ELAFIN IN INTESTINAL BARRIER FORTIFICATION: A POTENTIAL ADJUVANT THERAPY FOR GLUTEN INTOLERANCE

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TITLE: Elafin in Intestinal Barrier Fortification: A Potential Adjuvant Therapy for Gluten Intolerance

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ABSTRACT

Background: The intestinal barrier plays an important role in protecting the internal milieu from potentially harmful substances within the intestinal lumen. A breakdown in the protective capacity of this barrier has been implicated as a contributing factor in the development of gastrointestinal diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and celiac disease. Celiac disease and other, milder forms of gluten intolerance such as gluten sensitivity (GS), are triggered by the passage of gluten peptides across the intestinal barrier. Modulation of the barrier therefore represents an important therapeutic target for limiting uptake of gluten in celiac disease and GS. Elafin is a potent serine protease inhibitor that is abnormally expressed in patients with IBD. Administration of elafin in murine models of colitis has been found to reduce disease severity although the mechanism of this protective effect remains elusive. It is unknown if abnormal expression of elafin plays a role in the development of celiac disease or GS.

Study Objectives: Given the promising results of elafin therapy in murine models of IBD, I sought to investigate if elafin therapy could attenuate markers of gliadin sensitization in two gluten-sensitive mouse models: HCD4/DQ8 and NOD/DQ8 mice. I also sought to explore potential mechanisms through which elafin may enact its protective effect within the gut.

Methods: Male HCD4/DQ8 and NOD-DQ8 mice were sensitized with a mixture of peptic-tryptic (PT) digest of gliadin plus cholera toxin via oral gavage once per week for three weeks. Non-sensitized controls were gavaged with cholera toxin alone. Anti-proteolytic therapy was performed by oral administration of either *L. lactis* wild type (*Ll*-WT), *L. lactis* elafin (*Ll*-E) (10^9 cfu each) or 15% PBS/ glycerol (PBS) on a daily basis for 15 days beginning 24 hours after the final sensitization dose. Mice were challenged with 2mg/kg PT-gliadin on day 7 and day 15 of bacterial treatment. Animals were

sacrificed 18-48 hours after the final gliadin challenge. Ussing and metabolic chambers were used to assess intestinal barrier function. Intestinal damage and inflammation were assessed by histology. Additional parameters such as proteolytic activity, zonula occludins-1 (ZO-1) expression and the microbial composition of small intestinal contents were also assessed.

Results: Sensitized HCD4/DQ8 and NOD/DQ8 mice treated with PBS-glycerol experienced intestinal barrier dysfunction in response to gliadin challenge as measured *ex vivo*. Treatment of sensitized animals with *Ll*-E attenuated barrier dysfunction in both HCD4/DQ8 and NOD/DQ8 mice. This effect was also confirmed *in vivo* in NOD/DQ8 mice. *Ll*-E therapy prevented intraepithelial lymphocytosis in the jejunum of both HCD4/DQ8 and NOD/DQ8 mice. Gliadin challenge did not induce proteolytic imbalance in intestinal tissues of NOD/DQ8 mice either *in vivo* or *in vitro*. *Ll*-E maintained ZO-1 distribution at the intestinal epithelium but did not effect its relative expression at the genetic level. Small intestinal microbiota profiles in mice challenged with gliadin and treated with *Ll*-E had 80% similarity index compared to mice that remained unchallenged.

Conclusions: *Ll*-E therapy protects intestinal barrier function and limits intraepithelial lymphocytosis in sensitized HCD4/DQ8 and NOD/DQ8 mice following acute gliadin challenge. The protective effect of elafin does not appear to be mediated through the correction of a proteolytic imbalance. *Ll*-E's protective effect on the intestinal barrier may be due to maintenance of ZO-1 expression at the intestinal epithelium. The similar microbial profiles observed between mice challenged with gliadin and treated with *Ll*-E and mice free of gluten challenge suggests a possible modulatory effect of elafin on the small intestinal microbiota.

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LIST OF ABBREVIATIONS AND SYMBOLS

IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
GS	Gluten sensitivity
CD- 4, 8, etc.	Cluster of differentiation 4, 8, etc.
PT-gliadin	Peptic-tryptic-digest of gliadin
Ll	*Lactococcus lactis*
***Ll*-WT**	*Lactococcus lactis* wild type, *Ll* NZ9000
***Ll*-E**	*Lactococcus lactis* elafin, *Ll* NZpSEC:elafin
ZO-1	Zonula occludins-1
Ig (A/G)	Immunoglobulin (A/G)
sIgA	Secretory IgA
AMP	Anti-microbial peptide(s)
Muc-2	Mucin 2
CF	Cystic fibrosis
SFB	Segmented filamentous bacteria
Th1/2/17	T helper 1/2/17 response
IEL	Intraepithelial lymphocytes
ROS	Reactive oxygen species
DSS	Dextran sodium sulfate
TJ	Tight junction(s)
TLR	Toll-like receptors
NLR	Nucleotide-binding site and leucine-rich repeat containing molecules
RIG	Retinoic acid-inducible gene 1-like receptors
MHC I/II	Major histocompatibility complex class I/II
MIC-A/B	MHC class I polypeptide-related sequence A/B
IL- 1β, 6, etc.	Interleukin-1β, 6, etc.
PP	Peyer's patches
MLN	Mesenteric lymph nodes
DC	Dendritic cells
CCR9	C-C chemokine receptor type 9
ATP	Adenosine triphosphate
cAMP	cyclic Adenosine monophosphate
IFN-γ/β	Interferon γ/β
TNF-α	Tumor necrosis factor-α
NSAIDs	Non-steroidal anti-inflammatory drugs
IBS-D	Diarrhea-predominant IBS
GFD	Gluten-free diet
tTG2	Tissue transglutaminase-2 antibodies
DGP	Deamidated-gliadin peptide antibodies
EMA	Endomysial antibodies
HLA	Human leukocyte antigen
CXCR3	CXC chemokine receptor 3

AGA	Anti-gliadin antibodies
PI3	Proteinase Inhibitor 3
SNPs	Single nucleotide polymorphisms
WAP	Whey acidic protein
SKALP	Skin-derived antileukoprotease
HIV	Human immunodeficiency virus
LPS	Lipopolysaccharides
NFκB	Nuclear Factor kappa-B
AP-1	Activator protein-1
SLPI	Serine leukocyte protease inhibitor
PAR	Protease-activated receptor(s)
CXCL1	CXC chemokines receptor ligand 1
MCP-1	Monocyte chemotactic protein-1
MDA-5	Methyl dianiline 5
ARDS	Adult respiratory distress syndrome
COPD	Chronic obstructive pulmonary disorder
TNBS	2,4,6-Trinitrobenzene sulfonic acid
Ad-elafin	Adenoviral elafin construct
SPF	Specific pathogen-free
NOD	Non-obese diabetic mice
PBS	Phosphate buffered saline
FITC-dextran	Fluorescein isothiocyanate-dextran
^{51}Cr-EDTA	^{51}Cr-labelled ethylenediaminetetraacetic acid
PD	Potential difference
Isc	Short circuit current
BSA	Bovine Serum Albumin
PMSF	Phenylmethylsulfonylfluoride
ELISA	Enzyme-linked immunosorbent assay
HRP	Horseradish peroxidase
TMB	Tetramethylbenzidine
DAPI	6-diamino-2-phenylindole
MFI	Mean fluorescence intensity
ROI	Regions of interest
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
cDNA	complementary DNA
TAE	Tris-acetate EDTA
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
CT	Cycle threshold
PCR	Polymerase chain reaction
DGGE	Density gradient gel electrophoresis
PCI	Phenol-chloroform-isoamyl
UPGMA	Unweighted pair group method with arithmetic mean
ANOVA	Analysis of variance
OD	Optical Density

TEER	Transepithelial electrical resistance
CBA	Cytokine bead array

DECLARATION OF ACADEMIC ACHIEVEMENT

The initial concept for this project was conceived by Dr. Elena Verdú and Dr. Philippe Langella. The overall design of this study and all individual experiments was undertaken by Michelle Wiepjes under the advisement of Dr. Verdú. Unless otherwise stated, all experiments were conducted by Michelle Wiepjes with help from more experienced technicians where necessary. Data analysis and the interpretation of results were conducted by Michelle Wiepjes with guidance from Dr. Verdú. Insightful direction was provided by Dr. Philippe Langella, Dr. Elyanne Ratcliffe and Dr. John Bienenstock all of who were members of the Michelle Wiepjes' thesis advisory committee.

1. INTRODUCTION

1.1 The Intestinal Barrier

The intestinal barrier plays a critical role in the maintenance of homeostasis within the gut. The epithelium forms a selectively permeable barrier between the intestinal lumen and the internal milieu[1]. It is responsible for regulating the uptake of water, ions and essential nutrients from the intestinal lumen while at the same time it must exclude bacteria, viruses and harmful molecules from crossing the barrier[1]. The intestinal epithelium, along with underlying structures in the lamina propria (Figure 1), also play a key role in controlling the host immune response to luminal antigens that find their way across the intestinal barrier[1].

1.1.1 The Anatomy and Physiology of the Intestinal Barrier

1.1.1-i Extracellular Protection

The intestinal epithelium secretes several products that contribute to the overall protection of the intestinal barrier. These elements – which include mucus, secretory immunoglobulin A (sIgA) and anti-microbial peptides (AMP) – represent the first line of defense against foreign antigens. In addition, commensal bacteria are also important extracellular contributors to the defense and maintenance of the intestinal barrier.

Specialized epithelial cells known as goblet cells secrete mucins and glycoproteins that form a thick layer of mucus, which separates the epithelium from the intestinal lumen[1]. This mucus plays a key role in limiting the direct interaction of intestinal bacteria with the epithelial layer and in concentrating the metabolites of the intestinal brush border enzymes, thereby increasing the absorptive capacity of the epithelium[2]. The mucus also allows for the development of a gradient of sIgA and AMP, thus enhancing the degree of protection for the epithelium[1,3]. In addition, the glycoproteins themselves also provide anchoring sites for beneficial commensal organisms, thus enabling various forms of epithelial microbiota crosstalk[3,4]. Mice deficient in muc-2 (a mucin component of mucus) have been shown to spontaneously develop colitis, suggesting mucus plays an important

role in protecting the intestinal epithelium[5]. Furthermore these animals display increased epithelial permeability and spontaneous intestinal inflammation[5,6]. The decreased thickness of the mucus layer in these animals is thought to allow extensive microbial access to the epithelium, thereby increasing the potential for adverse immune reaction to commensal microbiota. Abnormally thick mucus can also cause problems in the intestine, as seen in the case of the hyperviscous mucus produced in cystic fibrosis (CF)[7]. The mucus layer in patients with celiac disease is also much thicker than in healthy individuals[8]. This increased mucus depth and/or viscosity may contribute to the generation of a malabsorptive state within celiac disease and CF[1]. It is also possible that it may have a negative impact on the health of the intestinal barrier by limiting the epithelial crosstalk with the microbiota – a necessary process in the maintenance of epithelial cell integrity[4]. The results in these animal models and disease states emphasize the critical role of mucus in the initial protection of the intestinal barrier and in concentrating other defense molecules secreted by the epithelium.

sIgA is produced by mucosal plasma cells and secreted by intestinal epithelial cells into the lumen[9]. Binding to sIgA enables the uptake of foreign antigens for immune processing within the mucosa-associated lymphatic tissues[9]. While this generally aids in regulating appropriate reactions to components of the luminal environment, it can sometime go awry[9]. For example, dietary gluten, the disease-triggering group of proteins in celiac patients, can be transported across the intestinal epithelium in association with sIgA to the lamina propria where it can then initiate disease[10]. sIgA secretion also promotes clearance of antigens and microorganisms by preventing their interaction with the epithelium[9]. These specialized antibodies sequester harmful antigens within the mucus thereby facilitating their clearance by the gut's peristaltic motion[9]. sIgA can also influence the composition of the microbiome through fab-dependent and independent mechanisms or by facilitating the adherence of beneficial bacteria to the outer mucus layer[9]. Deficiency in sIgA in mice can lead to the overexpansion of harmful bacterial species such as segmented filamentous bacteria (SFB)[11]. SFB closely associates with intestinal epithelial cells and promotes generation of Th17 responses[12]. Increased

mucosally adherent bacteria have also been observed in patients with ulcerative colitis[13]. Although secretion of immunoglobulin M can compensate somewhat for a lack of sIgA in patients with IgA deficiency, they are at an increased risk for the development of gastrointestinal disorders such as celiac disease[14] and allergies[15]. This underscores the importance of sIgA production to the protection of the intestinal barrier; however, secretory IgA is not the only secreted molecule protecting the intestinal barrier.

AMP are produced by Paneth cells and intraepithelial lymphocytes (IEL)[16,17]. These proteins directly impact bacteria and other microbial constituents within the intestinal lumen and help to control their growth, thereby shaping the overall composition of the intestinal microbiome[16,18]. Mice deficient for, or overexpressing, the AMP α-defensins for example, have been shown to have completely different microbial compositions than their normal counterparts[18]. Although the concentrations of these peptides are relatively low within the lumen, the mucus layer helps to concentrate them and generates a gradient that increases towards the intestinal crypts where AMP levels can be high enough to induce bacterial lysis[19]. Crohn's disease, a form of IBD is associated with insufficient production of defensins[20,21]. Furthermore, decreased levels of defensins have also been noted in intestinal biopsies from patients with active celiac disease[22]. This suggests that AMP are important components in the defense of the intestinal mucosal surface.

It has recently been revealed that commensal bacteria are essential to the overall health of the intestinal barrier (reviewed in 23). These organisms contribute to intestinal health by displacing and competing with pathogenic species, producing bacterial anti-microbial factors, and inducing the production of sIgA by enterocytes[4,23]. In addition, studies in germ-free animals suggest that commensal bacteria communicate with enterocytes and help to maintain appropriate cell turnover rates[23-28]. This in turn ensures the overall health of the intestinal barrier by encouraging apoptosis of old or damaged enterocytes and ensuring sufficient proliferation to replace these dying cells. Commensal bacteria are also believed to play an important role in epithelial restitution and wound healing; possibly through the production of reactive oxygen species (ROS)[29]. In murine

models of colitis, germ-free animals and those with commensal bacterial depletion have increased susceptibility to dextran sodium sulfate (DSS) -induced colitis and experience a greater degree of mucosal damage[28,30]. Altogether, these findings suggest that the intestinal commensal bacteria are critical components in intestinal barrier protection and overall barrier health.

Various disease states and animal models emphasize the importance of mucus, sIgA, AMP and the commensal microbiota to the maintenance and protection of intestinal barrier function. These elements work in concert to protect the intestinal epithelium from exposure to harmful luminal antigens and maintain a balance between the protective and absorptive responsibilities of the intestine.

1.1.1-ii The Intestinal Epithelium

The intestinal epithelium is comprised of a single layer of cells that physically separates the intestinal lumen from the underlying internal mileu[1] (Figure 1). This cell layer is mainly composed of columnar epithelial cells, known as enterocytes, together with a scattering of more specialized cells such as goblet cells, enteroendocrine cells and paneth cells[1]. The enterocytes play a crucial role in regulating both the absorption and secretion of water, ions, nutrients and wastes from and to the intestinal lumen[1,31]. This occurs via two distinct pathways: transcellularly and paracellularly.

The transcellular pathway utilizes cellular machinery to transport substances through the enterocytes themselves[31,32]. This pathway is predominately used to transport essential nutrients and electrolytes across the epithelial barrier, but may also be involved in the uptake and presentation of foreign antigens[9,31,32]. The transcellular pathway is closely regulated via cellular mechanisms controlling the transcription, translation and expression of particular channels, receptors and protein pores at the apical and basolateral membranes of the enterocytes, thus allowing the epithelium to tightly control which substances have access to the internal mileu[32].

The paracellular pathway in contrast, allows molecules to “leak” through the space between adjoining cells[31]. In order to restrict the movement of harmful substances through this pathway, the cells forming the epithelial layer are tightly joined together via

a number of molecular complexes including tight junctions and adherens junctions (Figure 2)[1,31-33]. Of particular importance for the protective capacity of the intestinal barrier are the tight junctions (TJ)[1,31-33]. As indicated in Figure 2 TJ join adjacent cells together at the intersection of the apical and lateral cellular membranes[32,33]. They form a ring around each cell that dramatically restricts the free movement of macromolecules across the epithelial barrier via the paracellular pathway[32,33]. The TJ complexes are composed of members of several different protein families including the occludins and claudins[1,31-33]. These molecules adhere to one another and to the actin cytoskeleton to closely tether the complexes to the structure of the epithelial cells[1,31-33]. The TJ are particularly important to overall health and decreased expression of a number of the molecular components of TJ has been tied to IBD[34] and celiac disease[35].

The intestinal epithelium also plays a critical role in the recognition and initial immune response to foreign antigens[2,36]. The intestinal enterocytes express a variety of pattern recognition receptors such as the toll-like receptors (TLR), nucleotide-binding site and leucine-rich repeat containing receptors (NLR) and retinoic acid-inducible gene-1-like receptors (RIG)[4]. Furthermore, the enterocytes themselves are capable of acting as antigen presenting cells and of producing cytokines and chemokines, which can recruit additional immune cells and initiate either a tolerogenic or effector response[36]. The enterocytes themselves therefore represent a bridge between the innate and adaptive immune response in the mucosa.

In summary, enterocytes are critical components of the intestinal barrier as a result of their ability to regulate the passage of molecules across the intestinal epithelium as well their ability to act as sentinels for harmful molecules within the gut.

1.1.1 – iii The Mucosal Immune System

The mucosal immune system plays a critical role in the regulation of the local immune response to antigens within the gut[37]. It is composed of a collection of diffuse immune cell populations as well as organized lymphoid structures within the mucosa and lamina propria[37]. The combined efforts of these cells allow a measure of regional control

over the immune response within the gut and help to discriminate between harmless and harmful antigens[37].

Intraepithelial lymphocytes (IEL) represent one of the first lines of defense encountered by luminal antigens that manage to penetrate the intestinal epithelial barrier[38]. The majority of IEL are $CD8^+$ T cells that express the early activation marker CD69 but lack expression of CD25 suggesting that this population of cells exists within a heightened state of activation[38]. This is beneficial in that it avoids the delay in response that is incurred as a result of the need for a priming step prior to full activation of the T cell. The majority of these cells also express lytic granule component granzyme B and have cytolytic capacity[38]. There are two main subsets of IEL within the intestine which can be distinguished on the basis of the chains which make up their T cell receptors: the $\gamma\delta^+$ T cells and the $\alpha\beta^+$ T cells[38].

$\gamma\delta^+$ T cells represent approximately 10% of the IEL population within the human intestine[39]. They express antigen receptors of limited diversity and display a more "innate" phenotype than other IEL subsets[38,40]. These cells are responsible for the rapid production of cytokines that regulate pathogen clearance, inflammation and intestinal homeostasis[38,40]. They are also capable of producing AMP and chemokines, which recruit other innate immune cells (neutrophils, macrophages, etc.) to aid in the defense of the epithelium[38,40]. In addition, $\gamma\delta^+$ T cells play an important role in the maintenance of the epithelial barrier through the production of keratinocyte growth factor, which promotes epithelial healing[41,42]. Interestingly, they are also capable of triggering the apoptosis of epithelial cells expressing the stress induced non-classical MHC class I molecules MIC-A and MIC-B[43]. $\gamma\delta^+$ T cell maintenance and activation are dependent on IL-15[44], the overexpression of which is associated with CD and $\gamma\delta^+$ T cell expansion within the small intestine[45].

In contrast to $\gamma\delta^+$ T cells, $\alpha\beta^+$ T cells are believed to possess a greater degree of antigen specificity[38]. They closely resemble many aspects of effector memory $CD8^+$ T cells[38]. $\alpha\beta^+$ T cells migrate into the intestinal tissues and integrate into the epithelial cell layer following antigen exposure[38,46]. The $\alpha\beta^+$ population of T cells expands with age

consistent with the belief that they are antigen-specific, migratory cells that are acquired in the gut with experience[38]. Once activated, these IEL become licensed cytotoxic cells that are capable of inducing apoptosis within cells expressing their cognate antigen in the context of MHC class I[37,38].

Peyer's patches (PP) are specialized lymphoid structures found within the lamina propria[37]. Together with the mesenteric lymph nodes (MLN), PP serve as sites for antigen presentation as well as the generation and maintenance of effector and memory responses within the gut[37,38]. The PP in particular play an essential role in initiating tolerance and effector responses[37]. Specialized enterocytes, known as microfold or M cells, line the follicular epithelium directly above the PP[37]. These cells are capable of capturing and transporting luminal antigens directly into the lamina propria[37,38]. Just below the M cells, the antigens encounter different populations of dendritic cells (DC) in the subepithelial dome[37,38]. DC take up antigens within the PP and present them in the context of Human MHC class I or II to T cells within the thymus dependent area[37]. Presentation of antigen by DC triggers T cell priming and activation[37]. DC can further direct the generation of either an effector or tolerogenic response by altering their expression of costimulatory signals such as cytokines and surface receptors[37,38]. A similar process also occurs when DC encounter antigen within the MLN[37]. DC encountering antigens in the lamina propria however, with the exception of some subpopulations of IEL generally migrate back to the PP or MLN before they are capable of generating an immune response[37,38]. DC also play a key role in directing the migration of mature T cells to their effector sites. Within the gut, DC generally promote the expression or CCR9 and integrin $\alpha_4\beta_7$ on T cells[47,48]. This ensures that T cells will home back to the intestinal tissues once they exit the lymphoid tissue and are recirculated through the blood[49]. PP and MLN also contain B cell follicles[37]. Once B cells are exposed to their cognate antigen, they migrate to the marginal zone of the follicle where they can then be activated with the help of an activated T cell sharing specificity for its cognate antigen[28]. The B cell can then migrate out of the PP or MLN to the tissue where it matures into a plasma cell and begins to produce antigen-specific antibodies[37].

The cell populations described above, together with other innate immune cells such as macrophages, neutrophils, eosinophils and mast cells cooperate to protect the intestine from pathogens and other harmful molecules that manage to penetrate the preliminary defenses of the intestinal barrier. The mucosal immune system represents a critical final layer of defense in the intestinal barrier and dysfunction within this immune compartment is associated with generation of gastrointestinal diseases such as celiac disease and IBD.

