CHARACTERIZATION OF A NOVEL MODEL OF GLUTEN SENSITIVITY

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LAY ABSTRACT

People with certain predisposing alleles, that consume gluten found in cereals like rye, wheat and barley, can develop a serious condition called celiac disease. The disease is characterized by damage to the lining of the upper gut and has many intestinal and non-intestinal symptoms such as anaemia, poor bone and dental health, depression, neurological problems, and infertility. Having the celiac predisposing alleles (called HLA-DQ8 and DQ2) is necessary, but not sufficient to develop celiac disease, and thus other factors have been proposed. Among those, microbes are suspected to trigger active disease in some patients. Animal models that reproduce some features of the celiac disease can help us understand mechanisms by which microbes increase risk. For this reason, I set out to characterize a novel mouse model that expresses the same human risk gene (DR3-DQ2) that is carried by the majority of celiac patients. I determined that these mice develop inflammation and blood positive tests after exposure to gluten, and reproduces some characteristics seen in patients with celiac disease. My research will allow others to use this model to study mechanisms that trigger celiac disease and test preclinically therapies adjuvant to the gluten-free diet.

ABSTRACT

The gastrointestinal tract forms the largest mucosal interface between the host and the external environment and is therefore exposed to antigens from food and microbes as well as nutrients and environmental toxins. Gut functions necessary for the survival of the host, such as digestion and nutrient absorption, must be completed while ensuring protection from pathogenic microorganisms and potential toxins. This is achieved through highly coordinated interactions between the intestinal barrier, the gut immune system, neuromotor and endocrine systems, and the gut microbiota. An example of breakdown in this balance is celiac disease (CeD), an immune mediated enteropathy that is triggered by the ingestion of gluten proteins by individuals that carry either the HLA-DQ8 or DQ2 allele. Currently, the only treatment for CeD is a strict, lifelong gluten free diet and drug development in CeD has been delayed due to a lack of preclinical models. Further, most preclinical murine models are based on the expression of the HLA-**DQ8** allele, the CeD risk allele that is carried by only 10% of patients. Therefore, the overall goal of my thesis is to characterize a model of gluten sensitivity based on the transgenic expression of the HLA-**DQ2** allele.

In **Chapter 3** of this thesis, I characterized the physiological and immunological response to gluten using transgenic DR3-DQ2 mice. I determined that following gluten sensitization and an acute challenge, DR3-DQ2 mice developed a moderate level of enteropathy, anti-tissue transglutaminase 2 and anti-gliadin antibodies, and had increased *il15* expression. In **Chapter 4** of this thesis, I explored the impact of gluten sensitization and a long-term exposure to gluten using DR3-DQ2 mice. I demonstrated that without

gluten sensitization, DR3-DQ2 mice spontaneously begin to develop anti-tissue transglutaminase antibodies, mild enteropathy, and increased paracellular permeability when exposed to gluten for a longer period of time. Together, the findings in both **Chapter 3** and **Chapter 4,** show that mice transgenic for the DR3-DQ2 haplotype mimic different responses to gluten seen in CeD patients and will be a valuable tool in preclinical CeD research in the future. These findings also show the importance of utilizing a well-defined model to study the complex interactions between the host, immune system, and microbiota in the context of CeD.

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LIST OF ABBREVIATIONS

^{51}Cr	Chromium
AGA	Anti-gliadin antibodies
AGU	Axenic gnotobiotic unit
AJ	Adherens junction
AMP	Antimicrobial peptides
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATI	Amylase trypsin inhibitor
BSA	Bovine serum albumin
Breg	Regulatory B cell
C-section	Caesarean section
CeD	Celiac disease
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein sussinimidyl ester
CFU	Colony forming units
cm	Centimetre
CT	Cholera toxin
Ctrls	Controls
d	Day
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EC	Epithelial cell
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorting
$Foxp3^{+}$	Foxhead box p3
g	Gram
GALT	Gut-associated lymphoid tissue
GC	Goblet cell
GFD	Gluten free diet
GI	Gastrointestinal
GRD	Gluten-related disorders
h	Hour
H&E	Hematoxylin and eosin
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IE-CTL	Intraepithelial cytotoxic T lymphocytes
IEC	Intestinal epithelial cells
IEL	Intraepithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cells
i.p.	Intraperitoneal
Isc	Short-circuit current
kDa	Kilodalton
Kg	Kilogram
L	Litre
LPS	Lipopolysaccharide
M	Molarity, Mol/L
M Cells	Microfold cells
mAb	Monoclonal antibody
mg	Milligram
MHC	Major histocompatibility complex
Min	Minute
mL	Millilitre
MLN	Mesenteric lymph node
mM	Millimolar
mol	Mole
N	Normality
NCGS	Non-celiac gluten sensitivity
NOD	Non-obese diabetic
NS	Non-sensitized
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween 20
PCR	Polymerase chain reaction
PT	Pepsin-trypsin
RBC	Red blood cell
RPMI	Roswell Park Memorial Institute medium
rRNA	Ribosomal ribonucleic acid
SCFA	Short chain fatty acids
SD	Standard deviation
SEM	Standard error of the mean
SIgA	Secretory IgA
SPF	Specific pathogen free
Spp	Species, plural
TCR	T-cell receptor
TG2	Tissue transglutaminase-2
Th	T helper
TJ	Tight junction
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tr1	Regulatory type 1
Tregs	Regulatory T cells
Vol	Volume
V	Volume
Wk	Week
WT	Wild-type
ZO	Zonula occludens
˚C	Degrees Celsius
α	Alpha
β	Beta
δ	Delta
γ	Gamma
μ	Micro
μA	Microampere
μg	Microgram
μL	Microlitre
μm	Micrometre

DECLARATION OF ACADEMIC ACHIEVEMENT

I, Alexandra V. Clarizio, declare that I am the sole author of this document. The work presented in Chapter 3 and Chapter 4 of this thesis was completed by myself, with technical support provided by my supervisor, other members of the laboratory, and collaborators. Writing this thesis was completed by myself, with feedback provided by Dr. Elena Verdu, Heather Galipeau, Josie Libertucci, and Julie Godbout.

CHAPTER 1: INTRODUCTION

INTRODUCTION

1.1 Structure, immunity, and function of the small intestine

The gastrointestinal (GI) tract makes up the largest mucosal interface between the external environment and the host. The primary functions of the GI tract are the chemical and mechanical digestion of food, absorption of nutrients, vitamins, and minerals; and the elimination of waste products. The GI tract also plays an essential role in forming a barrier, a general term that includes physical, immune, and functional aspects that regulate the uptake of nutrients and secretions within the GI tract (König *et al*., 2016). While its morphology changes depending on location, the intestinal barrier is always composed of a single layer of epithelial cells (ECs) linked together via tight junctions (TJs), a mucus layer on the apical surface of the ECs, and immune cells throughout the barrier (Rescigno, 2011). The small intestine is involved in the digestion of food, through chemical and physical digestion. However, it's primary function within the GI tract is in the absorption of nutrients from food leaving the stomach. This exposes the small intestine to a wide range of dietary and microbial antigens ingested with the food. Thus, the interactions between the physical, immune, and functional aspects of the small intestinal barrier are vital to ensure efficient absorption of nutrients is occurring while excluding toxins and pathogens. In contrast, the colon primarily regulates water absorption and waste elimination. While some complex carbohydrates are absorbed in the colon, the number of antigens from food the colon is exposed to is significantly lower than that in small intestine (Tortora & Derrickson, 2017). This thesis focuses on mimicking dysregulated host-dietary interactions within the small intestine using a murine model expressing the human leukocyte antigen (HLA) molecule

DR3-DQ2, necessary for the development of celiac disease, an immune-mediated food sensitivity.

1.1.1. Small intestinal anatomy and microscopy

The small intestine is characterized by long, thin villi lined by a single layer of intestinal epithelial cells (IECs) with apical microvilli to maximize the absorption of dietary components. Alongside these villi are crypts that house pluripotent stem cells that give rise to the four types of ECs that make up the small intestinal barrier and each play a distinctive role in maintaining homeostasis. These include enterocytes (absorption), goblet cells (mucus production), Paneth cells (anti-microbial peptide production), and enteroendocrine cells (hormone production) (Ménard et al., 2010). IECs are linked together through Adherens junctions (AJs), tight junctions (TJs), and desmosomes. Together, these epithelial junctional complexes help to maintain IEC polarity while regulating intestinal paracellular permeability (Turner, 2009).

1.1.2. Small intestinal barrier

The small intestinal barrier includes physical and immune components to mediate selective permeability, permitting secretion and absorption processes necessary for homeostasis. The proper maintenance of the barrier allows the small intestine to absorb nutrients from food, while preventing pathogens or toxins from moving from the lumen into the host in a dysregulated manner.

The *physical* barrier is provided by the monolayer of intestinal epithelial cells and the mucus layer. These epithelial cells and mucus layer provide the first structural defense against possible pathogens, microbial products, and dietary antigens within the lumen (Y. S. Kim & Ho, 2010). Large glycoproteins called mucins form this mucus layer, with goblet cells (GCs) within the epithelial layer secreting the majority of MUC2, the predominant gel-forming mucin within the intestinal tract. In the small intestine, the majority of GCs are found within the crypts, and the mucus makes up a single, thin, layer that is porous (Johansson & Hansson, 2016; Y. S. Kim & Ho, 2010). In contrast, within the colon the mucus forms a double layer. Under steady conditions, the inner layer is thick, free of bacteria, and contains antimicrobial peptides (AMPs). The outer layer is looser and is penetrable by bacteria. These differences ensure that nutrients can be absorbed from the small intestinal lumen, however, this leaves the small intestine more vulnerable to invasion by pathogens compared to the colon. The intestinal epithelium mediates selective permeability via two routes. Transcellular permeability allows for the movement of larger molecules across the epithelial barrier, such as amino acids, short chain fatty acids (SCFAs), sugars, and electrolytes (Groschwitz & Hogan, 2009). Movement of larger molecules through IECs can be achieved using either aqueous pores or through active transport via endocytosis. Ultimately, the transcellular pathway allows larger dietary components to be absorbed after digestion occurs in the lumen. As well, this pathway allows IECs and specialized microfold (M) cells to randomly uptake peptides or microbial antigens along the length of the intestine and release them at the basolateral membrane, where they can then interact with the immune system (Ménard *et al*., 2010). It is believed

that this sampling contributes to the development of tolerance towards both dietary antigens and commensal microbial antigens, allowing homeostasis to be maintained allowing the length of the small intestine (Perrier & Corthésy, 2011).

The paracellular pathway is regulated by cellular junctions and allows the diffusion of smaller molecules between IECs (Figure 1.1). TJs are the most apical junction between IECs and act as the rate-limiting molecule in paracellular permeability. TJs are composed of transmembrane proteins (claudin, occludin, and junctional adhesion proteins) that are connected to actomyosin via zonula occludin (ZO) proteins. Under homeostatic conditions, TJ pores allow the movement of ions and molecules less than 600 Da in size to cross the epithelial barrier via the paracellular pathway (Ménard *et al*., 2010). TJs are also dynamic structures and rearrange themselves to help protect the host from possible pathogens or epithelial stress, modifying paracellular permeability to maintain this homeostasis. Under pathophysiological conditions, paracellular permeability can increase significantly and larger molecules can indiscriminately cross the epithelial barrier through this pathway (König *et al*., 2016; Ménard *et al*., 2010).

