EXPLORING THE MECHANISMS OF ESOPHAGEAL KERATINOCYTE HOMEOSTASIS IN THE CONTEXT OF EOSINOPHILIC ESOPHAGITIS

A Thesis

Submitted to

the Temple University Graduate Board

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

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May 2019

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ABSTRACT

Eosinophilic esophagitis (EoE) is a chronic allergic pathology of the esophagus characterized by infiltration of eosinophils into the esophageal mucosa. EoE results in considerable impact on patient quality of life, substantiating the need to better understand the pathobiology of the disease in order to define novel approaches to diagnosis, monitoring and therapy. Our previous studies indicate an increase in circulating mitochondrial DNA in patients with active EoE and extracellular structures consistent with mitochondria in esophageal epithelium of patients with active EoE inflammation. While published studies provide evidence of a genetic link between mitochondrial dysfunction and development of EoE, the functional role of mitochondria in EoE pathophysiology remains unclear. In this thesis, we use immunohistochemistry on human patient biopsies, mouse models of EoE-like inflammation, and complementary *in vitro* and *ex vivo* models to explore the effects of the EoE inflammatory milieu on mitochondria in esophageal keratinocytes. We report that mitochondrial content is increased in human patients and mice with EoE inflammation. We also provide evidence that the EoE-associated cytokine, interleukin-13 increases mitochondrial DNA level and mitochondrial activity *in vitro*. To explore the role of autophagy in mitochondrial regulation in esophageal keratinocytes, we began generation of an autophagy-deficient cell line. This thesis provides foundation for further studies evaluating the role and mechanisms of mitochondrial regulation in the context of eosinophilic esophagitis.

ACKNOWLEDGMENTS

I would first like to thank my thesis mentor, Dr. Kelly A. Whelan for her tremendous support and guidance through this process. She continually inspires me with her spirit and attitude towards academic success in addition to helping me grow tremendously as a scientist and a person. The lessons I have learned in her lab I will carry throughout my career.

I also wish to express my gratitude for the members of the Whelan lab especially Mary Grace Murray, Anne-Laure Monégar, and Anbin Mu. It was always a pleasure to work by their side and without their help, none of this would have been possible.

I next want to thank the members of my committee Dr. Italo Tempera, and Dr. Dale Haines. Their support was integral to my success and development through the past year.

I would also like to thank my colleagues and friends Marcus Wagner, Matthew Chrzanowski, and Jeremy Wurtzel. Throughout my graduate career, they have always been to there to offer encouragement and guidance for any laboratory or personal issue I had experienced. They instilled the confidence in me that I needed to succeed.

Finally, nobody supported me more than my parents. With their love and help, I was able to successfully pursue and accomplish my academic goals.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ANKRD27	Ankyrin repeat domain-containing protein 27
ANOVA	Analysis of Variance
APC	Antigen presenting cell
ATP	Adenosine Triphosphate
ATG7	Autophagy-Related 7
CAPN14	Calpain 14
Cas9	CRISPR associated protein 9
CCL26	Chemokine (C-C motif) ligand 26
CRISPR	Clustered regularly interspaced short palindromic repeats
DAMP	Damage-associated molecular pattern
DHTKD1	Dehydrogenase E1 and transketolase domain containing 1
Drp1	Dynamin-1-like protein
DTT	Dithiothreitol
EoE	Eosinophilic Esophagitis
Eos	Eosinophils
ESCC	Esophageal squamous cell carcinoma
EPC2	Esophageal Primary Cell 2
GERD	Gastroesophageal reflux disease
GFP	Green fluorescent protein
gRNA	Guide RNA
GWAS	Genome-wide association study
HBSS	Hank's Balanced Salt Solution
hTERT	Human telomerase reverse transcriptase
hpf	High power field
IgE	Immunoglobulin E
IL-13	Interleukin 13
IL-1β	Interleukin 1 beta

IL-4	Interleukin 4
IL-5	Interleukin 5
KFSM	Keratinocyte serum free medium
LB	Lysogeny Broth
LC3	Microtubule-associated proteins 1A/1B light chain 3B
LRRC32	Leucine rich repeat containing 32
MC903	Calcipotriol
MID49/51	Mitochondrial dynamics proteins of 49 and 51 kDA
Mfn1	Mitofusin 1
Mfn2	Mitofusin 2
MTCO1	Cytochrome c oxidase subunit I
mtDNA	Mitochondrial DNA
NADPH	Nicotinamide adenine dinucleotide phosphate
ND6	NADH-ubiquinone oxidoreductase chain 6
NLRP3	NLR family pyrin domain containing 3
OGDHL	Oxoglutarate dehydrogenase like
OPA1	Dynamin-like 120 kDA protein
OVA	Ovalbumin
P63	Tumor protein 63
PBS	Phosphate-buffered saline
PGC1-α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Pink1	PTEN-induced kinase 1
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
RPM	Round per minute
shRNA	Short-hairpin RNA
SNP	Single nucleotide polymorphism
Sox2	Sex determining region Y-box 2
STAT3	Signal transducer and activator of transcription 3
STAT6	Signal transducer and activator of transcription 6

STI	Soy-trypsin inhibitor
Tfam	Mitochondrial transcription factor A
TGF-β	Transforming growth factor beta
Th2	T helper 2
TLCV2	Tetracycline lenticrispr version 2
TNFα	Tumor necrosis factor alpha
TSLP	Thymic stromal lymphopoietin

CHAPTER 1

INTRODUCTION

1.1 Esophageal Development and Homeostasis

The esophagus is the tubular organ that connects the pharynx (throat) to the stomach and is responsible for transportation of sustenance. The esophagus is anatomically located posterior to the trachea and heart and transverses the diaphragm. In early embryonic development, the esophagus and the trachea are conjoined with the esophagus subsequently becoming an extension of the foregut. This extension begins after respiratory appendages, such as the lungs and trachea, develop and separate (Fausett et al., 2012). After complete development, the esophagus is formed by multiple layers of mucosal, submucosal, muscularis propria, and adventitial tissues (Rosekrans et al., 2015). The mucosal membrane of the esophagus is comprised of a superficial layer of nonkeratinized stratified squamous epithelium. This squamous epithelium spans the entire length of the esophagus and consists of esophageal keratinocytes. Beneath the epithelium is the submucosal connective tissue as well as resident immune cells, nerve cells, and the associated vasculature (Rosekrans et al., 2015). In addition, secretions from glands in the submucosa aid food clearance from the esophagus and protect the tissue from acid injury (Long et al., 1999). The muscularis propria is primarily responsible for the peristaltic contractions of the esophagus that move food into the stomach. Finally, the adventitia is a fibrous layer connecting the esophagus with adjacent structures (Goyal et al., 2008).

1.1.1 Structure of the Esophageal Epithelium

The most studied layer of the esophagus is the epithelium due in part to the many pathologies that arise from it. Structurally, the esophageal epithelium is comprised of keratinocytes that can be compartmentalized into three separate layers: a basal layer of undifferentiated cells, several suprabasal layers displaying a differentiation gradient, and a luminal layer of terminally differentiated cells (Rosekrans et al., 2015). Graphical representation of mouse and human esophageal epithelium structure is described in **Figure 1**. Cell classification is identified by relative location of the cells as well as the expression of specific markers associated with differentiation status. One of the main types of functional proteins found in esophageal keratinocytes are keratins, which are abundantly expressed and support the cytoskeleton (Grace et al., 1985; Karantza, 2011). Basal cells are situated at the base of the epithelium, display square-like morphology and highly express keratin 15, keratin 14 and keratin 5 (Whitbread et al., 1998; Wilson et al., 1992). As basal esophageal keratinocytes initiate squamous differentiation, expression of keratin 15, keratin 14, and keratin 5 decreases. In addition, differentiating cells exhibit expression of keratin 4 and keratin 13 (van Muijen et al., 1986). Thus, keratin subtypes can be utilized as markers for esophageal keratinocytes, especially in distinguishing those with phenotypic differences. Differentiated epithelial cells will then begin to synthesize involucrin, a protein that helps maintain epithelial barrier function (Banks-Schlegel et al., 1981). In addition, they produce keratohyalin granules, a precursor of fillagrin, which promotes cross-linking of the epithelium and a flattened cellular morphology (Makino et al., 2003). In order for the esophageal epithelium to maintain homeostasis it undergoes complete cellular turnover. This is a function of the basal cells migrating towards the

luminal surface, where they begin to rapidly proliferate in the suprabasal layer, and finally differentiate (Rosekrans et al., 2015). After differentiation, cells will shed off into the lumen, and the process will repeat with an average turnover time of seven days in mice (Eastwood, 1977). Debate still exists over whether a resident stem cell replenishes the epithelium and is present in the basal cell layer (Giroux et al., 2017), or if the basal cells are comprised of a homogenous population capable of self-regeneration (Doupé et al., 2012). There are about twenty layers of epithelial cells in the human esophagus, and about six layers in the mouse esophagus (Barbera et al., 2015). In addition to the difference in layer count, the mouse lacks papillary structures that human basal cells are organized around and submucosal glands (Rosekrans et al., 2015). The murine esophageal epithelium also contains a superficial layer of keratin that presumably protects the esophagus from insult. Proliferation is limited to the basal layer in mouse, whereas proliferation primarily occurs in the suprabasal cells in humans (Barbera et al., 2015).

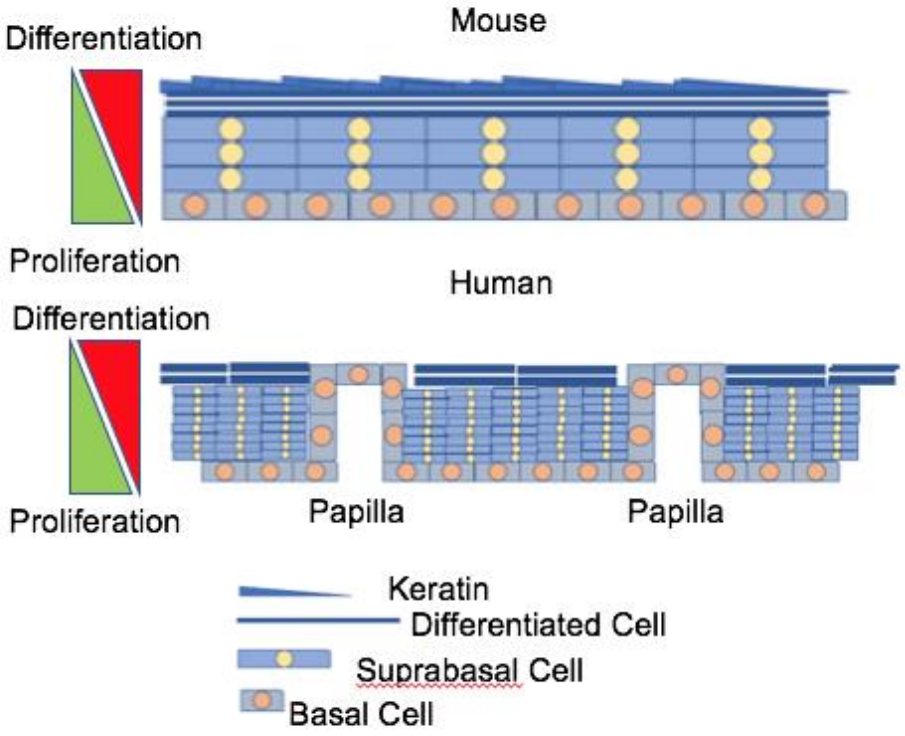

Figure 1: Structure of Mouse and Human Esophageal Epithelium.

(**A**) The mouse esophageal epithelium contains a layer of linearly-aligned basal cells. A six-cell thick layer of suprabasal and differentiated cells lies above the basal cells. A superficial layer of keratin is present in the mouse esophageal epithelium which may provide protection from ingested insults. (**B**) Human esophageal epithelium consists of the same cell subtypes, but is approximately twenty layers thick and organized around papillary structures. Papillary structures are submucosal protrusions which separate the epithelium into interpapillary regions. The exact function of these papillae remains unclear. Basal cells which are less differentiated with distinct morphology, migrate upwards into the suprabasal layer where proliferation increases rapidly until complete

differentiation occurs. After differentiation, keratinocytes ultimately die and shed off into the esophageal lumen.

1.1.2 Regulation of the Proliferation/Differentiation Gradient in Esophageal Epithelium

There are various regulators of esophageal epithelial proliferation, differentiation, as well as barrier function. Many of the cellular pathways that are activated during embryonic development of the esophageal epithelium also play a role in maintaining homeostasis. Sox2 is a transcription factor that is associated with pluripotency and is critical for the development of the esophageal epithelium (Trisno et al., 2018). In adult esophageal epithelium, Sox2 is expressed in the basal layer, with Sox2-positive cells having stem-like characteristics. Genetic ablation of Sox2 causes an absence of the esophageal basal cells in mice (Arnold et al., 2011). Reciprocally, if Sox2 is overexpressed, there is an increase in suprabasal proliferating cells (Liu et al., 2013). Another transcription factor that regulates development and homeostasis of the epithelium is Tumor Protein 63 (p63) (Daniely et al., 2004). It has been shown that deletion of p63 results in inhibition of basal cell renewal in organotypic culture (Jeong et al., 2016). Other pathways involved in the regulation of the esophageal epithelium include Hedgehog, and Notch signaling (Isohata et al., 2009; Ohashi et al., 2010).

1.2 Esophageal Pathologies

Multiple pathological conditions can affect the esophagus including gastroesophageal reflux disease (GERD), in which acidic stomach contents are

regurgitated through the lower esophageal sphincter into the esophagus, leading to damage to the gastroesophageal epithelium and cause inflammation of the esophagus (esophagitis). GERD predisposes patients to development of specialized intestinal metaplasia termed “Barrett’s esophagus”, a precursor to esophageal adenocarcinoma (Martinucci et al., 2016). Esophageal adenocarcinoma is the most prevalent in the US, but the most common esophageal malignancy worldwide is esophageal squamous cell carcinoma (ESCC which is associated with alcohol consumption and tobacco use (Napier et al., 2014). In addition to GERD and esophageal malignancies, various nonmalignant conditions are found in the esophagus, including EoE.