1.1.2 Regulation of the Intestinal Permeability

As mentioned previously, solutes can cross the intestinal epithelial barrier via either the transcellular or paracellular pathways[31-33]. The transcellular pathway is primarily involved in the selective transport of sugars, amino acids, short-chain fatty acids and electrolytes[32]. Regulation of the transcellular pathway is achieved via direct regulation of factors affecting the expression and localization of various receptors, channels, pumps and transporter molecules at both the apical and basolateral membrane of the enterocyte[32].

In contrast to the transcellular pathway, movement of solutes along the paracellular pathway is less selective and is controlled primarily by the size of the intercellular space[31-33]. Regulation of paracellular permeability therefore is predominantly mediated via alteration of the TJ, which restrict the size of particles that can move between cells and act as electrically charged fences that favor cationic molecules[33]. TJ are dynamic structures that can be influenced by a variety of intrinsic and exogenous stimuli and therefore play a key role in determining the overall degree of intestinal permeability[31-33].

The structure and stability of the TJ are typically controlled by intrinsic factors. Various intracellular processes are known to influence the TJ[31-33]. For example, the expression of the molecular components of TJ is known to vary during different stages of the enterocyte cell cycle and TJ stability is known to be adversely affected by decreased availability of ATP, cAMP or calcium[33]. TJ structure is also regulated by phosphorylation

of its component molecules[33]. This allows the enterocyte to directly control the stability of the tight junctional complex[33]. Extracellular signals derived from the surrounding tissue can also influence TJ stability. Oxidative stress within the epithelium for example, is known to contribute to the destabilization of TJ[33]. Proteases released by resident leukocytes can trigger the breakdown of TJ via macromolecular cleavage[31,32]. In addition, the degranulation of mast cells or eosinophils during an allergic response within the gut can also negatively impact the TJ[31,33]. Certain cytokines also play a key role in TJ regulation[31], the release of pro-inflammatory such as IL-4[50], IL-13[50], IFN-γ[51,52] and TNF-α[51,52] is known to have a dissociative effect on TJ leading to increased permeability while in contrast, IL-10[53] protects the TJ and maintains the intestinal barrier.

Regulation of the paracellular pathway through TJ can be greatly influenced by a variety of exogenous stimuli[31,33]. Certain probiotic bacteria for example have been shown to maintain intestinal barrier function through the upregulation of ZO-1 and myosin light chain kinas expression[34,35]. In contrast, pathogenic bacteria such as *Vibiro cholerae*, *Shigella flexneri*, *Clostridium perfringens*, *Clostridium difficile* and Enteropathogenic *Escherichia coli* commonly directly or indirectly target TJ[33]. Infection with such pathogenic bacteria, or in certain cases, merely the ingestion of their toxins leads to TJ destabilization and increased paracellular permeability[31,33]. Exposure to food antigens may also influence TJ structure[56]. Gliadin, a glycoprotein found in wheat has been shown to trigger the release of zonulin by enterocytes[35]. This compromises interactions between ZO-1 and occludins leading to TJ instability and a resultant increase in permeability[35]. Although other food antigens are believed in impact barrier function none have been studied to the same depth as gliadin and there remains a great deal to learn about how food can alter gut function[56]. Chronic alcohol consumption can also lead to barrier dysfunction[31]. This process is believed to be triggered by the increased production of acetaldehyde and nitric oxide production, which leads to increased phosphorylation of the TJ proteins ZO-1, E-cadherin and β-catenin[57,58]. Finally, there is substantial evidence suggesting that chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) can cause intestinal barrier dysfunction and gastrointestinal damage[59,60]. Studies with gastric

epithelial cell lines revealed that acute exposure to aspirin leads to an increase in permeability that was correlated with decreased expression of claudin-7[61]. Furthermore, NSAIDs use can lead to an increase in oxidative stress and nitric oxide production, both of which can decrease the stability of TJ[62]. Use of NSAIDs may also trigger the uncoupling of mitochondrial oxidative phosphorylation, thereby decreasing the production of ATP, which is critical to maintaining TJ stability[63].

1.1.3 Intestinal Barrier Dysfunction in Gastrointestinal Disease

Intestinal barrier dysfunction occurs when there is a breakdown in the integrity or regulatory capacity of the intestinal epithelium leading to an increase in barrier permeability, particularly towards large macromolecules. Barrier dysfunction has been associated with increased oxidative stress, inflammation, tissue damage and abnormal reactions to luminal antigens[31,33,35,50-52,62,63]. Transient or localized increases in barrier permeability are generally not sufficient to cause disease[1]; however, chronic barrier dysfunction is believed to contribute to the development of several gastrointestinal conditions in predisposed individuals including IBD, IBS and celiac disease[31,33].

IBD is a chronic inflammatory condition that includes Crohn's disease and ulcerative colitis. IBD is believed to be caused by an inappropriate inflammatory response to constituents of the gut microflora[64]. Patients with IBD have long been known to experience intestinal barrier dysfunction; however, it remains unclear whether this state is a cause or consequence of the ongoing inflammation characteristic of the disease[64]. Measurements of intestinal permeability collected from first-degree relatives of patients with IBD have proved to be higher than those of the general population, suggesting that the barrier defect in patients may have been present prior to development of disease[65,66]. Furthermore, long term follow-up with those relatives experiencing increased epithelial permeability found that several went on to develop IBD[65,67]. Although not conclusive, this points to a possible contribution of barrier dysfunction in the pathogenesis of IBD.

IBS is a functional disease characterized by chronic abdominal pain or discomfort, bloating and altered bowel habits in the absence of organic abnormality[68,69]. The cause of

IBS remains unknown but the condition has been linked to infection, psychological stress and various forms of food sensitivity[68]. Increased intestinal permeability and low-grade inflammation have been identified in patients from several subgroups of IBS[68-73]. Furthermore, these alterations seem to be correlated with symptom severity in patients[72] and with the generation of visceral hypersensitivity in animal models[68]. Interestingly, in patients from the diarrhea-predominant subgroup of IBS (IBS-D), scientists have identified alterations in the normal expression and regulation of key TJ proteins, most notably ZO-1[72]. It is possible that in patients with IBS, barrier dysfunction allows for increased transit of bacterial and food antigens across the intestinal barrier leading to the generation of the various symptoms of IBS; however, further investigation is required to identify the exact mechanisms involved within this process.

Celiac disease is a chronic inflammatory condition in which the absorptive surface of the small intestine is damaged by an abnormal response to gluten[74]. Increased intestinal permeability has been described in patients with celiac disease as well as their first-degree relatives[74]. This increased level of permeability is highest in newly diagnosed patients and improves when patients are placed on a gluten-free diet (GFD); however, even with treatment, there is some evidence to suggest that intestinal permeability in celiac patients remains elevated relative to the general population[75]. The evidence collected from relatives of celiac patients and those patients following a GFD suggest that an inherent defect in the intestinal barrier may precede the development of celiac disease. Further evidence of the role of intestinal barrier dysfunction in the development of celiac disease comes from gene expression studies, which indicate that celiac patients have reduced expression of ZO-1[35]. Gluten itself has also been implicated in the development of barrier dysfunction in celiac patients. Some groups have hypothesized that gluten exposure in celiacs leads to the release of zonulin, which reversibly modulates the structure of the TJ leading to increased intestinal permeability[35]. This increase in paracellular permeability may lead to an increase in the flux of gluten and its fragments across the intestinal barrier where they can then initial the abnormal immune response in celiac patients.

Barrier dysfunction may also lead to the increased uptake of additional luminal antigens. Of particular concern, is the potential for increased migration of harmful bacteria across the intestinal barrier. Celiac patients have been shown to have an altered microbiome characterized by increased Gram negative bacteria and reduced Bifidobacteria relative to healthy controls[76-81]. Evidence from the HCD4/DQ8 murine model of gluten intolerance indicates that intestinal bacteria may enhance the response to gluten through bystander activation of immunity[82]. Furthermore, certain bacteria have been shown to enhance the inflammatory immune response to gliadin[83]. Taken together, this evidence suggests that intestinal bacteria make an important contribution to the pathogenesis of celiac disease. Given that celiac disease is triggered by dietary gluten and that the response to this antigen may be enhanced by increased uptake of harmful luminal antigens such as bacteria, it stands to reason that the intestinal barrier represents and an important therapeutic target within celiac disease.

The examples of IBD, IBS and celiac disease indicate the critical role of intestinal barrier integrity in the protection of the gastrointestinal tract. The intestinal barrier plays a crucial role in the regulation of both immunity and homeostasis within the gut and its breakdown may be the determining factor in the pathogenesis of disease within predisposed individuals.

Unlike IBD and IBS, the environmental trigger of celiac disease, gluten, is known. Furthermore, the passage of gluten across the intestinal barrier is recognized as being essential for the development of disease. As a result, celiac disease represents an important model in which we can explore the role of the intestinal barrier in gastrointestinal disease.

1.2 Celiac Disease

Celiac disease is a chronic small intestinal enteropathy triggered by an abnormal immune response to dietary gluten in genetically susceptible individuals[84,85]. This condition is traditionally characterized by chronic inflammation of the proximal small intestine leading to atrophy of mucosal tissues and malabsorption [84,85]; however, a much larger spectrum of clinical presentation is now recognized within the disorder[85]. Diagnosis is made based on elevated levels of antibodies against tissue transglutaminase-2 (tTG2) and deamidated-gliadin peptides (DGP) as well as endomysial antibodies (EMA), in combination with a small intestinal biopsy to look for overt enteropathy[84,85]. Currently the only known treatment for celiac disease is strict adherence to a gluten-free diet[84-86]. The complete exclusion of all gluten-containing grains and their derivatives generally results in the resolution of symptoms and entrance into a remission-like state [84-86].

1.2.1 *Prevalence*

Historically, celiac disease was believed to be a rare disease presenting mainly in children of northern European descent[84,85]; however, it is now believed to be much more common. The prevalence of celiac disease in North America has recently been estimated to be as high as 1%[87]. Similar rates have also been found in Europe[88], the Middle East and North Africa[89], Northern regions of India[89,90]. Serological testing and comparison of serum samples taken between 1948-1954 with cohorts from the present day has suggested that the incidence of celiac disease in North America has increased approximately 4-fold over the past fifty years[87]. Cases of celiac disease have also been reported in various parts of the world including North and South America, Europe, Australia, south and west Asia and North Africa suggesting that the disorder is not confined to any specific geographical region[84].

1.2.2 *Pathogenesis*

1.2.2– i *Genetics*

Studies of monozygotic twins have revealed a high level of concordance in the development of celiac disease resulting in heritability estimate of approximately 80%[91]. Furthermore, studies of relatives of celiac patients have revealed that increased rates of celiac disease in first (1/22) and second-degree (1/39) relatives compared to the general population (~1/100)[92]. This suggests that there is a strong genetic component responsible for the development of this condition.

Celiac disease has been primarily linked to specific alleles of the human leukocyte antigen (HLA) component of the class II major histocompatibility complex (MHC)[93]. Approximately 90% of celiacs express the HLA-DQ2 (DQA1*05 and DQB1*02) heterodimer while the remaining cases are positive for HLA-DQ8 (DQA1*03 and DQB1*0302)[93]. These HLA genes are believed to be responsible for 35-40% of the genetic heritability of celiac disease with the remainder being attributed to various known and unknown non-HLA genes[84,93]. Celiac disease cannot develop in the absence of HLA-DQ2/8 risk factors and although approximately 30% of the population expresses these alleles, many will never develop the condition[84,93]. This suggests that HLA-DQ2/8 is necessary but not sufficient to develop celiac disease.

Recent studies have identified 39 non-HLA risk genes many of which are immune-related[93]. Definite associations with the condition have been reported for 26 of these identified genes while the remaining 13 have been identified as specific pre-disposing factors for celiac disease[93]. Interestingly, celiac disease shares many of these genes with various other disorders, many of which are known to occur concurrently with celiac disease, including type 1 diabetes, Crohn's disease and rheumatoid arthritis[93]. Together, these genes explain only 5% of the heritable risk of celiac disease suggesting that other factors remain to be identified[93]. It is possible that much of the variation in clinical presentation of celiac disease may be explained in part by the polygenic nature of the disorder.

1.2.2 – ii *Gluten*

Celiac disease represents a unique model of autoimmune disease in that, the environmental trigger of the abnormal immune response is known. Ingestion of gluten is a necessary step in the pathogenesis of celiac disease. Gluten is a storage protein found in the grains wheat, rye and barley as well as their derivatives[94]. It is composed of gliadins and glutenins, both of which have high proline and glutamine content[94,95]. This renders them particularly resistant to complete proteolytic digestion by gastric, pancreatic and brush border enzymes[95].

In the context of celiac disease, it is the gliadin fraction of gluten that is of particular interest. Peptide fragments of α-gliadin have been identified as being involved directly in the pathogenesis of celiac disease[95]. These fragments can be divided into either 'toxic' or 'immunogenic' categories depending upon their specific composition[95]. So-called 'toxic' fragments such as the P 31-43 α-gliadin peptide (19-mer) likely exert effects directly upon the epithelium via an as-yet-unidentified mechanism resulting in the activation of the innate immune response as seen in the production of IL-15[45]. 'Immunogenic' peptides on the other hand, such as the P 56-68 α-gliadin peptide, are responsible for activating the humoral immune system through interaction with HLA DQ2/8[95]. The immunogenicity of such peptides is increased by deamidation by tissue trans-glutaminase although the exact location and timing of this enzymatic interaction remains unknown[95,96].

1.2.2– iii *Aberrant Immune Response*

The pathogenesis of celiac disease is ultimately dependent upon an abnormal immune response to the large peptide fragments generated during gluten digestion[95]. This response generates both the characteristic small intestinal enteropathy and the antibody response used to diagnose celiac disease.

This abnormal immune response begins with the passage of undigested gliadin peptides across the intestinal epithelium. The manner in which this occurs remains controversial and there are two schools of thought regarding how gliadin gains access to the lamina propria. The first possibility is that gliadin peptides cross the intestinal

epithelium via the transcellular pathway: retrotranscytosis in association with sIgA that is recognized and taken up by the transferrin receptor CD71[10]. The second possibility is that gliadin gains access to the lamina propria via the paracellular pathway. Gliadin has been shown to interact with CXCR3 on the cell surface thereby inducing the production of zonulin[97]. Zonulin then triggers rearrangement of the actin cytoskeleton and TJ leading to increased paracellular permeability and an increase in the influx of gliadin peptides into the lamina propria[97].

Once in the lamina propria, gliadin peptides such as the 33mer p 56-68 α-gliadin, are deamidated or transamidated by tissue transglutaminase-2[95,96]. The process of deamidation introduces negative charges to the peptides that increase the affinity of HLA-DQ2/8 for the peptides[98]. Once bound to HLA-DQ2/8 on antigen presenting cells, the deamidated gliadin peptide is presented to $CD4^+$ T cells[96]. The primed and activated $CD4^+$ T cells then release proinflammatory cytokines that induce inflammation (TNFα, IL-21, IL-6, IL-18)[95,96]. They also activate fibroblasts and lamina propria mononuclear cells, which release metalloproteases that degrade the underlying ECM, support structure of the epithelium[95,96,99]. $CD4^+$ cells activate IEL such as $CD8^+$ and natural killer cells leading to enterocyte apoptosis[95,96]. The activated $CD4^+$ T cells also activate B cells via IFN-y leading to the production of antibodies against tTG2 and DGP as well as anti-gliadin antibodies (AGA) and EMA[96,99].

Enterocytes themselves can also initiate parts of the abnormal response to gluten. Enterocytes have been shown to produce IL-15, which can activate the populations of IEL responsible for triggering epithelial apoptosis[95,96]. Enterocytes also upregulate the expression of stress markers such as MIC-A, MIC-B and HLA-E, as well as NKG2D – their cognate receptor on IEL – thereby allowing them to be targeted for apoptosis[100,101].

The combined effects of this aberrant immune response to gluten lead to the development of the classic celiac lesion. Chronic exposure to gluten leads to hyperplasia of intestinal crypts in attempt to replace cells damaged by the Th1 arm of the immune response to gluten. At the same time, there is an overall decrease in villus height due to matrix remodeling and enterocyte death. Simultaneously, serum levels of tTG2, AGA,

DGP and EMA antibodies increase due to activation of the Th2 arm of the immune response.

1.3 Milder Forms of Gluten Intolerance: Gluten Sensitivity

Gluten sensitivity (GS) encompasses a variety of conditions in which ingestion of gluten triggers symptoms, immunological and morphological changes in the absence of celiac disease[102]. It is estimated that up to six percent of the general population complains of some form of GS; however, the symptoms of these patients have remained unrecognized by the medical community until very recently[103,104]. In 2009, Verdù *et al* proposed the existence of gluten sensitivity (GS) as a separate clinical entity existing in the "no man's land" between celiac disease and IBS[105]. This proposal was based on clinical observations of apparent symptomatic response to the GFD in a subset of patients with IBS, as well as findings in the transgenic HLA-DQ8 and HCD4/DQ8 murine models of gluten-intolerance that pointed to factors beyond genetics as important contributors to disease[105]. In 2011, Biesiekierski *et al*. confirmed the existence of GS when he demonstrated in a double-blind randomized trial that patients with GS truly did experience symptoms in response to gluten exposure[106].

The mechanisms responsible for the development of GS are currently unknown. It is thought that patients with GS may simply be "innate responders" to gluten[107]; however, the relationship between GS and conditions such as type I diabetes[108,109] and psorasis[110] suggest some link to the adaptive immune system. Unlike celiacs, patients with GS experience symptoms in the absence of overt enteropathy, increased intestinal permeability and elevated levels of tissue tTG2, DGP or endomysial EMA antibodies that are characteristic of celiac disease[111]. Diagnosis of GS therefore, currently relies on first ruling out celiac disease then placing a patient on the GFD to determine if their symptoms respond to the GFD and reappear when challenged with gluten[107,112]. This diagnostic process is both expensive and time consuming. As a result, a great deal of research is currently focused on identifying key characteristics that distinguish celiac disease and GS. Recent studies have suggested that GS patients may develop subtle morphological

changes within the intestine such as increased numbers of IEL[113]. Furthermore, serological testing has revealed that over half of GS patients develop elevated levels of IgG anti-gliadin antibodies (AGA) suggesting that these antibodies may represent a biomarker of GS[114].

Unlike celiac disease, there is no formally indicated treatment for GS, although gluten-sensitive patients frequently elect to follow a gluten-free or gluten-reduced diet. It remains unclear whether GS is a lifelong condition like celiac disease or if patients with GS eventually develop some level of tolerance to gluten. More research is necessary to better understand exactly how to diagnose and treat GS. Further research also promises to increase the medical community's understanding and acceptance of this potentially very common emerging condition.

1.4 Alternative Therapies for Gluten Intolerance

1.4.1 The Problem with the Gluten-free Diet

Currently, the only accepted treatment for celiac disease and other gluten-sensitive conditions is strict adherence to a GFD; however, the efficacy of this therapy is limited by the ability of patients to comply with their dietary restrictions[115,116]. Despite increasing public awareness of the disease and the recent increase in the availability of gluten-free products, maintaining a diet that is completely gluten-free remains extremely difficult. Patients and their families must contend with a variety of challenges such as cross-contamination, unclear ingredient labeling and relatively higher food costs[115,116]. To make matters worse, they must also contend with a general lack of medical, industrial, political and public awareness of celiac disease, gluten-sensitivity and the inherent nutritional challenges of the gluten-free diet.

Although relatively safe, there is also mounting evidence that the GFD may have some adverse effects. Studies of the intestinal microbiota of healthy volunteers found that their microbial composition changed as a result of the GFD[117]. Furthermore, gluten-free foods often contain higher levels of fat and sugar relative to the standard gluten-

containing fare[118-122]. This can lead patients transitioning onto the diet to gain weight while at the same time leaving them deficient in key nutrients and fibre[118-122]. This poses a problem for the medical community as it becomes unclear whether the GFD, with its associated financial burden and potential adverse effects, should be indicated for the treatment of patients with GS or with clinical presentations that fall just below the threshold of diagnosis for celiac disease. It is therefore prudent that we develop safe alternatives to the GFD for the treatment of milder forms of gluten-intolerance.

Eating is a highly emotional activity that is often associated with various elements of culture and family. Food is often used as the centre point for many social activities and as a result patients following a gluten-free diet often feel that it has a negative impact on various aspects of their daily lives including their career, family, social lives and ability to travel [115,116]. Patients may find that they restrict themselves from large social gatherings surrounding food and as a result may suffer from social isolation[121,122]. These negative social and emotional implications of the diet are some of the largest challenges faced by patients and may explain why dietary non-compliance is often triggered within social settings[116]. As a result of the social impact of the GFD, there is increasing demand within the celiac community for alternative therapies and treatment options to help minimize the negative impact of the GFD on celiac patients quality of life.

1.4.2 Alternative Therapies to the Gluten-free Diet

As a result of the imperfect nature of the gluten-free diet as a treatment for celiac disease and GS, it is imperative that new therapeutic targets are identified and new treatments are developed for these complex conditions. Several different strategies are currently being investigated as alternatives for the GFD in the treatment of gluten intolerance as shown in Figure 3. These strategies have been extensively reviewed (see 123-125) and include alternatives such as: the reduction of the gluten content of flour prior to ingestion, the oral administration of prolyl endopeptidases that are capable of breaking down digestion-resistant fragments of gluten, the sequestration of gluten within the lumen by binding to either synthetic polymers or IgG anti-gluten antibodies from

cow's milk, modulators of paracellular permeability, inhibitors of tissue transglutaminase 2 and HLA-DQ2/8, gluten peptides that downregulate or alter the typical progress of the gluten-induced immune response, restoring immune tolerance to gluten via oral "vaccination" and biological agents which directly target specific cytokines or cell populations[123-125].

None of the therapies currently in development are perfectly suited to the treatment of celiac disease and GS or have the same level of efficacy as strictly following the GFD[123-125]. Furthermore, it seems unlikely that any one treatment will work for all the various clinical manifestations of GS and celiac disease. The amount of variation both in clinical presentation and in patients' tolerance of minute exposure to gluten suggest that in the future, a combination of different therapies that can be tailored to suit the patient may be the best approach to the treatment of celiac disease and GS. It is therefore important that we continue to identify novel therapeutic targets and develop new methods to protect celiac disease and GS patients from exposure to dietary gluten.