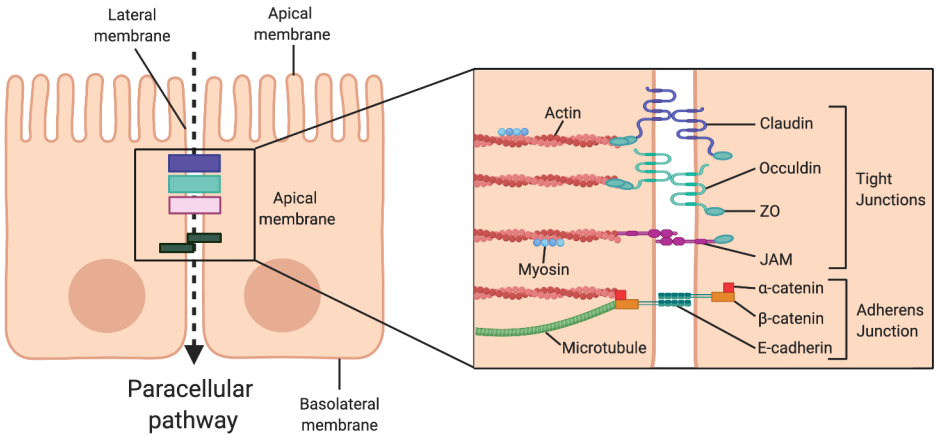


Figure 1.1 Apical junctions and paracellular permeability in the intestinal tract

Tight junctions (TJs) and adherens junctions (AJs) connect neighbouring epithelial cells to regulate paracellular permeability. TJs consist of claudin, occludin, and junctional adhesion molecules (JAM) that are anchored by ZO proteins. AJs consist of E-cadherin proteins that are anchored by α and β-cadherin proteins. Made using BioRender software.

IECs also secrete antimicrobial peptides (AMP)s, which are part of the innate immune function of the barrier, to control pathogens that may be present within the lumen. In particular, Paneth cells are granule-rich ECs found exclusively within the small intestinal crypts of most mammals and are responsible for majority of AMP secretion, although other cells in the intestinal tract can secrete them as well (Chairatana & Nolan, 2017). AMPs are believed to play two roles within the small intestine: protecting the host from pathogens that may be present within the intestinal lumen and to help shape the community of commensal microbes that colonize the gut (Clevers & Bevins, 2013). Paneth cells secrete defensins, lysozyme C, phospholipases, and REG3α (or REG3γ in mice) in response to bacteria or bacterial products. However, defensins are the most abundant family of AMPs within the GI tract and are the first chemical line of defense against a variety of pathogens (Chairatana & Nolan, 2017; Clevers & Bevins, 2013). AMPs play an important role in not

only providing the first defense against pathogens but may also provide a link between the innate and adaptive immune response within the intestinal tract. Lastly, secretory IgA (sIgA) is the most abundant antibody within the intestinal tract and a key immune component of the intestinal barrier (Mantis et al., 2011). SIgA is produced by B cells in Peyer's patches throughout the intestine after being activated by DCs. Currently, the exact signaling pathways induced by sIgA are unknown. However, it is believed that sIgA acts as a link between the innate and adaptive immune responses in the gut by helping to train the immune system to recognize pathogenic microbes while promoting tolerance of commensals (Brown et al., 2013; Mantis et al., 2011).

Collectively, the physical and immune aspects of the barrier created by the five types of epithelial cells present in the small intestine and components of the mucosal immune system play an important role in protecting the host. These aspects allow the proper absorption of nutrients and helps to shape the gut microbiota composition. The careful regulation of this barrier plays an important role in maintaining host homeostasis, and dysfunction of this intestinal barrier plays a role in various GI disorders (Y. S. Kim & Ho, 2010; König *et al*., 2016; Perrier & Corthésy, 2011).

1.1.3. Small intestinal immunity

Working in concert with the physical and immune aspects of the intestinal barrier is an underlying immune network known as the gut-associated lymphoid tissue (GALT). The GALT has a dual purpose within the small intestinal tract. First, the GALT must be able to mount an appropriate inflammatory response against pathogens that may be invading the

epithelial barrier. At the same time, the GALT plays an important role in the development and maintenance of immune tolerance towards innocuous dietary antigens and commensal microorganisms present within the lumen. The careful balance and tight regulation of these two functions ensures homeostasis is established and conserved within the small intestine (Figure 1.2)

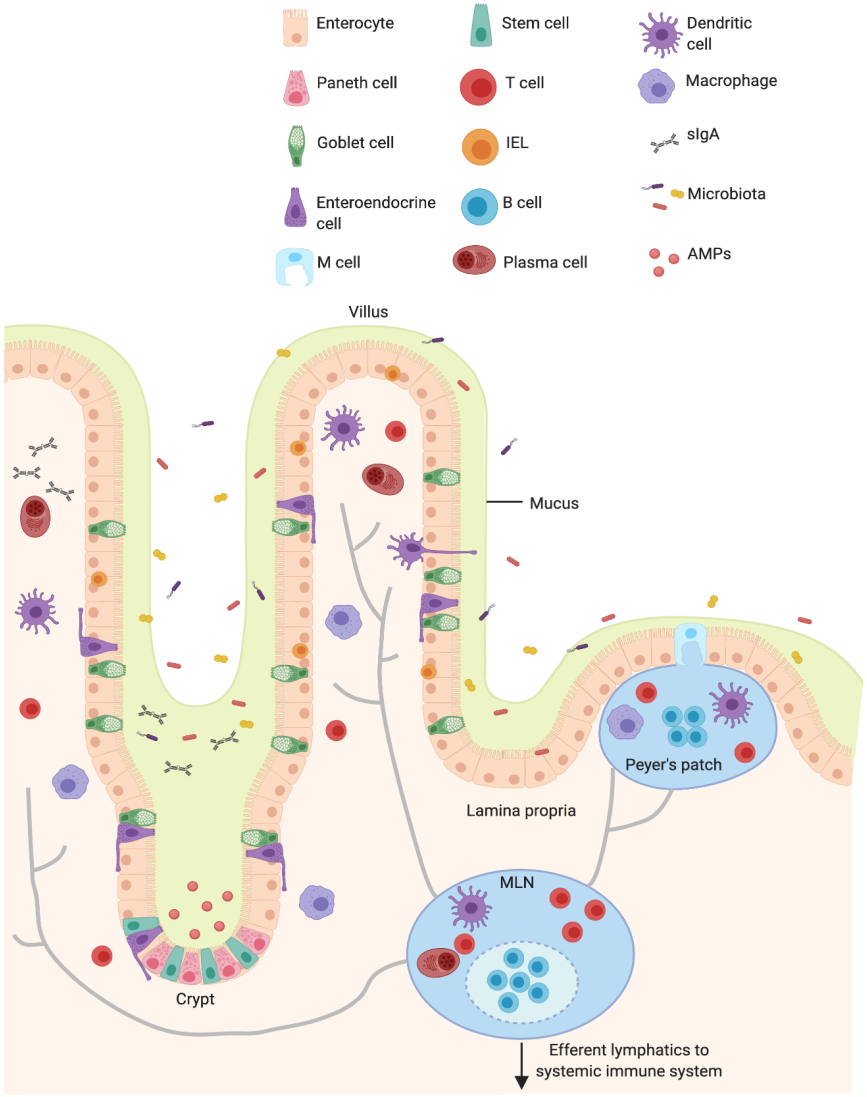


Figure 1.2 Overview of the mucosal immune system & barrier of the small intestine

The intestinal immune system is made up of effector sites (lamina propria, epithelial cells) and inductive sites (Peyer's patches and the mesenteric lymph nodes (MLN)) to create immune responses within the small intestine. The single layer of epithelial cells, chemicals released from secretory cells, and IELs and other immune cells work to provide the physical, immune, and functional properties that make up the small intestinal barrier. AMPs, antimicrobial peptides; IEL, intraepithelial lymphocyte; sIgA, secretory IgA. Made using BioRender software.

The GALT can be divided into two compartments, inductive and effector sites. Inductive sites are vital in the uptake of exogenous antigens, the transport of these antigens to antigen-presenting cells (APCs), and the generation of antigen-specific immune responses (Brandtzaeg et al., 2008). Inductive sites within the intestinal tract include Peyer's patches, isolated lymphoid follicles, and the mesenteric lymph nodes (MLN) that drain the gut tissue. Effector sites within the intestinal tract include the lamina propria and epithelial cells that make up the intestinal barrier (Pabst & Mowat, 2012).

Under steady state, the epithelium overlaying isolated lymphoid follicles and Peyer's patches contains ~10% M cells (Mabbott et al., 2013). These antigens are then passed on to underlying APCs, such as dendritic cells (DCs), which can then activate naïve T lymphocytes and begin mounting an immune response. Activated T cells can then activate B cells within the Peyer's patches or isolated lymphoid follicles to begin secreting antibodies (Macpherson *et al*., 2000). Once activated, T cells and B cells, along with DCs, can travel to effector sites through lymphatic vessels or to MLNs for further expansion of T and B cells. The MLNs then act as the last line of immune defense against potential pathogens before dissemination occurs, resulting in a systemic immune reaction (Macpherson & Uhr, 2004; Pabst & Mowat, 2012). Through the tight regulation of GALT responses, tolerance towards both commensal microbes and dietary antigens can be achieved while appropriate inflammatory responses can be mounted towards pathogens within the small intestine.

While the adaptive immune response mounts a response to specific pathogens or antigens, innate immune cells act as the first line of defense towards pathogens that may

invade the small intestine epithelium. The innate immune system refers to the cells, pathways, and functions that are fast-acting and non-specific to combat infection within the first few days of exposure to a pathogen or antigen. Being able to combat a wide range of pathogens remains vital within the small intestine, as exposure to pathogens and toxins from the lumen is consistent. To accomplish this, the innate immune system's primary functions are pathogen recognition, immune cell recruitment to sites of infection, and activation of adaptive immune responses through antigen uptake and presentation. The cell types involved in innate immunity include phagocytic cells (macrophages, neutrophils, DCs), innate leukocytes (natural killer cells, mast cells, basophils, and eosinophils), and epithelial cells. Within the small intestine, the epithelial barrier (discussed above) acts as the first line of defense against pathogens by acting as a physical block between the host and the luminal contents (Coates et al., 2018).

1.1.1.1 Intraepithelial lymphocytes

Intraepithelial lymphocytes (IELs) are a heterogenous population of specialized T cells that reside within the intestinal epithelial barrier. Due to their close proximity to the lumen and their close interaction with IECs, they provide one of the first immune responses when pathogens are detected within the barrier and therefore act as "innate-like" T cells (Abadie et al., 2012). IELs are antigen-experienced, unlike conventional naïve T cells, and majority derive from either the T cell receptor (TCR)-$\gamma\delta^+$ or TCR$\alpha\beta^+$ lineages (Table 1.1). IELs can further be divided into two major groups, induced (type A) or natural (type B), where the former develops in the periphery and the latter develops thymically (Olivares-

Villagómez & Van Kaer, 2018). Induced IELs include TCR$\alpha\beta^+$ cells, with majority of this subset being made up of TCR$\alpha\beta^+$CD8$\alpha\beta^+$ cells, which become activated upon the recognition of exogenous antigens within the peripheral lymphoid tissues. Induced IELs arise from conventional T cells that are derived in the thymus and are absent at birth, suggesting that the expansion and establishment of these IELs are microbial driven (Abadie *et al*., 2012; Olivares-Villagómez & Van Kaer, 2018). In contrast, natural IELs express either TCR$\alpha\beta^+$ or TCR$\gamma\delta^+$, but do not express the CD4 or CD8$\alpha\beta$ receptors like induced IELs. Majority of natural IELs express the CD8$\alpha\alpha$ heterodimer (Abadie *et al*., 2012). Natural IELs develop within the thymus and undergo a self-antigen-based activation process, in contrast to induced IELs. After leaving the thymus, natural IELs directly enter the intestinal epithelium. Natural IELs are present at birth, which suggests that these IELs provide protective immunity against invasive pathogens while the repertoire of induced IELs is developing (Cheroutre et al., 2011).