1.3 Eosinophilic Esophagitis

EoE is an allergic inflammatory condition of the esophagus characterized by infiltration of eosinophils into esophageal mucosa and submucosa. EoE patients typically present with symptoms including dysphagia, food impaction, and esophageal dysfunction. Other symptoms include failure to thrive, vomiting, and abdominal pain. EoE patients will usually undergo treatment for GERD which includes acid reducing medications, however this is frequently ineffective at reducing symptomology. If EoE is suspected, esophageal biopsies will be taken during esophago-gastroduodenal endoscopy. Macroscopic findings suggestive of, but not specific to, EoE include esophageal corrugated rings and strictures observed during endoscopy (Straumann et al., 2018). Confirmation of diagnosis is based on esophageal biopsy with histological staining showing greater than or equal to 15 eosinophils per high powered field (hpf) (Straumann & Katzka, 2018). In addition, the patient must have a complete evaluation for other

conditions which may be causing eosinophil infiltration such as medication or infection. If ≥15 eosinophils/hpf are observed, the patient is considered to be in an active state of EoE. When patients with a previous diagnosis of active EoE have a follow-up biopsy showing fewer than 15 eosinophils/hpf, the disease is classified as inactive. Flow diagram of clinical evaluation and diagnosis of EoE is represented in **Figure 2**. Notably, histological classification of inactive disease does not necessarily indicate a lack of symptoms (Carrasco et al., 2017; Cianferoni et al., 2016). In addition to the presence of eosinophils, a classical histological finding in EoE is basal cell hyperplasia, an increase in the total epithelial height and a greater percentage of basal cells (Carrasco et al., 2017). In addition, patients can develop progressive epithelial and sub-epithelial fibrosis which can lead to esophageal strictures and persistent symptoms (Aceves, 2014). As such, EoE can have a long term negative impact on patient quality of life (Mukkada et al., 2018). The disease frequently affects young Caucasian males; however, individuals of both sexes as well all races and ethnicities can develop this condition at any age (Prasad et al., 2009). The incidence of newly diagnosed EoE is approximately 1/10,000 per year with the prevalence continuing to increase (Dellon, 2014). EoE is also the most common cause of food impaction.

The exact pathophysiology of EoE is unclear. It is an allergic disease with the most commonly associated trigger foods being milk, gluten, eggs, and nuts (Spergel et al., 2012). Complete elimination of suspected triggers helps reduce symptoms, but will not cure the disease (Cianferoni & Spergel, 2016). Because a patient may have multiple dietary triggers, the best method to ensure allergen elimination is switching to a diet consisting of only elemental formula (Markowitz et al., 2003). Patients can also be

treated with corticosteroids to reduce inflammation, but these agents may have adverse side effects if taken long term. Specifically, long term use of corticosteroids can result in hyperglycemia, hypertension, and Cushing's syndrome (Poetker et al., 2010). As both dietary elimination and steroid therapy are non-specific and have the potential to negatively impact patients (Mukkada et al., 2018), it is of great clinical interest to discover novel methods of diagnosis, monitoring and therapy for EoE.

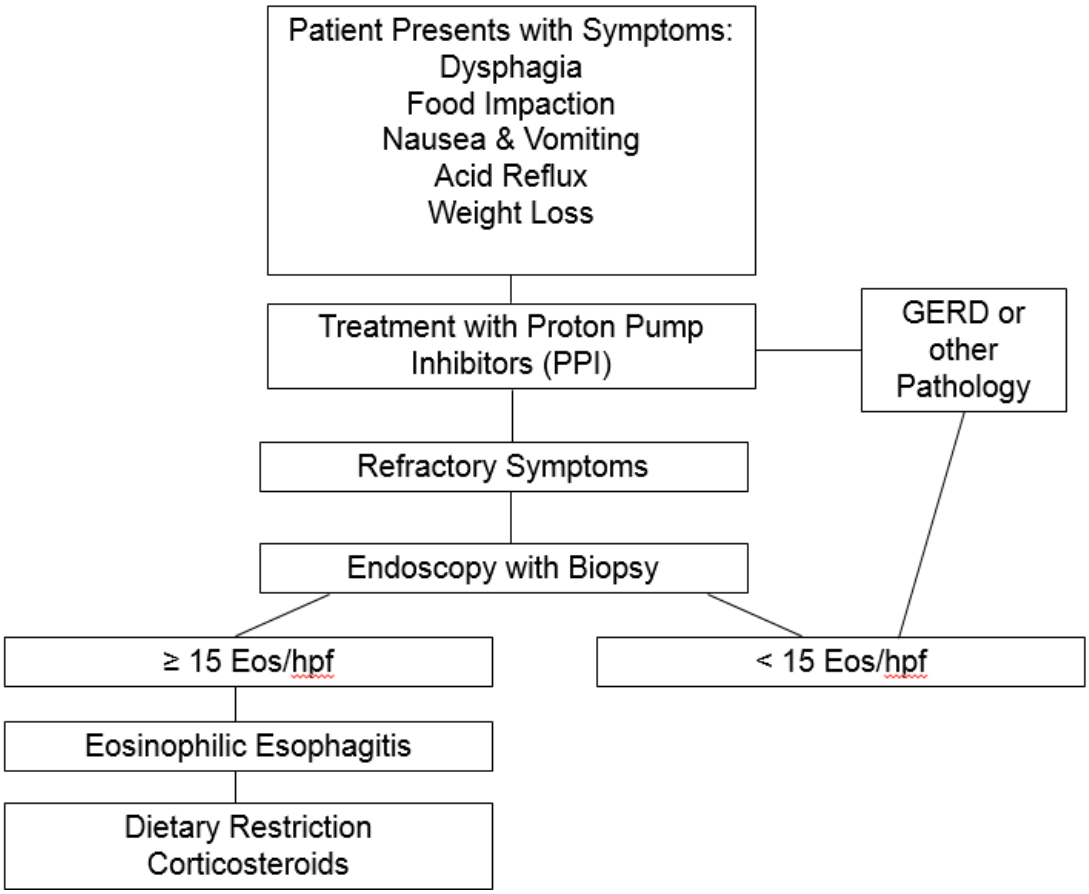

Figure 2: Diagnosis and Treatment of Eosinophilic Esophagitis.

Patients with eosinophilic esophagitis (EoE) are predominantly young Caucasian males who present with non-specific symptoms such as dysphasia, food impaction, nausea &

vomiting, acid reflux, and weight loss. Patients will generally undergo treatment for gastroesophageal reflux disease (GERD) which includes acid-reducing medications such as proton pump inhibitors. If symptoms resolve, patients are diagnosed as having GERD, or another pathology with reflux as a secondary symptom. Patients with refractory symptoms will undergo endoscopy where an esophageal tissue biopsy will be collected. If biopsy reveals ≥ 15 Eosinophils (Eos)/high power field (hpf) the patient will be diagnosed with active eosinophilic esophagitis provided that no other causes for eosinophilia can be identified. Patients with < 15 Eos/hpf will be considered for other possible diagnosis. Treatment for EoE mainly consists of dietary restrictions to eliminate possible food triggers, corticosteroids to reduce inflammation, follow-up endoscopies, and symptom monitoring. Patients with EoE diagnosis and follow-up endoscopy with < 15 Eos/hpf are considered to be inactive.

1.3.1 Eosinophilic Esophagitis Genetics

Most patients with EoE also present with comorbid atopic diseases such as asthma, allergic rhinitis, and atopic dermatitis (Durrani et al., 2018). Although environmental risk factors contribute to disease process, the 41% concordance rate between monozygotic twins is suggestive that genetic factors play a critical role in predisposing a patient to developing EoE (Alexander et al., 2014). Genome Wide Association Study (GWAS), Single Nucleotide Polymorphism (SNP), and familial genetic studies reveal hits on genes alterations associated with increased risk of EoE. Frequently associated alterations include those in *CCL26*, the gene that encodes the potent eosinophil chemoattractant, eoxtaxin-3, the epithelial barrier associated gene,

FLG, and the cytokine encoding gene *TGF-β1*. Variants in thymic stromal lymphopoietin (*TSLP*), another factor associated with epithelial barrier function and T helper 2 (Th2) skewing, are also highly associated with EoE predisposition (Kottyan et al., 2014; Kottyan et al., 2017). The function of the epithelial barrier is to prevent antigen and pathogen translocation which can result in disease, epithelial remodeling, or hyperproliferation. Alterations in *CAPN14*, the gene that encodes Calpain 14, a calcium-activated protease, are also associated with an increased risk of EoE. Recent studies have begun to explore Calpain 14 as a key regulator of epithelial repair, but further functional studies are required to fully assess its role (Davis et al., 2016; Litosh et al., 2017). In addition, variants in *STAT6*, *LRRC32/C11orf30*, and *ANKRD27* are linked to EoE (Kottyan & Rothenberg, 2017). Signal Transducer and Activator of Transcription 6 (STAT6) is the main transcription factor associated with activating the EoE-related transcriptome. Although there are genetic associations between *LRRC32* and *ANKRD27* with EoE, the mechanistic relationship is unclear. Most of the genetic alterations in EoE are associated with aberrant immune activities and loss of epithelial barrier function. Genes associated with EoE are summarized in **Table 1**.

Table 1: Genes associated with EoE

Mutations, variants, and single nucleotide polymorphisms (SNPs) have been associated with increased risk of developing eosinophilic esophagitis (EoE). Associated genes are mainly involved in epithelial barrier function, recruitment of the immune system, and T helper 2 (Th2) skewing.

Genes Associated with EoE				
Gene	**Encodes**	**Function**	**Alteration**	**References**
CCL26	Eotaxin-3	IL-13-inducible chemokine that recruits eosinophils	Variant in 3'Untranslated Region (UTR) to promote mRNA stability, increased expression	(Sherrill et al., 2014)
CAPN14	Calpain-14	IL-13-inducible calcium-dependent protease which regulates epithelial repair	Promotor variant which may lead to genotype-dependent CAPN14 expression	(Kottyan et al., 2014)
TSLP	Thymic stromal lymphopoietin	Regulator of epithelial barrier and can activate Th2 response and eosinophils	SNPs in promotor region and intron	(Sherrill & Rothenberg, 2014)
LRRC32/C11orf30	Leucine rich repeat containing 32/EMSY	TGF-β binding protein which may regulate fibrosis	Intergenic, Intron SNPs	(Kottyan et al., 2014)
STAT6	Signal Transducer and Activator of Transcription 6	Downstream of IL-13 and IL-4 receptor activation	SNP rs167769 [T]	(Sleiman et al., 2014)
ANKRD27	Ankyrin repeat containing protein 27	Inhibitor of SNARE complex	SNP rs3815700 [C]	(Sleiman et al., 2014)
FLG	Filaggrin	Barrier function	Loss of Function Mutations	(Blanchard et al., 2010)
TGF- β1	Transforming growth factor beta	Barrier function	SNP in promotor	(Aceves et al., 2010)

1.3.2 Eosinophilic Esophagitis Immunology

EoE is generally considered to be an Immunoglobulin E (IgE)-mediated disease, due to the associated IgE-mediated comorbidities such as atopic dermatitis. However, recent studies show that this may not be the case due to the lack of symptom suppression based on putative food triggers identified through IgE testing (Simon et al., 2016). Multiple cytokines and chemokines are implicated in the development and chronicity of EoE. The major cytokines associated with EoE are those of the Th2 class. T helper 2 cells are T cells that were prompted to switch phenotypic expression profile after costimulatory signaling from antigen-presenting cells (APC). In EoE, these T helper 2 cells become activated and begin to secrete the Th2 cytokines interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-13 (IL-13). Together, these contribute to the type 2 inflammatory response that ultimately results in eosinophil infiltration (Hill et al., 2016). In addition to APC-mediated activation of the immune system, TSLP can be secreted by the esophageal epithelial cells of EoE patients, triggering a Th2 immune response (Cianferoni et al., 2014). Cytokines have limited potential as a serum biomarker as EoE patients are not found to have detectible serum concentration of these Th2 cytokines (Ishihara et al., 2017). Both IL-4 and IL-13 primarily transduce through similar receptors and the transcription factor, signal transducer and activator of STAT6 which leads to expression of eotaxin-3 (Matsukura et al., 2001). IL-13 is the most well-characterized cytokine involved in EoE mice deficient in IL-13 do not develop EoE (Blanchard et al., 2005). In fact, IL-13 alone can elicit a large portion of the EoE-related transcriptome in esophageal epithelial cells (Blanchard et al., 2007). In addition, IL-13 and IL-4 have been classically associated with epithelial barrier dysfunction (Saatian et al., 2013). IL-5 is

another Th2 cytokine that has been shown to promote eosinophil infiltration, survival and activation. Patients with EoE display IL-5-positive T helper cells in their serum and esophageal tissues (Blanchard et al., 2011). In addition, topical steroids lower IL-5 expression in the esophagus of EoE patients (Lucendo et al., 2008). Animal studies also reveal that global dysregulation and overproduction of IL-5 induces esophageal eosinophilic infiltration (Akei et al., 2005). In clinical trials, targeting IL-5 or IL-13 with monoclonal antibodies had success in reducing eosinophilia in EoE patients, but limited to no success in reducing symptoms (Assa'ad et al., 2011; Rothenberg et al., 2015; Stein et al., 2006). Therefore, new approaches outside of cytokine depletion must be explored to treat EoE patients.

1.4 Mitochondrial Dynamics

There have been recent discoveries showing that mitochondrial dysfunction may play a role in atopic diseases including EoE (Iyer et al., 2017). Understanding mitochondrial biology can provide insight into the process that may be effected in EoE. Mitochondria function as the organelle responsible for the generation of cellular energy in the form of adenosine triphosphate (ATP). This is closely associated with substrates of the Krebs cycle. Mitochondria have inner and outer membranes which enclose a hydrogen ion gradient and electrochemical buffer system. This membrane system allows for efficient energy production as well calcium transport into the mitochondria. Mitochondria contain their own deoxyribose nucleic acid (mtDNA), which is an approximately 16 kilobase circular genome present in multiple copies per mitochondria. MtDNA encodes 13 proteins that are constituents of the mitochondrial electron transport

chain, although many mitochondrial proteins are nuclear encoded. The mitochondrial electron transport chain lies on the inner mitochondrial membrane and contains four complexes responsible for transferring electrons from nicotinamide adenine dinucleotide phosphate onto oxygen, and coupling this reaction with adenosine diphosphate (ADP) phosphorylation to generate ATP. Electrochemical potential is generated through this process, and functional mitochondrial are classically considered to be in a polarized state (Friedman et al., 2014). Mitochondria depolarization can act as an intracellular signal that promotes mitochondrial repair/degradation mechanisms. However, some studies show that mitochondrial depolarization may not necessarily be due to dysfunctional mitochondria (Ziegler et al., 2018). In addition, mitochondrial dysfunction has been associated with aging and defects in stem cells (H. Zhang et al., 2018).