1.5 Trappin-2/Elafin Therapy: A Novel Approach to Gluten Intolerance?

Trappin-2/elafin is encoded by a gene that is specific to eutherian mammals and has been detected in various species throughout this lineage with the exception of mice, rats and rabbits[126,127]. Trappin-like genes have also been identified in marsupials, monotremes, birds, and fish suggesting that the ancestral form of this gene may have served an important function in our evolutionary past[126].

In humans, trappin-2/elafin is encoded by the peptidase inhibitor 3 (PI3) gene on human chromosomal region 20q12-13[128,129]. The PI3 gene is highly polymorphic due to its large number of single-nucleotide polymorphisms (SNP)[130]. It is believed that the variation generated from these SNP may help to account for the variable expression of trappin-2/elafin within different tissues. The human PI3 gene encodes for three different exons encoding for a signal peptide, as well as the characteristic cementoin and whey-acidic protein (WAP) domains[131,132]. The cementoin domain allows trappin-2/elafin to interact with tissue transglutaminase and become cross-linked to various fibers within the

extracellular matrix[133]. It is thought to allow for the concentration of Trappin-2/elafin at its respective sites of action[121-123]. The WAP domain in contrast is known to be responsible for the protease inhibitor capacity of trappin-2/elafin[133-135]. The final protein product of the PI3 gene has a molecular weight of 9.9 kDa and is known as trappin-2, pre-elafin or elafin[135]. During secretion, the signal peptide and cementoin domain are often cleaved producing an approximately 6 kDa molecule called elafin, peptidase inhibitor 3 or skin-derived antileukoprotease (SKALP)[135]. Few studies within the literature make the distinction between the 9.9 kDa and 6 kDa molecular forms of trappin-2/elafin and it is therefore often difficult to ascertain which form is being used in any given study[135]. Within this investigation, the use of the term elafin is meant to specify the 9.9 kDa secreted form of Trappin-2/elafin.

1.5.1 The Expression of Elafin in the Human Body

Elafin has been identified in both adult and fetal[139,140] tissues and can be found at various sites throughout the human body[139,132]. It is most strongly expressed at mucosal surfaces regularly exposed to inflammatory stimuli such as the respiratory tract, the female reproductive tract and the gut[129,132,140]. In fact, many of these mucosal tissues often display constitutive expression of elafin suggesting that it may play an important role in their protection from noxious stimuli[132].

Elafin can also found at high levels at sites of inflammation and is rapidly induced in response to injury or infection where it is believed to help moderate the inflammatory response[132,140]. In the skin for example, elafin is typically found at low levels but has been observed to increase dramatically in response to various forms of insult[132,141]. Similar observations have also been made in serum[142,143] endothelium[144] and joints[145].

Various molecules associated with inflammation have been found to upregulate elafin expression. For example, both neutrophil elastase and mast cell tryptase, proteases released by immune cells during the course of inflammation, have been found to rapidly increase the levels of elafin[146,147]. Neutrophil elastase may also inactivate elafin when it reaches excessively high levels within the tissue allowing for greater progression of the

inflammatory response[148]. Elafin production has been shown to be stimulated in various cell lines by proinflammatory cytokines such as IL-1β[149], TNF-α[150] *in vitro*. Exposure to poly i:c viral double stranded RNA *in vitro* has also been shown to induce elafin expression in human genital epithelial cell lines[151]. Various molecules associated with innate immunity and anti-microbial defense have also been suggested to increase elafin expression suggesting that elafin may play a key role in innate immunity[152]. Elafin levels in the female reproductive tract have also been found to vary throughout the menstrual cycle suggesting the elafin production may also be hormonally regulated[153].

1.5.2 Elafin: A Potent Protease Inhibitor

Elafin is a serine protease inhibitor and may be classed as an alarm protease inhibitor[150]. Elafin inhibits serine proteases via a tight-binding reversible mechanism with a 1:1 molar inhibitory ratio[137,138]. Elafin has potent inhibitory capacity against various forms of human leukocyte and pancreatic elastases as well as proteinase-3 and their murine analogs[137,138,154]. Elafin does not inhibit trypsin, chymotrypsin or cathepsin-G[154]. Biochemical studies have indicated that the protease inhibitory active centre of elafin is located at the exposed loop of its WAP domain core[136]. Further investigations also indicate that oxidizing elafin decreases its affinity for its target proteases as wells as its inhibitory capacity[155].

Within inflamed tissues, elastase and proteinase 3 are known to cause degradation of extracellular matrix proteins, generate chemotactic peptides, activate matrix metalloproteinases, trigger the production and activation of pro-inflammatory cytokines, and aide in the phagosomal degradation of bacterial proteins[146]. While in the right context, these actions help to protect the tissues from infection, when uncontrolled, they can quickly cause large amounts of damage. The role of elafin as a protease inhibitor is thought to be to protect tissues from the effects of uncontrolled inflammation[140]. Furthermore, there is some evidence to suggest that elafin may also aid in the resolution of inflammation and promotion of wound healing[140,157].

1.5.3 The Anti-Microbial Activity of Elafin

Elafin has been shown to have antibacterial capacity against both Gram-positive and Gram-negative bacteria at physiologically relevant concentrations (1-25μM)[158]. Elafin's anti-bacterial capacity has been proven against *P aeruginosa*, *Staphlococcus aureus*, *Klebsiella pneumoniae*, *E coli*, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Branhamella catarrhalis*[135,159,160]. Elafin has also been shown to have anti-fungal effects against *Aspergillus fumigatus* and *Candida albicans*[159].

Elafin's anti-microbial activity has been shown to be independent of its protease inhibitor capacity[158,159]. Biochemical investigations have indicated that the N-terminus domain of elafin seems to host most but not all of the molecule's anti-microbial capacity[140, 161]. The N-terminus domain of elafin has been shown to adopt an α-helical conformation in a membrane mimetic environment[161]. This characteristic conformation is shared with other AMP such as the family of defensins and is thought to interfere with the membranes of bacteria[161]. The anti-microbial capacity of elafin is decreased in the presence of heparin and NaCl suggesting that, similar to other AMP, it is partly a result of the cationic nature of the molecule[159]. Studies of elafin in the context of *Pseudomonas aeruginosa* infection of respiratory cell lines suggest that elafin may also be capable of altering the expression of virulence factors via some unidentified intracellular mechanism[161]. It is theorized that elafin may impact bacterial cellular machinery of potentially interfere with the protease mediated activation of bacterial signaling pathways[140]. Secreted elafin has also been shown to trigger the dispersion of biofilms in *P aeruginosa* and (to a lesser extent) *Escherichia coli* infection of respiratory cell lines[161].

Elafin has recently been identified as having a prominent role in the resistance to human immunodeficiency virus (HIV) infection following the observation that HIV resistant female sex-workers in Kenya had higher levels of elafin in cervico-vaginal lavage fluids than their infected and unexposed counterparts[162]. Elafin prevents HIV attachment and transcytosis in genital epithelial cells[163]. This anti-viral capacity appears to be dependent on the direct interaction of elafin with the HIV virion rather than any alteration of host expression of cell surface receptors[163]. Higher elafin expression has

been associated with decreased expression of TLR-2, TLR-4 and RIG-1; however no direct causal link between elafin and these surface receptors has been established[151].

Elafin is constitutively expressed at the mucosal sites within the human body that are most likely to be exposed to microbial assault. This, coupled with its proven broad anti-microbial capacity, suggests that it plays an important role in the innate defense of the human mucus membranes. Further support for this theory is provided by the observation that particular bacteria, parasites and viruses have evolved strategies to downregulate or inactivate elafin suggesting that these pathogens have developed counter-evolutionary measures to incapacitate a key-protective molecule of the mucus membranes[140,164].

1.5.4 Immunomodulation by Elafin

Elafin appears to be capable of altering the expression of various cytokines and influencing the recruitment and activation of inflammatory cells. These immunomodulatory effects have been noted at a physiologically relevant nanomolar range[158,159,165]. Part of the immunomodulatory effect of elafin may be due to its effect on the ubiquitin-proteosome pathway[166]. Elafin was shown to delay the ubiquitination of proteins in response to lipopolysaccharides (LPS)[166]. Elafin has also been proven to be capable of inhibiting nuclear factor kappa-B (NFκB) and activator protein 1 (AP-1) although the mechanism by which this occurs is currently unknown[167]. Secretory leukocyte protease inhibitor (SLPI), a serine protease inhibitor which shares several characteristics with elafin, has been shown to be capable of crossing the cell membrane and binding to DNA suggesting that elafin may also exert its immunomodulatory effects intracellularly[140]. This is further supported by the fact that elafin has been shown to be capable of interacting with the cell membrane[168]. Elafin may also act extracellularly on matrix molecules or at the cellular surfaces to prevent activation of TLR-4 and protease-activated receptors (PAR) although it is currently unclear how this might affect the immune response[140].

Elafin can have a proinflammatory effect in response to specific stimuli. Overexpression of PI3 gene in murine lung has been shown to cause increased influx of inflammatory cells in response to LPS stimulation[169]. Elafin overexpressing mice appear to have increased populations MHC II^{high} $CD11c^{high}$ dendritic cells (DC) with high levels of the cellular surface markers CD80 and CD86 indicating increased activation status[169]. Elafin also induced upregulation of chemokine CXCL1 with triggered an increase in neutrophil recruitment in response to *P aeruginosa* infection[170]. Elafin has further been shown to have an adjuvant effect that enhances innate and adaptive immune response with certain antigens such as LPS or adenoviral infection[169]. In these cases, elafin induces a Th1-biased response that is associated with increased production of IL-12, IFN-γ and IgG2a[169]. Elafin's interaction with LPS augments LPS-induced TNF-α response in murine macrophage via CD-14[170]. In the case of *P aeruginosa* infection, LPS exposure or adenoviral infection, the upregulation of inflammatory response may aid in the rapid clearance of the pathogens during early stages of infection.

In contrast, elafin has also been shown to be anti-inflammatory and to promote the resolution of inflammation. Elafin inhibits MCP-1 production in response to LPS in mononuclear cells by preventing activation of NFκB and AP-1[167]. Elafin can complex with LPS in the serum leading to decreased LPS stimulation of macrophages and consequently decreased production of TNF-α[156]. Secretion of elafin by human endometrial cells triggered a reduction in the production of the proinflammatory cytokines IL-8, IL-6, TNF-α and IFN-β following stimulation with poly (I:C) viral double stranded DNA[150]. This reduction in proinflammatory cytokines was associated with reduced expression of innate viral sensors RIG-1 and methylene dianiline 5 (MDA-5)[150]. A reduction in proinflammatory cytokines has also been noted in animal models of colitis and within the endothelium[171,172]. Elafin appears to be involved in the resolution of inflammation by preventing cleavage of macrophage CD14 by neutrophil elastase thus allowing phagocytosis of apoptotic leukocytes[157]. It also abolishes elastase induced keratinocyte activation and hyperproliferation[173], which may in turn reduce fibrosis as seen in the cardiovascular system[174].

The effects of elafin may be pro-inflammatory or anti-inflammatory depending on the context in which it is released. It is possible that this dual nature allows elafin to promote the rapid and effective clearance of pathogens by first initiating a proinflammatory response and then triggering an anti-inflammatory response to control the resultant inflammation and promote would healing once the threat has been eliminated. Further research is necessary to isolate not only what determines whether elafin enhances or attenuates the inflammatory response, but also how these effects are generated within the inflamed or infected tissues themselves.

1.5.5 The Protective Capacity of Elafin Therapy

The protective capacity of elafin has been well established in animal models of infection and inflammation in the cardio-vascular system as well as the respiratory and reproductive tracts.

Elafin has been studied extensively in the cardiovascular system and has been shown to have a potent protective effect against the damage caused by various forms of inflammation and ischemia (reviewed in 174). Elafin expression has been detected within the human endothelium, serum and the intima of coronary arteries[174]. Elafin has proven to be protective in animal models of balloon angioplasty, wire induced injury, pulmonary hypertension, atherosclerosis, limb and cardiac reperfusion injury, myocardial infarction, myocarditis and cardiac transplantation[174]. The protective effect of elafin in these models was generally mediated by a combination of reduced infiltration of neutrophils and macrophages, the suppression of MMP and myleoperoxidase activity as well as reduced tissue remodeling and fibrosis[174].

Most of the studies focusing on the cardiovascular system have administered elafin via an intravenous route[174,156]. Intravenous elafin has been proven to have low toxicity with short plasma and activity half-lives in both animal models and humans[174]. The proven safety profile of intravenous elafin has opened the possibility of using elafin to treat human disease. Several phase I and II clinical trials to investigate the potential of elafin in the peri-operative treatment of transthoracic esophagectomy, coronary artery

bypass and kidney transplantation are currently in development[156]. One phase II clinical trial, the elafin myocardial protection from ischemia, reperfusion or EMPIRE study (Eudra CT no:2010-019527-58) is currently underway[174].

The role of elafin in the respiratory tract has also been extensively examined and has been shown to be critical in protecting the lung from infection and inflammatory damage[135]. Polymorphisms of the PI3 gene have been associated with increased risk for conditions such as adult respiratory distress syndrome (ARDS)[143]. Imbalance between proteases and antiproteases within the lung are implicated in a variety of respiratory conditions including ARDS, asthma, pulmonary fibrosis, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF)[135]. The anti-protease capacity of elafin therefore seems to be crucial to the protection of the lung. Elafin variants with decreased anti-protease capacity have been shown to be ineffective in protecting the lung in animal models of elastase-induced emphysema[175]. *In vitro*, elafin has been shown to protect respiratory cell lines such as A549 lung epithelial cells from cell detachment induced by neutrophil elastase (NE) and proteinase 3[176]. Elafin also reduced NE activity and protected ZO-1 expression and collagen adhesion capacity following exposure of A549 cells to LPS[160]. These effects help to protect the lung from inflammation-induced injury; however elafin also has another important protective effect within the lung. Elafin has been shown to have potent anti-microbial effects against a variety of Gram-positive and Gram-negative respiratory pathogens including *P aeruginosa* and *S aureus* common bacterial causes of pneumonia in CF patients[135,158,159]. It has also been noted that elafin transfection in animal models also protected mice from further challenge with adenoviral constructs suggesting that elafin may also have anti-viral capacity within the lung although this remains to be fully investigated[169].

The protective role of elafin within the female reproductive tract is an emerging area of interest in infectious disease, especially in relation to HIV. Studies attempting to identify the potential cause of HIV resistance identified a number of anti-microbial compounds, including elafin, within the cervical-vaginal secretions of resistant women[162]. Elafin was found to be overexpressed in HIV resistant women relative to their infected

counterparts[162]. Further studies of elafin in the female reproductive tract demonstrated that a 30 percent immunodepletion of elafin in culture accounted for a reduction of up to 60% of its HIV inhibition capacity[163]. This suggests that elafin may be the predominant compound responsible for HIV resistance. Interestingly, decreased elafin levels have also been found to be associated with bacterial vaginosis and the preterm rupture of amniotic membranes during pregnancy, suggesting that elafin's protective activity in the female reproductive tract extends beyond its anti-viral capacity[177].

There is a growing body of evidence from systems throughout the human body which indicates that elafin is a critical molecule used as protection both from external threats as wells as uncontrolled inflammation. Elafin's protective capacity in such a wide variety of conditions and bodily systems suggests that it may be a powerful therapeutic tool for the treatment of inflammatory and infectious disease.

1.5.6 Elafin in Gastrointestinal Disease

Elafin expression has been confirmed throughout the gastrointestinal tract from the oral cavity to the colon[178]. Within the small intestine, elafin is predominantly expressed by the epithelium[178,179] although elafin production by $\gamma\delta^+$ T cells has also been detected *in vitro*[172]. Several gene expression studies have identified that elafin expression and induction is abnormal in patients with IBD[179, 181-183].

In order to further explore the role of elafin in the pathogenesis of IBD, Motta et al. induced acute DSS and trinitrobenzene sulfonic acid (TNBS) colitis in elafin transgenic and transfected mice[171]. When compared with controls, the animals expressing elafin experienced reduced severity of colonic inflammation in response to DSS and TNBS[171]. Transgenic and Ad-Elafin transfected mice had decreased inflammatory cell infiltrate and lower microscopic damage scores than the controls receiving DSS or TNBS[171]. Similar results were seen in mice receiving a daily gavage of $5x10^9$ cfu of *Lactococcus lactis* or *Lactobacillus casei* over the 7 days of DSS colitis induction[184]. Elafin expression and bacterial therapy were also correlated with decreased elastase- and trypsin-like activity within the gut and decreased levels of proinflammatory cytokines and

chemokines[171,184] Transfection of Caco-2 intestinal epithelial cells with Ad-elafin in vitro attenuated TNF-α induced increase in monolayer permeability[171]. Supplemental data collected by Motta et al. from DSS-treated animals suggests that Ad-elafin prevents rearrangement of the tight junctional molecule ZO-1 during colitis, suggesting that elafin may protect intestinal barrier function via ZO-1[171]. Further study in this model is necessary to determine if elafin can protect animals from more chronic forms of colitis. In order to evaluate the potential of bacterial elafin therapy in for human treatment of IBD it would also be prudent to examine the long term efficacy of such orally administered forms of elafin in protecting mice from colitis. Given the importance of intestinal barrier dysfunction in the pathogenesis of gastrointestinal disease it would also be very interesting to investigate how elafin over-expression in this model impacts barrier integrity *in vivo*.

The exploration of elafin's role in the gastrointestinal tract is currently in its infancy but the findings are very promising. The protective effect of elafin within the murine model of DSS and TNBS colitis as well its deficit in patients with IBD suggests that abnormal elafin expression may play an important role in the pathogenesis of similar gastrointestinal diseases such as celiac disease.

1.6 Hypothesis and Aims

The combined results of investigations in animal models and clinical trials suggest that elafin may represent a powerful therapeutic tool for the treatment of a variety of inflammatory conditions including celiac disease. Given the promising results of elafin therapy in murine models of IBD, we sought to investigate if elafin therapy could attenuate markers of gliadin sensitization in two gluten-sensitive mouse models.

1.6.1 Hypothesis

We hypothesized that elafin therapy would ameliorate intestinal barrier dysfunction and acute small intestinal inflammation induced by gliadin challenge in gluten-sensitive mice.

1.6.2 Aims

The overall aims of this study were as follows:

1) To test the efficacy of elafin therapy in attenuating the inflammation, intestinal damage and functional changes induced by acute gliadin exposure in gluten-sensitive mice.
2) To explore potential mechanisms through which elafin may exert its protective effect in the gastrointestinal tract

2. METHODS

2.1 Mice

All experiments were conducted with the approval of the McMaster University Animal Research Ethics Board (AREB) and were in accordance with the Canadian Guidelines for Animal Research set by Canadian Council of the Use of Laboratory Animals. Mice were bred and housed within McMaster University's Central Animal Facility (CAF). Mice were housed in sterilized ventilated rack (HCD4/DQ8, see section 2.1.1) or filter topped (NOD-DQ8 see section 2.1.2) cages under specific pathogen free (SPF) conditions. Animals were maintained on a 12h:12h light/dark cycle and were provided with access to low-fat gluten-free chow (Harlan, ON, Canada) and sterile water *ad libitum*. Experiments were conducted on male mice aged 6-12 weeks upon commencement of each protocol. Mice were housed according to treatment group with a maximum of four animals to a cage.

2.1.1 HCD4/DQ8 Mice

The HCD4/DQ8 transgenic mouse expresses both human HLA-DQ8 and CD4 in the absence of endogenous mouse MHC-II[82,185-187]. These animals have been shown to develop intestinal barrier dysfunction[82,186,187] and intraepithelial lymphocytosis[186,187] in response to gluten exposure although no overt enteropathy (intestinal atrophy) is demonstrated in acute administration models (one gluten challenge after sensitization). Recent findings from the Verdú lab indicate that these mice do develop partial villous atrophy as well as an IgA anti-gliadin antibody response following chronic exposure (2 months) to gluten[187].

2.1.2 NOD-DQ8 Mice

NOD-DQ8 mice express human HLA-DQ8 in the absence of endogenous MHC-II and in predisposing autoimmune background of the non-obese diabetic (NOD) mouse[188,189]. Although prone to insulitis, these mice do not appear to spontaneously

develop diabetes as seen in NOD mice[188]. When sensitized and repeatedly (3 times/week) challenged with gluten for one to three weeks, these animals develop moderate enteropathy with increased intraepithelial lymphocytosis and decreased villus/crypt ratios[188]. Furthermore, NOD/DQ8 mice develop antibody responses to both gliadin and tissue-transglutaminase[188] as well as small intestinal barrier dysfunction following exposure to gluten[188].

2.2 Gliadin Sensitization

Mucosal sensitization was achieved via intragastric gavage of 25 μg of cholera toxin (List Biological Laboratories, CA, USA) and 500 μg of peptic-tryptic-digest of gliadin (PT-gliadin) dissolved in 0.02 M acetic acid. Cholera toxin is used to break tolerance to gluten and mimic sensitization occurring in adult onset CD. The volume of each gavage was adjusted to 100 μl with sterile phosphate buffered saline (PBS). Each mouse was administered with this solution once weekly for a total of three weeks to achieve the desired level of sensitization.

As a control for mucosal sensitization to PT-gliadin, certain treatment groups were gavaged with a solution of 25 μg of cholera toxin alone that had been adjusted to a volume of 100 μl with PBS. Animals belonging to treatment groups receiving only cholera toxin during sensitization are henceforth referred to as non-sensitized controls.

2.3 Gliadin Challenge

Gliadin challenges were administered via intragastric gavage to mice from all treatment groups. Each challenge dose consisted of 2mg of PT-gliadin dissolved in 0.02M acetic acid and adjusted with PBS to a total volume of 100 μl. Gliadin challenges were administered on a weekly basis for two weeks starting one week after the final dose in the sensitization regime.