Under homeostatic conditions, induced and natural IELs play an important role in providing immunity against invading pathogens and in promoting tissue healing after injury to the intestinal barrier. For example, TCR$\gamma\delta^+$ IELs are critical in repairing the epithelial barrier, controlling epithelial growth, IgA production, and in clearing necrotic epithelial tissue in the intestinal tract (Cheroutre *et al*., 2011). In addition to their protective functions, TCR$\gamma\delta^+$ IELs have various cytotoxic and proinflammatory responses in order to provide immunity against a number of infections (Božić *et al*., 1998; Dalton *et al*., 2006). Similarly, the primary function of TCR$\alpha\beta^+$CD8$\alpha\beta^+$ IELs, also known as intraepithelial cytotoxic T cells (IE-CTLs), is to prevent infection through cytolytic responses against infected cells

and by producing proinflammatory cytokines to induce an inflammatory response. Interleukin (IL) 15 is a cytokine that acts as a potent proinflammatory stimulant of IE-CTL proliferation, and may contribute to excessive inflammation (Pagliari *et al*., 2013). In contrast, IE-CTLs under homeostatic conditions express the natural-killer (NK) inhibitory receptor (NKG2A) and are believed to be regulated by other intestinal cues in order to prevent abnormal inflammatory responses in the intestinal tissue (Cheroutre *et al*., 2011). The tight regulation of IELs and their responses remains integral to the overall health of the host. The abnormal activation and proliferation of cytotoxic IELs is believed to play a role in a number of GI diseases, including celiac disease (discussed further below) and inflammatory bowel disease (IBD).

Table 1.1 Major IEL subsets in mice

IEL subset	TCR phenotype	Frequency	Development	Functional role
Induced (Type A)	$TCR\alpha\beta^+CD4^+$ $CD8\alpha\alpha^+$ $CD8\alpha\alpha^-$	< 15%	"Conventional" T cell development in thymus; moves to peripheries; unknown MHC restriction	Development of oral tolerance; antimicrobial responses; protect barrier
	$TCR\alpha\beta^+CD8\alpha\beta^+$ $CD8\alpha\alpha^+$ $CD8\alpha\alpha^-$	20-30%		NKG2D-mediated cytotoxicity; pathogenic in CeD
Natural (Type B)	$TCR\alpha\beta^+$ $CD8\alpha\alpha^{+/-}$	20-50%	"Alternative" self-antigen selection; migrates directly into intestinal tissue	Anti-inflammatory responses; immune regulation
	$TCR\gamma\delta^+$ $CD8\alpha\alpha^{+/-}$	40-70%		Antimicrobial; protect gut integrity through epithelial growth and repair

CeD, celiac disease; IEL, intraepithelial lymphocyte; ILC, innate lymphoid cells; TCR, T cell receptor

1.1.4. The small intestinal microbiome

The human gastrointestinal tract is home to a diverse ecosystem of trillions of microorganisms, termed the microbiota, which we are colonized with at birth. The microbiota forms a symbiotic relationship with the host and is crucial for the proper functioning of the gastrointestinal tract. These organisms have been found to provide defense against pathogens, aid in developing the immune system, and help with the digestion of food (Lozupone et al., 2012). In early life, the microbiome's composition is highly variable and changes rapidly from birth until weaning. At two years of age, the microbiota stabilizes and resembles that of an adult (Clemente et al., 2012). The majority of gut bacteria is found in the colon, and the small intestine is colonized by a relatively low number of bacteria (~10^3 bacteria cells/gram) due to the harsh conditions within the small intestine. Despite its small population, the small intestinal microbiota plays an important role in maintaining host health. (Aidy et al., 2015).

More is known about the microbes colonizing the large intestine due to ease of collection, but a few studies have investigated the overall composition of the small intestinal microbiota. The composition of the small intestinal microbiome appears to be highly dependent on the nutrients available within the lumen and is less stable than the colon microbiome as a result (Aidy *et al*., 2015; Martinez-Guryn *et al*., 2018; Zoetendal *et al*., 2012). The dominant genera within the small intestine appear to contribute to a significant portion of carbohydrate metabolism within the lumen, indicating that these bacteria are important commensals that benefit host digestion (Aidy *et al*., 2015; Zoetendal *et al*., 2012).

Alongside their ability to aid in the digestion of food, the gut microbiome within the small intestine helps with the proper maturation of the immune system. Studies in germ-free mice highlight the importance of the gut microbiome on immune development. For example, germ-free mice have underdeveloped GALTs, including smaller Peyer's patches and MLNs, altered crypt structure, and reduced mucosal thickness. B cells in germ-free mice produce low amounts of sIgA, further disrupting barrier integrity in these animals. In addition, germ-free mice have a higher abundance of T helper (Th) 2 cells, fewer Th1 cells, and low peripheral $CD4^+$ T cells. Interestingly, once colonized with microbes, germ-free mice have a rescued Th1-Th2 balance and $CD4^+$ T cell counts, showing that the gut microbiome is necessary for proper T cell maintenance (Rooks & Garrett, 2016).

1.1.5. Development and maintenance of oral tolerance

Within the GI tract, mechanisms exist to ensure that ingested food proteins do not induce an inflammatory response to allow for proper nutrient absorption. Oral tolerance is defined as a state of immunological unresponsiveness, or a lack of proinflammatory reactions, to innocuous dietary antigens and commensals that are present in the intestinal lumen (Tordesillas & Berin, 2018; Wambre & Jeong, 2018). The development of oral tolerance is typically established within the small intestine, since majority of nutrient absorption occurs within this portion of the GI tract and there is closer contact between the immune system and commensals. The GALT plays a key role in limiting inflammatory responses to innocuous food proteins. The development and maintenance of oral tolerance relies on DCs, the induction of induced regulatory T cells (iTregs) and regulatory B cells

(Bregs), and to a lesser extent, the gut microbiota (Mauri & Menon, 2017; Tordesillas & Berin, 2018).

To begin, antigens are taken up from the lumen of the small intestine through M cells or through dendritic-like extensions of DCs. These antigens are then transferred from M cells to DCs within the lamina propria through goblet-cell-associated antigen passages (GAPs). $CD103^+$ $CXCR1^-$ DCs have tolerogenic properties and promote expansion of iTregs by migrating to MLNs via the CCR7 chemokine following uptake of an antigen. Once in the MLN, $CD103^+$ $CXCR1^-$ DCs produce TGF-β that help promote the development and expansion of iTregs (Pabst & Mowat, 2012; Tordesillas & Berin, 2018). Lastly, $CD103^+$ $CXCR1^-$ DCs produce retinoic acid through expression of RALDH2 and this retinoic acid induces the expression of α4β7 and CCK9 that home iTregs to the gut itself (Tordesillas & Berin, 2018).

The best described iTreg is the $CD25^+$ forkhead box p3 $(Foxp3)^+$ iTreg. These iTregs are essential for oral tolerance to develop (Wambre & Jeong, 2018). These iTregs are able to suppress inflammatory responses by producing the inhibitory cytokines IL-10 and TGF-β, inhibiting antigen presenting cells, preventing other effector T cell proliferation by binding to IL-2, and producing granzyme A and B to induce cytolysis of other T cells. Active $FOXP3^+$ Tregs are able to home to the gut, where they are able to expand within the lamina propria to achieve oral tolerance (Tordesillas & Berin, 2018). Bregs also play an important role in the development of oral tolerance to dietary antigens, although they make up less than 10% of the B cell population. It has been shown that Bregs help to suppress proinflammatory responses by producing IL-10, TGF-β, and IL-35. However, their exact

mechanisms of action and their complete role in promoting oral tolerance remains to be elucidated (Mauri & Menon, 2017). Lastly, the gut microbiota may also play a role in the development of oral tolerance to food antigens by helping to induce iTregs. It has been shown that SCFAs produced by bacterial metabolism may enhance DC activity in the gut and can induce Tregs and IL-10 production (Tan *et al*., 2014). Similar to Bregs, the exact mechanisms and extent of the gut microbiota's role in the development of oral tolerance to dietary proteins remains to be elucidated.

Oral tolerance is necessary to establish in order to prevent abhorrent inflammatory reactions to innocuous food proteins and commensals that are constantly present within the lumen of the GI tract. Disruptions to any of the pathways that regulate inflammatory responses within the small intestine can severely impact the health and wellbeing of the host. Indeed, small intestinal homeostasis relies on the careful regulation of the physical intestinal structure, immune responses, host factors, and the gut microbiota. The breakdown of any of these pathways can lead to dysregulation and improper functioning of the small intestine. This dysregulation may lead to the breakdown of oral tolerance towards typically innocuous antigens within the lumen, including the breakdown of tolerance towards a specific dietary antigen (termed food sensitivities). Celiac disease (CeD) is one of the most well-defined food sensitivities and exemplifies how dysregulation in all pathways contributing to intestinal homeostasis can lead to disease pathogenesis.

1.2. Celiac disease: Overview

CeD is a systemic, immune-mediated disorder that primarily causes small intestinal enteropathy upon the ingestion of gluten, a group of proteins found in wheat, barley, and rye (Verdu, Galipeau, & Jabri, 2015). Though it was previously believed to be a condition that exclusively affected small children of European descent, CeD has been found to develop at any age and has been reported around the globe (Ahadi *et al*., 2016; Parra-Medina *et al*., 2015; Singh *et al*., 2018). The prevalence of CeD around the world has risen rapidly over the past 60 years, with an approximate prevalence of 1% across Europe and North America (Leonard et al., 2017; Lionetti et al., 2015; Reilly & Green, 2012; Tack et al., 2010). "Classical", or typical, symptoms of CeD include gastrointestinal manifestations related to malabsorption, such as weight loss and diarrhea, as well as flattening of the villi within the small intestine. However, an increasing fraction of patients present with atypical symptoms, due to the multisystemic nature of CeD. These symptoms include iron-deficiency anemia, neuropathy, decreased bone density, infertility, migraines, and ataxia. A subset of CeD patients are asymptomatic or "silent" and are often diagnosed incidentally while under investigation for other autoimmune disorders or due to a first-degree relative having CeD (Green, 2005). Finally, potential CeD patients are characterized by having normal villous-crypt architecture but one positive serology test. Together, these three groups of patients represent the celiac iceberg, where the overall size of the iceberg is the total number of CeD patients. Due to the wide-range of symptoms and since CeD can develop at any point throughout life, CeD remains a challenge to diagnose clinically (Green, 2005; Lionetti *et al*., 2015).

CeD belongs to a group of gluten-related disorders (GRD), which includes wheat allergy, non-celiac gluten sensitivity (NCGS), dermatitis herpetiformis, and gluten ataxia. NCGS encompasses patients who experience symptoms upon the ingestion of foods containing gluten and whose symptoms improve when gluten is withdrawn from their diet, but who do not have the serological or villus damage seen in CeD or wheat allergy. While there has been an increased interest in exploring the differences in the etiology and pathophysiology of CeD and NCGS, gaps within the literature and confusion between the different conditions remains (Lebwohl et al., 2015). In this thesis, I explore physiological and immunological features mainly related to CeD, rather than NCGS, as the latter is only characterized symptomatically.