Mitochondria are dynamic organelles that constantly undergo quality control. Mitochondria have a proteolytic system that is responsible for degrading misfolded proteins and dysfunctional outer membrane proteins. They can also undergo degradation, fusion, and fission depending on cellular energy requirements, and environmental stressors; with oxidative stress being the most frequent environmental stressor associated with mitochondrial damage (Youle et al., 2012). This is due in part by the natural production of reactive oxygen species (ROS) generated by the mitochondria as a byproduct of respiration. ROS can directly damage mtDNA which can ultimately lead to mutations. Fusion between mitochondria can limit the effects of these mtDNA mutations through complementation. Mitochondria containing mutant mtDNA can fuse with wild-type mitochondria to account for defects. In addition, fusion occurs during mitochondrial biogenesis and during periods of increased energy demand and stress (Youle & van der

Bliek, 2012). The main proteins associated with mitochondrial membrane fusion are mitofusin 1 (Mfn1), mitofusin 2 (Mfn2) and optic atropy1 (OPA1). Mfn1 and Mfn2 are GTPases that can fuse outer mitochondrial membranes to one another. OPA1 is another GTPase but is responsible for fusion of the mitochondrial inner membrane. Mitochondrial fission occurs to extend the mitochondrial network by creating more mitochondria as well as separating parts of dysfunctional mitochondria (Youle & van der Bliek, 2012). Mitochondrial fission is mediated primarily by the large GTPase dynamin-related protein 1 (Drp1). Drp1 is localized to the cytosol but is recruited to the outer mitochondrial membrane where it interacts with multiple mitochondrial receptor proteins. These include mitochondrial dynamics protein of 49/51 kDA, as well as fission 1, and mitochondrial fission factor. Fusion and fission are considered the main quality control mechanisms to conserve energy and eliminate dysfunctional mitochondria (Scott et al., 2010). Mitochondria that are presumably damaged beyond repair are degraded through a process called mitophagy, a mechanism by which mitochondria are targeted and ultimately degraded by lysosomes. The most studied mechanism of mitophagy is Parkin-dependent mitophagy. In Parkin-mediated mitophagy pathway, PTEN-induced kinase 1 (Pink1), a serine/threonine kinase localizes to the depolarized outer membrane. In the absence of active mitophagy, Parkin, an E3 ubiquitin ligase, resides in the cytosol. When mitochondria become coated by Pink1, Parkin is recruited and directly phosphorylated by Pink1. Parkin will then begin to ubiquitinate the mitochondria leading to targeting by the lysosome-associated protein p62/SQSTSM which identifies autophagic cargo to facilitate complete degradation of the mitochondria by lysosomes (Jin et al., 2012).

1.5 Mitochondria and Eosinophilic Esophagitis

Emerging findings suggest a link between mitochondrial function and allergic diseases, such as asthma and potentially EoE. In allergic airway disease, exposure to ROS has been associated with an inflammatory response. A landmark paper showed that an isolated antigen, ragweed extract, was able to increase ROS production by inducing dysfunction in mitochondrial respiratory complexes I & III (Aguilera-Aguirre et al., 2009). This led to an exacerbation of allergic disease and eosinophil accumulation in mouse airways (Aguilera-Aguirre et al., 2009). In an *in vitro* study, it was found that human bronchial epithelial cells underwent mitochondrial fragmentation when exposed to dust-mite antigens. This mitochondrial fragmentation subsequently led to the secretion of Interleukin 1 beta (IL-1β) and Interleukin 8. Upregulation of Drp1 was mainly responsible for this mitochondria-mediated reaction, suggesting that fractionated mitochondria or increased mitochondria level may be a marker of disease (VA., 2015). A recent article using whole-exome sequencing in sixty families with documented predisposition toward EoE uncovered novel variants in oxidoreductase dehydrogenase E1 and transketolase domain-containing-1 (*DHTKD1*) and oxoglutarate dehydrogenase-like (*OGDHL*), linking mitochondrial biology to EoE for the first time (Sherrill et al., 2018). Specifically, this study identified 5 damaging variants in *DHTKD1* and seven in *OGDHL*. *DHTKD1* is a nuclear-encoded gene responsible for mitochondrial lysine metabolism as well as ATP production. Nonsense mutation (arginine [Arg] 834*) and a splicing mutation (c.1897-1 guanine [G] > adenine [A]) identified in *DHTKD1* in a 2-generation family with EoE are suggestive of a link between EoE and mitochondrial disease. These mutations result in nonsense-mediated decay and haploinsufficiency. In addition, it was

revealed that missense mutations in *OGDHL* (c.406 cytosine [C] > thymine [T], p. proline [Pro] 136 serine [Ser]) cause mitochondrial dysfunction and the generation of ROS. Depletion of *DHTKD1 in vitro* promotes increased expression of radical S-adenosyl methionine domain-containing 2, a gene that induces Th2 skewing. In addition, small hairpin ribonucleic acid (shRNA) knockdown of *DHTKD1* causes production of ROS. Mitochondrial functional testing in the *DHTKD1* knockdown esophageal epithelial cell line (EPC2) revealed a decrease in basal and maximal respiration as well as ATP production (Sherrill et al., 2018).

In addition to alterations in mitochondrial number and function, circulating mitochondrial DNA has been demonstrated in a variety of diseases, including inflammatory bowel disease where it has recently been shown to serve as a biomarker (Boyapati et al., 2018). Eosinophils have been shown to react to pathogen associated lipopolysaccharide which can activate eosinophils to "catapult" mtDNA traps as an antimicrobial tactic. In addition, eosinophils are able to react to the EoE-associated cytokine IL-5 to perform this same mtDNA extrusion (Yousefi et al., 2008). Mast cells, another EoE-related immune cell, release mitochondrial components and mtDNA after exposure to the inflammatory trigger IgE (B. Zhang et al., 2012). We have demonstrated the presence of structures consistent with mitochondria in the extracellular space of EoE epithelium (**Figure 3**). Additionally, we have found that levels of mtDNA are elevated in the serum of patients with active EoE as compared to those with inactive EoE and GERD as well as non-EoE controls. (**Figure 4**). This may indicate that a process of increased mtDNA is occurring in the tissue of EoE patients with unknown genesis. Thus, it would be of great interest to further investigate this process to discover the cellular origin of

mtDNA generation and the mechanism through which this occurs. In addition, understanding why mtDNA is synthesized and what role mitochondria serve in EoE pathophysiology may lead to novel therapeutic interventions. Based upon these preliminary data and emerging studies linking mitochondria to EoE, we have begun to further investigate the role of mitochondria in EoE biology.

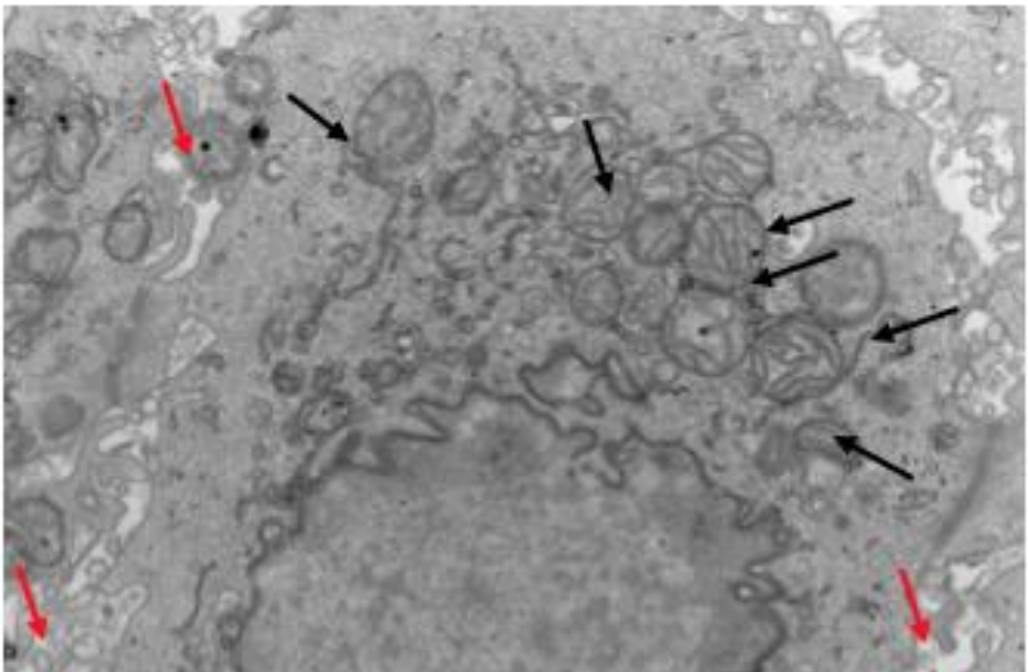

Figure 3: Electron Micrograph of Esophageal Keratinocytes with Extracellular Mitochondrial Structures.

Transmission electron microscopy in biopsy from patient with active eosinophilic esophagitis. Red arrows show structures consistent with mitochondria in extracellular space. Black arrows indicate intact intracellular mitochondria.

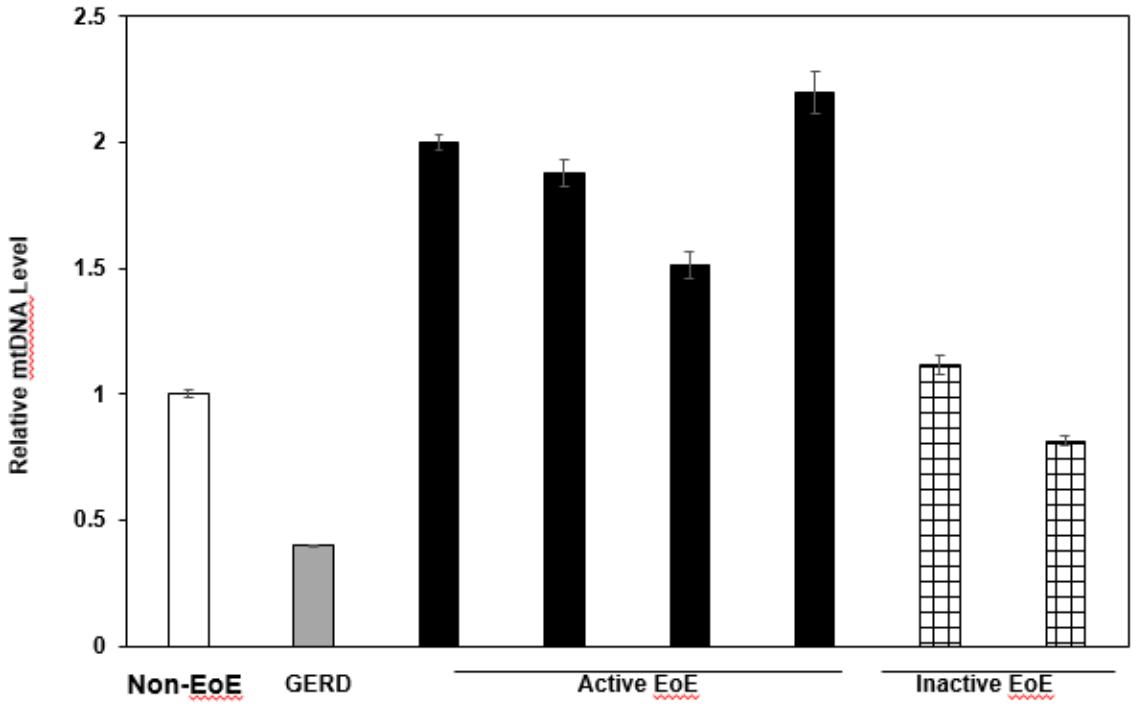

Figure 4: Evidence of Increased mtDNA in Adult EoE Patient Serum.

Relative abundance of mitochondrial DNA (mtDNA) in serum from patients of the indicated diagnoses was determined by quantitative PCR. The ratio of circulating mtDNA (ND6) to nuclear DNA (HGB1) was evaluated for each patient. Four active eosinophilic esophagitis (EoE) patients had a higher ratio of mtDNA to nuclear DNA as compared to Non-EoE and inactive EoE. In order to examine whether increased circulating mitochondrial DNA was specific to EoE, a patient with gastroesophageal reflux disease (GERD), an additional inflammatory disorder of the esophagus, was tested. Further statistical power is necessary and will be performed in future experiments after curation of samples.

1.6 Objectives and Hypothesis

Studies show that mitochondrial dysfunction in epithelial cells plays a role in the development of allergic airway diseases and eosinophilic esophagitis (Sherrill et al., 2018) (Aguilera-Aguirre et al., 2009). Our preliminary data shows an increase in serum mtDNA in active EoE patients **(Figure 3)** in addition to an electron micrograph suggesting that mitochondria may be extruded by esophageal keratinocytes **(Figure 4)**. Therefore, we hypothesized that mitochondrial level may increase in the esophageal epithelium in response to specific microenvironmental cues.

The objectives of this thesis are:

1) To determine whether the EoE microenvironment affects mitochondrial content *in vivo* and *in vitro*
2) To investigate mechanisms of EoE-mediated alterations in mitochondria function.

CHAPTER 2

MATERIALS AND METHODS

2.1 Culture of Human Immortalized Esophageal Keratinocytes (EPC2-hTERT) and Primary Esophageal Keratinocytes

EPC2-hTERT cells are derived from a 55-year old male that underwent esophagectomy due to Barrett's metaplasia and were gifted from the laboratory of Anil K. Rustgi of the University of Pennsylvania Perelman School of Medicine where the cell line was immortalized via expression of human telomerase. Primary esophageal keratinocytes from normal and EoE patients were kindly gifted from the laboratory of Hiroshi Nakagawa of the Division of Gastroenterology at the University of Pennsylvania. Human esophageal keratinocytes are grown in Keratinocyte Serum Free Media (KFSM) (Thermofisher Catalog #17005042) containing L-glutamine and 0.06mM $CaCl_2$. KFSM is supplemented with 1ng/mL of Epidermal Growth Factor 1-53, 25mg Bovine Pituitary Extract (with proprietary constituents), and 1000 units/mL penicillin, 10,000 ug/mL streptomycin. Cells are plated on pretreated tissue flasks at a confluence between 30%-70%, unless otherwise noted. Incubation is in a 5% CO_2 chamber at a constant temperature of 37°C. Cells are grown until 70% confluence and then split into separate flasks. Cells are maintained at a confluency that is between 30% and 70% to prevent potential phenotypic changes. KFSM was changed every 48-72 hours. Splitting cells requires 0.05% trypsin and soy-bean trypsin inhibitor (STI; 250 ng/mL). Flasks are coated with 0.05% trypsin for approximately five minutes at 37C to allow detachment. Afterward cells are pipetted into a conical tube containing STI at a ratio of 3:1 inhibitor

to trypsin. Cells are then centrifuged at 1000 revolutions per minute (RPM) for 3 minutes at room temperature. Cells are re-suspended in KFSM and seeded at appropriate concentrations. If freezing cells, resuspension is in 10% dimethyl sulfoxide in fetal bovine serum. They are then slowly frozen in cryogenic vials by Mr. Frosty Freezing Container with 100% isopropanol at -80°C overnight. To test viability, one vial is then thawed by placing it in a 37°C water bath and then washed with 14mL KFSM. The conical tube is then centrifuged at 1000 RPM, the supernatant is suctioned off and the pellet re-suspended in KFSM and plated. After confirmation of cell viability, remaining cell vials are placed in liquid nitrogen at -200°C for long term storage. Cells are routinely tested for Mycoplasma with the MycoAlert Mycoplasma Detection Kit (Lonza Catalog #: LT07-318).