2.4 Recombinant *Lactococcus lactis*

Recombinant *Lactococcus lactis* (*Ll*) was prepared as described previously in World Intellectual Property Organization (WIPO) patent application WO/2011/086172 A1[184]. The codons for the PI3 gene signaling peptide were excised and replaced with those encoding for $U_{sp}45$, the main protein secreted by *Ll*[184]. Elafin expression was then placed under the control of the P_{nisA} promoter, which responds to nisin induction[184]. Together these elements formed the plasmid pSEC:elafin, which was then introduced into *Ll* NZ9000 to generate the recombinant bacterium, NZpSEC :elafin (*Ll*-E) used in this investigation[184]. *L.l.* NZ9000 (*L.l.*-WT) was used as wild type control within this investigation. All bacteria were suspended in %15 PBS-glycerol (abbreviated as PBS in all graphs).

The recombinant and wild type *L. lactis* were generously prepared and aliquoted by members of Dr. Philippe Langella's research team at the MICALIS (Microbiologie de l'Alimentation au service de la Santé) Institute at INRA in Jouy-En-Josas, France. The bacteria were shipped frozen on dry ice to McMaster University and were stored at -80°C upon receipt. Each aliquot was thawed approximately 20-30 minutes prior to use.

2.5 Overall Experimental Design

As depicted in Figure 4, male HLA-DQ8 $HCD4^+$ and NOD-DQ8 mice were sensitized with a mixture of PT-gliadin plus cholera toxin via oral gavage once weekly for three weeks. Non-sensitized controls were gavaged with cholera toxin alone. Mice then received oral administrations of wild type *L. lactis* (*Ll*-WT), *L. lactis* elafin (*Ll*-E) (10^9 cfu each) or 15% PBS-glycerol on a daily basis for 15 days beginning 24 hours after the final sensitization. Mice were challenged with 2mg/kg PT-gliadin on day 7 and day 15. 18-24 hours following administration of the final gliadin challenge mice were prepared for endpoint investigations. Intestinal barrier function was assessed via Ussing chambers and *in vivo* clearance of ^{51}Cr-labeled ethylenediaminetetraacetic acid (^{51}Cr-EDTA) and Fluorescein isothiocyanate dextran (FITC-dextran) in the urine. Intestinal damage and

inflammation were assessed based on histology. Testing for AGA was also preformed in serum and intestinal lavage samples collected at endpoint.

2.6 Assessing *L. lactis*-Elafin Treatment Efficacy

2.6.1 Ussing Chambers

Ussing chambers were assembled and the automated voltage/current clamp apparatus (World Precision Instruments, FL, USA) was zeroed using 37°C Krebs buffer to account for the inherent electrical properties of the fluid as well as to correct for any drift by the electrical sensors during periods of inactivity. The voltage clamp apparatus was then set to standby and the chambers were drained of Krebs while the required tissue specimens were prepared.

Animals were sacrificed via cervical dislocation 18-24 hours following gliadin challenge. Beginning approximately 6 cm below the gastro-duodenal juncture, a 5 cm long section of jejunal tissue was carefully excised and placed in a shallow dish of oxygenated Krebs buffer containing mannitol in place of glucose. The tissue segment was cut in half and each half was opened longitudinally along the mesenteric border. Any adherent intestinal contents were gently removed from the mucosal surface. Each piece of tissue was carefully pinned over the opening of one half chamber of the setup. Extra care was taken to keep the tissue moist, to avoid sections containing PP and to prevent extensive stretching or ripping that could compromise the experimental behavior of the tissue. During each experiment, all mounting was performed by the same individual as an attempt to minimize variations in the amount of stretch placed upon the tissue.

The Ussing chambers were assembled and the tissue was bathed in 37°C oxygenated Krebs buffer on its mucosal surface. This buffer contains glucose as an energy source for the tissue and is osmotically balance by mannitol in the modified Krebs buffer bathing the serosal surface of the tissue. The tissue was then allowed to equilibrate within the chamber for 10 minutes at which point a 100 µl baseline sample was taken from the serosal buffer. At this time, baseline values for the potential difference (PD) and

short circuit current (Isc) were also recorded. 6 µl/ml of the paracellular probe ^{51}Cr-EDTA (PerkinElmer, MA, USA) was added to the mucosal side of the Ussing chamber and a 100 µl "hot" sample was then taken from the same side. 100 µl samples were taken from the serosal side of the Ussing chamber every thirty minutes for 2 hours after the addition of the probe. Corresponding PD and Isc values were also recorded at the time of each sample collection.

The ^{51}Cr-EDTA level in each sample was counted using a Beckmann Coulter LS600 multi purpose scintillation counter (Beckman Coulter, ON, Canada). The counts for each 30-minute period were averaged and compared to the "hot" sample for each piece of tissue. The data was then expressed as %flux/cm^2/hr. Conductance was calculated via Ohm's law from the recorded PD and Isc values.

2.6.2 Metabolic Chambers and In Vivo Permeability

Metabolic chambers (Fischer Scientific, ON, Canada) were set-up in a quiet room with restricted access for the duration of the experiment in order to minimize disturbance and stressing of the animals. Mice were orally challenged with 2mg/kg PT-gliadin 24 hours prior to the administration of the probes. Four hours prior to the placement of the mice in the metabolic chambers, food and water were removed from the animals' home cages. Following this four-hour fast, mice were gavaged with a 200ul dose of saline solution containing approximately 18mg of FITC-dextran (3000-5000 KD) (Sigma-Aldrich, ON, Canada) and 5µCi ^{51}Cr-EDTA (PerkinElmer, MA, USA). The mice were then placed in their individual metabolic cages without food or water. After an additional two hours, the animals were provided with water and they were then left in their cages for 24 hours with minimal to no disturbance.

After 24 hours, animals were removed from the metabolic cages and sacrificed. The collection surfaces of the metabolic chambers were rinsed with 1ml of distilled water to dissolve any dried urine. The combined volume of urine and this wash was then measured. After gentle mixing, two 200ul samples of urine from each mouse were aliquoted into a fluorometric plate (Sarstedt, QC, Canada). Emission of the samples was

read at 490-520nm using a Spectramax M3 Multi-mode microplate reader and Softmax pro 5.4 software (Molecular Devices, CA, USA) and compared to a standard curve in order to quantify the % FITC-dextran clearance. Similarly, a Beckman Coulter LS600 scintillation counter (Beckman Coulter, ON, Canada) was used to determine the % hot sample clearance of ^{51}Cr-EDTA in 200ul aliquots of urine from each mouse compared to 200ul aliquot of the original gavage solution.

2.6.3 Morphological Analysis

Small intestinal sections were preserved in 10% buffered formalin for 48 hours. Samples were then transferred to 50% ethanol for 15 minutes and finally to 70% ethanol. Tissues were embedded in paraffin and cut into 4 μm sections by McMaster University Centre for Gene Therapeutics' Core Histology Research Services.

2.6.3-i Immunohistochemistry and CD3$^+$ Lymphocyte Counts

Slides were placed in a 60°C oven for 30 minutes to soften the paraffin The slides were then immersed in CitriSolv (Fischer Scientific, ON, Canada), followed by decreasing concentrations of ethanol and ending in distilled water. This removed the paraffin and rehydrated the tissue. Next, the sections were then covered with peroxidase blocking reagent (Dako, ON, Canada) for 10 minutes to block any endogenous peroxidase activity. After washing with water and PBS the slides were treated with Proteinase K (Dako, ON, Canada) for 15 minutes to unblock the antigen-binding site. Following an additional wash cycle the samples were incubated in a 1% bovine serum albumin (Sigma-Aldrich, ON, Canada) diluted in PBS (1% BSA) for 15 minutes to block undesired antibody binding sites. The slides were then incubated overnight at 4°C with a 1:2000 dilution of rabbit anti-CD3 antibody (Dako, ON, Canada) in 1% BSA. On the following day, slides were washed in PBS and before applying Envision, a horseradish peroxidase-coupled anti-rabbit secondary reagent (Dako, ON, Canada), for 30 minutes. Following a second wash cycle in PBS, the tissue was incubated for ten minutes in 3-Amino-9-Ethylcarbazole (AEC) Substrate-Chromogen (Dako, ON, Canada). The substrate is bound to the antibody complex, thus allowing for visualization of the CD3+ antigen binding

sites. After washing in water and PBS the slides were counterstained with Mayer's hematoxylin for 1 minute before a final wash in water then PBS and mounting with gel/mount (Biomeda Corporation, CA, USA) aqueous mounting medium.

IEL counts were preformed in a blinded fashion on 10 randomly selected villous tips from each slide. Villi were examined at 200X magnification and the number of $CD3^+$ cells falling within the 10 uppermost enterocytes to either side of the villus tip was quantified as previously described[190].

2.6.3-ii Villus Crypt Ratio

Sections were stained with hematoxilyn and eosin by McMaster University's Core Histology Research Services. Slides were examined at 100X magnification. Images were captured using an Olympus BX51 microscope (Olympus, ON, Canada) equipped with a micropublisher 3.3 RTV color camera (QImaging, BC, Canada) and villus height was measured from the apical surface of the villus tip to the approximate tissue baseline using Image-Pro 6.3 imaging software (Mediacybernetics, MD, USA). Using the same software, crypt depth was measured from the approximate tissue baseline to the basal surface of the intestinal gland. The ratio of villus height to crypt depth (V/C) was then calculated for each paired set of measurements.

Five well-preserved and well-oriented villi with adjacent well-preserved/-oriented crypts were measured in two different tissue sections for each slide. Villi were considered well preserved and well oriented if they were relatively straight and continuous with a single layer of intact enterocytes indicating the apical margin of the villus tip. The villi measured also were free of obvious histological artifacts due to sectioning or poor preservation. Crypts were considered to be well oriented if they appeared as a longitudinal cross section with an identifiable base and mouth. The mucosal tissue surrounding crypts used for measurements appeared to be well preserved and was relatively free of histological artifacts.

2.6.4 Anti-gliadin Antibodies (AGA) ELISA

At the time of sacrifice, animals were anesthetized with Isofluorane (Abbott, IL, USA) and blood samples were collected via cardiac puncture. The animals were then sacrificed via cervical dislocation. The blood was centrifuged at 13,000 rpm for 10 minutes to allow for sedimentation of its cellular components and the serum was then carefully removed, aliquoted and stored at -20°C for further investigation.

Following sacrifice and collection of tissue for Ussing chambers, the remainder of the small intestine was carefully excised. Using a sterile gavage needle, the intestine was flushed twice with 5 ml of ice cold PBS containing 0.05 M EDTA (pH 8) and 0.1 mg/ml soybean trypsin inhibitor (Sigma-Aldrich ON, Canada) and 40ul of 100 mM phenylmethylsulfonylflouride (PMSF) (Sigma-Aldrich, ON, Canada) in 95% ethanol. The intestinal wash was centrifuged at 3700 rpm and 4°C for 30 minutes. The supernatant was then aliquoted and stored frozen at -20°C.

A stock solution of gliadin for coating AGA ELISA plates was prepared in advance aliquoted and stored frozen at -20°C. To prepare the stock solution, 50mg of Gliadin from wheat (Sigma-Aldrich, ON, Canada) was slowly dissolved in 10ml of 70% ethanol at room temperature. The solution was gently stirred by hand for one hour to encourage complete dissolution. 40ml of PBS were added and the solution was stirred for an additional 20-30 minutes at room temperature.

To prepare microplates for AGA ELISA, the stock gliadin solution was diluted 10 times with PBS. 50µl of this solution was then pipetted into each well of a Nunc-Immuno maxisorp surface 96 microwell plate (Thermo Scientific, ON, Canada). The plates were then incubated overnight at 4°C to allow for adequate coating of the well bottom.

The following morning, the solution was aspirated and plates were washed with pbs-0.05% Tween-20 (PBS-Tween). Plates were incubated for 1 hour at room temperature with 100µl per well of 1% BSA to block unwanted antigen binding sites. The blocking solution was then aspirated and samples were added (50µl /well). Serum samples were diluted in 1% BSA (1:5 for IgA assay; 1:50 IgG assay) while samples of intestinal wash were not diluted prior to their addition to the plate. The ELISA plate was

then incubated with the samples for 2 hours at room temperature. The samples were then aspirated and the plate was washed in PBS-Tween prior to the addition of a secondary antibody conjugated to the horseradish peroxidase enzyme (HRP). Secondary antibodies were diluted in 1% BSA to a concentration of 1:8000. The plate was then incubated with 50µl per well of either rabbit anti-mouse IgA (α-chain specific)-HRP conjugated (Sigma-Aldrich, ON, Canada) or rabbit IgG (γ-chain specific)-HRP conjugated (Sigma-Aldrich, ON, Canada) antibodies for 1 hour at room temperature. Following incubation, the solution was aspirated and plates were washed with PBS-Tween. 50µl of the HRP substrate solution 3,3',5,5'-Tetramethylbenzidine (TMB) (Biofx Laboratories, MD, USA) was added to each well and the plate was then incubated at room temperature for 15-30 minutes. The HRP enzymes conjugated to the secondary antibodies cleave the TMB forming a blue-colored product. The production of this tinted byproduct is directly related to the amount of secondary antibody bound within each well and is therefore an excellent indicator of the relative amount of AGA present within each sample. To stop the HRP catalysis of TMB, 50µl of 1N HCl was added to each well. The plate was then read at 450nm using a PowerWave x340 microplate reader and Gen5.1 Software (BioTek Instruments, MN, USA).

2.7 Investigating Potential Mechanisms of Action

2.7.1 Proteolytic Activity

2.7.1-i In Vivo *Sample Collection*

Mice were sacrificed by cervical dislocation18-24 hours following challenge with gliadin. A 4 cm section of small intestine was collected from approximately 2.5 cm below the gastroduodenal juncture. Using a sterile gavage needle, this section was carefully flushed with approximately 1 ml of cold, sterile PBS. The wash liquid was then flushed through the tissue for a second time before both the tissue and wash were snap frozen in liquid nitrogen.

2.7.1-ii Organ Culture

Animals were sensitized with PT-gliadin and cholera toxin as described in section 2.2. One week after administration of the final sensitization dose animals were sacrificed via cervical dislocation. The animals were then sprayed with 70% ethanol and the abdomen was dissected using separate sets of sterile instruments for the outer layer of skin and muscle, the abdominal wall and the internal organs. A segment of the duodenum and upper jejunum was removed starting approximately 2 cm below the gastro-duodenal juncture. The intestine was carefully opened longitudinally along the mesenteric border and flattened in a sterile Petri dish over ice. The tissue was kept moist with a small amount of cold PBS while sections were carefully cut using a 4 mm biopsy punch. The tissue biopsies were then washed individually for 30 s in 3 separate rounds of ice-cold PBS containing 40 μg/ml of gentamicin (Sandoz, QC, Canada). The biopsies were then transferred into separate wells of a 48 well plate (BD Biosciences, ON, Canada) containing 500 μl per well of sterile RPMI 1640 medium enriched with 10% inactivated fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin and 2mM L-glutamine. The media in each well was enriched with 100 ug/ml of the following common gliadin digest peptides: 19-mer (p31-49), 20-mer (p120-139) or 33-mer (p56-89) (Thinkpeptides, FL,USA). Some wells were left unsupplemented to act as controls. Following the addition of the biopsies, the plates were incubated for 3 h at 37°C in a sterile incubator with 95% O_2 and 5% CO_2 humidified air. After three hours, tissue biopsies were removed and quickly snap frozen in liquid nitrogen. The supernatants were then collected and briefly centrifuged to remove any suspended cells or tissue fragments. The supernatant was then aliquoted and flash frozen. Samples were then stored at -80°C and shipped on dry ice to the Dr. Nathalie Vergnolle's lab at INSERM in Tolouse, France for further analysis.

2.7.1-iii Measurement of Trypsin-like and Elastase-like Activity

Measurement of the trypsin-like and elastase like activity of the samples from the *in vivo* and organ culture studies was conducted by members of Dr. Nathalie Vergnolle's Research Team at INSERM in Tolouse, France as previous described by Motta *et al*[163].

2.7.2 Zonula Occuldins-1 Expression

2.7.2-i ZO-1 Immunohistochemistry and Mean Fluorescence Intensity

Small intestinal sections were preserved in 10% buffered formalin for 48 hours. Samples were then transferred to 50% ethanol for 15 minutes and finally to 70% ethanol. Tissues were embedded in paraffin and cut into 4 μm sections by McMaster University Centre for Gene Therapeutics' Core Histology Research Services. Each slide was prepared with 2 serial sections to enable direct comparison of the positive and negative staining of each piece of tissue.

Slides were warmed in a 60°C oven for 20 minutes to soften the paraffin before emersion in 2 washes of Xylene. The tissue was then rehydrated by emersion in a series of decreasing concentrations of ethanol followed by distilled water. In order to unmask the antigen-binding site, slides were immersed in 10mM sodium citrate buffer at room temperature for 2 minutes. The buffer was then brought to a boil in a microwave for 5 minutes and maintained at a temperature of 95-99°C for an additional 10 minutes. Slides were then allowed to cool in the buffer for 20 min before rinsing with distilled water and PBS. Each section was circled with a delimiting pen (Dako, ON, Canada) to allow for the application of different reagents to each section. Next, both sections were covered with 2% BSA in PBS to block unwanted binding sites. Rabbit anti-ZO-1 antibody (Invitrogen, ON, Canada) was diluted in 2% BSA to a concentration of 1:500 μl, then applied to one section per slide. The remaining sections, the negative controls, were covered with 2% BSA. Slides were then incubated at room temperature for 2 hours. Following incubation, the slides were washed in PBS and a 1:500 dilution of the secondary antibody, Alexa fluor 488 (Invitrogen, ON, Canada), was applied to all sections. The slides were then incubated in the dark for 2 hours at room temperature before being washed once more in PBS. The slides were then mounted and coverslipped with Prolong Gold Anti-fade with 4',6-diamidino-2-phenylindole (DAPI), a fluorescent nuclear stain (Invitrogen, ON, Canada).

Using Image-Pro Plus 6.3 software (Mediacybernetics, MD, USA), a total of 10 images of both the positively and negatively stained sections were obtained for each slide. Images were captured using an Olympus BX51 fluorescent microscope (Olympus, ON, Canada) and QImaging Micropublisher 3.3 RTV camera (QImaging, BC, Canada). Images were captured at 200x magnification using the same exposure time for each image. Using Image J software (National Institutes of Health, MD, USA) the mean fluorescence intensity (MFI) of 5 identically sized regions of interest (ROI) was determined for each image. Each ROI was centered on the epithelial border. The average MFI for the negatively stained section was then subtracted from that of the positively stained sections for each slide to account for the background fluorescence (staining present in both the positive and the negative) thus generating an overall MFI for each slide. To avoid bias in imaging and analysis slides and images were assessed blindly and identified by reference number only prior to statistical analysis.

2.7.2-ii ZO-1 qRT-PCR

Small 1-2 cm segments of jejunum were excised at the time of sacrifice, placed in RNA later stabilization buffer (Qiagen, ON, Canada) and stored at 4°C overnight before transfer to -20°C for long term storage. RNA extraction was preformed using the commercially available RNeasy mini Kit (Qiagen ON, Canada). Samples were prepared as per the manufacturer's instructions. As the samples were to be used for quantitative reverse transcription polymerase chain reaction (qRT-PCR), we elected to perform the optional DNase treatment indicated within the manufacturer's instructions to remove any contaminating strands of DNA. The RNA samples generated were stored at -80°C when not in used to prevent degradation.

Following RNA extraction, the concentration and 260/280 ratio of the samples was verified using a Bio photometer (Eppendorf, ON, Canada). Only samples with a 260/280 ratio of between 1.65 and 2.01, indicating low protein contamination, were used to generate cDNA for qRT-PCR.

The integrity of the RNA samples was also checked via aragose gel electrophoresis before proceeding with cDNA synthesis. To prepare the aragose gel,

100ml of Tris-Acetate EDTA (TAE) buffer was mixed with 1.5g of aragose powder. The mixture was heated in a microwave to the point of translucence and vigorously swirled to evenly dissolve the aragose into the TAE buffer. The mixture was subsequently allowed to cool to around 50°C at which point one drop of Ethidum bromide was added to the mixture. The solution was again swirled to ensure even deposition of the ethidium bromide throughout the gel. The gel solution was then quickly poured into a prepared tray and combs were placed to form the wells. The gel was then allowed to cool and solidify. Once cooled, the gel was cut to size and immersed in TAE within the gel apparatus. Small quantities of each RNA sample were then mixed with 10x loading dye and added to separate wells of the prepared gel. The apparatus was then set at a voltage of 80-100V and the samples were allowed to migrate through the gel towards the positive pole for approximately 30 minutes at which point the samples should have separated into two distinct bands. After the apparatus was shut down, the gel was carefully removed and placed on the reading surface of a Geldoc apparatus (BioRad, CA, USA). The bands of RNA were imaged using the trans-UV setting and the images of the gel were then analyzed using Quantityone Software (Biorad, CA, USA). Only samples that separated into two distinct bands during electrophoresis were used to generate cDNA as extensive blurring and a lack of these bands indicated possible degradation of the RNA sample.

First strand cDNA synthesis was preformed as indicated in the manufacturer's protocol for the commercially available M-MLV Reverse Transcriptase Kit (Invitrogen ON, Canada) and the resulting samples were stored at -20°C when not in use to prevent degradation. The concentration and purity of the cDNA was verified using a Bio photometer (Eppendorf, ON, Canada) as described above for RNA prior to beginning qRT-PCR. The integrity of the cDNA was also verified using TAE aragose gel electrophoresis as described previously for RNA with the sole exception being that the presence of only one distinct band was sufficient for the sample to be deemed worthy of proceeding to qRT-PCR.