Currently, the diagnosis of CeD in adult patients is based on histological scoring of villous atrophy and crypt hyperplasia, as well as the presence of high levels of antibodies to tissue transglutaminse-2 (TG2), deamidated gliadin peptides (DGP), and endomysium (Rubio-Tapia et al., 2013). Most commonly, the Marsh-Oberhuber scoring system is used to assess the level of small intestinal architectural damage. Typically a score of IIIa or higher is considered high enough architectural damage for a diagnosis with CeD, though some clinicians consider a Marsh score of II to indicate CeD (Table 1.2) (Adelman *et al*., 2018; Rubio-Tapia *et al*., 2013).

Table 1.2 Marsh histological scoring in CeD

Score	Histological description
0	Normal – Villous architecture appears normal
I	Infiltrative – Normal mucosal and villous architecture; increased IEL numbers in villi
II	Hyperplastic – Similar to I. Enlarged crypts and increased crypt cell division
IIIa	Partial villous atrophy – Shortened, blunt villi; mild lymphocyte infiltration, enlarged hyperplastic crypts
IIIb	Subtotal villus atrophy – Clearly atrophic villi, enlarged crypts, significantly increased epithelial cell generation, influx of inflammatory cells
IIIc	Hypoplastic – Total villous atrophy, complete loss of villi, severe crypt hyperplasia, infiltrative inflammatory lesion

The only treatment for CeD is the complete and total removal of gluten from the diet for life, so termed a gluten-free diet (GFD). This remains a challenge for the majority of CeD and is discussed in greater detail below (Verdu *et al*., 2015).

1.3. Celiac disease pathogenesis

The development of CeD relies on the complex interaction between genetic, immunological, and environmental factors. The major susceptibility genes that contribute to CeD pathogenesis have been identified and include the HLA-DQ2 and -DQ8 alleles. While 30-40% of the general population carries these risk alleles, only about 2-4% of this group will go on to develop CeD during their lifetime. This discrepancy shows that these risk alleles are required but not sufficient to cause CeD.

CeD is a unique disorder since the main trigger is known. Gluten acts as the main trigger of CeD and is an alcohol-soluble protein complex comprising of gliadins and glutenins. Gliadin is the best studied of these proteins and is rich in proline and glutamine.

Their unique structure, and proline placement, makes them proteolytic resistant. This leaves large protein fragments in the lumen of the small intestine, including sequences that can illicit immune responses in CeD patients (Shewry et al., 2002). Throughout this thesis, gluten is used as a global term for related prolamines that contain sequences that trigger immune responses in CeD patients. Specific reference to gliadin or glutenin will be made where applicable.

In addition to gluten, unknown environmental risk factors may influence the initial development of CeD (Tack *et al*., 2010; Verdu *et al*., 2015). Variations in CeD prevalence across sex, age, and geographic location further suggests that risk factors outside of genetic background are important to disease development (Tye-Din et al., 2018).

1.3.1. Genetic risk factors

CeD remains a unique immune-mediated disorder, as the main risk alleles associated with CeD development have been identified. The genetic factors most strongly associated with celiac disease are the alleles that encode the major histocompatibility (MHC) class II molecules HLA-DQ8 and -DQ2. The HLA-DQ2 molecule is more strongly associated with CeD development. Approximately 90% of celiac patients carry the HLA-DQ2 allele, while the rest carry the HLA-DQ8 allele. Moreover, the specific isoform and dosage effect further changes the risk of a genetically individual developing CeD.

The HLA-DQ2.5 isoform of the HLA-DQ2 molecule is correlated with the highest risk of CeD development, compared to the HLA-DQ2.2 and HLA-DQ8 molecules. Throughout this thesis, for simplicity, the HLA-DQ2.5 isoform will be referred to as simply

the HLA-DQ2 allele or molecule, as it carries the highest CeD risk. Individuals that are homozygous for the DR3-DQ2 haplotype (DQ2.5/DQ2.5) or heterozygous for the DR7-DQ2 haplotype (DQ2.2/DQ2.5) express the highest levels of HLA-DQ2.5 molecules (Ploski et al., 1993; Ludvig M Sollid & Thorsby, 1993). The DR3-DQ2 and DR7-DQ2 haplotypes are associated with the highest risk of CeD. The majority of CeD patients that do not respond to a GFD and go on to develop enteropathy-associated T-cell lymphoma carry the homozygous DR3-DQ2 haplotype, with the minority carrying other genotypes (Al-Toma *et al*., 2006).

Moreover, differences in the physicochemical properties of the HLA-DQ2.5 and HLA-DQ8 MHC class II molecules themselves influences the risk associated with CeD development. In particular, the HLA-DQ2.5 molecule has a lysine amino acid at position β71, allowing for a preference for negatively charged residues such as glutamate to bind at positions P4, P6, and P7 within the binding groove. The HLA-DQ2.5 molecule also preferentially binds to positively charged residues such as proline at position P1. Together, these properties allow the HLA-DQ2.5 molecule to bind with a strong affinity to a variety of proline and glutamate rich gliadin peptides following deamidation (C. Y. Kim et al., 2004).

The HLA-DQ8 molecule is missing an aspartic acid at position β57. This change allows the P9 binding pocket in the HLA-DQ8 molecule to preferentially bind to negatively charged residues, such as glutamate. The P1 binding pocket in the HLA-DQ8 molecule also has an affinity towards negatively charged residues (Todd & Bell, 1988). Together, these properties allow the HLA-DQ8 molecule to bind to gliadin peptides with a similar affinity

as the HLA-DQ2.5 molecules. In contrast, the HLA-DQ8 molecule can only bind to a smaller range of gliadin fragments due to P1 and P9 preferentially binding to glutamate, in comparison to the wide range of gliadin fragments HLA-DQ2.5 can bind to (Abadie et al., 2011). Overall, both the HLA-DQ2.5 and HLA-DQ8 molecules play an important role in celiac disease development and pathogenesis.

1.3.2. Environmental risk factors in CeD

Approximately 30% of the general population carries either the HLA-DQ2 or -DQ8 CeD risk alleles; only about 2-4% of these individuals will go on to develop CeD in their lifetime. Because of this gap, it is now believed that external environmental factors may play a role in initially triggering the immune responses that lead to CeD development (Tack *et al*., 2010; Verdu *et al*., 2015). There has been growing interest in exploring different environmental factors that may be contributing to CeD risk, including dietary components, early life factors, and gut microbiota changes.

1.3.2.1. Feeding patterns in early life

Timing of gluten introduction has been investigated as having either a deleterious or protective effect on CeD development. It has been previously suggested that early gluten introduction (prior to 4 months of age) and late gluten introduction (after 7 months of age) increases CeD risk in infants carrying the HLA-DQ8 or -DQ2 alleles. However, recent studies and meta-analyses have found no significant association between the timing of

gluten introduction and CeD development in at-risk children (Aronsson *et al*., 2015; Lionetti *et al*., 2014; Pinto-Sánchez *et al*., 2016; Szajewska *et al*., 2016).

There appears to be an association between gluten dosage during gluten introduction and CeD development. This is best exemplified by the Swedish "celiac epidemic", where Sweden experienced a large increase in CeD incidence in children under the age of 2 between 1984 and 1996. This surge coincided with changes in infant feeding guidelines, which included an increase in the amount of gluten added to formula available for infants. This large intake of gluten was introduced during a critical time where oral tolerance towards dietary antigens is being developed, and it is believed that this high gluten dosage ultimately contributed to the surge of CeD in Sweden (Al Nabhani et al., 2019; Ivarsson et al., 2013; Cecilia Olsson et al., 2008). Further research is needed to confirm if dosing at the time of gluten introduction impacts CeD development in at-risk infants.

Recently, there has been an increasing belief that dietary components other than gluten itself may be playing a role in exacerbating CeD or triggering the innate immune system. Of significance are amylase trypsin inhibitors (ATIs), proteins found within wheat that do not belong to the gluten fraction. It has been shown that these ATIs can stimulate the innate immune system through CD14-MD2 toll-like receptor (TLR) 4 signaling (Schuppan & Zevallos, 2015). As well, a recent study has found that adding ATIs to the diet of non-obese diabetic (NOD) DQ8 mice, a model of gluten sensitivity, exacerbates wheat-induced immunopathology (Caminero *et al*., 2019). These findings suggest that other dietary components, such as ATIs, may be contributing to CeD risk and pathogenesis, but more studies are needed to determine this.

1.3.2.2. Microbial factors

There has been growing interest in the possibility that perturbations in the gut microbial community influences CeD development, as manipulation of this community may be exploited as new therapeutic targets. Studies have found that patients with active CeD have differences in their overall microbiome composition compared with healthy individuals. For example, some studies have found that adults with active CeD have a decrease in protective bacteria, such as *Bifidobacterium*. As well, these patients have an increase in Gram-negative bacteria that are believed to be proinflammatory, including *Bacteroides* and *Escherichia coli* (M. C. Collado et al., 2009; Maria Carmen Collado et al., 2008; Di Cagno et al., 2011). Other studies have found that children with CeD have high numbers of *Staphylococcus* and *Clostridium*, and decreased amounts of *Lactobacillus spp* (De Palma *et al*., 2010; Di Cagno *et al*., 2011).

Importantly, *in vivo* studies from our laboratory utilizing animal models of gluten sensitivity have begun to show the critical role the microbiota can play in influencing gluten-induced enteropathy. First, germ-free NOD-DQ8 mice developed more severe gluten-induced pathology compared with specific-pathogen free (SPF) mice, suggesting that the gut microbiota may modulate gluten-induced inflammation. NOD-DQ8 mice colonized with a microbiota devoid of opportunistic pathogens were protected from gluten-induced pathology, compared to the SPF mice (Galipeau *et al*., 2015). In addition, our group demonstrated that *Pseudomonas aeruginosa*, an opportunistic pathogen present in the small intestine of patients with CeD, partially degraded the 33-mer gluten peptide. Metabolism of this peptide by *P. aeruginosa* resulted in the release of shorter immunogenic

peptides that better translocated across the intestinal barrier in mice. In contrast, *Lactobacillus spp.* from control patients detoxified gluten peptides produced by host enzymatic processing and through *P. aeruginosa* metabolism (Caminero *et al*., 2016). These studies highlight that the presence and the composition of the small intestinal microbiota, and their metabolism of gluten, influence gluten immunogenicity and availability of gluten peptides to the lamina propria and through these mechanisms could influence the development of CeD.

There have been limited studies investigating other members of the gut microbiome community, such as fungal species or bacteriophages, and their contributions to CeD development. Further research is required to determine the exact influence these microorganisms may have on CeD pathogenesis.

1.3.3. Small intestinal permeability in CeD

Patients with active CeD have an increase in intestinal permeability, as measured by the lactulose/mannitol test. It is believed that this increase allows immunogenic gliadin peptides to reach the lamina propria without being completed degraded. These immunogenic peptides then sustain the immune responses that ultimately lead to the villous atrophy that is a hallmark of CeD (Duerksen et al., 2005; Menard et al., 2012). In animal studies, gliadin can enhance intestinal permeability by increasing the release of zonulin, a protein that was proposed to increase permeability by modulating TJ structure (König *et al*., 2016). The initial trigger that leads to an increase in intestinal permeability and allows the sustained movement of these immunogenic peptides into the lamina propria remains

controversial. Furthermore, accurately measuring intestinal permeability in patient cohorts is difficult, leading to conflicting results and poor translation from mouse and *in vitro* studies to clinical studies. For example, one study showed that most patients that remain on a GFD for over a year normalize intestinal permeability, suggesting that intestinal permeability could be secondary to active inflammation (Duerksen *et al*., 2005). However, other studies have found that CeD patients developed only a partial decrease in intestinal permeability when placed on a GFD, but their barrier structure remained dysregulated in comparison with healthy volunteers (Ciccocioppo *et al*., 2006; Schulzke, Schulzke, Fromm, & Riecken, 1995; Schulzke, Bentzel, Schulzke, Riecken, & Fromm, 1998). Genetic studies have identified several genes whose expression are associated with CeD. While these loci are less strongly associated with CeD development compared to the HLA-DQ2 or -DQ8 alleles, many of these genes are predicted to play a role in cell-cell adhesion and are important for regulating barrier function (Cardoso-Silva *et al*., 2019; Trynka *et al*., 2011). The presence of changes in the expression, or polymorphisms, in these genes may explain why some studies found that intestinal permeability does not completely recover in CeD patients on a GFD and may contribute to eventual CeD development in potential CeD patients. These studies suggest changes in intestinal permeability in some individuals may thus be conducive to inflammation, and participate in CeD development, when other important risk factors are present. Once inflammation and mucosal damage is present, further secondary damage that is functional and structural of the intestinal barrier will develop. The exact pathways of gluten peptide uptake and early events leading to initial permeability changes remain unclear.