2.2 Immunohistochemistry for MTCO1

Paraffinized histological slides of 29 active EoE, 26 inactive EoE, and 7 normal control patients were kindly gifted from the laboratory of Amanda Muir of the Division of Gastroenterology, Hepatology and Nutrition at The Children's Hospital of Philadelphia. Slides were deparaffinized by first warming them in a 60°C incubator for 10 minutes. Then, they were completely submersed in 100% xylene 3 times for 5 minutes each. They were then submersed in 100% ethanol 2 times for 2 minutes each. Subsequent submersion of slides in 95%, 80%, 70% and deionized water (dH_2O) were then carried out. Slides were then immersed in 10mM citric acid buffer (pH 6.0) and incubated in a pressure cooker for 2 hours. Slides were then gently rinsed with dH_2O and placed into a hybridization box. Hydrophobic pens were used to circle tissue. Next, endogenous

peroxidases were quenched by using a solution of 3% Hydrogen Peroxide in dH_2O on the slides for 5 minutes. Slides were washed by placing dH_2O on each slide for 5 minutes. The slides were then washed with Phosphate-buffered saline (PBS) for 5 minutes. Next, the tissue was blocked with Avidin D blocking reagent for 15 minutes and subsequently washed quickly with PBS. The tissue was then also blocked with Biotin Blocking Reagent for 15 minutes and washed quickly with PBS. MTCO1 antibody (abcam ab14705) was diluted at 500 ng/mL in PBS-T (0.1% Tween-20). Slides were then incubated overnight at 4°C. Slides were then washed with PBS 2 times for 5 minutes each to remove primary antibody. Secondary mouse antibody conjugated to Biotin is diluted in PBS-T at 1:200 dilution and placed on the tissue for 30 minutes in a 37°C incubator. Slides were then washed with PBS 2 times for 5 minutes each. A 20 uL solution of 1:1 Reagent A and B of HRP-conjugated ABC reagent (Vector Elite Kit, Vector Laboratories) were mixed together and pipetted into 1 mL PBS-T. Slides were then incubated for 30 minutes at 37°C and then washed with PBS and dH_2O for 5 minutes each. Slides were then developed with DAB substrate kit (Vector Laboratories) for 2 minutes and each slide washed with dH_2O to stop reaction. After all slides were developed, counter stain with hematoxylin for 30 seconds. Slides were then rinsed in dH_2O and submerged into 70%, 95% ethanol for 2 minutes each. After, slides were submerged in 100% ethanol and Xylene 2 times for 2 minutes and 1 minute respectively. Slides were then mounted with coverslip and sent to for pathologic analysis by Dr. Andres Klein-Szanto at Fox Chase Cancer Center.

2.3 Cytokine Treatment Assays

For experiments that began without confluence, 50,000 EPC2-hTERT cells were seeded onto 6-well plates and left to adhere to the plate overnight. The next day, cells were separately/simultaneously treated with human recombinant IL-13 (R&D Systems CN# 213-ILB-005), with IL-4 (R&D Systems), with IL-5 (R&D Systems CN# 205-IL-005), with IL-1β (R&D Systems CN# 201-LB-005) at a concentration of 10 ng/mL, and/or TNFα (R&D Systems CN# 210-TA-005) at a concentration of 40 ng/mL. For all experiments, medium containing fresh cytokines was changed every 48-72 hours. Multiple experiments were performed including time courses of cells treated with only IL-13 at points of 0, 4, 24 , 48 , 72 , 96 , 120, 168, and 240 hours. Cells in multiple cytokine treatments were seeded on 6 well plates and treated with IL-13/IL-4, IL-13/IL-5, and IL-13/TNFα respectively for 7 days. Cells were also seeded and grown to 100% confluence before beginning IL-13 treatment for 7 days.

2.4 DNA/RNA Collection, Quantitative Polymerase Chain Reaction (qPCR) for mtDNA

For all experiments performed that require DNA isolation QIAGEN kits (DNAeasy Blood & Tissue Kit), manufacturer protocols were followed. DNA concentrations were quantified using Nanodrop. For Quantitative Polymerase Chain Reaction (qPCR), SYBR-Green Reagents (Thermofisher Catalog #A25741) were used in combination with StepOnePlus Real-time PCR system 2-hour SYBR-Green standard thermocycling profile. For all DNA-isolation qPCR experiments, 25uL reactions of 2.5ng DNA, 12.5μL SYBR-GREEN, 2.5 μL 10uM forward primer, 2.5 μL 10uM reverse

primer, and completed to 25μL with nuclease-free water. qPCR experiments were performed in triplicate reactions to ensure consistent pipetting. Results are analyzed by delta-delta-cT. Control for DNA-isolation experiments was GAPDH. For determination of mtDNA content, ratio of GAPDH-MTCO1/ND6 delta-delta cT was performed, with COXIV being used as a secondary control. Primer sequences are as follows: GAPDH forward 5'-CCAGGTGGTCTCCTCTGACTTC-3', GAPDH reverse 5'-GTGGTCGTTGAGGGCAATC-3'; MTCO1 forward 5'-CCCACCGGCGTCAAAGTAT-3', MTCO1 reverse 5'-TGCAGCAGATCATTTCATATTGC-3'; ND6 forward 5'-CCCCCATGCCTCAGGAT-3', ND6 reverse 5'-GGAATGATGGTTGTCTTTGGATATACT-3'; COX IV forward 5'-GGGCGGTGCCATGTTCT-3', COX IV reverse 5'-CATAGTGCTTCTGCCACATGA-3'.

2.5 *In vitro* Autophagy Related 7 (ATG7) CRISPR Cas9 Cleavage Assay

Multiple kits were utilized in this CRISPR Cas9 cleavage assay (GeneArt Precision gRNA Synthesis Kit – Invitrogen A29377) and Guide-it singe-guide ribonucleic acid (sgRNA) Screening Kit (Takarabio Clontech). First, ATG7 as a target gene was selected and guide ribonucleic acid (gRNA) candidates were obtained through the gRNA design tool at www.thermofisher.com/crisprdesign. Five candidate gRNAs were used with respective gRNA synthesis and genomic cleavage detection primers ordered by standard de-salt (Integrated DNA Technologies). gRNA synthesis target oligonucleotide is required for *in vitro* synthesis of gRNA; genomic cleavage primers are primers that flank the target sequence of the gRNA by 200-400 nucleotides 5' and 3'.

gRNA is synthesized and purified as per GeneART Precision gRNA Synthesis Kit protocol. After gRNA synthesis, DNA from EPC2-hTERT was isolated, and PCR amplified (see Quantitative Polymerase Chain Reaction) with the respective genomic cleavage detection primers. Next, following Guide-it sgRNA Screening Kit, gRNA was hybridized with Recombinant Cas9, then added to solution, and incubated with the amplified genomic targets in concentrations listed in the protocol. Cleavage efficiency of genomic PCR products was then calculated by gel electrophoresis on a 1% agarose gel. ATG7 genomic cleavage primers forward 5'-TAAATGGGGGAAAAGAGCTG-3', ATG7 genomic cleavage primer reverse 5'-CCACAAGTGGTCTTTAAGTTCCC-3', Target sequence 5'-TTATACAGTGTTCCAATAGC-3'.

2.6 Plasmid Cloning and Transduction of TLCV2

Plasmid TLCV2 was obtained from the Addgene repository in a Stbl3-E-coli bacterial stab. TLCV2 is a doxycycline inducible Cas9-2A-eGFP-Puro lentiviral plasmid. ATG7 gRNA target sequence 5'-TTATACAGTGTTCCAATAGC-3' was selected to be cloned into the TLCV2 backbone. TLCV2 was plated on LB agar plates with 100mg/mL ampicillin and grown overnight at 37°C. Single colonies were then stabbed by pipette tip and placed into 500mL LB Broth flasks containing 100mg/mL ampicillin. Plasmid DNA was isolated and purified by GeneJET Plasmid Maxiprep Kit (Thermofisher Catalog # K0491) as per protocol. Concentration of resulting plasmid was quantified by nanodrop. Cloning of gRNA into backbone required a set of single stranded oligonucleotides both ordered by standard desalt (Integrated DNA Technologies). The oligonucleotide that contains the target sequence must have a 5'-CACCG; ATG7-Oligo 1: 5'-

CACCGTTATACAGTGTTCCAATAGC-3’ as one oligonucleotide. The second oligonucleotide requires a 5’AAAC; then the reverse complement of the target sequence; followed by a 3’C; ATG7 oligo 2: 5’-**AAAC**GCTATTGGAACACTGTATAA**C**-3’. The TLCV2 plasmid is then digested and dephosphorylated in a reaction containing: 1 μg TLCV2, 3 μL FastDigest Esp3I, 3 μL FastAP, 10X FastDigest Buffer, and 0.6 μL 100 mM DTT. After adding the plasmid and reagents to a reaction tube, it is digested in a thermocycler for 30 minutes at 37°C. After digestion, the reaction is directly purified by the QIAquick Gel Extraction Kit (Qiagen Catalog #28104) and DNA concentration quantified by nanodrop. Lentivirus was generated with lentiviral packaging plasmids and transduced into HEK293T cells. HEK293T cells were then treated for 3 days with 1 μg/mL doxycycline. Plasmid expression was confirmed by green fluorescent protein (GFP) expression.

2.7 Organoid Formation and Treatment Assays

Seven-week old C57BL/6 mice were euthanized in a CO_2 chamber. They were then cut longitudinally to open the thoracic cavity. Forceps were used to remove adjacent organs such as the liver, heart, and lungs, exposing the stomach. The esophagus is first located at top of the stomach where it is carefully separated from connecting tissues. The esophagus was cut with sterile scissors and was washed in sterile PBS. After washing with PBS, the esophagus was opened longitudinally, exposing the inner epithelium. Esophageal epithelium was then placed into a 1.7 mL Eppendorf with a 1:5 solution of Dispase:PBS and incubated in a 37°C shaker for 10 minutes. Afterwards, the esophagus was transferred to a 1.7 mL Eppendorf containing 0.25% trypsin in EDTA and incubated

at 37°C with at 800 RPM for 10 minutes. The esophagus was then placed in a fresh 1.7 mL Eppendorf tube containing 0.25% trypsin in EDTA and incubated at 37°C with at 800 RPM for 10 minutes. After incubation in trypsin, the cells were filtered through a 70 μM cell strainer into a 50 mL conical tube containing 3 mL STI to inhibit trypsin activity. The remaining tissue on the surface of the cell strainer was forced through with the plunger of a 1 ml syringe. Cells were then centrifuged for 1000 rpm for 5 minutes and resuspended in 100 μL KFSM. 1000 primary mouse esophageal keratinocytes were then mixed in 50 μL matrigel per well and pipetted into the center of a 24 well plate, forming a bubble of matrigel with embedded keratinocytes. Cells were then bathed with 500 μL of organoid growth medium containing 47mL DMEM/F12, Glutamax, HEPES, Penicillin/Streptomycin and supplemented with 500 μL N2, 1mL B27, 100 uL N-Acetylcyestine 0.5M, 70 μL human epidermal growth factor, and conditioned medium containing Noggin and R-Spondin. In addition, 50 μL of Y27632 was added. Cytokines (IL-13, IL-1β, IL-4, IL-5, TNFα) were then added at a concentration of 10 ng/mL. Organoids were incubated at 37°C. Medium was changed every 2-3 days and after 7 days, organoids were counted by Lionheart LX Automated Microscope (Biotech). DNA was collected by first digesting the matrigel in a 1:5 solution of dispase:HBSS. Afterwards, DNA purification was performed according to the manufacturer's protocol for Qiagen Blood & Tissue Kit. Organoids that were embedded and sent for histopathology were first digested in a 1:5 solution of dispase:HBSS. They were then added to a 1.5 mL tube and spun at 3000 RPM for 2 minutes. Next, the pellet was washed with PBS and spun at 3000 RPM for 2 minutes. The pellet was re-suspended in 500 μL of 4% paraformaldehyde and fixed overnight at 4°C. The solution was then spun for 3000

RPM for 2 minutes and then washed with PBS. Afterwards, cell pellets containing organoids were embedded in a solution of 2% Bacto-Agar and 2.5% gelatin. The embedded organoids were left to solidify at 4°C for 30 minutes. They were then placed within histo-cassettes and stored in a 70% ethanol solution. Sectioning was performed at the Fox Chase Cancer Center Histopathology Core.

2.8 Murine Eosinophilic Esophagitis Experiments

A modified version of a MC903-OVA model of EoE was performed (Noti et al., 2013). 7-week old C57BL/6 mice (Jackson Laboratory) were separated into control and experimental groups. Both mice underwent epicutaneous scraping on their ear with subsequent exposure to 20 μL of 2 nmol MC903 (calcipotriol) dissolved in 100% ethanol to induce atopic dermatitis. The mouse in the experimental group was also exposed to 100 μg of ovalbumin dissolved in 10 μL of PBS on its ear after scraping. This was performed every day from days 1 to 12 for mouse sensitization. The mice then received no experimental manipulation for two days. Afterward, 15 g/L ovalbumin was dissolved into the drinking water of the experimental mouse for the remainder of the experiment. Additionally, a series of oral gavages with 50 mg ovalbumin dissolved in water were performed every two days over the course of fourteen days. Total experimental time course was 28 days. Mice were then euthanized as previously discussed in the organoid formation protocol. DNA and protein were harvested from esophagi for subsequent assays.

2.9 Microscopy for Mitochondrial Content

50,000 EPC2-hTERT cells were plated onto two wells of a 6-well plate. Cells were subsequently treated for 7 days with or without 10n g/mL IL-13. After 7 days, measurement of mean-fluorescent intensity was acquired by the Lion-Heart LX Automated Microscope. Medium was then removed and replaced with fresh KFSM containing a 1:2500 dilution of MitoTracker Deep Red (Thermofisher Catalog #M22426) and incubated for 30 minutes at 37°C. After 30 minutes of incubation, medium was replaced with fresh KFSM. Representative pictures were acquired through fluorescence microscopy to observe localization and morphological alterations.