2 µl of cDNA from each sample was mixed with 36µl of autoclaved, distilled water 8 µl of Evagreen Ssofast supermix (Biorad, CA, USA) and 1 µl each of the forward

and reverse primers for the gene of interest. Forward (5'-GTTGGTACGGTGCCCTGAA AGA-3') and reverse (5'-GCTGACAGGTAGGACAGACGAT-3') sequence primers were used for ZO-1 amplification. In a separate reaction glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene that is expressed at near constant levels within the intestinal tissue, was amplified using the following Forward (5'-CCATGGAG AAGGCTGGGG-3') and reverse (5'-CAAAGTTGTCATGGATGACC-3') primers. The samples were added in triplicate to the PCR reaction plate and carefully sealed to prevent evaporation of the reagents. The plate was then placed in a 1Q5 Multi-color real time PCR thermocycler (BioRad, CA, USA) and cycled once for 30 s at 95°C, 30 times at 95°C for 6 s followed by 60°C for 10 s. Following this step, data collection began and the samples were cycled once at 95°C for 1 min followed by 55°C for 1 minute and finally 61 30 s cycles oscillating between 65°C and 95°C. Melt curve and cycle threshold (CT) data was analyzed using IQ5 software (BioRad, CA, USA). The CT mean of ZO-1 was normalized to that of GAPDH for each sample respectively in order to determine the relative expression of ZO-1.

2.7.3 Composition of the Small Intestinal Microbiota

In order to determine if *Ll*-E therapy affects the resident microbiota in the small intestine of NOD/DQ8 mice, cages of four male littermates aged 8-12 weeks were sensitized with PT-gliadin and cholera toxin (section 2.2) followed by a series of 2 gliadin challenges on day 7 and day 15 (section 2.3). To minimize the cage effect on intestinal microbiota composition, all mice in a single cage were allocated to different treatment groups. One animal from each cage (n=3) did not receive gliadin challenges (figure 5). Challenged mice were treated daily for fifteen days with PBS, *Ll*-WT or *Ll-E.* During treatment, these animals were housed in individual cages to prevent microbial transfer due to coprophagy. Mice were sacrificed 24 h after the administration of the final gliadin challenge. Upon sacrifice, samples of the small intestinal contents were collected in sterile microtubes from the bottom 4 cm segment of the terminal ileum just above the caecum. These samples were immediately flash frozen and stored at -80°C.

2.7.4-i Denaturing Gradient Gel Electrophoresis (DGGE)

Small intestinal contents were homogenized in 900 µl of basic PBS and 100 µl of guanidine-EDTA-sarkosyl solution for 5 min at 30 revolutions/sec. The samples were then briefly centrifuged to reduce the amount of bubbles in the solution. Next, 50µl of lysozyme (100mg/ml) (Sigma-Aldrich, ON, Canada), 10 µl of RNase A (10 mg/ml) (Invitrogen, ON, Canada) and 50 µl of mutanolysin (10 U/µl) (Sigma-Aldrich, ON, Canada) were added to each sample to lyse any cells present. The samples were then vortexed to mix the solution and allowed to incubate at 37°C for 1 hour. To continue the enzymatic lysis process, 25 µl of 25% Sodium dodecyl-sulfate, 100 µl of 5M NaCl and 25 µl of proteinase K (Sigma-Aldrich, ON, Canada) were added to each sample. Again, the samples were briefly vortexed and allowed to incubate at 65°C for approximately 1 hour. The samples were then centrifuged for 5 min at 13000 rpm. 900 µl of the supernatant was then transferred to a new 2 ml tube for Phenol-choloroform-isoamyl (PCI) extraction. Approximately 900 µl of PCI was added to the supernatant and the tube was gently shaken to generate a precipitate layer at the interface between the two liquids. The samples were then centrifuged for 10 min at 13000 rpm. The supernatant was transferred to a new 1.5 ml tube while being very careful not to disturb the layer of precipitate. The DNA was then isolated from the reaction solution according the manufacturer's instructions for the commercially available DNA clean and concentrator kit (Zymo, CA, USA). The samples were then amplified via polymerase chain reaction (PCR) in an IQ5 Multi-color real time PCR thermocycler (Biorad, CA, USA) using the universal bacterial primers HDA1-GC (5'- CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGG GGGGACTCCTACGGGAGGCAGCAG T-3') and HAD-2 (5'-GTATTACCGCGGCT GCTGGCAC-3'). The optimized cycle used for amplification was as follows: 1 cycle at 95°C for 5 min; 40 cycles at 95°C for 30 s, 57°C for 30s and 68°C for 1 min; followed by 1 final cycle at 68°C for 5 min. The concentration, purity and integrity of the generated DNA mixture were then verified as described in section 2.7.2-ii. DGGE was preformed using the DCode Universal Mutation Detection System (BioRad, CA, USA) as per the manufacturer's instructions for linear denaturing gels and DGGE. The resulting gels

were imaged using a Geldoc apparatus and Quantityone Software (BioRad, CA, USA). The resulting image was then imported to GelComparII software (Applied Maths, TX, USA) for calculation of the Dice similarity coefficient and UPGMA clustering analysis to determine the percent homology between different samples. In order to minimized cage effects within the analysis, comparisons were conducted between animals from the same original cage.

2.8 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism software (Version 4, 2004, CA, USA). Statistical significance was determined by one-way ANOVA using Bonferroni's post-hoc test to compare the multiple columns. Significance was reported for p-values <0.05 at a 95% confidence level. Outliers were determined to be those values greater than 2 standard deviations from the mean of a give sample group. All data are presented as mean ± standard error of mean.

3. RESULTS

3.1 The Protective Effect of *Ll*-Elafin Therapy

3.1.1 Gliadin Sensitization and Challenge Increased Intestinal Permeability and Inflammation in Gluten-Sensitivity Models

Previous studies have shown that both HCD4/DQ8 and NOD/DQ8 mice develop barrier dysfunction following sensitization and challenge with gluten[82,185-189]. The results of my investigation confirmed that gluten-sensitization and challenge increased small intestinal paracellular permeability – as indicated by increased ^{51}Cr-EDTA flux *ex vivo*, in sensitized HCD4/DQ8 and NOD/DQ8 mice (fig. 6A, 8A). Sensitized NOD/DQ8 mice also demonstrated increased conductance following gliadin sensitization and challenge (fig 8B). My investigations also confirmed an increase in permeability *in vivo*. Sensitized NOD/DQ8 mice displayed increased percent clearance of ^{51}Cr-EDTA and FITC-dextran over 24 hours *in vivo* relative to nonsensitized, untreated controls (fig 8C,D). This effect was not seen in HCD4/DQ8. This suggests that NOD/DQ8 mice experience a greater increase barrier dysfunction following gliadin-sensitization and acute challenge relative to the HCD4/DQ8 model.

Both HCD4/DQ8 and NOD/DQ8 mice have previously been found to develop various degrees of intraepithelial lymphocytosis and intestinal enteropathy following gliadin sensitization and challenge[185,188]. I confirmed that sensitization and acute gliadin challenge increased the IEL population within the jejunal tissues of sensitized HCD4/DQ8 and NOD/DQ8 relative to nonsensitized controls (Fig 7,9). Gliadin sensitization and acute gliadin challenge did not produce any appreciable change in villus/crypt ratio of either HCD4/DQ8 or NOD/DQ8 mice (Fig 11).

3.1.2 Ll-Elafin Therapy Reduces the Impact of Gliadin Challenge on Sensitized HCD4/DQ8 Mice

In order to investigate the potential of *Ll*-E therapy for the treatment of gluten-intolerance, I first tested the therapy in the HCD4/DQ8 model. Treatment of sensitized animals with *Ll*-E led to a significant reduction in ^{51}Cr-EDTA flux ($p<0.05$; fig 6A) relative to those treated with PBS alone. The small intestinal tissue conductance of the HCD4/DQ8 mice did not vary significantly between the treatment groups as shown in figure 6B.

As part of my evaluation of the efficacy of Ll-E therapy, I next assessed the degree of inflammation ($CD3^+$ cell count) and damage (villus/crypt ratio) within the jejunum of the HCD4/DQ8 mice. Sensitized animals receiving PBS-glycerol or *Ll*-WT were found to have significantly higher numbers of $CD3^+$ IEL within their villous tips relative to the nonsensitized controls ($p<0.05$; fig 7A). Sensitized mice receiving *Ll*-E treatment in contrast had significantly lower IEL lymphocyte counts ($p<0.001$; fig 7A) which were comparable to those of the nonsensitized controls suggesting that *Ll*-E therapy prevents intra-epithelial lymphocytosis in sensitized HCD4/DQ8 mice following exposure to gliadin. In contrast to the IEL count, no significant difference was observed in the villous crypt ratios between the various treatment groups (similar to findings from NOD/DQ8 mice presented in Fig 11).

3.1.3 Ll-Elafin Therapy Attenuates the Effect of Gliadin Challenge on Sensitized NOD-DQ8 Mice

After determining that *Ll*-E therapy could exert a protective effect within the HCD4/DQ8 mouse model of gluten-sensitivity, I chose to investigate its efficacy within another model of gluten sensitivity, the NOD/DQ8 mouse. Mice treated with *Ll*-E therapy had significantly lower ^{51}Cr-EDTA flux relative to sensitized animals receiving either PBS-glycerol ($p<0.001$) or *Ll*-WT ($p<0.01$; fig 8A). *Ll*-E therapy also significantly reduced small intestinal tissue conductance in sensitized mice relative to those receiving PBS-glycerol ($p<0.01$) or *Ll*-WT ($p<0.05$; fig 8B).

Figure 8C indicates that sensitized mice treated with PBS-glycerol experienced the highest clearance rate of ^{51}Cr-EDTA of all the groups *in vivo*. This rate was significantly higher than in the nonsensitized controls treated with PBS-glycerol ($p<0.05$; fig 8C). Sensitized animals treated with either *Ll*-WT or *Ll*-E experienced significantly lower levels of ^{51}Cr-EDTA clearance relative to the sensitized PBS-treated mice ($p<0.05$ and $p<0.01$ respectively; fig 8C). A similar pattern was observed in the percent clearance of FITC-dextran. Sensitized animals treated with PBS had a significantly higher percent clearance of FITC-dextran after 24 hours ($p<0.05$; fig 8D) relative to nonsensitized controls receiving PBS. Administration of *Ll*-E or *Ll*-WT therapy was able to partially attenuate the gliadin-induced increase in FITC-dextran clearance in sensitized animals. Unlike the protective effect observed with the ^{51}Cr-EDTA probe however, the reduction in FITC-dextran clearance mediated by *Ll*-E and *Ll*-WT was not statistically significant (fig 8D).

As in the HCD4/DQ8 model, I next assessed the degree of inflammation and damage within jejunal sections from NOD/DQ8 mice. Sensitized NOD-DQ8 mice treated with PBS-glycerol had significantly higher IEL counts than their nonsensitized counterparts ($p<0.01$; fig 9A). Furthermore, sensitized animals receiving *Ll*-E treatment had significantly lower IEL count that those treated with PBS-glycerol ($p<0.001$; fig 9A). Similarly to the HCD4/DQ8s, no significant differences in villous crypt ratio were noted in the NOD-DQ8 mice (Fig 11).

Gliadin challenge elicited the production of low levels of both IgG AGA in the serum and intestinal lavage of all NOD-DQ8 mice (fig 10). Although no significant differences were noted in the titers produced by the various treatment groups, individual animals from the sensitized groups treated with PBS-glycerol or *Ll*-WT, but not *Ll*-E, were found to have particularly high levels of IgG AGA within their serum (fig 10A). The majority of NOD/DQ8 mice were found to have levels of IgG AGA that were below the limit of detection of our assay; however, three of the four sensitized mice treated with PBS-glycerol were found to have detectable levels of IgG AGA antibodies in their

intestinal lavage (fig 10B). There were levels of IgA AGA in the serum and intestinal lavages from the NOD/DQ8 mice were undetectable.

3.2 Potential Mechanisms Behind the Protective Effect of *Ll*-Elafin

3.2.1 Gliadin Challenge Does Not Impact Small Intestinal Trypsin-like or Elastase-like Proteolytic Activity

Proteolytic imbalance has been implicated in the development of several chronic gastrointestinal conditions[176]. Given the potent protease inhibitory capacity of elafin, I chose to investigate whether the protective effect of *Ll*-E could be mediated through its impact on intestinal proteases. Analysis of the trypsin- and elastase-like activity in samples from small intestinal tissue and intestinal lavage from NOD/DQ8 and HCD4/DQ8 mice did not reveal significant differences in the levels of either trypsin-like proteases following gliadin sensitization and challenge or treatment with PBS, *Ll*-WT or *Ll*-E (fig 12 A, B) while elastase-like activity was undetectable.

Stimulation of small intestinal biopsies *in vitro* with the gliadin digestion fragments 33-mer, 20-mer and 19-mer also did not evoke the production of a proteolytic imbalance. Elastase-like activity was not detected and Trypsin-like activity was not significantly affected in either the small intestinal biopsies or culture supernatant following *in vitro* stimulation with the gliadin fragments (fig. 13, A.B).

3.2.2 Ll-Elafin Maintained Zonula Occludens-1 Distribution Following Gliadin Challenge in Sensitized NOD/DQ8 Mice

Given that both the HCD4/DQ8 and NOD/DQ8 models experience a reduction in their ^{51}Cr-EDTA flux (a measure of paracellular permeability), I hypothesized that the protective effect of elafin may be mediated through a direct effect on tight junctions. This theory is supported by the findings of Q. Li *et al,* who observed that airway epithelial cells which over-expressed elafin were protected from LPS-induced suppression of ZO-1[160] as well as those of Motta et al who noted that elafin prevented the redistribution of ZO-1 immunofluorescence within their murine models of colitis[171].

Visualization of ZO-1 by immunofluorescence in the small intestine of NOD/DQ8 mice showed concentration of ZO-1 protein at the junction of the apical and lateral membranes of the enterocytes (fig 14, B-E). Staining for ZO-1 was greatly decreased in gliadin sensitized mice treated with PBS-glycerol (fig 14 A). Although some areas of their small intestinal tissue expressed ZO-1, the expression in sensitized PBS-glycerol treated mice was patchy with much of the expression being limited to the villus base and crypts. I confirmed this apparent reduction in ZO-1 staining via calculation of MFI (fig 15). Sensitized animals treated with PBS-glycerol had significantly lower ZO-1 MFI than the unsensitized controls treated with PBS-glycerol ($p<0.05$; fig 15, 14 A, D). Sensitized animals receiving *Ll*-E therapy also displayed significantly higher ZO-1 MFI than those treated with PBS-glycerol ($p<0.05$; fig 15, 14 A, C). In contrast to ZO-1 MFI, the relative genetic expression of ZO-1 in the small intestine of NOD/DQ8 mice did not vary significantly between treatment groups (fig 16).

3.2.3 Gliadin-challenged NOD/DQ8 Mice Treated with *Ll*-Elafin had Similar Microbiota Profiles to Unchallenged Mice

Given the importance of the microbiome to gastrointestinal health and elafin's known anti-microbial capacity, I hypothesized that *Ll*-E's therapeutic effects may be due to its ability to alter the intestinal microbiota.

DGGE analysis in three separate groups of littermates revealed that the small intestinal microbial composition of NOD/DQ8 mice treated with *Ll*-E was most similar to that of mice that had received no gliadin challenge (fig 17). In contrast, the microbial composition of small intestinal contents from sensitized animals treated with PBS-glycerol or *Ll*-WT showed a greater degree of divergence from the sensitized control (no gliadin challenge) (fig 17).

4. **DISCUSSION**

Proper function of the intestinal barrier is critical to maintenance of homeostasis. Intestinal barrier dysfunction has been implicated in the development of IBD[64], IBS[68-73] and celiac disease[74,75]. This breakdown in the capacity of the intestinal barrier to regulate the passage of molecules between the lumen and intestinal tissues may lead to increased uptake of harmful luminal antigens that in turn may promote disease development[82,83]. Because the principal environmental trigger of celiac disease, gluten, is known, this condition represents a powerful model to study the effects of barrier dysfunction in the development of gastrointestinal disease.

Elafin is a serine protease inhibitor produced at epithelial surfaces throughout the human body[132,139]. It has been widely studied in the lung, cardiovascular system and reproductive tracts where it appears to play an important role in the protection of the epithelium[129,132,140]. Little is currently known about the role of elafin in the gut; however, the abnormal expression of elafin in patients with IBD[179,181-183] and elafin's ability to reduce disease severity in murine models of colitis[171,184] suggests that it may be an important part of the innate defense of the gut.

In this thesis, I sought first to test the efficacy of *Ll*-E therapy in attenuating barrier dysfunction, inflammation and intestinal damage within two animal models of gluten intolerance. I then sought to explore potential mechanisms through which *Ll*-E mediates its protective effect within the small intestine. I demonstrated that *Ll*-E therapy effectively attenuates the development of a paracellular permeability defect and intraepithelial lymphocytosis following acute gliadin challenge in sensitized HCD4/DQ8 and NOD/DQ8 mice. I found that the protective effect of *Ll*-E therapy correlated with the protection of normal ZO-1 distribution at the apical junctional complex, suggesting that *Ll*-E targets pathways involved in TJ regulation. The microbiota changes associated with elafin therapy suggest a possible modulatory effect of elafin on the microbiota; however, the significance of this change in our model will require further investigation.

4.1 The Effect of Gliadin Sensitization and Challenge

Barrier dysfunction is a hallmark response to gluten exposure in both HCD4/DQ8 and NOD-DQ8 mice[82,185,188]. Increased Horseradish peroxidase (HRP) (a measure of transcellular permeability) and ^{51}Cr-EDTA flux have been shown to develop in sensitized HCD4/DQ8 mice following challenge with gliadin 3 times per week over the course of seven weeks[82,185]. Similarly, increased tissue conductance has been noted in sensitized NOD/DQ8 mice following 3 weeks on a gluten-containing diet[188]. In the experimental model used in this thesis, I examined a much more acute time period than in previous studies, with animals receiving only one gliadin challenge per week over 2 weeks. Even in this acute model, I found that gliadin sensitization and challenge triggered the development of intestinal barrier dysfunction within both HCD4/DQ8 and NOD/DQ8 mice. This increased permeability was characterized by increased ^{51}Cr-EDTA flux in both models and increased tissue conductance in the NOD/DQ8 mice. Increased intestinal permeability was also confirmed *in vivo* in sensitized NOD/DQ8 mice gavaged with ^{51}Cr-EDTA and FITC-dextran. To my knowledge, this represents the first time that the gliadin-induced barrier dysfunction measured in Ussing chambers has been confirmed *in vivo* in the NOD/DQ8 model.

Gliadin exposure had a pronounced effect on the flux of ^{51}Cr-EDTA, both *ex vivo* and *in vivo*. ^{51}Cr-EDTA is a paracellular probe, suggesting that HCD4/DQ8 and NOD/DQ8 mice develop a specific paracellular permeability defect in response to acute gliadin exposure. The development of a paracellular permeability defect in this acute model is consistent with findings surrounding the earliest stages of the response to gluten in celiac disease. Acute gluten challenge has been shown to increase paracellular permeability in celiac patients within 36 h of gluten ingestion[191]. This rapid response was also observed in intestinal biopsies from celiac patients where gliadin stimulation dramatically decreased transepithelial electrical resistance (TEER) within 15 minutes[35]. Furthermore, examination of celiac patients and their relatives have suggested that increased intestinal permeability may precede the development of overt disease in genetically susceptible individuals[74]. My findings coupled with these earlier results

support the hypothesis that increased paracellular permeability is one of the first abnormalities to develop in the pathogenesis of gluten intolerance.

Immunohistochemistry for $CD3^+$ cells revealed that sensitized HCD4/DQ8 and NOD/DQ8 mice had increased populations of IEL relative to nonsensitized controls. This finding is consistent with the previously published results in HCD4/DQ8 and NOD/DQ8 mice[185,188] and with human studies of celiac disease and GS, both of which are characterized by increases in the IEL population of the intestinal tissues[113,190]. Villus/crypt ratios in preserved jejunal segments of both HCD4/DQ8 and NOD-DQ8 mice were unchanged in sensitized animals (fig 11). Low levels of both IgG and IgA AGA were detected in the serum and intestinal lavage of NOD-DQ8 mice 18-24 hours following an acute gliadin challenge (fig 10). Although there was a tendency for higher OD IgG AGA values in sensitized mice, no significant differences were observed between the different treatment groups. The lack of atrophy in both models coupled with the low, yet detectable production of IgG AGA suggests that this experimental design captures the very early stages of disease development and may in fact represent a window into the transition from an innate to adaptive immune response triggered by gluten.

4.2 The Protective Effect of Elafin

4.2.1 Ll-*Elafin Maintains Intestinal Barrier Function*

Given the importance of the exclusion of gluten-peptides from the internal milieu in celiac disease, the intestinal barrier represents a key therapeutic target for disease prevention. One of the primary objectives of this study therefore was to determine in *Ll*-E therapy could protect the intestinal barrier of gliadin-sensitized mice following acute challenge. My results indicate that treatment of sensitized mice with *Ll*-E was able to attenuate gliadin-induced barrier dysfunction in both HCD4/DQ8 and NOD-DQ8 mice. The effect of *Ll*-E was most consistent and pronounced in measurements of ^{51}Cr-EDTA flux suggesting that *Ll*-E acts in a manner that protects the paracellular pathway in sensitized HCD4/DQ8 and NOD-DQ8 mice. These results are consistent with the findings

of Motta et al (2011) who demonstrated that Caco-2 cell monolayer transfected with an adenoviral construct encoding for elafin were protected from increased paracellular permeability triggered by TNF-α exposure *in vitro*[171].

Although the protective effect of *Ll*-E therapy on the paracellular pathway is promising, its impact on transcellular permeability remains to be elucidated. Increased transcellular permeability has been observed in HCD4/DQ8 mice following gliadin challenge 3 times a week for seven weeks[82]. Furthermore, the Verdù lab has previously shown that increased bacterial translocation in these animals occurs independently of the paracellular permeability defect[185]. Biopsies from gluten sensitive Rhesus macaques and celiac patients stimulated with gliadin *in vitro* have also shown that transcellular permeability rapidly increases in response to gliadin, is highest during active disease and is decreased following commencement of a gluten-free diet[10,192-194]. It is well established that gliadin can cross the intestinal barrier via interaction with sIgA and the transferrin receptor, although exactly when and how this mechanism develops during the course of celiac disease pathogenesis remains unknown[10,195]. Evaluation of the effect of *Ll*-E on HRP flux in both HCD4/DQ8 and NOD/DQ8 mice is important in order to establish the full therapeutic potential of *Ll*-E therapy. Furthermore, investigation of transcellular permeability both *ex vivo* and *in vivo* in acute models of gluten exposure may help to establish the relative importance of the transcellular transport of gliadin to early stages of celiac disease pathogenesis.