1.3.4. The adaptive immune response in CeD

In the context of CeD, the adaptive immune response has been well characterized and is triggered by the ingestion of gluten (overview of adaptive response is shown in Figure 1.3). Both gliadin and glutenin proteins have been implicated in initiating the adaptive immune response, however, epitopes within the α-gliadin protein is preferential in activating T cells (Rostom et al., 2006; Shan et al., 2002; Shewry et al., 2002). Due to the unique placements of proline (P) residues within gliadin and glutenins, gastrointestinal digestion of these proteins is poor. The result is large, partially digested peptides that are rich in P and glutamine (Q) residues that are immunogenic to celiac patients, such as the 33-mer peptide that results from α-gliadin digestion (Shan *et al*., 2002).

Following partial digestion within the lumen, these gliadin fragments cross the small intestinal mucosal barrier via paracellular and transcellular transport (Caminero *et al*., 2016). Of these partially-digested peptides, the 33-mer fragment is highly studied and is immunogenic in the majority of CeD patients (Camarca *et al*., 2009). Once in the *lamina propria*, these peptides serve as the substrate for TG2, which converts glutamine residues to glutamic acid (E). The physiological role of TG2 has only been partly explored, and it is not understood why gliadin fragments act as a preferred substrate for this enzyme (Schuppan *et al*., 1998). Deamidation by TG2 is a crucial step in the initiation of the adaptive immune response (Tye-Din *et al*., 2018). This post-translational modification increases gliadin peptide's binding affinity to HLA-DQ8 or -DQ2 molecules present as part of the MHC class II machinery on DCs. Ultimately, DCs present these fragments to $CD4^+$

T cells, which become gliadin-specific and are HLA-DQ2 or -DQ8 restricted (Abadie *et al*., 2011). These T cells display a Th1 profile and produce a variety of cytokines such as IL-21. Importantly, these T cells produce large amounts of interferon (IFN) γ (Nilsen *et al*., 1995). Furthermore, gliadin-specific $CD4^+$ T cells are believed to help B cells produce gliadin and TG2-specific antibodies. Both of these antibodies are hallmarks of the adaptive immune response seen in CeD, and measurement of anti-TG2 antibodies in the serum remains a highly specific and sensitive test for celiac disease (Abadie et al., 2011; L. M. Sollid et al., 1997). All-together, the activation of these gliadin-specific $CD4^+$ T cells leads to inflammation of the small intestine, but for intestinal atrophy to occur, the innate immune response needs to be activated as well (Green & Cellier, 2007).

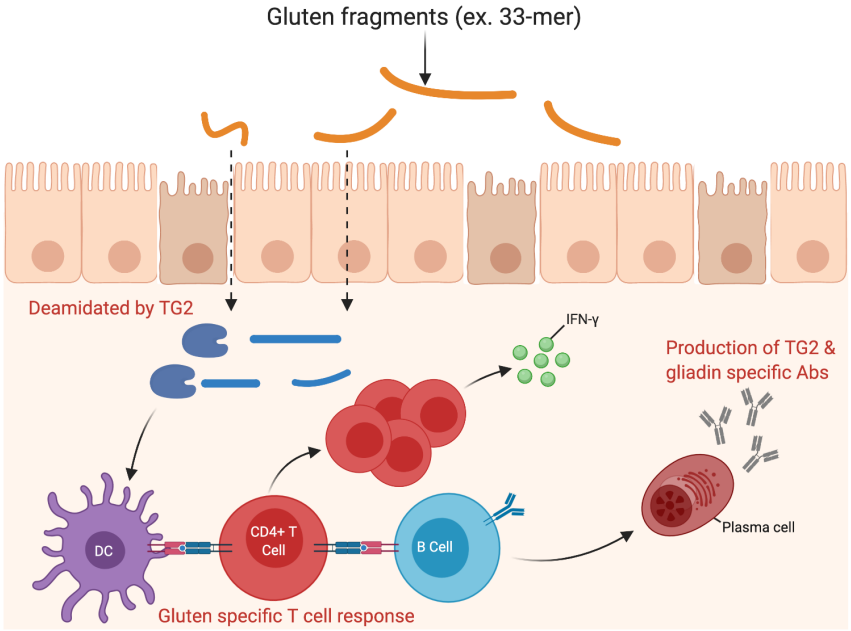


Figure 1.3 Overview of the adaptive immune response in CeD

Partially digested gluten fragments (ex. the 33-mer peptide) translocate across the small intestinal barrier, where they are deamidated by TG2. The deamidated peptides are taken up by DCs, where they are then presented to $CD4^+$ T cells via HLA-DQ2/8. This leads to specific T cell responses and the production of TG2 and gliadin antibodies. Made using BioRender software.

1.3.5. The innate immune response in CeD

The innate immune system plays a critical role in CeD pathogenesis. In contrast to the adaptive immune response, the factors inducing the innate response in CeD are highly debated (overview of innate response is shown in Figure 1.4). Previous work has shown that the gliadin peptide p31-43 may act as an innate immune system trigger, through MyD88 and type I IFN pathways. However, whether this peptide is responsible for initiating the innate immune response seen in patients with CeD remains unclear (Araya *et al.*, 2016). An increase in IL-15 is seen in most CeD patients and plays a critical role in the upregulation of IL-21, the suppression of regulatory T cells, and promoting the expansion

of cytotoxic T cells. However, the mechanisms contributing to the initial upregulation of this cytokine is not yet defined (Abadie & Jabri, 2014; Pagliari *et al*., 2013; Sarra *et al*., 2013). IL-15 has also been implicated in promoting the upregulation of MIC-A, a stress molecule produced by enterocytes. MIC-A is then recognized by the NKG2D receptor present on cytotoxic lymphocytes and induces increased epithelial apoptosis and destruction of the small intestinal barrier (Pagliari *et al*., 2013).

Proliferation and activation of IELs within the villi tips of the small intestine occurs before villous atrophy is seen in CeD patients and is a key component of the innate immune response (Järvinen *et al*., 2003). As previously discussed, under homeostatic conditions, IELs are tightly regulated to ensure that abhorrent inflammation does not occur in the small intestine. In CeD, this regulation is lost and TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ IEL subsets rapidly increase (Abadie *et al*., 2012; Kutlu *et al*., 1993). The initial trigger for the induction of these IELs are unknown. IL-15 plays a critical role in promoting the upregulation of NKG2D on TCR$\alpha\beta^+$CD8$\alpha\beta^+$ IELs, which contributes to epithelial apoptosis, villous destruction, and refractory CeD in a small subset of patients. Interestingly, CeD patients on a GFD have a decrease in TCR$\alpha\beta^+$ levels while TCR$\gamma\delta^+$ remain slightly elevated, suggesting that these IELs may be promoting epithelial repair (Järvinen *et al*., 2003).

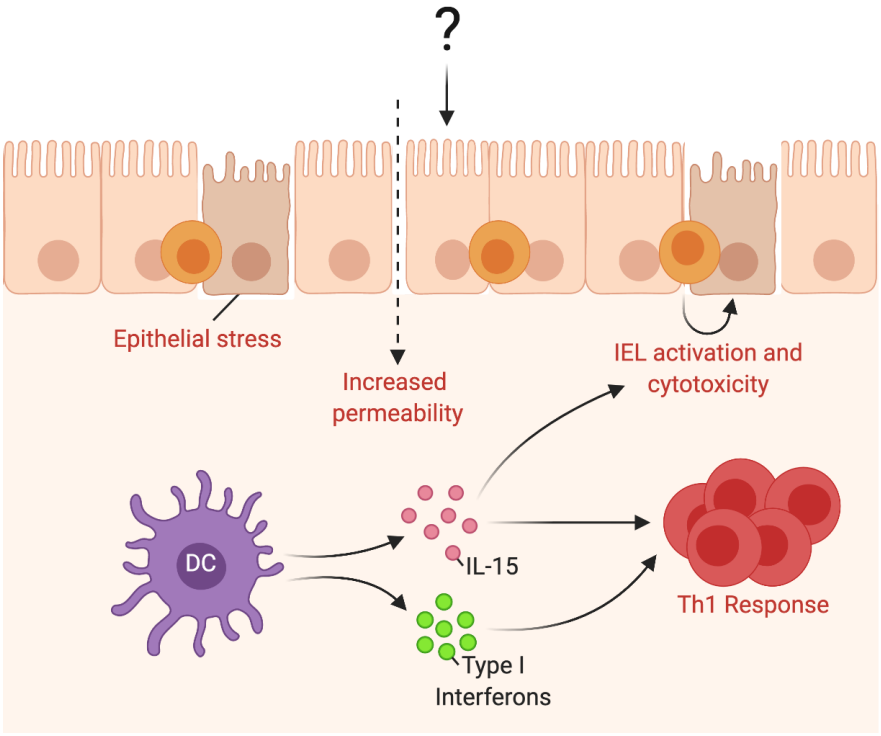


Figure 1.4 Overview of the innate immune response in CeD

Unlike the adaptive immune response, the initial trigger of the innate immune response in CeD is unknown. The innate immune response is characterized by an increase in intestinal permeability and epithelial stress. IL-15 and type I interferons are upregulated in the mucosa. IL-15 contributes to an increase in IEL numbers and cytotoxicity. Both IL-15 and type I interferons promote a Th1 response. Made using BioRender software.

1.4. Current treatment

The only treatment available for CeD is a strict, life-long adherence to a GFD that resolves mucosal damage and helps to relieve symptoms. However, adherence to the GFD and complete removal of gluten is difficult for many patients. Gluten-free foods remain over twice as expensive as their gluten-containing counterparts, creating a high financial cost on patients. Scarce availability of gluten-free products in supermarkets places another burden on individuals with CeD (A. R. Lee et al., 2007). Beyond financial burdens, CeD patients report a poorer quality of life, increased anxiety when eating out, and social isolation because of their diet. This social burden decreases willingness to adhere to the

GFD, particularly in adolescent patients (Ciacci & Zingone, 2015; C. Olsson et al., 2008; White et al., 2016). Finally, even patients that strictly follow a GFD to the best of their abilities report feeling a burden-level similar to that of patients with end-stage renal disease (Shah *et al*., 2014). A GFD is important for CeD patients to follow, as failure to adhere to one can increase the risk of severe complications (Rubio-Tapia *et al*., 2013). The financial and social burdens of the GFD highlight the importance of finding alternative therapies that can be used in conjunction with this diet to aid CeD patients.

There has been an increase in interest from pharmacological companies to become involved in CeD drug development. These novel drugs target various steps in CeD pathogenesis, including intraluminal therapies, therapies targeted at restoring gluten tolerance, and various biologics. Unfortunately, for many of these therapies, there has been little evidence showing positive outcomes in patients during clinical trials and a lack of adequate preclinical animal models has further hampered CeD drug development (Wungjiranirun et al., 2016).