2.10 SDS-PAGE and Western Blot

Cells collected from culture plates are first washed with PBS and then exposed to a solution of 1X lysis buffer (Cell Signaling Technologies), 1X protease inhibitor cocktail (Sigma). Cells are then scraped off the plate into solution and centrifuged at 14,000 RPM for 15 minutes. Supernatant is collected and protein quantified by the Qubit 4 (Invitrogen). 30 μg of protein samples were diluted in NuPAGE sample buffer, reducing agent and deionized water to a final volume of 50 μL. Samples were then heated to 95°C for a total of 5 minutes before being loaded onto an SDS-PAGE 4-12% Tris-Glycine gel (Novex) submerged in SDS running buffer (Novex). The gel was exposed to 100 volts for two hours for complete migrations. Gels were then transferred over to a PVDF membrane that has first been activated by soaking in methanol for 30 seconds. Protein transfer is performed at 4°C at a constant 100 volts. The membranes were then blocked in a 5%

milk solution (5 g non-fat dry milk in 100mL PBS-T: 0.05% of Tween-20 in PBS. The membranes were then cut and diluted in the appropriate concentrations of primary antibodies for respective experiments: MTCO1 (abcam ab14705) 1:1000, LC3B (Cell Signaling Technologies #2775) 1:3000, ATG7 (Cell Signaling Technologies #2631S) 1:1000, β-actin (Invitrogen #MA1-744) 1:10000. Membranes were then washed 3 times for 10 minutes each with PBT-T. Then the appropriate mouse or rabbit antibody is used at a concentration of 1:3000-10000 before being developed with ECL reagent kit and the iBright (Invitrogen). Densitometry was performed using iBright software.

2.11 Statistical Analysis

All statistical analysis was done using graph pad statistical software. Analysis of variance (ANOVA) was performed on all experiments with more that 2 groups. Unpaired t-test was performed for comparisons between two groups.

CHAPTER 3

RESULTS

3.1 Increased MTCO1 Expression in Active EoE Patients

Mitochondria and their constituent proteins have been shown to be important for many intracellular and extracellular processes. These processes can be highly variable between tissue types and have diverse functionality based on microenvironmental cues (Clay Montier et al., 2009). As previously discussed, eosinophils and mast cells are common cells observed in EoE that may contribute to extruded mtDNA as part of the immune response. It has recently been shown that mtDNA can be released by lung epithelial cells in exosomes to contribute to an inflammatory response (Szczesny et al., 2018). To determine how EoE microenvironment influences mitochondrial content in esophageal epithelium, we performed immunohistochemistry for MTCO1, a subunit of complex IV of the electron transport chain, in formalin-fixed paraffin-embedded esophageal biopsies. After examining 7 non-EoE, 29 active EoE, and 26 inactive EoE patient samples we found a significant increase in MTCO1 expression ($p<0.05$) in patients with active EoE as compared to non-EoE patients (Figure 5). Although there was a downward trend in MTCO1 staining when comparing active and inactive EoE, this change was not statistically significant. These results are consistent with our hypothesis that esophageal epithelial cells in EoE may have increased mitochondrial content.

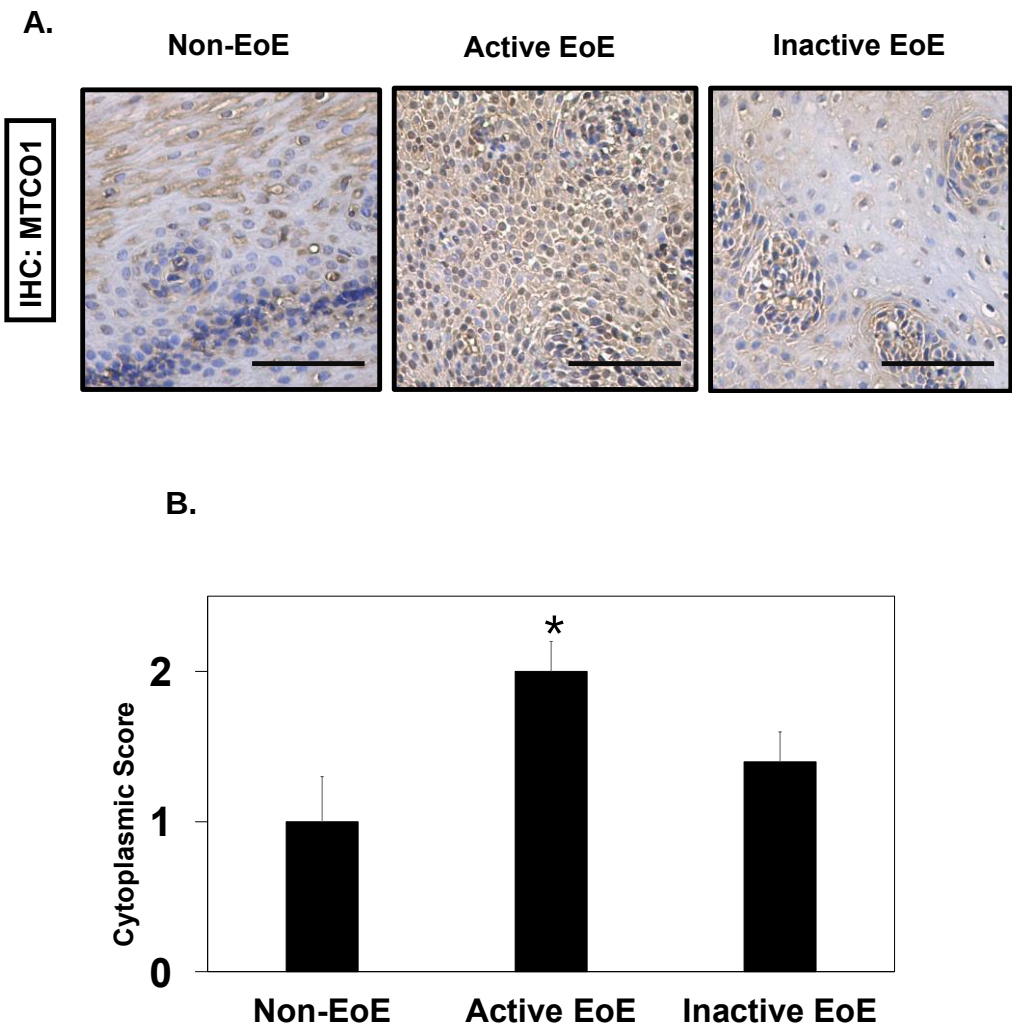

Figure 5: Increased MTCO1 Expression in Adult EoE Patients.

Immunohistochemistry for the mitochondrial encoded gene Cytochrome C oxidase subunit 1 (MTCO1), a subunit of complex IV of the electron transport chain, was performed in esophageal biopsies from patients with normal pathology (n=7), active EoE (n = 29), or inactive EoE (n = 26) (**A**) Representative histology of MTCO1 staining. Scale bars 100 μm. (**B**) Cytoplasmic staining in active EoE patients significantly greater than Non-EoE control ($p<0.05$; One way ANOVA). Non-EoE and inactive EoE did not

have a statistically significant difference when compared to each other ($p<0.05$). MTCO1 level in inactive EoE is trending towards the level seen in Non-EoE patients ($p=0.11$)

3.2 Increased Mitochondrial Levels in EoE Mouse Model

After observing an increase in histological staining for MTCO1 in human patients, we sought to further understand how EoE inflammation effects mitochondrial biology through use of a mouse model. Mouse systems that can be used to understand EoE pathophysiology have been generated and previously discussed in the literature (Jiang et al., 2015; Masterson et al., 2014; Noti et al., 2013). We adapted a version of the MC903/OVA protocol as described above to generate a more robust EoE phenotype. To determine whether there was an increase in mitochondrial content in the esophageal epithelium in the context of EoE inflammation, we performed western blot to examine MTCO1 expression in peeled esophageal epithelium (**Figure 6A)**. Indeed, there was a 2.3-fold increase in MTCO1 protein expression in the mouse with EoE inflammation as compared to control. Next, we further confirmed increased mitochondrial content by evaluating mtDNA level in the esophageal epithelium of the mouse with EoE inflammation. In order to estimate density - or number of mitochondria per cell - a ratio of nuclear DNA to mtDNA is determined. This is based on the concept that nuclear encoded genes have two copies per cell, whereas mtDNA copy number can be variable, depending upon mitochondrial replication and other dynamic processes (Jornayvaz et al., 2010). Using this methodology, we found that the esophageal epithelium of a mouse with EoE inflammation displayed increased mtDNA level as compared to a control animal (**Figure 6B**). Taken together, the increased mitochondrial content in the EoE mouse

suggests that this model can aid further investigation into mitochondrial contribution to EoE pathophysiology.

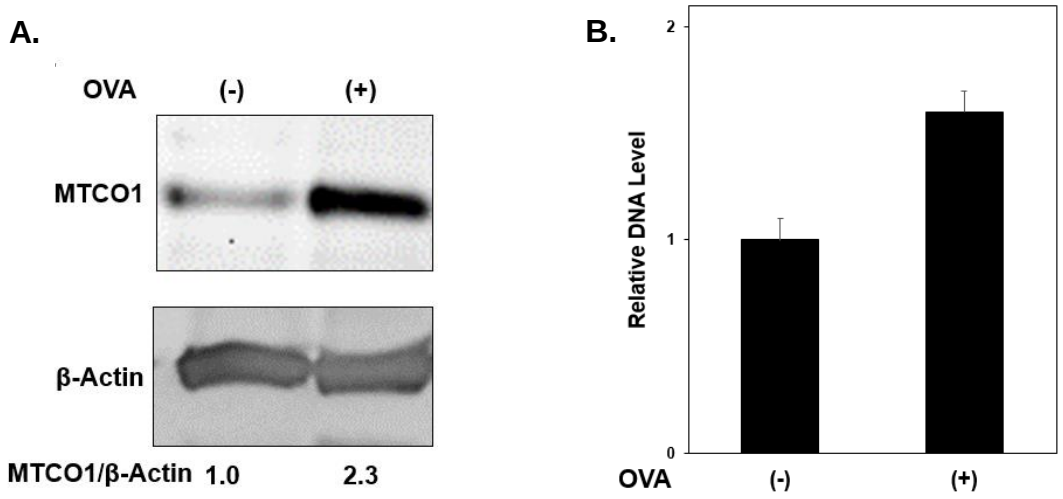

Figure 6: Evidence of Increased Mitochondrial Content in EoE Mouse Model. Mice (C57/B6) were treated with MC903 alone (OVA-) or MC903/OVA (OVA+) to induce eosinophilic esophagitis (EoE)-like inflammation over a 28-day time course. Protein and DNA were isolated from esophageal epithelium. (**A**) Immunoblotting to assess protein expression of MTCO1 adjusted to β-actin. Densitometry performed with iBright software; (n=1/group). (**B**) qPCR analysis to examine mtDNA levels in mouse with EoE-inflammation as compared to control (n=1).

3.3 EoE-relevant Cytokines Increases Murine Esophageal Organoid Formation

After increased MTCO1 expression was identified in esophageal epithelium of mice with EoE-like inflammation, an *ex vivo* three-dimensional (3D) organoid system was utilized to study the effects of the EoE inflammatory milieu upon mitochondria. The 3D organoid systems for esophageal keratinocytes has been well-established (Kasagi et al., 2018; Whelan et al., 2018). In this system, single cells isolated from mouse esophageal epithelium are grown in a growth-factor rich medium that facilitates cell proliferation and differentiation. The resulting structures display tissue architecture comparative to that seen in esophageal epithelium *in vivo*. Esophageal organoids are spherical and contain a single outer layer of basal-like keratinocytes surrounding multiple layers of keratinocytes with flattened morphology and a keratin core (Whelan et al., 2017). This experimental platform will help provide information into how the EoE inflammatory milieu effects mitochondria in an intact epithelial structure in terms of cell survival, proliferation, cellular interactions, differentiation, and gene expression. Therefore, we added cytokines involved in EoE processes, IL-13, IL-1β, IL-4, IL-5, TNFα, to the culture medium at the time of cell seeding. After 7 days of culture in the presence of EoE-relevant cytokines, absolute number of organoids formed were counted (**Figure 7A**). We found that IL-13 treatment promoted the greatest increased number of organoids formed as compared to control (49 vs. 90). IL-4 treatment induced the second greatest increased number of organoids formed, followed by IL-1β and IL-5, both of which promoted modest increases when compared to control. Fewer organoids formed upon treatment with TNFα as compared to control. Taken together, increased organoid formation appears to occur in response to IL-13, IL-1β, IL-4, IL-5 treatment, but not

specific to a particular cytokine. To understand whether mitochondria level changes may be affected by the EoE-inflammatory milieu, we examined mtDNA levels in the fully formed organoids (**Figure 7B)**. mtDNA level of an organoid will represent the heterogeneous population of cells contained. We found that IL-13, and IL-4 treated organoids had similar 1.25-fold decreases in mtDNA level when compared to control. IL-1β and TNFα treated organoids showed 1.7-fold and 2-fold respective decrease in mtDNA levels when compared to control. IL-5 treated organoids had increased mtDNA level of 1.2-fold. Taken together, IL-13, IL-1β, IL-4, and TNFα treatments had decreased mtDNA level in fully formed organoids, suggesting that no specific cytokine is inducing this phenotype. This result is in contrast to our *in vivo* observations that indicate increased mitochondria in esophageal epithelium in the context of EoE-like inflammation. It is possible that mtDNA level is negatively affected after the complete formation of organoids, but it is unclear if this is due to the time point of observation. This suggests that the current experimental model may require further optimization to facilitate our studies into cytokine-mediated increases in mtDNA levels in the context of EoE.