4.2.2 Ll-*Elafin Prevents Intraepithelial Lymphocytosis*

Gliadin sensitization and challenge was associated with increased IEL count in jejunal sections from both HCD4/DQ8 and NOD/DQ8 mice. IEL count was normalized with *Ll*-E treatment suggesting that elafin itself may modify the activation or recruitment of such immune cells in the small intestine. The capacity of elafin to affect the numbers and activity of immune cells has been demonstrated previously in the lung[169,170] and suggests a role for elafin in the modulation of the bridging of a response from innate to adaptive immunity.

Another interesting implication of *Ll*-E's ability to prevent intraepithelial lymphocytosis is its potential effect on intestinal barrier function. Homing of CD8$\alpha\beta^+$ T cells to the intestinal epithelium has recently been found to increase paracellular permeability and reduce transepithelial resistance in an *in vitro* coculture model[196,197]. These studies suggest that homing of $\alpha\beta^+$ $CD8^+$ T cells triggers the production of cytokines such as IFN-γ which in turn leads to a reduction in barrier integrity and a subsequent decrease barrier function[196,197]. This has interesting implications for celiac disease where gluten exposure leads to increased infiltration of both $\alpha\beta^+$ T cells and (to a lesser extent) $\gamma\delta^+$ T cells suggesting that IEL may play a role in increasing intestinal permeability following gluten exposure in celiac patients[198-201]. Attempts to quantify IFN-γ production by Cytokine Bead Array (CBA) (BD Biosciences, ON, Canada) in serum and intestinal homogenates from my animals detected only low levels of IFN-γ that were below the limit of detection for the assay. This suggests that increased IFN-γ production is not the primary mechanism responsible for triggering barrier dysfunction in this model but it does not exclude the potential for IEL infiltrates to contribute to the increased intestinal permeability in gluten intolerance.

Human and murine IEL subsets differ slightly in their frequency, phenotype and functionality (reviewed in 202). For example, while $\alpha\beta^+$ $CD8^+$ T cells comprise 70-80% and $\gamma\delta^+$ T cells 5-20% of all IEL in the human small intestine, they comprise 40-80% and 40-70% of the IEL population respectively in mice[202]. In addition, $CD8^+$ IEL in mice can be subdivided by their expression of homodimeric ($\alpha\alpha$) or heterodimeric ($\alpha\beta^+$) CD8[202]. This distinction also reflects a division in functionality between the two phenotypes of murine $\alpha\beta^+$ $CD8^+$ T cells[202]. While CD8$\alpha\beta^+$ T cells are conventional MHC I restricted cells, CD8$\alpha\alpha^+$ T cells are unconventional non-classical MHC I restricted[202]. This suggests that in mice, innate and adaptive immune functionalities are mediated by different cell subsets while in humans, the same IEL subset carries out both functions[202]. Given these differences in the relative proportions and functionality of different IEL subtypes in mice relative to humans, further characterization of the IEL phenotypes present in the inflammatory infiltrate of these animal models is necessary to clarify its potential

contribution to the generation of barrier dysfunction. In addition, the differences between murine and human IEL indicate that the therapeutic advantage of elafin in reducing IEL will need to be confirmed in human celiac disease.

4.3 Potential Mechanisms Explored in this Study

4.3.1 Ll-*Elafin and Proteolytic Imbalance*

Elafin has been shown to correct an endogenous proteolytic/anti-proteolytic imbalance within the gut[171,184]. Such imbalances have been identified in patients with IBD and IBS[203] where it is believed they contribute to disease pathogenesis. In IBS, it is believed that an increase in proteolytic activity may contribute to the generation of visceral hypersensitivity[203]. Currently, it is unknown whether such an imbalance between proteases and anti-proteases also affects patients with celiac disease. However, it is well established that celiac and IBS symptoms overlap[105] and recently, protease-activated receptors (PAR) have been implicated in the regulation of tight junctions in celiac disease[204]. I therefore investigated the potential that such an imbalance exists without gliadin-sensitized mice and that it may be a target for *Ll*-E therapy.

Measurement of trypsin- and elastase-like activity within the intestinal tissues and lavages did not detect alterations in the activity levels of trypsin-like or elastase-like proteases (fig.12, 13). The lack of increased trypsin-like or elastase-like activity distinguishes the murine models of gluten intolerance used in this thesis from those of colitis[171,184] and IBS[203]. Furthermore, it suggests that proteolytic imbalance does not play an important role in the development of barrier dysfunction and inflammation in early stages of gluten intolerance. Unlike the effect of elafin in models of DSS and TNBS colitis[171,184], the lack of a proteolytic imbalance also implies that *Ll*-E's therapeutic effect in HCD4/DQ8 and NOD/DQ8 mice is not dependent on correcting for increased protease activity.

Although the correction of a proteolytic imbalance does not appear to be the mechanism by which *Ll*-E exerts its protective effect in this experimental model, the

possibility that it may play a protective role in more chronic models of gluten intolerance cannot be excluded. The degree of overt inflammation in gluten-sensitized mice following acute gluten challenge was mild. In contrast, proteolytic imbalance is frequently identified in acute or chronic diseases that result in high levels of inflammation. For example, excess proteolytic activity has been identified as a contributing factor in the tissue damage observed in ARDS, COPD, asthma, pulmonary fibrosis, cystic fibrosis, atherosclerosis and IBD[135,175,203,205,206]. Given this association, it seems likely that although my results do not support a role for proteolytic imbalance in the initial pathogenesis of celiac disease, it may develop after a chronic inflammatory response has been established. Further investigation in a chronic model of gluten intolerance (~ 2 months of gliadin challenges) would potentially be better suited for the identification of proteolytic imbalance in this model. In addition, testing of luminal fluid and intestinal biopsies from both treated and active celiac and gluten sensitivity patients would provide a definitive answer as to whether or not proteolytic imbalance even exists within clinical presentations of gluten intolerance.

4.3.2 Ll-*Elafin and ZO-1: The Protection of Tight Junctions*

Given that both HCD4/DQ8 and NOD/DQ8 mice experience a reduction in their ^{51}Cr-EDTA flux, I investigated whether the protective effect of elafin was mediated through an effect on tight junctions. This theory is supported by the findings of Li *et al* (2010) who observed that airway epithelial cells which over-expressed elafin were protected from LPS-induced suppression of zonula occludins- 1 (ZO-1) a key tight junctional protein[160]. Observations made by Motta *et al* (2011) that elafin-transfected animals maintained normal ZO-1 distribution following the induction of colitis further supports this possibility[171]. Given these results, and the fact that the expression of ZO-1 is considered to be a measure of TJ intergrity[207], I chose to investigate the effects of gliadin and *Ll*-E on ZO-1 in gliadin-sensitized NOD/DQ8 mice.

Immunofluorescent staining of jejunal sections suggested that *Ll*-E therapy maintains normal ZO-1 distribution at the apical junctional complex of the epithelium (fig

14 C vs. A). This observation was further supported by quantification of the MFI (fig15). Variation in the relative genetic expression of ZO-1 was not observed following gliadin sensitization and challenge (fig 16) suggesting that these observations are the result of post-translational processes. This is consistent with observations that acute gliadin exposure in small intestinal biopsies from celiac patients on a GFD disrupted normal ZO-1 protein distribution without affecting the expression of ZO-1 mRNA[35]. Decreased expression of ZO-1 mRNA has been noted in patients with untreated celiac disease however suggesting that ZO-1 gene expression is depressed by chronic exposure to gluten[35].

ZO-1 redistribution is believed to occur as a consequence of increased zonulin signaling in patients with celiac disease. Stimulation of biopsies from celiac patients has revealed that they experience and increased and prolonged zonulin response to gluten exposure relative to biopsies from healthy controls[35]. Gliadin binding to CXCR3 has been shown to trigger the release of zonulin[97]. It has recently been demonstrated that zonulin binds to PAR on enterocytes and leads to the phosphorylation of myosin 1C and ZO-1[204]. This alters the interaction between ZO-1 and its binding partners the occludins and claudins leading to tight junction disassembly[204].

PAR are typically activated by proteolytic cleavage of their extracellular N terminus[204]. This cleavage allows the N-terminus to bind to the receptor as a tethered ligand triggering activation of PAR signaling[204]. I previously investigated the expression of PAR-1 and 2 within the small intestine of both HCD4/DQ8 and NOD/DQ8 mice. I hypothesized first that gliadin-sensitized mice would have higher expression of PAR due to the increase in PAR signaling mediated by gliadin and or excess proteases, and second that *Ll*-E treated mice would be prevent this increase in PAR . This hypothesis was disproved when qRT-PCR and immunohistochemistry revealed no notable differences in the expression or distribution of PAR-1 or PAR-2 within the jejunum of HCD4/DQ8 or NOD/DQ8 mice from the different treatment groups (data not shown). Although I did not observe alterations in PAR-1 or -2 expression within the gut, it remains possible that elafin prevents PAR activation by gliadin or exogenous proteases. One way to test this

would be to compare the relative effects of PAR-2 agonists (such as SLIGRL[single amino acid code])[208], PAR-2 antagonists (such as 5-isoxazoyl-Cha-Ile-spiro[indene-1,4′-piperidine]; GB88)[209], gliadin and *Ll*-Elafin on small intestinal permeability in HCD4/DQ8 and NOD/DQ8 mice.

Given vast array of stimuli that are known to modulate TJ, identifying the exact molecular pathway by which *Ll*-E protects ZO-1 is beyond the scope of this thesis. Finally, it is unknown how elafin may affect other proteins within tight junctional complex. Future investigations surrounding elafin's ability to protect TJ should focus therefore focus both on the expression the molecular components of the tight junction as well as the activation of the various receptors and signaling cascades implicated in the regulation of TJ.

4.3.3 Ll-*Elafin and the Small Intestinal Microbiome*

Changes in the composition of the gut microbiota have been shown to be associated with the generation of various disease states[3,4,76]. Patients with both childhood and adult onset celiac disease have been shown to have altered microbiota relative to healthy controls[76-81]. In HCD4/DQ8 mice, sensitization and gliadin challenge has also been shown to trigger a decrease in the number of *E.coli*, *Eubacteria rectale* and *Clostridium coccoides* present within the animals' intestinal microbiota[82]. Alterations in the composition of the microbiota may alter the manner in which the host's mucosal immune system interacts with the microbial residents of the gut[3,4]. Gram-negative bacteria, such as *Shigella* CBD8 and *E. coli* CBL2, isolated from celiac patients have been found to enhance the secretion of proinflammatory cytokine and thereby enhance activation of the deleterious immune response to gliadin[83]. In HCD4/DQ8 mice, it is believed that increased migration of bacteria across the intestinal barrier leads to bystander activation of immunity and an exacerbation in the response to gliadin[82].

Given the antimicrobial properties of elafin, I sought to determine if the protective effect of *Ll*-E was associated with modulation of the intestinal microbiota in NOD/DQ8 mice after gluten challenge. The microbiome is influenced by the maternal microbiota,

host genetics and by environmental factors such as diet[210-212]. In order to avoid variation in my animals introduced by environmental variation between cages (cage-effect), the protocol for this experiment was designed to evaluate overall alterations of the microbiota that occurred between treated and untreated sensitized animals that were both litter- and cage-mates (see section 2.7.3 and fig 5).

Sensitized animals that were challenged with gliadin and treated with PBS-glycerol or *Ll*-WT had microbial profiles that differed greatly from those of sensitized animals receiving no gliadin challenge (fig 17). This observation is consistent with previous findings in HCD4/DQ8 mice, which have been shown to develop alterations in their intestinal microbiota following gliadin sensitization and challenge[82]. The data also follows clinical findings, which demonstrate that untreated celiac patients have a different microbial composition from those of both treated celiacs and healthy controls[78,79,213]. Interestingly, two gliadin challenges were sufficient to induce differences in the small intestinal microbiome between groups. This has implications for celiac patients as it suggests that alterations in their microbiome may develop even with infrequent gluten-exposure. Gluten is ubiquitous in the western diet and it remains difficult for celiac patients to completely eliminate all traces of gluten from their diet – even when consuming "gluten-free" foods. Certain bacterial strains, which are more prevalent in untreated celiac disease, have been shown to be capable of accentuating the inflammatory effects of gluten on the small intestine[83]. Furthermore, bacteria are increasingly being identified as important modulators of gut function[27,33,214]. It is therefore possible that alteration of the intestinal microbiota can lead to the development of barrier dysfunction and inflammation. In light of my findings and the potential adjuvanticity of intestinal bacteria in CD[82,83], it is increasingly important that strategies are developed to limit the effect of incidental gluten exposure in celiac patients.

My results indicate that small intestinal microbial profiles of mice challenged with gliadin, but treated with *Ll*-E were more similar to those of unchallenged mice. This suggests that *Ll*-E therapy prevented alterations in the small intestinal microbiota associated with gliadin challenge. While elafin has been shown to have potent anti-

microbial activity against both Gram-positive and Gram-negative bacteria in the lung[158], it remains unclear what contribution elafin makes to the antimicrobial protection of the gut. Given the interdependence of gut function, inflammation and microbial composition, it is difficult to tease out whether the effect of *Ll*-E on the microbiota is due to the direct effect of elafin on the microbiota or if it is secondary to the preservation of barrier function or the prevention of inflammation. Further experiments to clarify how exactly elafin impacts the small intestinal microflora within this model and in other models of chronic inflammation are currently in development and may shed light on the protective capacity of elafin in the gut. These experiments will focus on *Ll*-E and elafin separately and measure their ability to inhibit the growth of small intestinal bacteria isolated from sensitized animals in culture. Additionally, I plan to measure the production of bacteriocidins produced by *Ll*-E and *Ll*-WT as these natural antimicrobials could potentially contribute to the alterations of the small intestinal microbiota observed within out animals[215]. This study should allow us to determine if *Ll*-E therapy is capable of directly modifying the microbiota and may allow us to identify exactly how these alterations are generated. In addition, further analysis with more in depth methods such as quantitative PCR and 454 pyrosequencing should confirm these results and may provide additional insight into the nature of the compositional change. Overall my results suggest that *Ll*-E therapy is one promising treatment that may help to limit the impact of potential gluten-induced changes in the microbiome of celiac patients.

4.4 Conclusions:

In summary, I have shown that acute gliadin exposure causes intestinal barrier dysfunction in the form of a paracellular permeability defect in HCD4/DQ8 and NOD/DQ8 mice. This defect is accompanied by intraepithelial lymphocytosis but not overt villous atrophy. *Ll*-E therapy was able to protect barrier function and prevent IEL lymphocytosis. *Ll*-E effect is most likely mediated through modulation of paracellular pathway and the protection of TJ structure as evidenced by preserved ZO-1 expression and possibly through the prevention of small intestinal dysbiosis. The correction of a

proteolytic imbalance is unlikely to contribute to the beneficial effects of elafin in the acute model used in this thesis.

The implications of this study are limited by its acute nature and by its focus on paracellular permeability. In order to determine the full scope of *Ll*-E therapeutic potential in gluten intolerance, this treatment should be tested in a chronic model of gluten intolerance. In addition, quantification of elafin production and expression in celiac and gluten sensitive patients (both active and on a GFD) need to be performed to determine the translational value of these results in human disease. Further investigation is also required to determine the exact mechanisms underlying *Ll*-E's effects on ZO-1 and the microbiome.

The intestinal barrier represents the body's primary defense against a variety of harmful luminal antigens found within the gut. It must balance the body's demand for the absorption of water and nutrients with the exclusion of pathogens, toxins and parasites. Intestinal barrier dysfunction is believed to play a key role in the development of gastrointestinal disease. Furthermore, barrier dysfunction is known to contribute to the genesis of disease at various other locations throughout the body. Given the importance of epithelial barrier integrity in the maintenance of health, *Ll*-E represents a promising potential therapy for the treatment, not only of CD, but also of any condition that is exacerbated by a leaky epithelial barrier.

5. APPENDIX

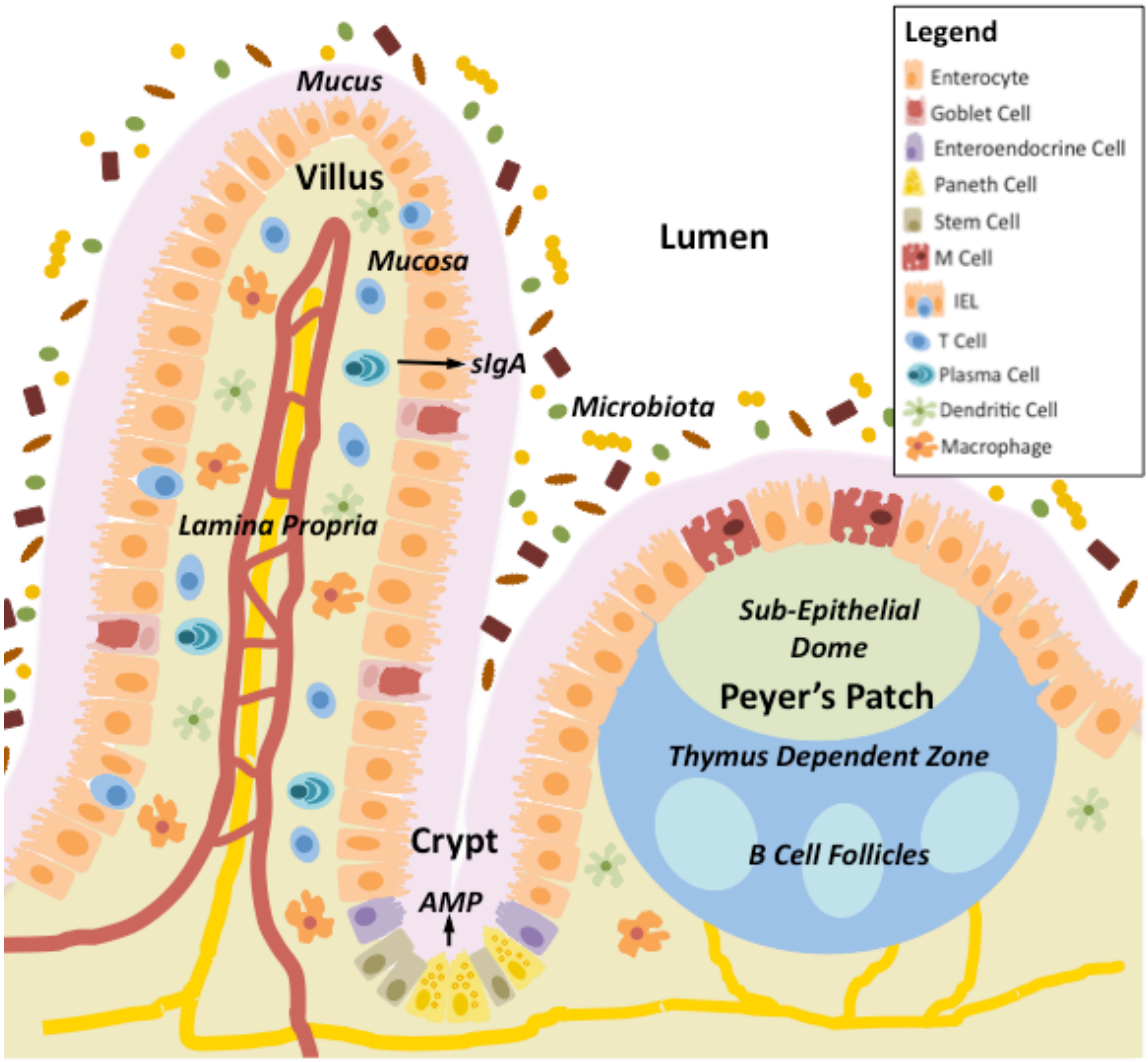

Figure 1: The Structural Organization of the Intestinal Barrier

The intestinal barrier can be organized into several distinct layers of protection that serve to limit the passage of luminal antigens into the internal milieu**.** Extracellular components of the barrier such as mucus, secretory IgA (sIgA) and anti-microbial peptides (AMP) serve as the first line of defense against luminal antigens. The cellular structure and selective permeability of the epithelium act as a second line of defense. The third and final layer of protection is generated by populations of immune cells residing within the epithelium, lamina propria or in specialized lymphoid tissues such as Peyer's patches.

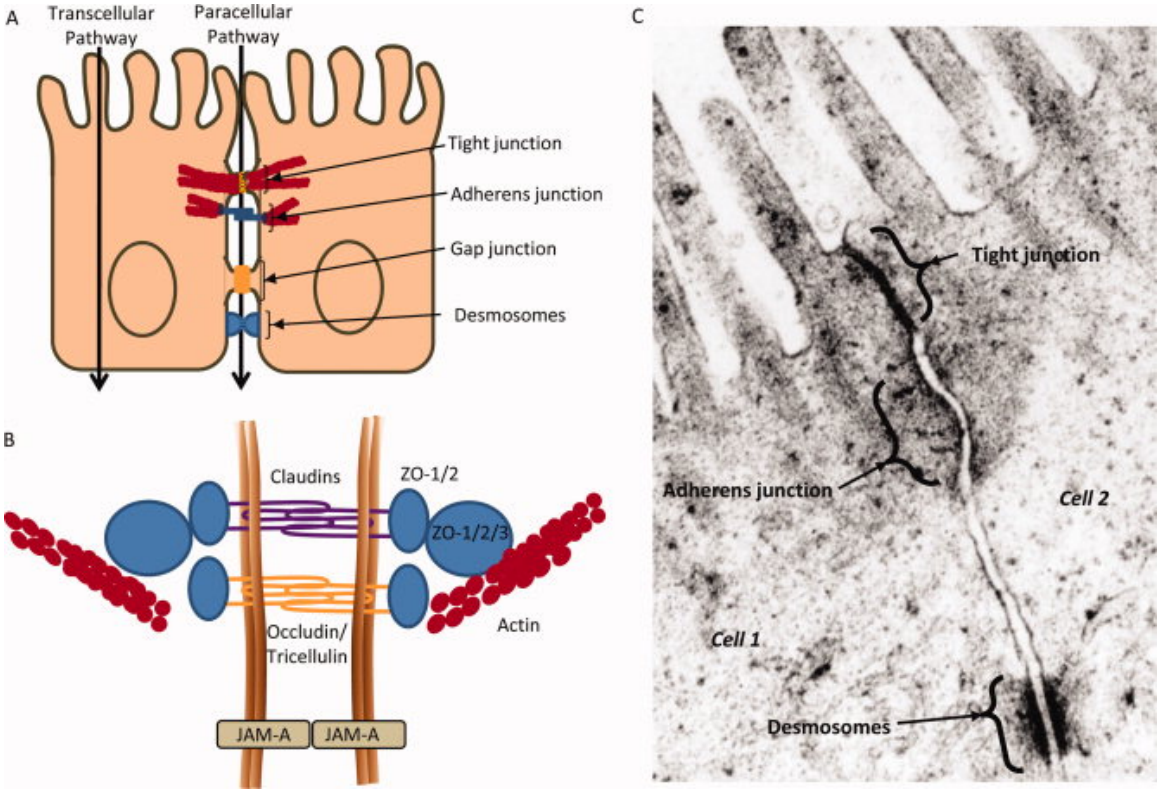

Figure 2: The Structure of Tight Junctions

Tight junctions are located at intersection of the apical and lateral membranes of adjacent epithelial cells. They are formed by the interaction of extracellular domains of integral transmembrane proteins (claudins, occludins or junctional adhesion molecules) on adjacent cells. Adaptor molecules such as ZO-1 anchor the junctional complex to the actin cytoskeleton of the epithelial cells. Tight junctions restrict the size of the paracellular gap thereby limiting the passive diffusion of large macromolecules along the paracellular pathway. Figure credit: Salim, S. Y., & Söderholm, J. D. (2011). *Inflammatory bowel diseases*, *17*(1), 362-81.]