1.5. Current animal models used in celiac disease research

For many years, animal modelling in CeD was elusive. Over the past 30 years, a variety of different animal models have been proposed and utilized in the study of CeD and other GRD conditions (Table 1.3). Gluten-dependent enteropathy has been described in animals outside of humans, including Irish Setters, Rhesus macaque monkeys, and some horses. The enteropathy seen in these animals is believed to be MHC class II independent, unlike in humans. Furthermore, using these animals within a laboratory is difficult and expensive,

making them poor models to study CeD (Korneychuk et al., 2015). Due to this, a number of different mouse models have been developed in order to study CeD development and to be used in preclinical drug testing.

Table 1.3 Animal models to study celiac disease

Animal	Model	Protocol	Histology	IELs	Antibodies		References
					AGA	Anti-TG2	
Dog	Irish Setter (no MHC II)	Spontaneous development	Partial villous atrophy	ND	+	ND	Batt *et al*. 1984; Hall & Batt, 1992; Polvi *et al*., 1998
Monkey	Rhesus macaque (MHC II unknown)	Spontaneous development	Partial villous atrophy	ND	+	+	Bethune *et al*, 2008; Sestak *et al*., 2011
Horse	MHC II unknown	Spontaneous development	Partial villous atrophy	ND	+	+	Van der Kolk *et al*., 2012
Germ-free rats	AVN Wistar	Gliadin gavage following birth	V/C reduction	Increase in IELs	-	-	Štepánková *et al*., 1996
Mouse	Balb/c	Gliadin/CFA i.p. then gluten containing diet	Normal	No change	-	-	Papista *et al*., 2012
	Rag$^{-/-}$	Transfer of T cells from C57Bl/6 to *Rag*$^{-/-}$	Partial villous atrophy	No change	-	-	Frietag *et al*. 2009
	HLA-DQ8	Gluten sensitization (i.p) + challenge	V/C reduction	Increase in IELs	+	-	Verdu *et al*., 2008
	HCD4-DQ8	Gluten sensitization (i.p.)	No atrophy	ND	+	-	Black *et al.*, 2002
	NOD-DQ8*	Oral gluten sensitization + challenge	V/C reduction	Increase in IELs	+	-	Galipeau *et al*., 2011
	HCD4-DR3-DQ2	Gluten sensitization (i.p. or s.c.) + gluten diet	No atrophy	No change	+	-	De Kauwe, *et al*., 2009
	DQ8-D^{d}-villin-IL15	Gluten exposure	V/C reduction	Increase in IELs	+	-	Abadie *et al*., 2020

* These mice also develop blistering similar to dermatitis herpetiformis following gliadin sensitization and challenge (Marietta *et al.,* 2004).

AGA, anti-gliadin antibodies; CFA, complete Freund's adjuvant; IEL, intraepithelial lymphocytes; i.p., intraperitoneal injection; MHC, major histocompatibility complex; ND, not determined; TG2, tissue transglutaminase-2

Previously, an $AB^{0/0}$ DQ8 transgenic mouse model of gluten sensitivity was developed. These mice do not express endogenous MHC class II molecules, ensuring that this model only expresses the human HLA-DQ8 molecules. In one investigation, these HLA-DQ8 transgenic mice also expressed human $CD4^+$ T cells (HCD4/DQ8 model). They found that these HCD4/DQ8 mice develop a strong, gliadin-specific T-cell response following sensitization to gliadin using intraperitonial (i.p) injection (Black et al., 2002). Building off of this study, mice transgenic only for the HLA-DQ8 allele have been found to develop anti-gliadin antibodies, a moderate increase in $CD3^+$ IELs, and an increase in ion transport across the intestinal barrier following sensitization and challenge (Verdu *et al.*, 2008). However, a major drawback of this mouse model is there are no changes to villus-crypt ratios and there is no development of anti-TG2 antibodies. (Black *et al*., 2002; Verdu *et al*., 2008)

Alongside the $AB^{0/0}$ DQ8 model, the transgenic NOD/DQ8 mouse model was developed and previously characterized in our laboratory. Similar to the $AB^{0/0}$ DQ8 mice, the NOD/DQ8 mice do not express endogenous MHC class II molecules. However, their non-obese diabetic background allows for an increased risk of autoimmunity and have been previously used to study type I diabetes pathogenesis. When sensitized and challenged with gluten, the NOD/DQ8 mice develop a gliadin-specific T-cell response, anti-gliadin antibodies, a mild increase in $CD3^+$ IELs, a moderate decrease in villus-crypt ratios, and changes in intestinal permeability (Galipeau *et al*., 2011). In another sample of NOD/DQ8 mice, Galipeau et al. (2011) also show that administration of anti-CD25 monoclonal antibodies before gliadin-sensitization induced the partial depletion of $CD25^+Foxp3^+$ T

cells. The loss of these Tregs in the NOD/DQ8 model ultimately led to the development of severe insulitis as well as gluten-induced immunopathology, suggesting that these mice could represent a model to study both CeD and type 1 diabetes. Another group found that NOD/DQ8 mice that were sensitized to gliadin and then continually exposed to gluten for a period of two to five months developed blistering similar to that seen in dermatitis herpetiformis (Marietta *et al.,* 2004). While the NOD/DQ8 mouse model has been integral to understanding celiac disease pathogenesis and management, there are two major drawbacks to utilizing this model. First, the NOD/DQ8 mouse does not develop anti-TG2 antibodies, like the $AB^{0/0}$ DQ8 model discussed above. Secondly, these mice have impaired IL-15 secretion and cannot recapitulate all aspects of the innate immune response seen in CeD patients.(Galipeau et al., 2011; Marietta et al., 2004; Suwanai et al., 2010)

Most recently, a mouse model of gluten sensitivity based on the transgenic expression of the HLA-DQ8 molecule and the overexpression of IL-15 in the lamina propria and epithelium has been described. These mice develop partial villous atrophy, anti-gliadin antibodies, anti-DGP antibodies, an increase in *Ifng* expression, increased IEL counts, and an increase in $CD4^+$ T cell proliferation (Abadie *et al*., 2020). Despite these features, the triple transgenic mouse model has limitations that restricts its use in a laboratory setting. First, these mice do not develop anti-TG2 antibodies after being put on a gluten-containing diet. Anti-TG2 antibodies remain the most specific and sensitive measurement in CeD patients and it is a major limitation that this model does not develop these antibodies. Similar to the $AB^{0/0}$ DQ8 model and the NOD/DQ8 model, these mice represent only a small fraction of CeD patients and cannot be used to study DQ2 specific

CeD immunopathology. Lastly, this model relies on the overexpression of IL-15 throughout the epithelium and lamina propria in the small intestine. IL-15 is a key component of CeD pathogenesis, however, not all active CeD patients show an overexpression of IL-15, and CeD patients on a GFD continue to have an increased expression of IL-15 despite having normalized small intestinal architecture (Abadie & Jabri, 2014). It also remains unclear if an overexpression of IL-15 is a cause of CeD or if this overexpression is an effect of the innate immune response. Because of these limitations, the triple-transgenic mouse model is a model that can be used to specifically study the role of IL-15 in CeD but has limited laboratory applications beyond this.

Currently, there is one humanized mouse model that represents CeD patients carrying the HLA-DQ2 allele. As with the other humanized mouse models, these mice do not express endogenous MHC class II molecules and instead express the DR3-DQ2 haplotype. These mice were developed to evaluate the preclinical effect of a vaccine based on the 3 main gluten epitopes that HLA-DQ2 positive CeD patients react to. This model was evaluated based on the double transgenic expression of the DR3-DQ2 haplotype and carrying expressing human $CD4^+$ T cells. While these mice did not develop gluten-induced atrophy after being sensitized to gluten using an i.p. injection, the authors did see an increase in gluten-specific $CD4^+$ T cells (de Kauwe et al., 2009). However, this model was never carefully evaluated immunologically and physiologically in response to different protocols of gluten exposure following mucosal sensitization to gliadin, as described in **Chapters 3** and **Chapters 4**.

CHAPTER 2: THESIS OBJECTIVES & SCOPE

THESIS OBJECTIVES & SCOPE

2.1. Thesis scope

CeD remains a unique immune-mediated disease to study as the risk alleles, main environmental trigger (gluten proteins and related prolamins), and most aspects of the adaptive immune response have been identified. Utilizing this knowledge has allowed for the creation of a variety of animal models that mimic different aspects of CeD pathophysiology. These have allowed further study into the environmental factors, such as the microbiota composition, that may increase CeD risk or may attenuate intestinal enteropathy. However, to date, there has been no animal models based on the transgenic expression of the DQ2 allele, the risk allele carried by the majority of CeD patients. **Thus, the overall goal of this thesis is to characterize a novel mouse model of CeD based on the transgenic expression of the DR3-DQ2 haplotype.**

The findings of this thesis are organized into three aims and two chapters. In **Chapter 3** of this thesis, I explored the impact of gluten on transgenic DR3-DQ2 mice using an established acute-exposure protocol. In **Chapter 4** of this thesis, I investigated the enteropathy of DR3-DQ2 fed wheat-containing food in a long-term exposure protocol. I also explored the impact of long-term exposure to wheat-containing food in DR3-DQ2 mice that were not previously sensitized to gluten peptides. The specific aims of each chapter are outlined below.

2.2. Thesis aims

Chapter 3: Effect of gluten sensitization and short-term gluten challenge on the development of immunopathology and gut dysfunction in DR3-DQ2 mice

Aims

1) To characterize gluten-induced immunopathology and barrier function in sensitized and characterized transgenic DR3-DQ2 mice.

Chapter 4: The effect of a long-term exposure on the development gluten-induced immunopathology in DR3-DQ2 mice

Aims

1) To characterize gluten-induced immunopathology in gliadin-sensitized transgenic DR3-DQ2 mice following long-term exposure to a wheat-containing diet.
2) To determine whether transgenic DR3-DQ2 mice develop gluten-induced immunopathology following long-term exposure to a wheat-containing diet without prior gluten-sensitization

The work in this thesis was presented in collaboration and with the support of various colleagues. Specific contributions of these colleagues are outlined in the prefaces at the beginning of each chapter.

CHAPTER 3: EFFECT OF GLUTEN SENSITIZATION AND SHORT-TERM GLUTEN CHALLENGE ON THE DEVELOPMENT OF IMMUNOPATHOLOGY AND GUT DYSFUNCTION IN DR3-DQ2 MICE

Preface: The work I performed in this chapter was carried out from September 2018 until present. I planned and conducted the majority of the experiments and analyzed the data with my supervisor E. Verdu. I received technical support and guidance from H. Galipeau (T cell proliferation assays, immunohistochemistry, ELISAs), J. Jury (Ussing chamber experiments), L. Rondeau, and A. Caminero. DR3-DQ2 mice were provided by R. Anderson.

3.1. Summary & significance

A number of previously characterized mouse models used in the study of celiac disease (CeD) have transgenically expressed the HLA-DQ8 allele. These mouse models only represent approximately 10% of CeD patients, with majority of CeD patients carrying the HLA-DQ2 allele. A mouse model that transgenically expresses the DR3-DQ2 haplotype, which includes the HLA-DQ2 allele, has been developed. There have been no previous studies that have characterized their immunological and physiological responses to gluten. Therefore, these experiments investigate whether DR3-DQ2 mice develop gut dysfunction following a standardized sensitization and challenge protocol. We show that following gluten sensitization and challenge, DR3-DQ2 mice develop key CeD features including increased *il15* expression in the small intestine, the development of anti-gliadin and anti-TG2 antibodies, increased $CD4^+$ T cell proliferation, increased intraepithelial lymphocyte (IEL) counts within the villi, and decreased villous-crypt ratios. We found that increased intestinal permeability is present in DR3-DQ2 mice prior to gluten treatment. These findings indicate that DR3-DQ2 mice are able to recapitulate key features of CeD pathogenesis following an easily reproducible gluten sensitization and challenge protocol. Overall, these findings demonstrate that DR3-DQ2 are a relevant model for future use in a laboratory setting to study CeD pathogenesis and treatment in DQ2 positive CeD patients.