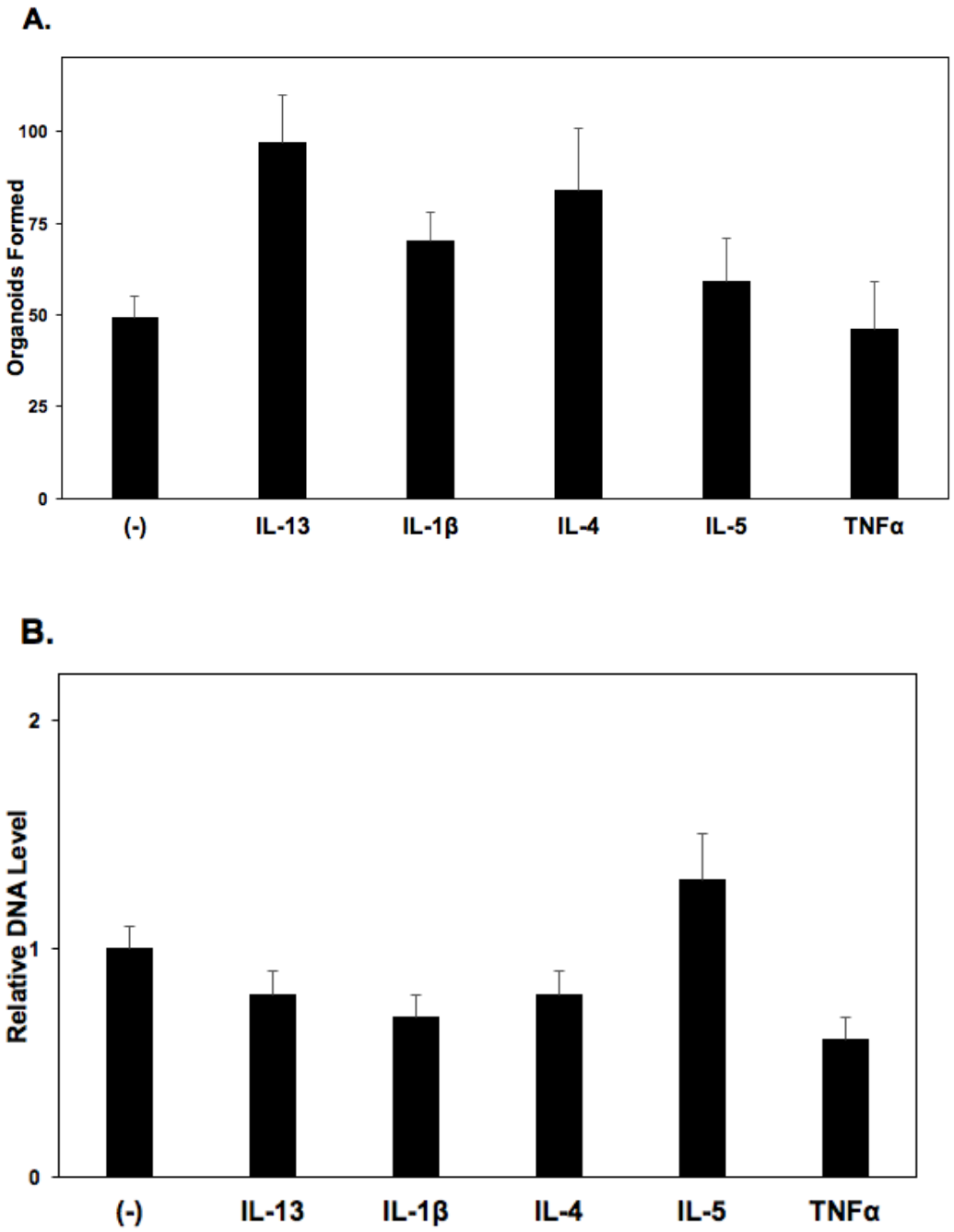

Figure 7: Organoid Growth and mtDNA Levels.

Organoids were established from C57/B6 wildtype primary mouse esophageal keratinocytes. Primary mouse cells were plated with IL-13 (10 ng/mL), IL-1β (10 ng/mL), IL-4 (10 ng/mL), IL-5 (10 ng/mL), TNFα (40 ng/mL). After 7 days of treatment,

organoid number was determined by Lionheart LX Microscope followed by DNA isolation. (**A**) Total organoids formed after 7 days of cytokine treatments (n=1) (**B**) PCR data indicating mtDNA levels in organoids after 7 days of cytokine treatment (n=1).

3.4 IL-13 Increases mtDNA Levels in Esophageal Keratinocytes *in vitro*

We then examined the effect of EoE-relevant cytokines on mtDNA level using an *in vitro* system. This will elucidate how cytokines effect mitochondrial level in human immortalized esophageal keratinocytes (EPC2-hTERT) with a basal cell-like phenotype. Under basal conditions, these cells do not undergo differentiation, provides information on mitochondrial changes in the basal-like population. We treated cells for 7 days with the described panel of EoE-relevant cytokines (**Figure 8A**). Treatment with IL-13 resulted in a 1.8-fold increase in mtDNA level when compared to control. Although IL-13 and IL-4 share the signaling intermediate STAT6, IL-4 treatment increased mtDNA level by only 1.3-fold when compared to control. Treatment with IL-1β, IL-5, and TNFα all failed to impact mtDNA level as compared to control.

After observing that IL-13 treatment induced the most robust change in mtDNA level, we focused on replicating the effects of IL-13 and found that IL-13 significantly increased mtDNA level after 9 independent experiments ($p<0.01$) (**Figure 8B**). To further examine mitochondrial content and function, we performed a 7 day IL-13 treatment of EPC2-hTERT and stained mitochondria with MitoTracker Deep Red, a dye that stains mitochondria with intact membrane potential. We found an increase in active mitochondria in IL-13 treated cells (**Figure 9A**) and preliminary evidence that mitochondrial localization is altered as compared to control cells (**Figure 9B**). Taken

together, these data suggest that IL-13 increases mtDNA levels and mitochondrial activity in esophageal keratinocytes *in vitro*.

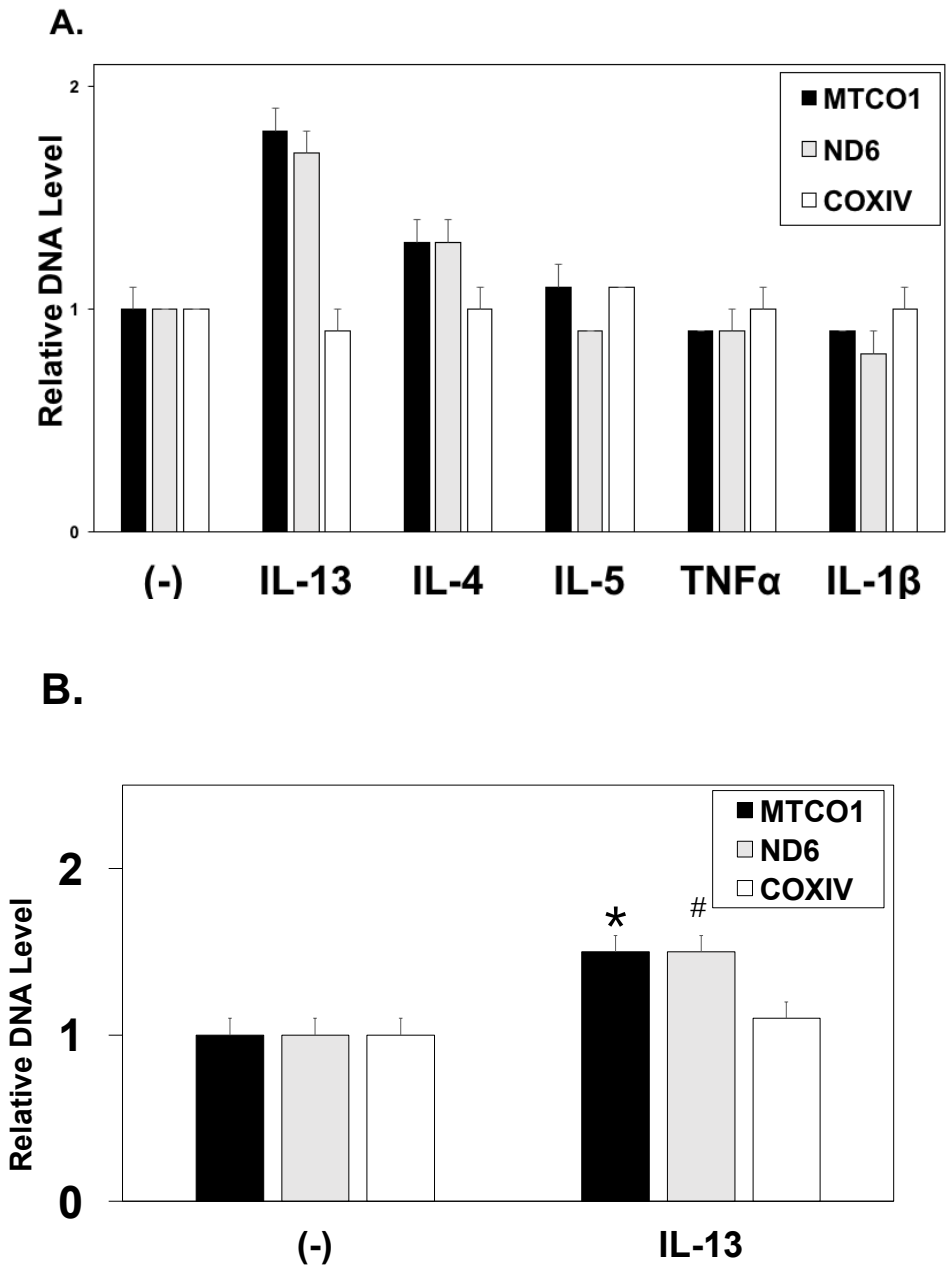

Figure 8: IL-13 Stimulates mtDNA Level Increase in EPC2-hTERT cells.

(A) qPCR measuring mtDNA levels in EPC2-hTERT cells treated with various cytokines for 7 days (n=1). IL-13 (10 ng/mL), IL-1β (10 ng/mL), IL-4 (10 ng/mL), IL-5

(10 ng/mL) or TNFα (40 ng/mL) for 7 days. N=1 (B) qPCR data measuring mtDNA levels in independent experiments trials of EPC2-hTERT cells treated with IL-13 (10 ng/mL) for 7 days. (n=9, $p<0.01$; Two-tailed t-test).

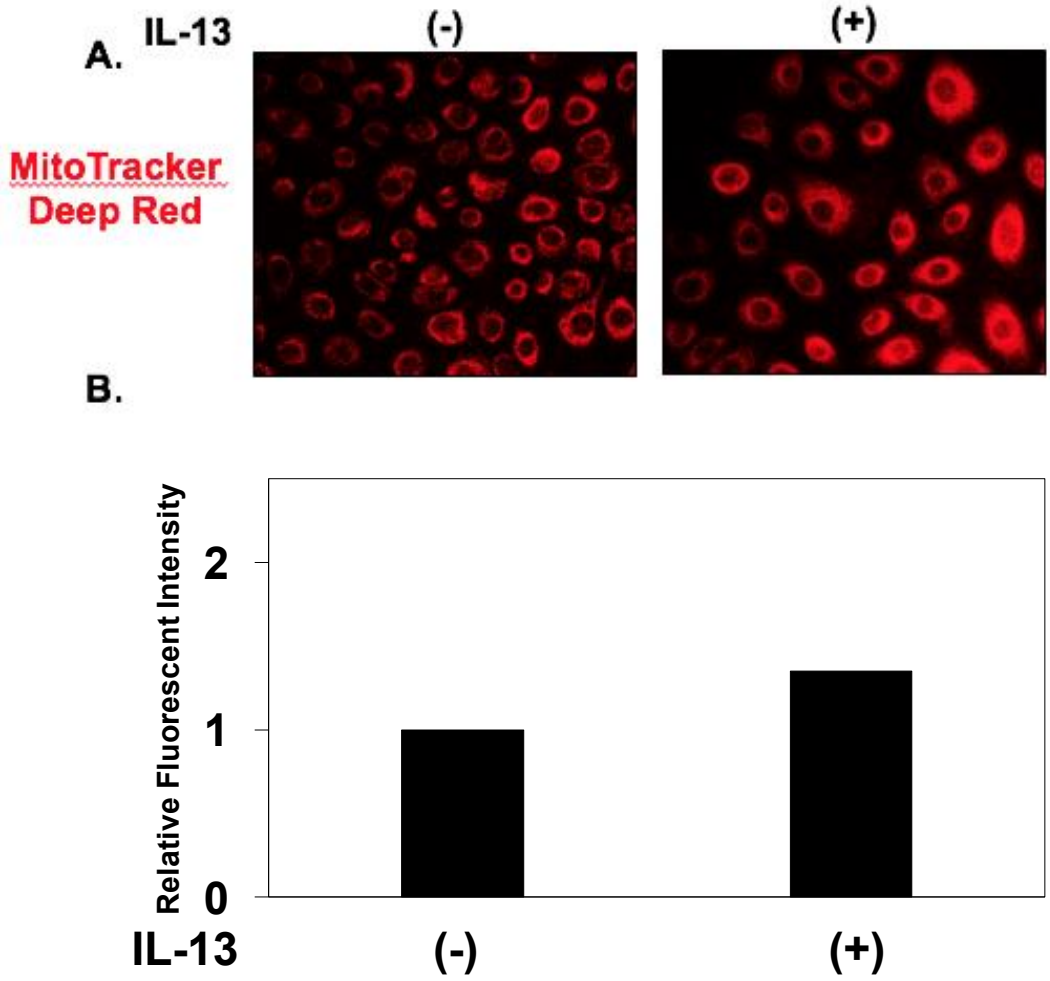

Figure 9: IL-13 Stimulates Mitochondrial Content and Function Increase.

Following 7 days of treatment with IL-13 (10 ng/mL), EPC2-hTERT cells were stained with MitoTracker Deep Red. (A) Representative staining of MitoTracker Deep Red in IL-

13 treated EPC2-hTERT cells. Add in magnification used. (B) Relative fluorescent intensity was determined in IL-13 treated cells and controls (n=1).

3.5 Kinetics of IL-13 treatment

After observing that 7-day treatment with IL-13 promotes an increase in mtDNA level *in vitro*, we examined how IL-13 affects mtDNA levels over time. This will elucidate critical points at which IL-13 is exerting maximum effects on mtDNA level and will aid further investigation into the mechanism by which this is occurring. We examined changes in mtDNA levels in EPC2-hTERT cells upon stimulation with IL-13 for 2, 3, 7, and 10 days **(Figure 10**). We found there to be no changes in mtDNA level after 2 days of IL-13 treatment when compared to day 0. At day 3, there was a 2.2-fold increase in mtDNA level when compared to day 0. This increase in mtDNA level is maintained at day 7 then declines at day 10 when mtDNA level is comparable to that observed at day 0. This data suggests that IL-13 promotes a maximal increase in mtDNA level between 3 and 7 days of IL-13 treatment. 10 days after plating is the point at which cells became fully confluent. As confluence induces differentiation of EPC2-hTERT cells (cite Hiro's papers), we hypothesized that confluence may influence IL-13 mediated mtDNA level increase. To evaluate this possibility, we treated EPC2-hTERT cells at 100% confluence with IL-13 for 7 days. We found that IL-13 did not affect mtDNA level after 7 days in cells undergoing contact-mediated differentiation (**Figure 11**). This suggests that differentiation or cellular contact may inhibit IL-13 mediated mtDNA level changes.