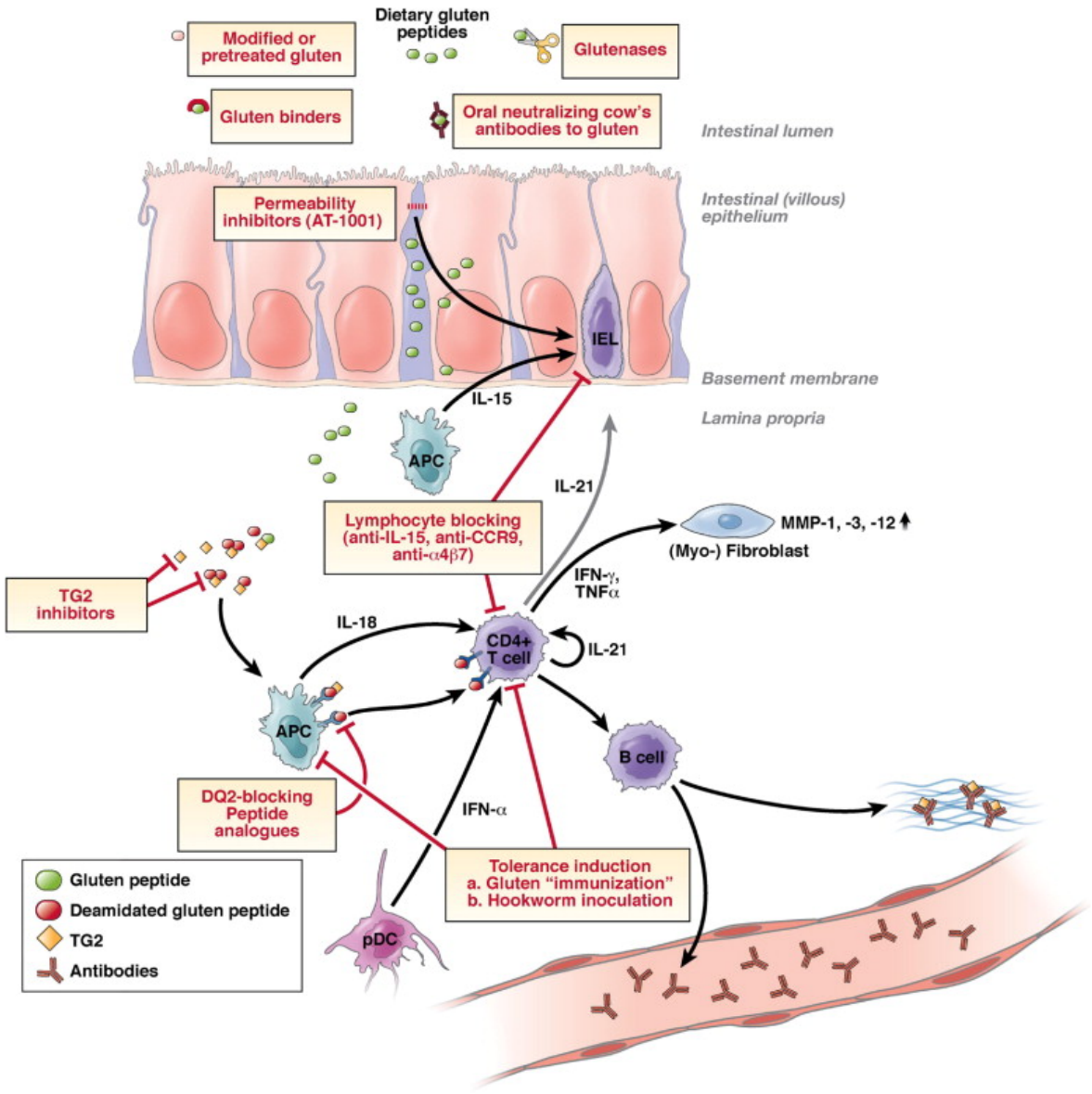

Figure 3: Investigational Therapeutic Approaches to the Treatment of Gluten Intolerance

Alternative therapies to the gluten-free diet currently focus on eliminating or sequestering gluten within the intestinal lumen, preventing the passage of gluten peptides across the intestinal barrier, preventing deamidation of gluten peptides by tissue transglutaminase-2 (TG2) or altering immune recognition of the gluten peptides. [Figure credit: Schuppan, D., Junker, Y., & Barisani, D. (2009) *Gastroenterology*, 137(6):1912-1933.]

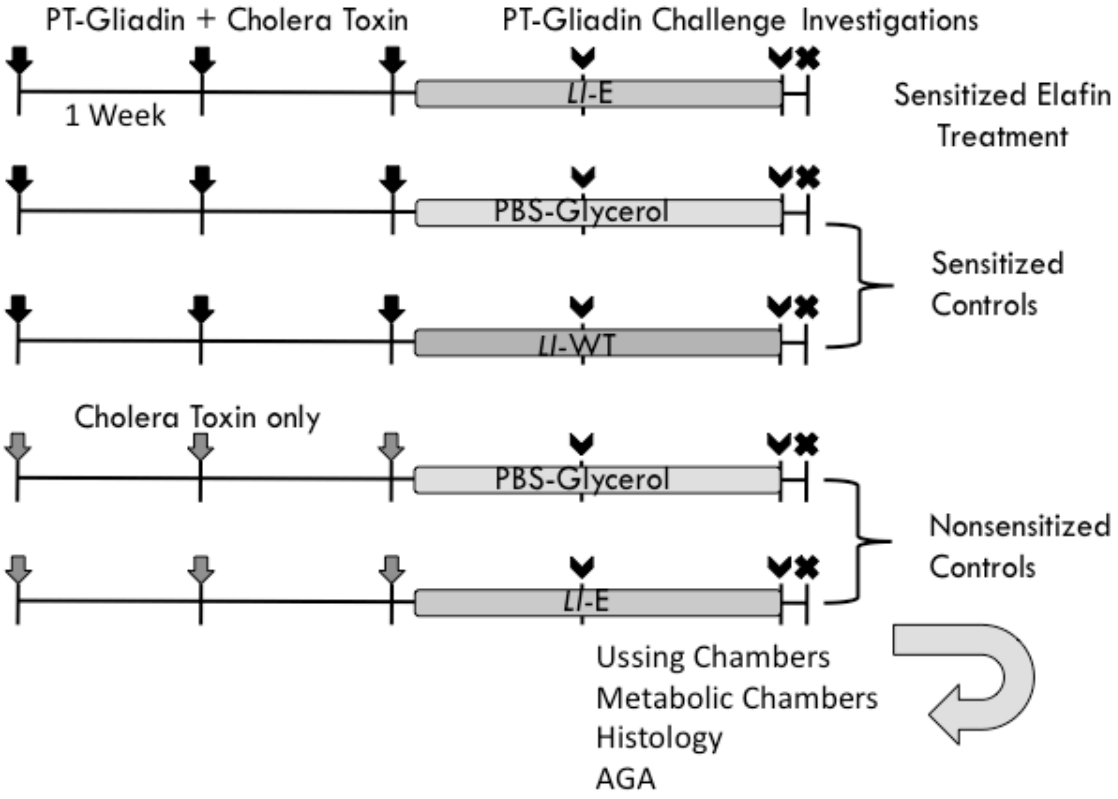

Figure 4: Experimental Design

Preparation of animals for investigation spanned a total of 31 days. Solid bars indicate 15 days of daily oral administration of PBS-glycerol, wild type *L. lactis* (*Ll*-WT) or *L. lactis* Elafin (*Ll*-E). Black arrows represent oral sensitizations with both PT-gliadin and cholera toxin, while the grey arrows represent sensitizations with cholera toxin alone. Black chevrons indicate oral PT-gliadin challenges. Black X's indicate initiation of endpoint investigations 18-24 hours following the final PT-gliadin challenge.

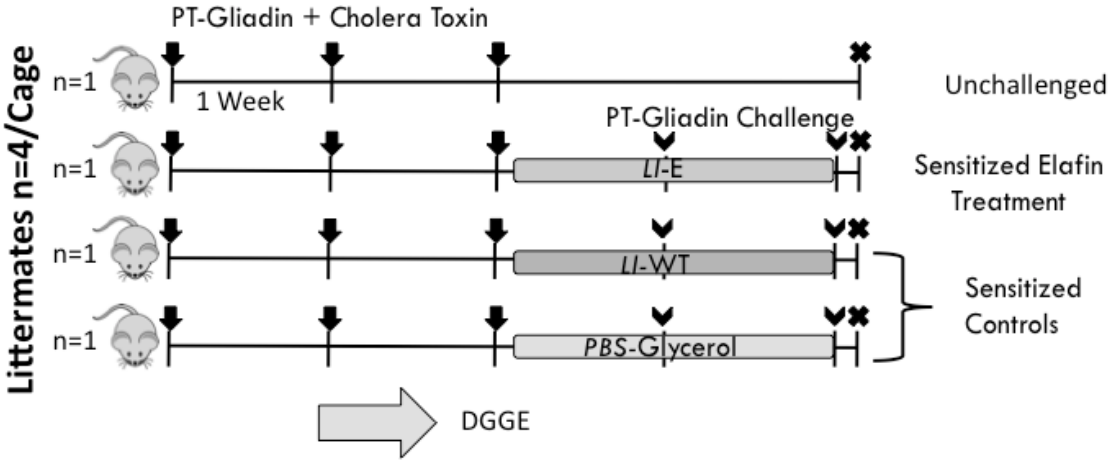

Figure 5: Experimental Design to test Effect of *Ll*-Elafin Therapy on the Intestinal Microbiota

One animal from each litter was assigned to each of the four treatment groups. Animals were housed in the same cage until commencement of 15 day treatment regime. During treatment animals were house individually to prevent coprophagy.

Preparation of animals for investigation spanned a total of 31 days. Solid bars indicate 15 days of daily oral administration of PBS-glycerol, wild type *L. lactis* (*Ll*-WT) or *L. lactis* Elafin (*Ll*-E). Black arrows represent oral sensitizations with both PT-gliadin and cholera toxin, while the grey arrows represent sensitizations with cholera toxin alone. Black chevrons indicate oral PT-gliadin challenges. Black X's indicate collection of small intestinal contents 18-24 hours following the final sensitization or PT-gliadin challenge. DGGE was used to compare the microbial composition of the small intestinal contents between animals.

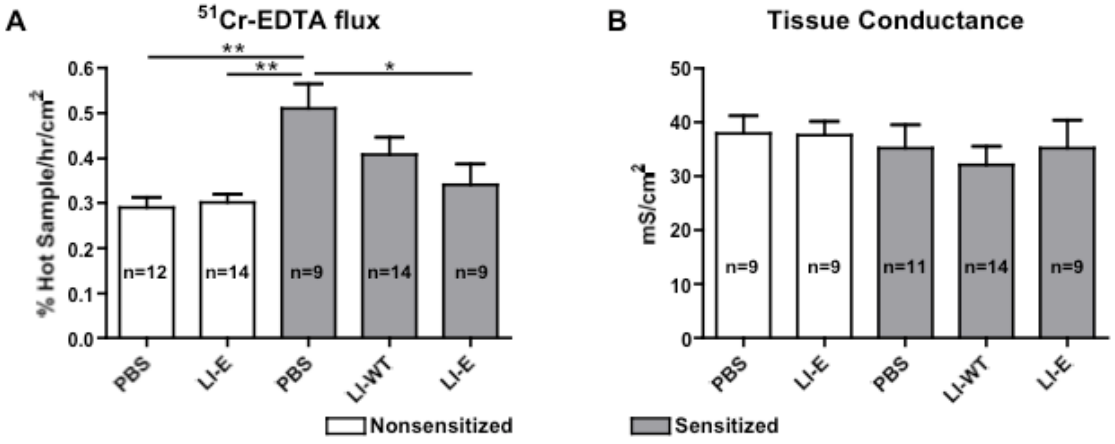

Figure 6: *Ll*-Elafin Therapy Attenuated Gliadin-induced Increase in ^{51}Cr-EDTA Flux in Gliadin-Sensitized HCD4/DQ8 Mice

Increased paracellular permeability in sensitized mice is attenuated with *Ll*-E Therapy (A). Gliadin Sensitization and challenge did not affect tissue conductance (B). Sections of small intestine were mounted in Ussing chambers. ^{51}Cr-EDTA flux (% Hot Sample/hr/cm^2) and tissue conductance (mS/cm^2) was measured 24 h after the final gliadin challenge. Data is represented as the mean ± SEM; *p* values were computed using an ANOVA with Bonferroni's post hoc test for multiple comparison

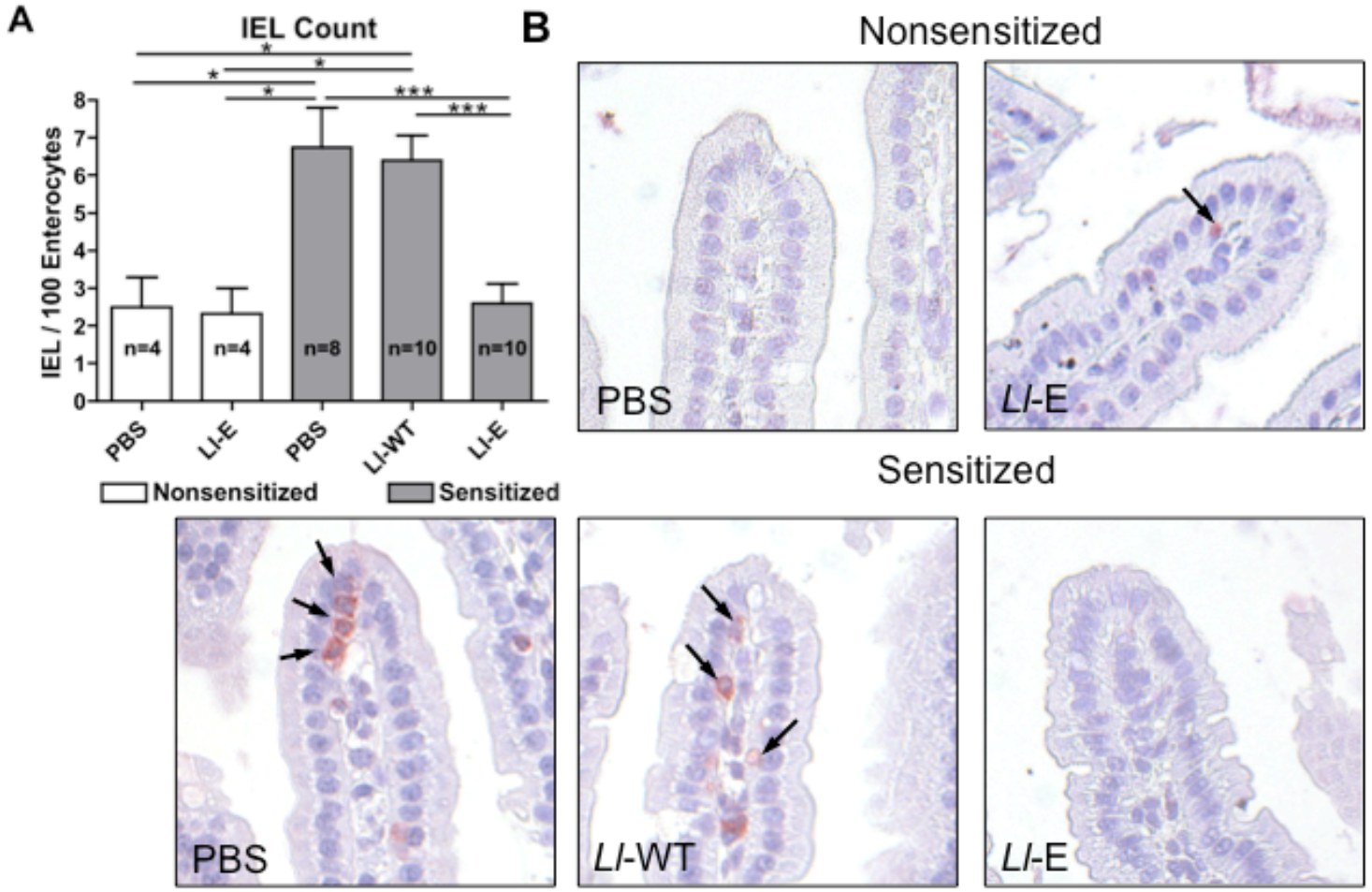

Figure 7: *Ll*–Elafin Treatment Attenuated Gliadin-induced Lymphocytosis in Sensitized HCD4/DQ8 Mice

Immunohistochemistry showing increased number of $CD3^+$ IELs in gliadin-sensitized mice and normalization with Ll-E therapy. $CD3^+$-stained sections of the proximal small intestine. Original magnification X20. Black arrows indicate IELs. Quantification of $CD3^+$ cells in villi tips, expressed as IEL per 100 enterocytes. Data is represented as the mean ± SEM; *p* values were computed using an ANOVA with Bonferroni's post hoc test for multiple comparisons.

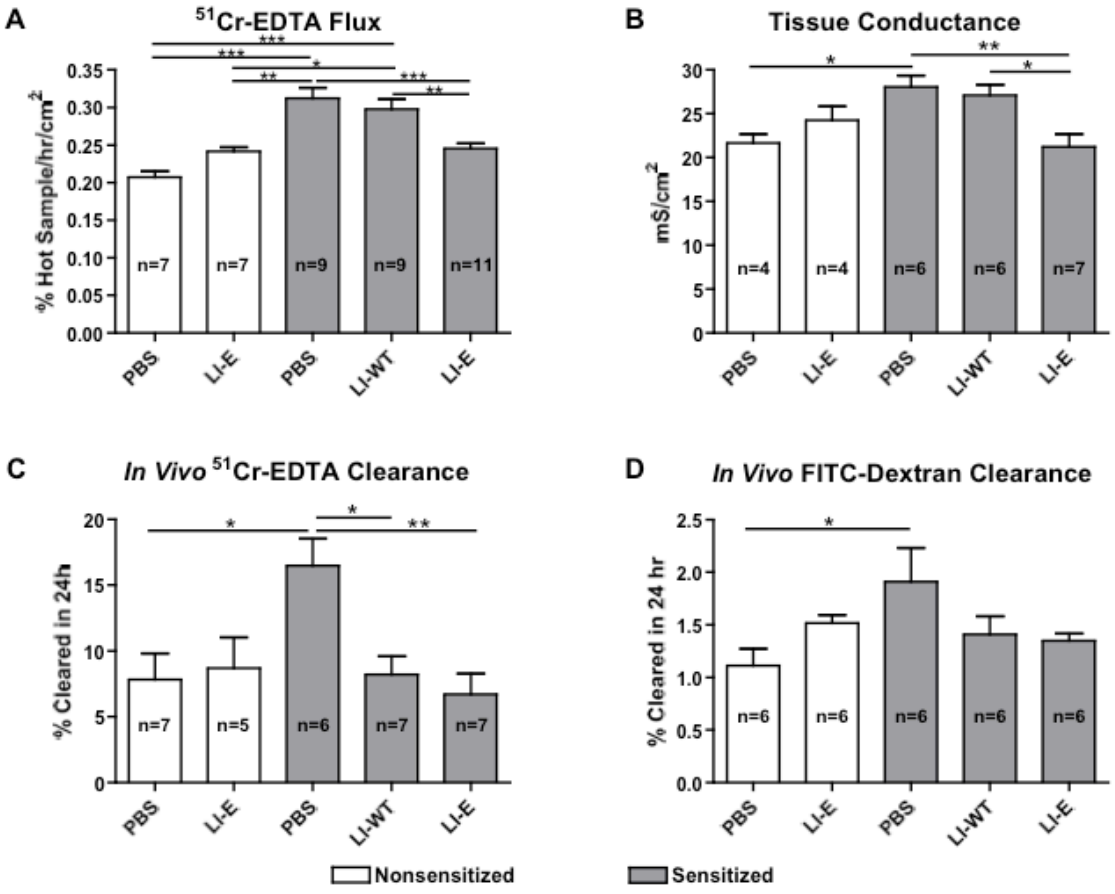

Figure 8: *Ll*-Elafin Therapy Protects Gliadin-Sensitized NOD/DQ8 Mice from Gliadin-Induced Increases in Paracellular Permeability