3.2. Introduction

Celiac disease (CeD) is an immune-mediated enteropathy triggered by the ingestion of gluten proteins in genetically susceptible individuals (Verdu *et al*., 2015). Both the human leucocyte antigen (HLA) DQ8 and DQ2 alleles are genetically linked to the development of CeD. While 30% of the general population carries these alleles, only 2-4% of those carrying either allele will go on to develop CeD. This discrepancy suggests that other environmental factors may play a role in CeD development in those carrying either allele (Tack *et al*., 2010; Verdu *et al*., 2015).

The immune response in CeD patients results in the infiltration of both adaptive and innate immune cells to the lamina propria and the epithelium of the small intestine. The adaptive immune response seen in CeD has been well characterized and is triggered by the ingestion of gluten. The HLA-DQ8 and DQ2 alleles encode for MHC class II molecules that present immunogenic gluten fragments to $CD4^+$ T lymphocytes, with each allele playing an important role in CeD pathogenesis. Presentation of gliadin peptides to T cells results in the activation gluten specific T-cell that produce interferon gamma (IFN-γ). The adaptive immune response also results in the production of gliadin and tissue transglutaminase (TG2) antibodies (Verdu *et al*., 2015).

In contrast, there is intense debate surrounding what factor or factors may initially trigger the innate immune response in CeD. The innate arm of CeD is marked by increased intestinal permeability, epithelial stress, intraepithelial lymphocyte (IEL) activation, and increased interleukin (IL)-15 production (Verdu *et al*., 2015). The proposed factors triggering these abnormalities include other dietary factors, such as amylase trypsin

inhibitors or portions of the gluten peptide itself, and viral or bacterial infections (Araya *et al*., 2016; Schuppan & Zevallos, 2015; Tye-Din *et al*., 2018). Currently, the only treatment for CeD is a strict, lifelong adherence to a gluten-free diet (GFD). This diet, which is a treatment not a cure, is socially and financially burdensome to those that are required to follow it and compliance to the diet is poor (Tack *et al*., 2010). For many years, relevant animal that allow us to investigate key aspects of CeD pathogenesis, to permit advancement in the development of therapies to complement the GFD, have been hampered by the lack of appropriate animal models.

Previously, we validated a model of CeD using mice on a non-obese diabetic (NOD) background that transgenically express the HLA-DQ8 allele. These mice develop moderate enteropathy and barrier dysfunction after gliadin sensitization (Galipeau *et al*., 2011). However, only 10% of CeD patients carry the HLA-DQ8 allele whereas 90% of patients carry the HLA-DQ2 allele (Tack *et al*., 2010). In this study, we used mice that lack all endogenous MHC class II molecules but express the DR3-DQ2 haplotype on a C57Bl/6 background. We show that, after gliadin sensitization and an acute gluten challenge, transgenic DR3-DQ2 mice develop moderate enteropathy, anti-TG2 and anti-gliadin antibodies, and show an increase in *il15* expression. These results suggest that mice that transgenically express the DR3-DQ2 haplotype mimic adaptive and innate immune responses seen in CeD pathogenesis.

3.3. Materials & methods

Animals

All experiments were approved by the McMaster University Animal Care Committee. Female and male (8-12 weeks old) specific pathogen-free (SPF) DR3-DQ2 mice on a C57BL/6 background were obtained from Dr. R. Anderson (ImmusanT, Cambridge, MA) and bred at McMaster University's Central Animal Facility. All DR3-DQ2 mice were fed a gluten-free diet (Harlan Laboratories, Indianapolis, IN). Female and male (8-12 weeks old) SPF C57BL/6 mice were purchased from Taconic Bioscience (Rensselaer, NY). All mice had unlimited access to food and water throughout the experiments.

Gliadin sensitization & challenges (gluten treatment)

Mice were sensitized with pepsin-trypsin digested gliadin (PT-gliadin). PT-gliadin was prepared as previously described (Thomas et al., 2006). Briefly, gliadin (Sigma-Aldrich,) was dissolved in endotoxin-free 0.2 N HCl for 2 hours in a 37°C water bath with 1g pepsin (Sigma-Aldrich). After two hours of digestion, the pH was adjusted to 7.4 using endotoxin-free 2 M NaOH. Trypsin (Sigma-Aldrich) was added, the solution was boiled vigorously for 30 min, and the PT-gliadin was stored at −20°C. Mice were gavaged with 500μg of PT-gliadin with 25μg of cholera toxin (CT) once per week for three weeks to sensitize them to gluten. Gluten sensitized mice were then gavaged with 10mg of gluten dissolved in acetic acid three times per week for three weeks to challenge them (Figure 3.1). The gluten treated group includes the DR3-DQ2 mice that were sensitized and

subsequently challenged with gluten. Controls were gavaged with only 25µg of CT during the sensitization phase and were given a vehicle (only acetic acid) during the challenge phase.

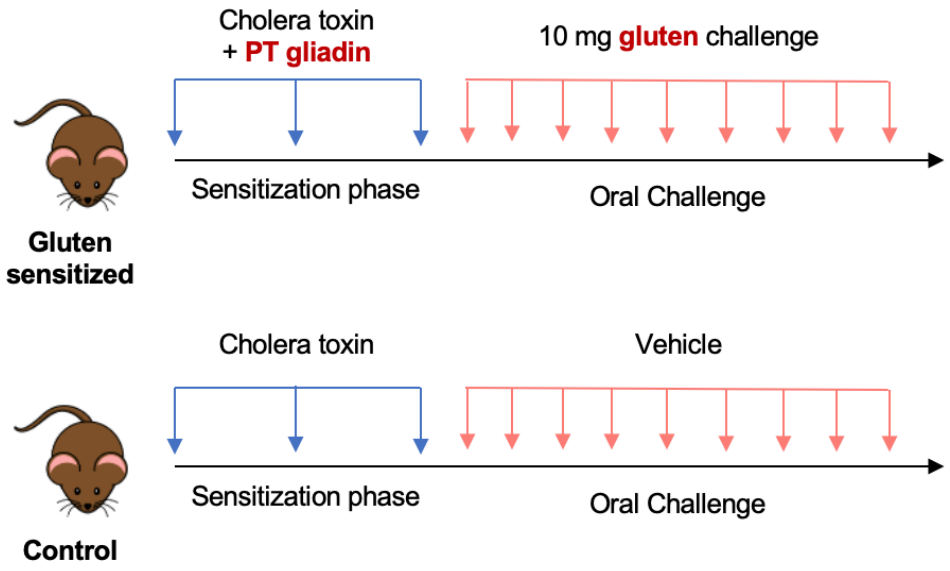


Figure 3.1 Experimental design

Adult SPF DR3-DQ2 mice were gavaged with cholera toxin and PT-gliadin or cholera toxin alone once a week for three weeks, this makes up the sensitization phase. Following this, they were gavaged with either 10mg of gluten or a vehicle three times a week for three weeks, this makes up the challenge phase. Mice were sacrificed at day 42 within 24h of last challenge.

Nanostring gene expression analysis

Small intestinal sections were collected in RNAlater (Thermo Fisher, Waltham, MA), kept at 4˚C overnight, and stored at -80˚C. Tissues were homogenized using a Tissue-Tearor homogenizer (Bispec, Bartlesville, OK). Total RNA was extracted from the sections using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's

instructions. RNA purity, quality, and concentration were verified using NanoDrop 2000 Spectrophotometer Bioanalyzer (Thermo Fisher). Gene expression was measured using a NanoString nCounter CodeSet (Mouse Inflammation Panel v2, 254 genes; NanoString Technologies, Seattle, WA) and analyzed with nSolver 4.0 (NanoString Technologies).

Anti-TG2 ELISA

Anti-TG2 antibodies were assessed in the intestinal content and serum using a previously described protocol (Galipeau *et al*., 2011). TG2 was derived from guinea pig liver (Sigma-Aldrich, Saint Louis, MO). TG2 was diluted in PBS and 0.1μg was added to each well of a 96-well Nunc-Immuno plate (Nunc, Waltham, MA). Plates were incubated overnight at 4˚C. Plates were washed five times with PBS and blocked with 1% BSA in PBS for 1h at room temperature. Serum or intestinal washes were added to the plates at 50μL per well and incubated for 1h at room temperature. The plates were washed five times, and secondary anti-mouse IgA or IgG (1:6000; Sigma-Aldrich) antibodies were added and incubated an additional 2h. Secondary antibodies were HRP conjugated, and tetramethylbenzidine was the substrate (SurModics, Eden Prairie, MN).

Anti-gliadin ELISA

Anti-gliadin antibodies were assessed in the intestinal content using a previously described protocol (Galipeau *et al*., 2011). In brief, gliadin (Sigma-Aldrich) was dissolved in 70% ethanol and then diluted in PBS. A 96-well Nunc-Immuno plate (Nunc) was coated with gliadin by adding 5μg to each well. The plate was then incubated overnight at 4˚C.

Plates were then incubated using 1% bovine-serum albumin (BSA) in PBS for 1h at room temperature. Intestinal content was diluted 1:10 into 1% BSA in PBS for detection of IgA before being added to plates for 2h at room temperature. Plates were then incubated with HRP conjugated secondary anti-mouse IgA (1:6000, Sigma-Aldrich). Antibodies were added to the plate for 1h at room temperature. Plates were washed with PBS-T, and tetramethylbenzidine (SurModics) was used as the substrate.

Villous-crypt ratio analysis

Cross-sections of proximal jejunum were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Histological evaluation of the villous-crypt architecture was completed using light microscopy under 10× magnification (Olympus, Richmond Hill, ON, Canada). Two sections of jejunum per mouse were used for villous-crypt evaluation. Twenty villous-crypt pairs were measured for each mouse in a blinded fashion and villous-crypt ratios were determined from these pairs.

Immunohistochemistry for $CD3^+$ cells

Immunostaining for $CD3^+$ cells was performed on paraffin sections to detect the presence of IELs in sections of proximal jejunum, as previously described (Verdu *et al*., 2007). Paraffin sections were incubated overnight at 4˚C with rabbit anti-mouse primary antibodies to CD3 (1:2000; Dako, Burlington, ON, Canada). Paraffin sections were then incubated with HRP conjugated anti-rabbit antibodies. The antibodies were visualized using 3-amin-9-ethylcarbazole and counterstained with Mayer hematoxylin. Negative

controls were performed in the absence of primary Ab. Slides were viewed by light microscopy under 20× magnification. $CD3^+$ IELs per 20 enterocytes in five randomly chosen villous tips were counted according to the previously described method. These were expressed as IELs/100 enterocytes (Biagi *et al*., 2004).

Intestinal permeability

Two sections of jejunum from each mouse were used for Ussing chamber experiments as previously described (Natividad *et al*., 2009; Verdu *et al*., 2007). A 4cm piece of jejunum was collected, cut into two sections, and placed in Krebs buffer aerated with 95% O_2 and 5% CO_2 (pH 7.3-7.4). Each segment of intestine was cut open along the mesenteric border to form a flat mucosal sheet and mounted into an Ussing chamber. The chamber exposed 0.25cm^2 of tissue surface area to 4mL of circulating oxygenated Krebs buffer containing 10mM glucose (serosal side) and 10mM mannitol (mucosal side), maintained at 37˚C. Net active transport across the epithelium was measured via short circuit current response (I_{sc}). Paracellular permeability was measured through mucosal-to-serosal flux of ^{51}Cr-EDTA. ^{51}Cr-EDTA was quantified in samples using a liquid scintillation counter and is expressed as % hot sample/h/cm^2. Tissue conductance (the passive permeability to ions) was calculated using Ohm's law and is expressed as ms/cm^2.