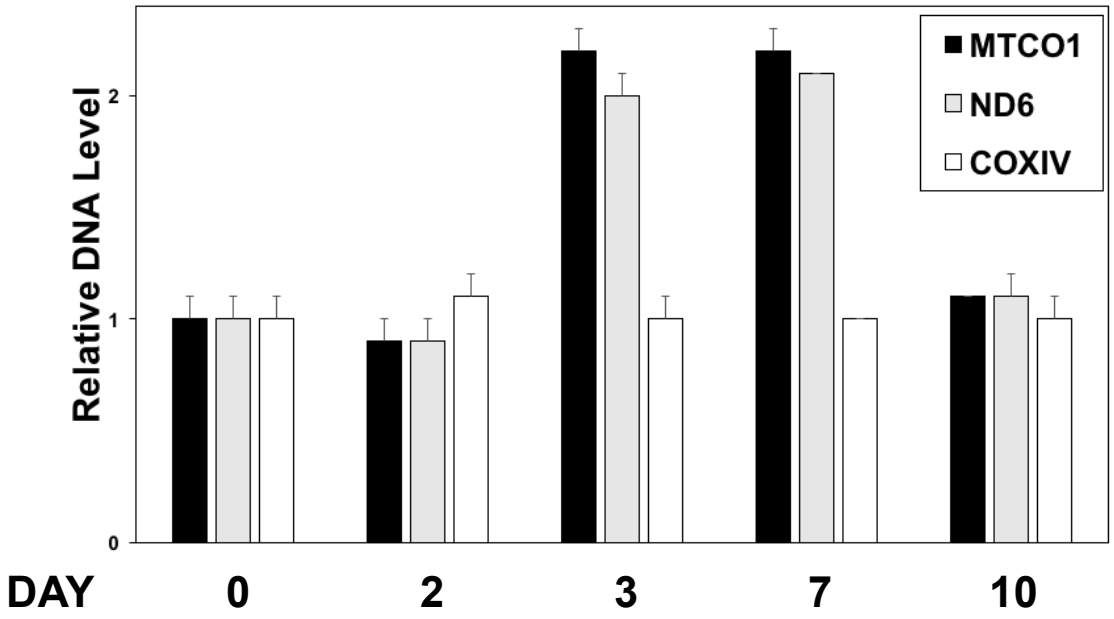

Figure 10: Kinetics of IL-13 Stimulated Mitochondrial Content Increase.

EPC2-hTERT cells were treated for 0, 2, 3, 7, 10 days with IL-13 (10ng/mL) then assessed for level of mitochondrial DNA by qPCR (n=1).

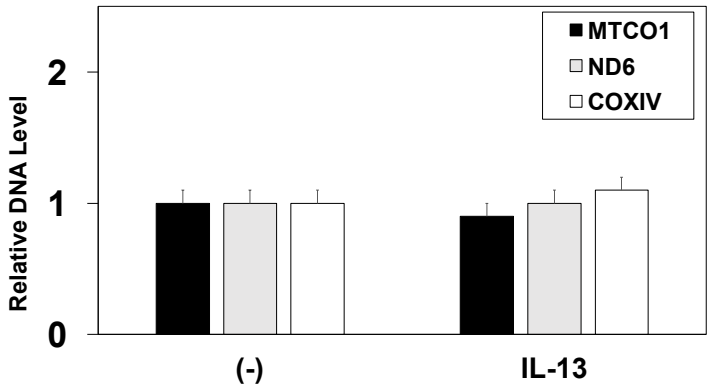

Figure 11: Confluence May inhibit IL-13 Induced mtDNA Level Increase.

EPC2-hTERT cells were grown to confluence then treated with IL-13 for 7 days. qPCR was performed to examine mitochondrial mtDNA level (n=1).

3.6 Co-treatment of IL-13 with EoE-relevant Cytokines

We next examined whether co-treatment of IL-13 with EoE-relevant cytokines, IL-4, IL-1β, or TNFα could alter the effects of IL-13 on mtDNA level. Previous studies have shown that TNFα enhances the effects of IL-13 on eosinophil activation (Luttmann et al., 1999). Within the EoE inflammatory microenvironment *in vivo,* multiple cytokines will simultaneously affect individual cells. Thus, it is important to address the effects of multiple cytokines on mtDNA level in esophageal keratinocytes. We treated EPC2-hTERT cells for 7 days with IL-13 along with IL-4, IL-1β, or TNFα (**Figure 12**). We predicted that IL-13 and IL-4 would synergize their effects on mtDNA level because IL-13 and IL-4 signal through similar receptors and transcription factors. In addition, IL-4 treatment alone showed modest increase in mtDNA in the previous experiment. However, we found that co-treatment of IL-13 and IL-4 did not influence mtDNA levels when compared to control. This suggests that a compensatory mechanism, or desensitization may be occurring to prevent IL-13 mediated mtDNA level increase. Co-treatment of IL-13 and IL-1β showed an increase in mtDNA level when compared to control. The increase of mtDNA level in cells co-treated with IL-13 and IL-1β is similar to the mtDNA level of IL-13 only in previous experiments. Thus, IL-1β may not affect IL-13-mediated mtDNA level increase. Co-treatment of IL-13 and TNFα showed a nearly 2-fold decrease in mtDNA levels when compared to control. These data raise the possibility that co-treatment of IL-13 and TNFα may promote degradation of mitochondria, but it is unclear how this may occur.

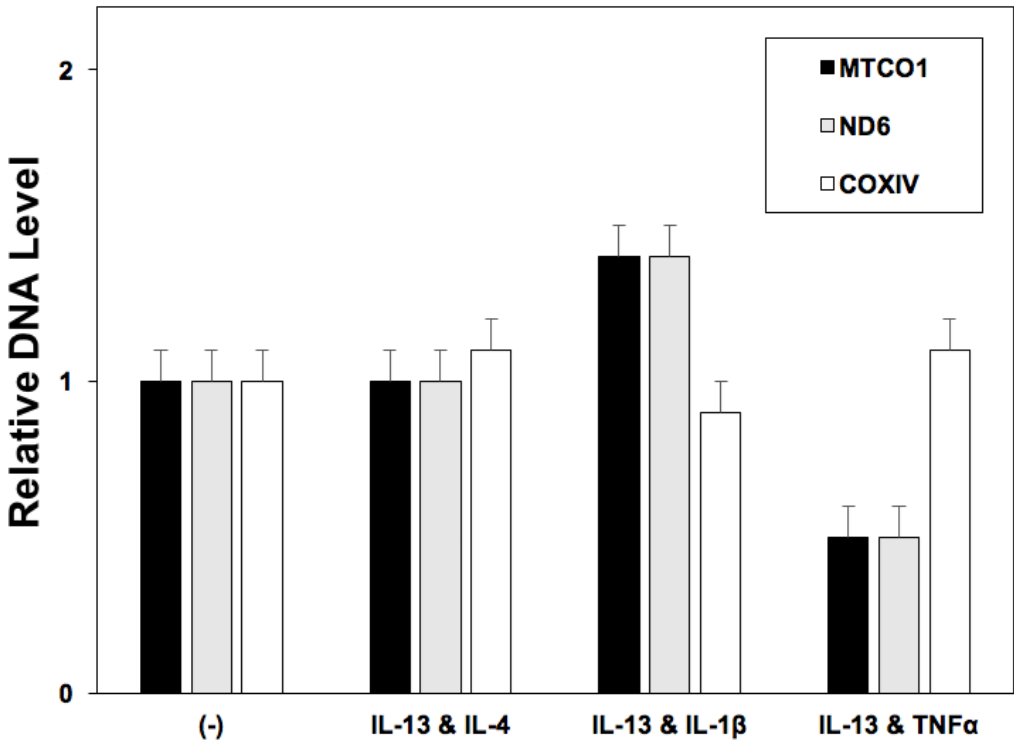

Figure 12: Simultaneous Cytokine Treatments.

EPC2-hTERT cells were stimulated for 7 days with IL-13 & IL-4 (10 ng/mL each), IL-13 & IL-1β (10 ng/mL each), IL-13 & TNFα (10 ng/mL & 40 ng/mL, respectively) then subjected to qPCR to determine mitochondrial DNA level (n=1)

3.7 Primary Esophageal Keratinocytes from EoE Patients Display Increased mtDNA levels

We then sought to determine if primary human esophageal keratinocytes isolated from patients with EoE had increased mtDNA level relative to non-EoE in two-dimensional culture. We procured 4 primary esophageal keratinocyte cell lines, 2 EoE patients and 2 non-EoE patients, and determined mtDNA levels. mtDNA levels were standardized to mtDNA level in EPC2-hTERT cells. We found that average mtDNA

levels of the EoE patient cell lines were greater than non-EoE patients (9.4 vs. 5.2-fold change) with this limited sample size (**Figure 13A**). We also found that the mtDNA level in all of the primary cell lines was greater than the immortalized cell line (EPC2-hTERT). This suggests that primary esophageal keratinocytes from EoE patients may maintain an increase in mtDNA levels *in vitro*.

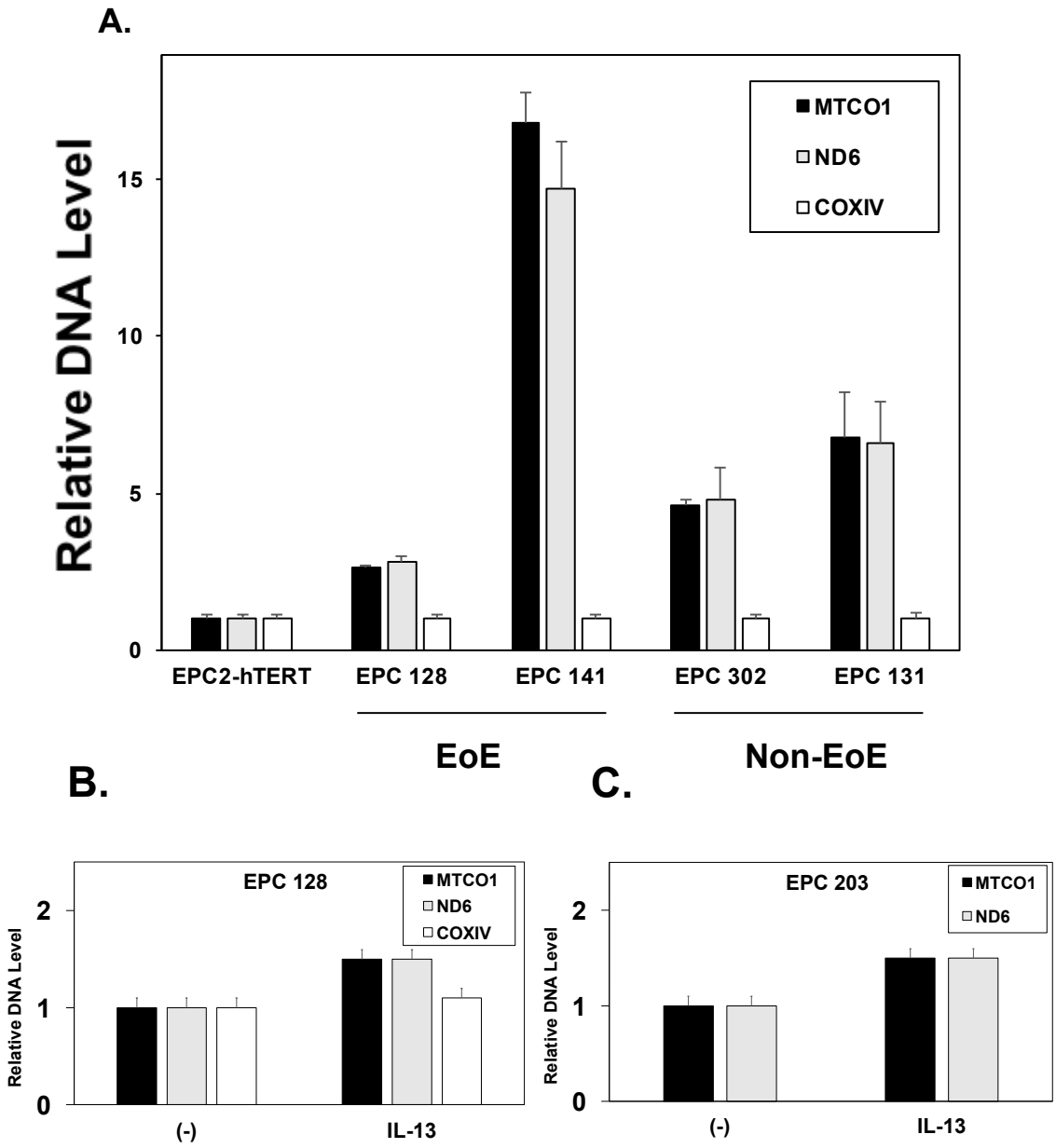

Figure 13: Primary Esophageal Keratinocytes of EoE patients and Non-EoE Controls

DNA was isolated from EPC2-hTERT, and primary human cell lines EPC 128 (passage 10), EPC 131 (passage 7), EPC 141 (passage 7) and EPC 203 (passage 8) cultured in the presence or absence of IL-13. qPCR was performed to determine relative mitochondrial

DNA level. EPC 128 and EPC 141 are cell lines derived from EoE patients. EPC 131 and EPC 203 are cell lines derived from Non-EoE patients. (**A**) mtDNA levels in listed cells at baseline. Both EoE (**B**) and Non EoE (**C**) cell lines showed increased mtDNA level upon stimulation with IL-13 (10 ng/mL) for 7 days.

3.8 IL-13 Increases mtDNA Levels in Primary Esophageal Keratinocytes

Next, we examined whether IL-13 affects mtDNA levels in primary cells from EoE and non-EoE patients. It is important to address whether the IL-13 mediated mtDNA level changes are either an artifact of immortalization or a finding specific to the EPC2-hTERT cell line. We treated 1 EoE derived esophageal keratinocyte cell line and 1 non-EoE derived esophageal keratinocyte cell line with IL-13 for 7 days. We found that both EoE and non-EoE primary cell lines displayed increased mtDNA level after treatment with IL-13 when compared to untreated controls (**Figure 13B, C**). This indicates that IL-13 mediated mtDNA level increase is not dependent on clinical diagnosis of EoE. Taken together, these results show that the effects of IL-13 on mtDNA of esophageal keratinocytes is neither an artifact of immortalization nor is unique to EPC2-hTERT cells.

