A restoration. As STAT6 expression was found to be unchanged in MID-1 silenced mice (whilst MID-1 expression was dependent on STAT6), these studies demonstrated how IL-13 could influence MID-1 expression in a STAT6 dependent manner independent of TRAIL.
The exact influence of IL-13 over MID-1 expression is unclear in antigen driven EoE. Both Chapter 1 and 2 demonstrated that IL-13, in addition to STAT6, was dependent on the expression of TRAIL and MID-1. While this suggests a simple cause and effect relationship, Chapter 3 expands on how TRAIL signalling can be influenced by IL-13, with elevated IL-13 expression promoting MID-1 expression through STAT6. While the common outcome observed as a result of both cytokines being upregulated involves eosinophilia, the multiple mechanisms by which this eosinophilia can occur demonstrates the multifaceted nature of EoE inflammation.

5.4 Therapeutic potential of modulating TRAIL associated molecules

Mainstream therapies for non-PPI responsive EoE are currently limited to the swallowing of corticosteroids (fluticasone or budesonide) in addition to or alternatively the implementation of dietary restrictions of foods that trigger EoE. While both forms of treatment have been shown to provide adequate relief from the symptoms associated with EoE and alleviation of detrimental remodelling and inflammation, there are several limitations associated with these therapeutic interventions. Swallowing corticosteroids can result in immunosuppression within the mouth, which can eventuate into adrenal insufficiency or candidiasis infection (165, 166). Dietary restrictions are limited by patient adherence, and a change of lifestyle can reduce patient quality of life, which was a significant concern in older children (167). Finally, there are subgroups of patients that present with persistent EoE that does not respond to these convention therapies (168, 169): these patients are at the highest risk of forming strictures within the oesophagus, resulting in a worsening of symptoms that can only be treated with dilation therapy (170-172).

As shown in Chapters 1 and 2, it was established that TRAIL deficiency and MID-1 inhibition resulted in a reversal of EoE hallmark features in vivo, in both Asp F and OVA
exposed mice. While the effect of modulating these factors clinically have not been explored, (only measured) it would suggest that exploring the clinical implications of supressing these factors could present itself as an alternative means of treating EoE. While targeted therapy for EoE may appear to be an attractive option to achieve EoE remission, there are fundamental issues for selecting TRAIL as a therapeutic target. TRAIL is a key apoptotic factor involved in the regulation of cancer (91, 93), eliminating TRAIL suppression as a viable therapeutic intervention. Silencing MID-1, however, remains to be a potential therapeutic target as there are no other known additional functions of this gene post foetal development (100).

In Chapter 3, STAT6 was demonstrated to have an upstream role in the induction of TRAIL and MID-1 in IL-13 induced EoE. This suggests that there is potential for STAT6 to be targeted therapeutically, as it has previously been explored in AAD (173). It is interesting to note, however, that Omeprazole (used in the treatment of GORD) has been shown in vitro to interfere with STAT6 binding to the eotaxin-3 promotor in EoE squamous epithelial cells after being stimulated by Th2 cytokines (13). As STAT6 was shown to have a downstream role in the allergen model shown in Chapters 1, it could explain why most EoE patients do not experience relief with PPIs as the disease is driven by TRAIL mediated inflammation. In contrast, PPI-REE pathology could be highly dependent on Th2-STAT6 interaction, which is in turn upstream of TRAIL mediated inflammation.

Chapter 2 presented the findings that, in addition to an induction of TRAIL and MID-1, PP2A is downregulated in EoE patients. Hence, upregulation of PP2A activity in the oesophagus of EoE patients could be an additional therapeutic strategy. Expanding from research done in allergic asthma, Chapter 2 also explored the effect of PP2A upregulation through the administration of salmeterol, which we found to reduce eosinophilia, Th2 cytokines and oesophageal enlargement. While the mechanism by which this occurs was not elaborated on in Chapter 2, the downregulation of CCL11 and CCL20 suggests that
salmeterol interferes with eosinophil chemotaxis and the regulation of dendritic cell and T cell recruitment as seen in rhinoviral exacerbation of AAD (123). Additionally, both salmeterol and formeterol have been demonstrated to upregulate PP2A activity in vitro (174), suggesting that β2-agonists in general may mediate anti-inflammatory effects by modulating the TRAIL pathway. This has demonstrated, for the first time, the potential of salmeterol as an alternate therapy to diet modification and steroid treatment in EoE. Given that salmeterol is a TGA approved drug for the treatment of asthma (albeit in combination with corticosteroid therapy), determining the therapeutic potential of salmeterol (and potentially other β2-agonists) in EoE clinically should be a highly considered option for exploring alternative EoE therapies.

5.5 Summary

With an increasing prevalence in the western world and a lack of targeted treatments for patients, EoE presents itself as a significant clinical challenge. The investigation of TRAIL and its associated factors in EoE in vivo models has highlighted the existence of a novel signalling pathway that is essential for the perpetuation of this disease. The upregulation of TRAIL and MID-1, as well as reduced PP2A activity, observed in EoE patient cohorts highlights the clinical significance of these factors and provides a novel avenue of alternate treatments for this disease. Through the submission of all these findings, the main outcomes that will hopefully be achieved include the further investigation of other key signalling pathways involved in the perpetuation of EoE and highlight the therapeutic potential of modulating TRAIL associated factors such as MID-1 and PP2A.
5.6. References


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