Gliadin sensitization and challenge increased paracellular permeability and barrier dysfunction as measured *ex vivo* (A, B). This was attenuated in sensitized animals receiving *Ll*-E therapy (A, B). Sensitized mice receiving PBS-glycerol had increased *in vivo* permeability (C, D) and ^{51}Cr-EDTA clearance was normalized in sensitized animals treated with *Ll*-E or *Ll*-WT (C). Sections of small intestine were mounted in Ussing chambers. ^{51}Cr-EDTA flux (% Hot Sample/hr/cm^2) and tissue conductance (mS/cm^2) was measured24 h after the final gliadin challenge (A, B). Animals were orally gavaged with ^{51}Cr-EDTA and FITC-dextran 24 h after the final gliadin challenge and % Clearance of the probes was measured in urine collected over a 24 h period. Data are represented as the mean ± SEM; *p* values were computed using an ANOVA with Bonferroni's post hoc test for multiple comparisons

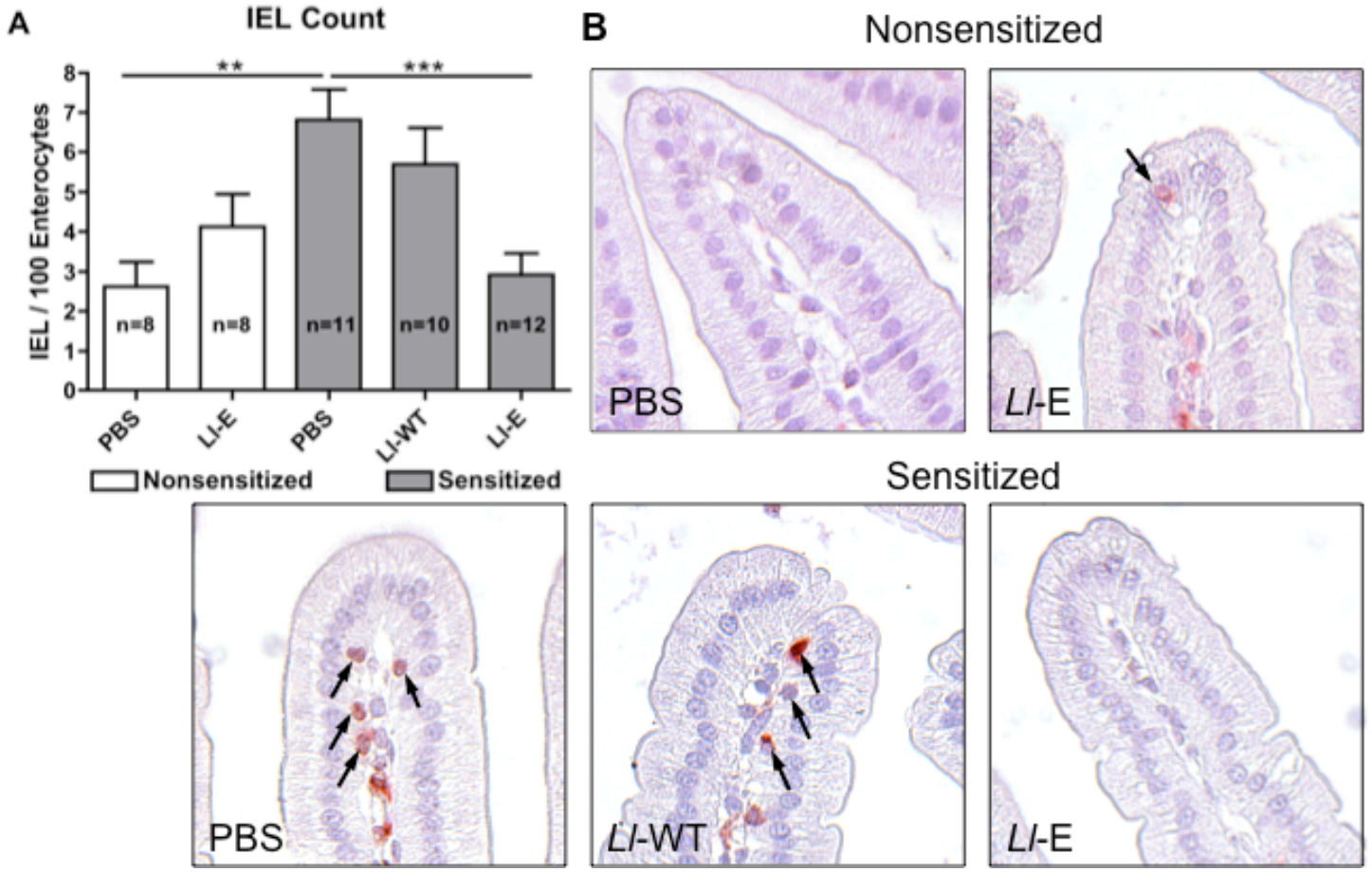

Figure 9: *Ll*–Elafin Treatment Attenuated Gliadin-induced Lymphocytosis in Sensitized NOD/DQ8 Mice

Immunohistochemistry showing increased number of $CD3^+$ IELs in gliadin-sensitized mice and normalized by Ll-E therapy. $CD3^+$-stained sections of the proximal small intestine. Original magnification X20. Black arrows indicate IELs. Quantification of $CD3^+$ cells in villi tips, expressed as IEL per 100 enterocytes. Data is represented as the mean ± SEM; *p* values were computed using an ANOVA with Bonferroni's post hoc test for multiple comparisons

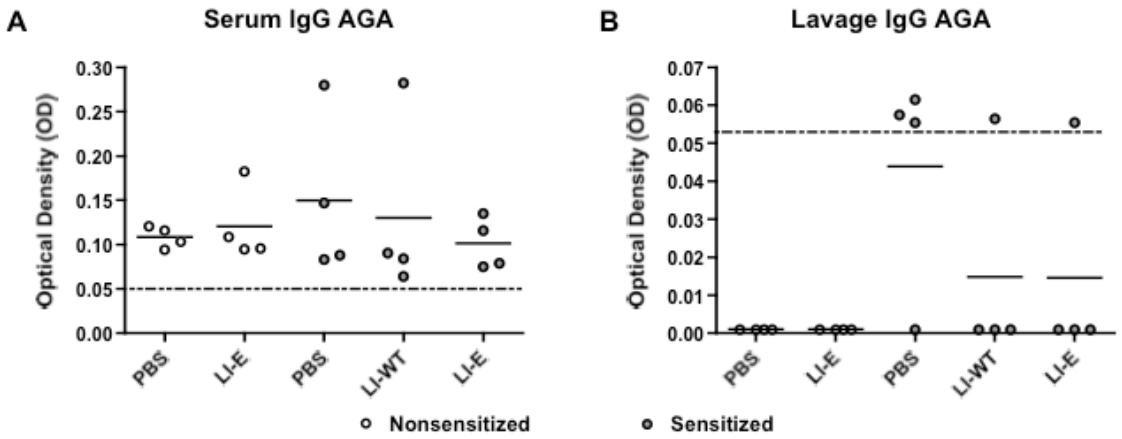

Figure 10: Acute Gliadin Challenge Elicits Low-level IgG AGA Production in NOD/DQ8 Mice

Gliadin sensitization and challenge elicit low-level production of IgG AGA in the serum of all mice (A). IgG AGA were detected in the intestinal lavage of 3 of 4 sensitized mice treated with PBS (B). Each dot represents an individual animal. Horizontal lines indicate the mean.

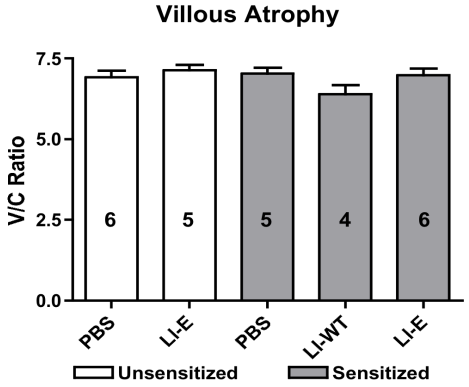

Figure 11: Acute Gliadin Challenge Did Not Induce Villous Atrophy in NOD/DQ8 Mice

Quantification of Villous Atrophy based on Villous/Crypt Ratio. No significant differences were seen in villous crypt ratio between treatment groups. Data is represented as the mean ± SEM; *p* values were computed using an ANOVA with Bonferroni's post hoc test for multiple comparisons.

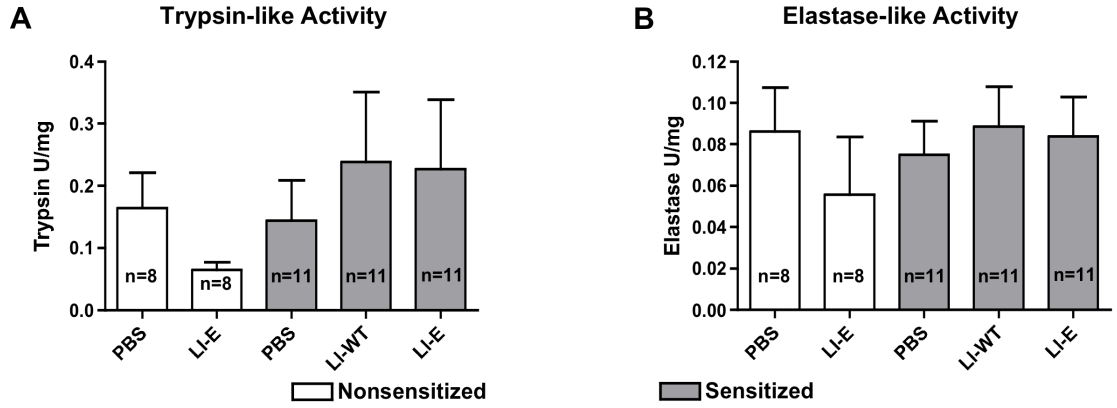

Figure 12: Gliadin Sensitization and Challenge Do Not Alter Trypsin- or Elastase-like Activity in NOD/DQ8 Mice *In Vivo*

No significant differences were seen in Trypsin- or Elastase-like activity of small intestinal tissues following gliadin sensitization and challenge (A, B). Tissues were collected 24 h after final gliadin challenge. Levels of trypsin- and elastase-like proteases were quantified in tissue homogenates and expressed as U/mg protein. Data are represented as the mean ± SEM.

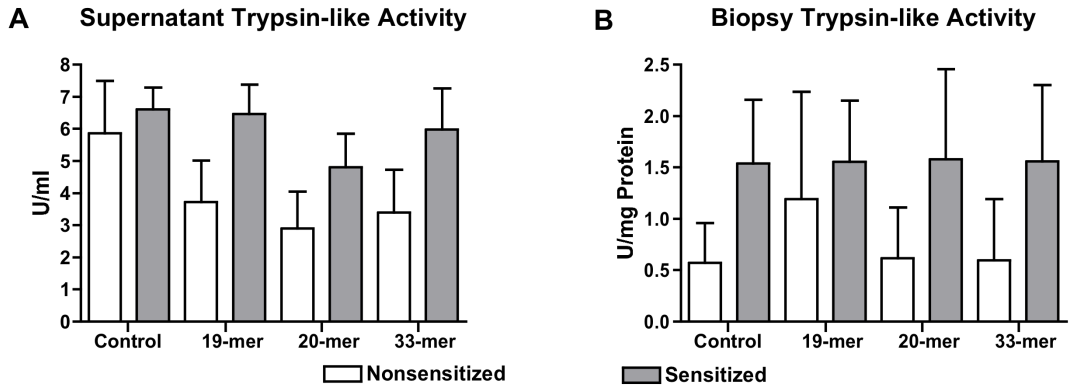

Figure 13: Gliadin Stimulation *In Vitro* Does Not Alter the Trypsin-like Activity of Small Intestinal Biopsies from NOD/DQ8 Mice

Stimulation with gliadin peptides *in vitro* failed to elicit an increase in trypsin-like activity of full thickness small intestinal biopsies from either sensitized or nonsensitized mice. Biopsies were stimulated with gliadin peptides for 3 hours *in vitro*. Trypsin-like activity was quantified in the culture supernatant (A) and biopsies (B) and expressed as U/ml and U/mg protein respectively. Data are represented as the mean ± SEM.

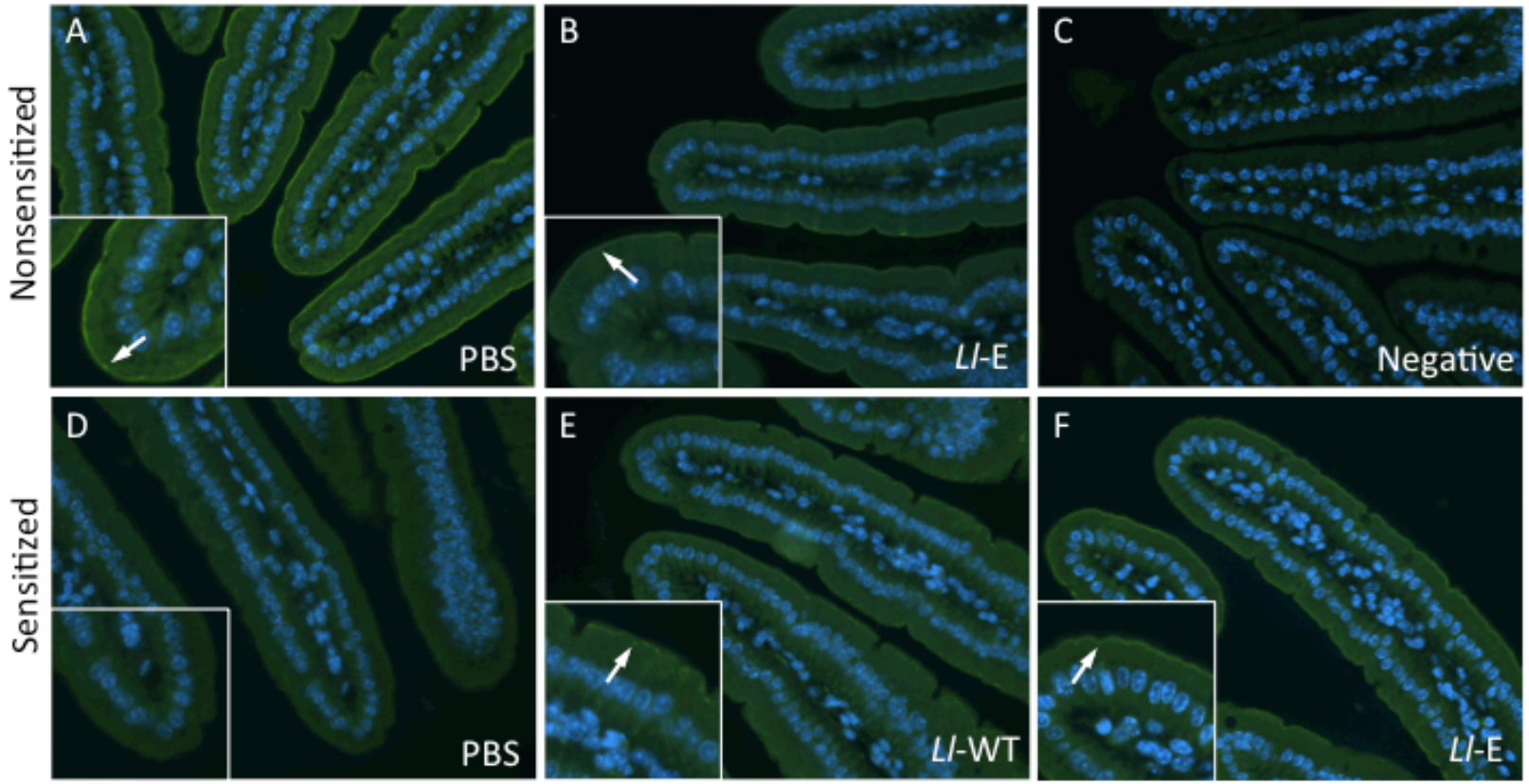

Figure 14: ***Ll*-Elafin Therapy Preserves ZO-1 Distribution Patterns at the Small Intestinal Epithelium in Sensitized NOD/DQ8 Mice Following Gliadin Challenge**

Gliadin sensitization and challenge reduced ZO-1 immunofluorescence in the small intestinal epithelium. Sensitized animals receiving *Ll*-E therapy maintained ZO-1 immunofluorescence following gliadin challenge. Original magnification X20. White arrows indicate strong immunofluorescence at the apical junctional complex.

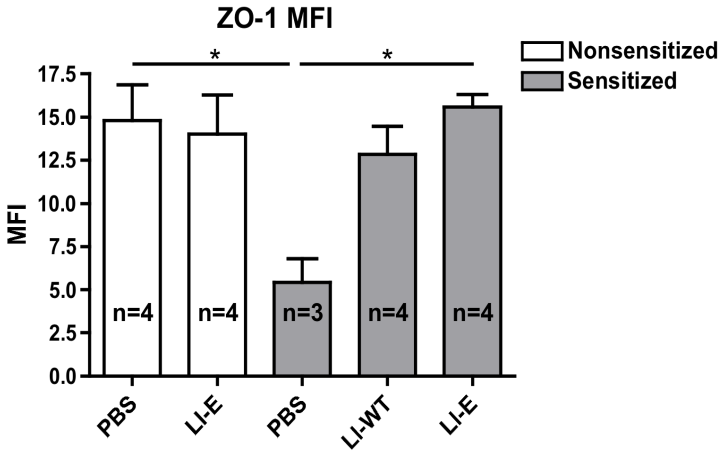

Figure 15: *Ll*-Elafin Therapy Maintains ZO-1 Protein Expression Following Gliadin-Challenge in Sensitized NOD/DQ8 Mice

Gliadin sensitization and challenge reduced ZO-1 immunofluorescence in the small intestinal epithelium. *Ll*-E therapy normalized ZO-1 immunofluorescence in gliadin-sensitized mice. Mean fluorescence intensity has been corrected for background fluorescence. Data is represented as the mean ± SEM; *p* values were computed using an ANOVA with Bonferroni's post hoc test for multiple comparison

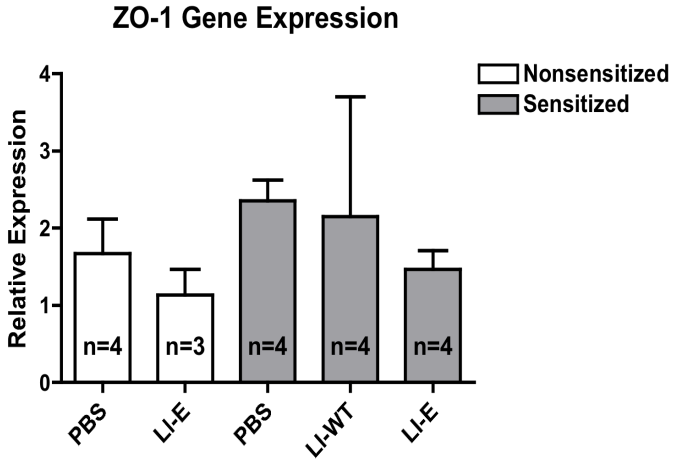

Figure 16: Gliadin Sensitization and Challenge Does Not Affect the Relative expression of the ZO-1 Gene in NOD/DQ8 Mice

The relative expression of ZO-1 compared to GAPDH was unaffected by gliadin sensitization and challenge. ZO-1 expression quantified by qRT-PCR from small intestinal tissue collected 48 hours after final gliadin challenge. Data is represented as the mean ± SEM.

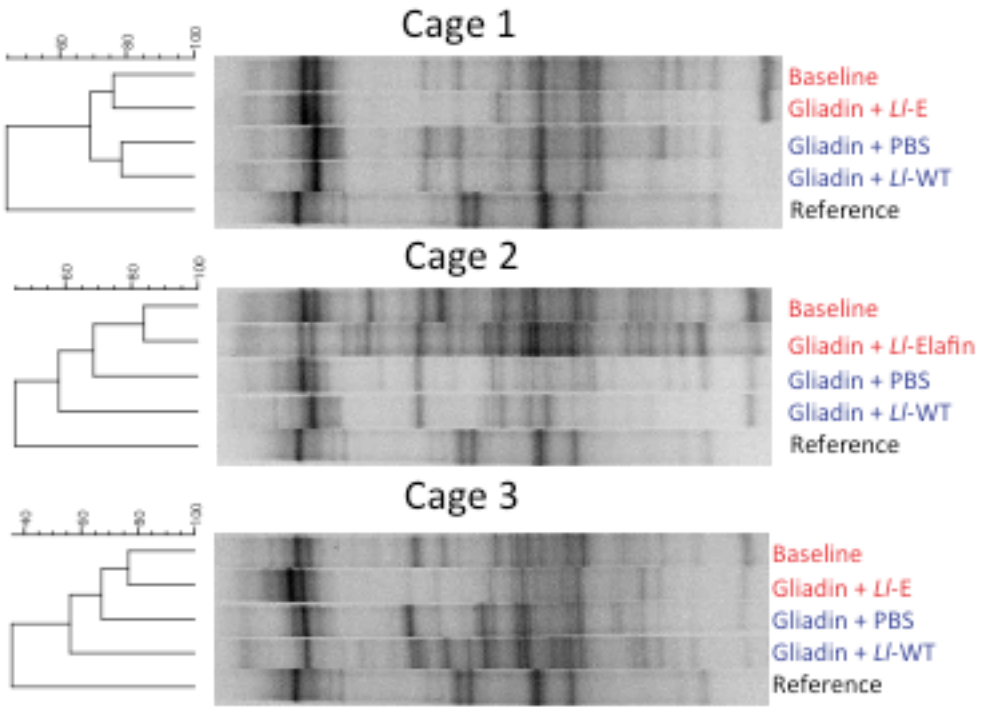

Figure 17: Small Intestinal Microbial Profiles of Gliadin-Sensitized NOD/DQ8 Mice
DGGE profiles and dendogram of small intestinal contents from 3 cages of littermates (n=4/group). Mice treated with *Ll*-E had a greater similarity index with unchallenged mice than with gluten-challenged mice treated with PBS-Glycerol or *Ll*-WT. This pattern was observed in the 3 separate cages analyzed. Each lane represents one animal. All mice were sensitized with gliadin and cholera toxin 1/week for 3 weeks. Unchallenged animals were sacrificed 24 hours after completion of sensitization regime. The remaining animals were then separated into individual cages to prevent coprophagy and treated with PBS-glycerol, *Ll*-WT or *Ll*-E for 15 days. Mice were challenged with gliadin on day 7 and day 15 of treatment then sacrificed 24 h after the final challenge. DGGE generated using universal bacterial primers HDA1-GC and HDA2.

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