3.9 Generation of an Autophagy-deficient Cell Line

Our results have shown that mitochondrial content and mtDNA level are upregulated *in vivo,* and our *in vitro* studies suggest that changes in mitochondria may be due to IL-13. However, the mechanisms responsible for these observations remain unclear. Previous studies have shown that IL-13 can induce the generation of ROS in

EPC2-hTERT cells and trigger autophagic flux (Whelan et al., 2017). In addition, a subtype of autophagy, mitophagy, is a quality control mechanism regulating mitochondria through their selective degradation. Therefore, we decided to investigate the functional role of autophagy as it pertains to IL-13-induced mtDNA increase. This can be accomplished through CRISPR Cas9 gene editing targeting a critical regulator of autophagy, ATG7. Autophagy is an evolutionarily conserved mechanism of intracellular degradation of proteins and other constituents. In the process, intracellular lipidated membranes, termed autophagosomes, form and encompass cellular cargo to be degraded (Glick et al., 2010)). ATG7, an E1-like ubiquitin ligase, conjugates cytosolic microtubule-associated protein 1A/1B-light chain 3 (LC3-I) to phosphatidylethanolamine forming LC3-II (Tanida et al., 2008)). This lipid conjugation is necessary for LC3 to be incorporated into autophagosomes. Loss of ATG7 will result in decreased LC-II levels and autophagosome formation. To test the functional role of autophagy, we sought to generate a cell line deficient in autophagy. We began by performing a CRISPR Cas9 *in vitro* cleavage assay to screen the cleavage efficiency of a gRNA specific to ATG7. In this assay, the gRNA is synthesized and hybridized to the Cas9 protein, then added to a solution containing donor DNA from EPC2-hTERT. Cleavage efficiency *in vitro* can predict whether the gRNA will efficiently cleave *in vivo* (Anders et al., 2014). We found that a gRNA targeting exon 3 of ATG7 was effective at cleaving EPC2-hTERT donor DNA (**Figure 14A).** To generate a cell line deficient in ATG7, we selected a doxycycline-inducible CRISPR Cas9 plasmid (TLCV2). The gRNA from the cleavage assay was cloned into the plasmid and transfected into HEK293T cells to detect if it was effective at editing ATG7. Protein expression of ATG7 in doxycycline-treated cells was

unchanged compared to untreated control (**Figure 14B**). However, expression of LC3-I was increased in doxycycline-treated cells compared to untreated. This data suggests that autophagy may be moderately limited in these cells.

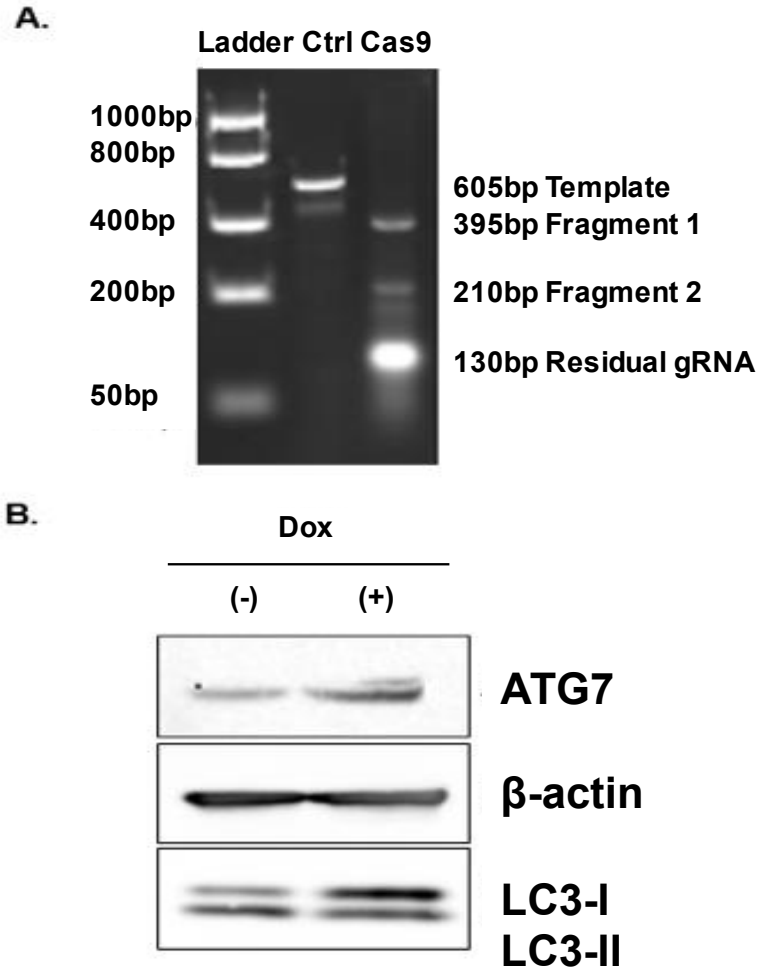

Figure 14: ATG7 CRISPR Cas9 gRNA efficiency

The plasmid selected for CRISPR Cas9 knockout is a Tet-inducible Lenticrisprv2 (TLCV2). This plasmid is a doxycycline inducible Cas9 with GFP expression to aid cell sorting. (**A**) The target sequence is to exon 3 of *ATG7* and is 5'-TTATACAGTGTTCCAATAGC-3'. To ensure efficient cleavage of the target an *in vitro*

cleavage assay was performed. This revealed the expected cleavage fragments at the target locus. (**B**) HEK293T cells were transduced with lentivirus to examine whether gene knockout can occur in a heterogeneous population. No ATG7 knockdown was observed; however, the LC3-II band (representing the cleaved and lipidated form of LC3 that is incorporated into autophagic vesicles) is less intense suggesting that ATG7-mediated lipidation of LC3 is limited.

3.10 Summary

This thesis provides preliminary data that mitochondrial level may be increased in the esophageal epithelium of active human EoE patients. In addition, primary esophageal keratinocytes from patients with EoE display increased mtDNA level in culture relative to non-EoE. It is also evident that mtDNA and MTCO1 protein expression is increased in the esophageal epithelium of mice with EoE-like inflammation. We propose that this increase in mtDNA level may be due, in part, to IL-13. IL-13-mediated increase in mtDNA level was observed in both immortalized and primary esophageal keratinocytes. IL-13 treatment elevated mtDNA levels maximally after 3 to 7 days and resulted in cells with more active mitochondria. Surprisingly, we find that cytokine co-treatment of IL-13 and IL-4 did not affect mtDNA levels and IL-13 with TNFα negatively affected mtDNA levels. We also show that that confluence may limit IL-13 mediated mtDNA level changes. In our 3D experiments, we found that the EoE-relevant cytokines IL-13, IL-4, IL-5 and IL-1β increased the formation of organoids. However, mtDNA level was not increased in the cytokine-treated organoids. To better understand the mechanism by

which IL-13 mediates mtDNA we began generating a cell line that is deficient in autophagy through CRISPR Cas9 gene editing.

CHAPTER 4

DISCUSSION

We have begun to address our objectives by providing data which suggests that the EoE microenvironment can affect mitochondria levels *in vivo* and *in vitro*. This supports our initial hypothesis and is consistent with preliminary data showing increased circulating mtDNA and extracellular mitochondrial structures in esophageal epithelium of EoE patients. We further initiated investigation into potential mechanisms underlying EoE-mediated mitochondrial changes, and show that IL-13 may be influencing the observed effects. We plan to explore the role of autophagy as it relates to IL-13-mediated effects on mitochondria using an autophagy-deficient cell line.

These observations provoke further studies into the mechanisms and significance of mitochondrial function in EoE. Immunohistochemistry analysis is an effective tool for observing histological changes in the esophageal epithelium. This technique enabled us to demonstrate mitochondrial protein upregulation in human patients. In the future, we will evaluate mtDNA levels in patient biopsies as well as expression of genes involved in mitochondrial biogenesis such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α). However, evaluation of mitochondrial activity and mechanisms regulating changes in mitochondrial biology remain difficult in human tissue samples. To address this, we have complemented human biopsy studies with *in vitro* and *ex vivo* experimental platforms.

We were able to test the effects of the EoE inflammatory milieu on mitochondria in esophageal keratinocytes *in vitro* and found that IL-13 treatment produced greater

mitochondrial levels and activity. Previous studies have shown that IL-13 can induce the generation of ROS and activate autophagy as a cytoprotective mechanism in EoE (Whelan et al., 2017). This seems counterintuitive in the context of esophageal epithelium exposed to the EoE microenvironment as mitochondrial levels are increased and show enhanced activity, whereas ROS is generally associated with damaged mitochondria and decreased mitochondrial biogenesis. However, our findings are in line with recent studies showing that mitophagy can occur concurrent with increased mitochondrial number and activity (Ziegler et al., 2018). Thus, future studies will seek to understand which mitochondrial processes IL-13 is affecting such as mitochondrial biogenesis, mitophagy, mitofusion, and/or mitofission. We also plan to explore whether inhibition of autophagy effects mitochondrial levels and mtDNA. CRISPR Cas9 editing of ATG7 in EPC2-hTERT will prevent autophagic flux and be more specific than siRNA strategies. Finally, we would determine whether autophagy is limiting EoE-inflammation in a mitochondrial-dependent manor.

The main intracellular signaling intermediate of IL-13 is STAT6 (Malabarba et al., 1996) and we will explore the functional role of STAT6 in IL-13-mediated effects on mitochondria. IL-4 and IL-13 share the receptor subunit IL-4 Receptor alpha (IL-4Rα) and can signal through STAT6 (Bao et al., 2015). Our findings showed that co-treatment of IL-4 and IL-13 did not increase mtDNA levels. This may be due to a competitive receptor engagement, and that mtDNA level increase is specific to IL-13. Studies have shown that IL-13 activates STAT3 (Bhattacharjee et al., 2013) and STAT3 can regulate mitochondrial activity (Wegrzyn et al., 2009). STAT3 can translocate to the mitochondria after upstream cytokine stimulation to upregulate mitochondrial activity and biogenesis

(Wegrzyn et al., 2009). In addition, it is possible that ROS are inducing quality control mechanisms to increase mitochondrial turnover resulting in generation of nascent functional mitochondria.

It also appears that IL-13-treated cells are heterogeneous with regard to mitochondrial activity. This suggests that subpopulations of cells may result in the collective phenotype. This question can be addressed through multiple techniques. Single-cell PCR can be utilized to examine the impact of IL-13 upon mtDNA in individual cells. In addition, flow sorting will facilitate exploration of differential gene expression in esophageal keratinocytes with high versus low mtDNA levels following IL-13 stimulation. IL-13 has also been shown to cause epithelial barrier dysfunction in airway epithelium contributing to airway inflammation in asthma and EoE (Saatian et al., 2013) (Wu et al., 2018).

We can postulate that IL-13-mediated mitocondrial activity may be contributing to barrier defect in EoE**.** To test this, we can manipulate mitochondrial function in a physiological setting by utilizing genetically engineered mice such as Transcription Factor A, mitochondrial (Tfam) knockouts. In addition, esophageal organoids deficient in Tfam have been previously generated (Srinivasan et al., 2017). It is shown that that depletion of mtDNA through Tfam knockout results in decreased organoid number. Thus, we can utilize Tfam depletion to explore whether mitochondrial depletion effects IL-13-mediated organoid formation. Notably, established organoids treated with EoE-relevant cytokines did not reveal increases in mtDNA levels. This may be due to the time point in which mtDNA level was analyzed. It is possible that the EoE inflammatory milieu may be damaging mitochondria in established organoids, resulting in an observed negative

change in mtDNA. In addition, the growth factor-rich medium in which organoids are cultured may be influencing cellular metabolism and subsequently mitochondrial levels. Manipulation of mitochondria prior to establishment will reveal how mitochondria affect esophageal epithelial development. In the future, immune cells isolated from patient biopsies can be co-cultured with organoids to explore the effect of immune cells on mitochondria in the esophageal epithelium.

To provide more robust evaluation of mitochondria *in vivo* we have made use of the MC903/ovalbumin. This non-genetic EoE model utilizes ovalbumin to induce EoE in the background of MC903-mediated atopic dermatitis. The use of ovalbumin as an EoE-trigger has physiological relevance as this is a protein found abundantly in eggs which are a common food trigger in EoE patients. Additionally, the MC903/OVA model recapitulates the disease state of human EoE, showing a robust Th2 skewing that is TSLP-dependent and not IgE-mediated (Noti et al., 2013). Moreover, this remains the only available EoE model that mimics the clinical symptomatic manifestation of food impaction. It must be noted that a drawback of the MC903/OVA model is that eosinophils are largely found in the lamina propria while human EoE displays transmural eosinophilia. In addition to the MC903/OVA model, we may utilize additional mouse models of EoE, including the L2-IL5/Oxazolone (Masterson et al., 2014), and Krt5-rtTA tetO-IL13/Doxycycline (Jiang et al., 2015) models. In the L2-IL5/Oxazolone model, IL-5 is constitutively active in the esophageal epithelium, and sensitization to oxazolone results in a robust EoE-like phenotype. These mice have upregulation of IL-13 and diffuse eosinophilia of the esophagus. This model can aid investigation into the physiological relationship between IL-13 and mitochondria. To further examine the

effects of IL-13 on EoE, we can utilize the Krt5-rtTA tetO-IL13/Doxycycline model, in which mice have inducible expression of IL-13 in the basal cells of the esophagus, and display robust basal cell hyperplasia phenotype. These additional models will help to eliminate the possibility of artefactual findings in the MC903/ovalbumin model. Overall, these systems serve as effective preclinical models, each with an advantage to exploring the role of mitochondria.

It is important to address the fundamental question of whether mitochondrial levels are a consequence of the inflammatory milieu and cytoprotective, or it is perpetuating the disease. Our preliminary data indicated that mtDNA level is increased and may be specific to active EoE patients. It remains unclear why there is circulating serum mtDNA and the origin of this mtDNA. Our *in vitro* studies focus on mtDNA levels as a surrogate for mitochondrial levels, but specific levels and functions of mtDNA may be independent of mitochondrial activity. Previous studies have shown that newly synthesized mtDNA can result in dysregulation of the NLRP3 inflammasome (Zhong et al., 2018). Since we observe increased mtDNA levels in esophageal epithelium, we can postulate that mtDNA is being synthesized and potentially promoting this inflammatory microenvironment. It is therefore possible that mtDNA is being released by esophageal keratinocytes resulting in the elevation in the serum. This aligns with a recent study showing that mtDNA can act as a pro-inflammatory Damage Associated Molecular Pattern (DAMP) potentially released from colonic mucosa to perpetuate inflammatory bowel disease (Boyapati et al., 2018). In addition, there was increased serum mtDNA level of patients with active inflammatory bowel disease (Boyapati et al., 2018). However, in a separate study, it was found that mitochondrial dysfunction contributes to

the destruction of the intestinal epithelial barrier in inflammatory bowel disease (Novak et al., 2015). Thus, it is possible that mitochondrial activity may be increased as a compensatory mechanism attempting to maintain the esophageal epithelial barrier and limit disease. Regardless, it appears that mtDNA may be serum biomarker for active EoE if it can be associated with a specific function of mitochondria or mtDNA in the disease process. Ultimately, these studies will elucidate the function of mitochondria in EoE and understanding the mechanisms could lead to better treatment or monitoring of disease state.

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