Statistical analysis

Data were analyzed in GraphPad Prism 8.0. Parametric data (normal distribution as determined by D'Agustino-Pearson omnibus normality test; Shapiro-Wilk test; and

Kolmogorov-Smirnov test) were analyzed using an unpaired T test. Non-parametric data was analyzed by Mann Whitney tests. Outliers were determined using the ROUT method with a maximum false discovery rate of 1%. A p-value of < 0.05 was considered to be significant.

3.4. Results

Gluten treatment upregulates proinflammatory genes in DR3-DQ2 mice

We first measured transcript levels of 254 genes involved in inflammatory pathways in proximal small intestinal sections from gluten treated or control DR3-DQ2 mice. Gluten treatment upregulated a number of proinflammatory genes, including *tlr3*, *il1a*, and *cfb* (Figure 3.2). *Il15* expression, a key cytokine involved in CeD pathogenesis, was increased in gluten treated DR3-DQ2 mice compared to controls. We also found a decrease in expression in *ccl2*, *irf3*, and *mmp9*. Altogether, these results suggest that gluten treatment in DR3-DQ2 mice upregulates proinflammatory genes, some of which play a role in CeD pathogenesis.

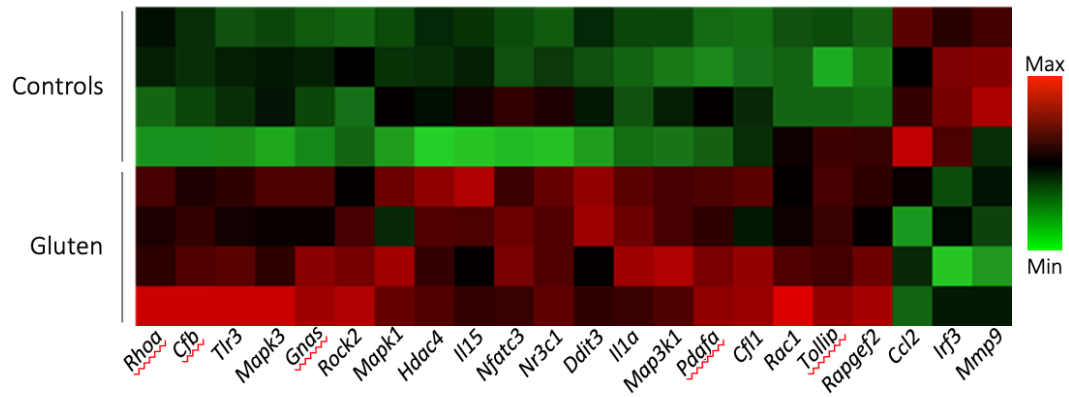


Figure 3.2 Gluten sensitization treatment upregulates proinflammatory genes in DR3-DQ2 mice

Heat map of gene expression in the proximal small intestine of gluten treated and control DR3-DQ2 mice. Only genes whose expression was significantly altered between the groups are shown. Each lane represents one mouse.

Gluten treated DR3-DQ2 mice develop adaptive immune responses to gluten

We evaluated adaptive immune responses to gluten in DR3-DQ2 mice measuring serological responses used in the clinical setting. Unlike anti-gliadin antibodies, anti-TG2 antibodies are highly sensitive and specific for CeD (Tack *et al*., 2010). We first measured anti-gliadin IgA antibodies in intestinal washes, as anti-gliadin IgA is found in many CeD patients and indicates a local immune response to gliadin (Volta *et al*., 1985). We found that anti-gliadin IgA antibodies were present in the intestinal content of most gluten treated DR3-DQ2 mice (Figure 3.3, A). Similarly, there was a trend for increased anti-TG2 IgA antibodies in the intestinal content of gluten treated DR3-DQ2 mice (Figure 3.3, B). These findings are in accordance with anti-gliadin and anti-TG2 antibody development seen in other mouse models of gluten sensitivity (Galipeau *et al*., 2011; Verdu *et al*., 2007). We also found a significant increase in anti-TG2 IgG antibodies in the serum of gluten treated DR3-DQ2 mice (Figure 3.3, C). These antibodies are not present in the serum of other transgenic DQ8 mouse models, suggesting that serum anti-TG2 IgG antibodies development is unique to gluten treated DR3-DQ2 mice.

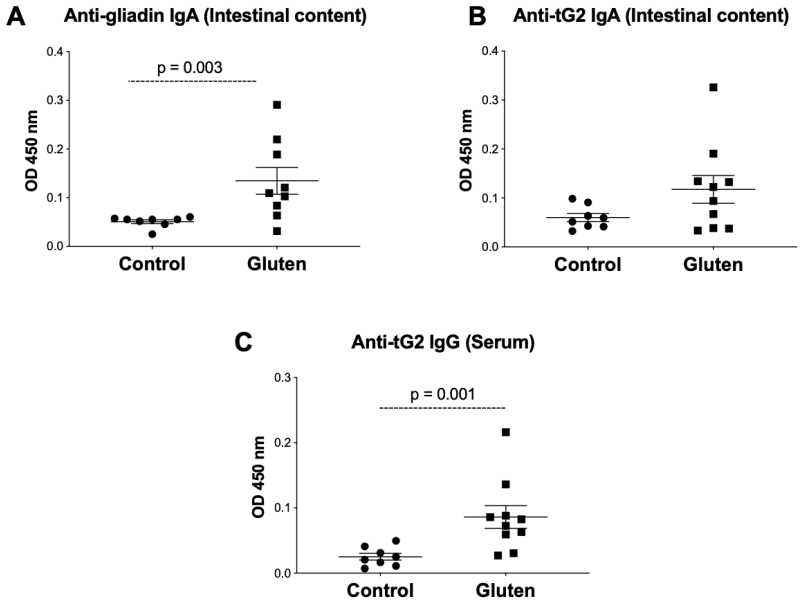


Figure 3.3 Development of anti-gliadin and anti-TG2 antibodies following gluten treatment

Serum, intestinal content, and spleens were collected from gluten sensitized and control DR3-DQ2 mice. The presence of anti-gliadin (**A**) and anti-TG2 (**B** and **C**) antibodies were determined using ELISAs. Each dot represents an individual mouse; horizontal line and whiskers depict mean ± SEM; p-values were determined using an unpaired t-test.

DR3-DQ2 mice develop moderate immunopathology following gluten treatment

To assess immunopathology, we measured $CD3^+$ IELs in villi tips and villous-to-crypt ratios. We found that gluten treated DR3-DQ2 mice develop significantly increased $CD3^+$ IEL counts within their villi tips compared to controls (Figure 3.4, A). We show that gluten treated DR3-DQ2 mice have 2x more $CD3^+$ IELs/100 enterocytes compared to controls, with gluten treated DR3-DQ2 mice having a mean of 6 $CD3^+$ IELs/100 enterocytes compared to a mean of 3 $CD3^+$ IELs/100 enterocytes in controls. This finding

suggests that there is an increase in IEL counts within the villi of gluten treated DR3-DQ2 mice.

Gluten treated DR3-DQ2 mice developed significantly lower villous-to-crypt ratios in comparison to control treated mice (Figure 3.4, B). Gluten treated DR3-DQ2 mice had a median villous-to-crypt ratio of 4.7 in comparison to controls that had a median villous-to-crypt ratio of 6.2. Therefore, DR3-DQ2 mice develop moderate immunopathology following gluten treatment.

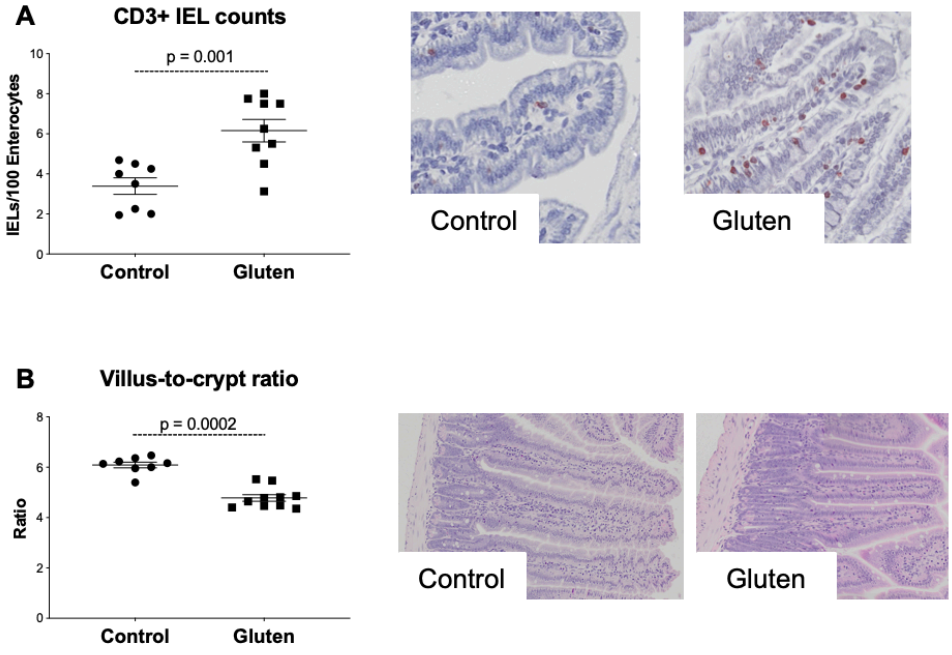


Figure 3.4 Gluten treatment increases CD3$^+$ IEL counts and induces mild enteropathy in DR3-DQ2 mice

The number of CD3$^+$ IELs and villous-crypt ratios in the proximal small intestine were determined in gluten sensitized and control DR3-DQ2 mice. (**A**) CD3$^+$ IEL counts were determined using immunohistochemistry. Representative photos are from gluten sensitized (*right*) and control (*left*) DR3-DQ2 mice. Each dot represents an individual mouse; data are represented as mean ± SEM; p-value was determined using an unpaired t-test. (**B**) Villous-

crypt ratios were measured from H&E stained slides. Representative photos are from gluten sensitized (*right*) and control (*left*) DR3-DQ2 mice. Each dot represents an individual mouse; horizontal line and whiskers depict median ± range; p-value was determined using a Mann-Whitney test.

DR3-DQ2 mice have higher paracellular permeability independent of gluten treatment

Lastly, we investigated intestinal permeability in DR3-DQ2 mice. For this we evaluated ion transport, conductance, and paracellular permeability to ^{51}Cr-EDTA in naïve DR3-DQ2 mice. There was no significant difference in small intestine ion transport or conductance in naïve DR3-DQ2 mice compared to naïve C57Bl/6 mice. Notably, we saw a trend for increased paracellular permeability to ^{51}Cr-EDTA in naïve DR3-DQ2 mice compared to the control C57Bl/6 mice (Figure 3.5, A-C). We then assessed if gluten treatment would further exacerbate this increase in paracellular permeability to ^{51}Cr-EDTA. We did not observe a significant increase between gluten treated DR3-DQ2 mice and control DR3-DQ2 mice when assessing ion transport, conductance, and paracellular permeability (Figure 3.5, D-F). Taken altogether, these results suggest that DR3-DQ2 mice have a baseline increased paracellular permeability compared to C57BL/6 mice that is independent of gluten treatment and is present before gluten-induced enteropathy occurs.

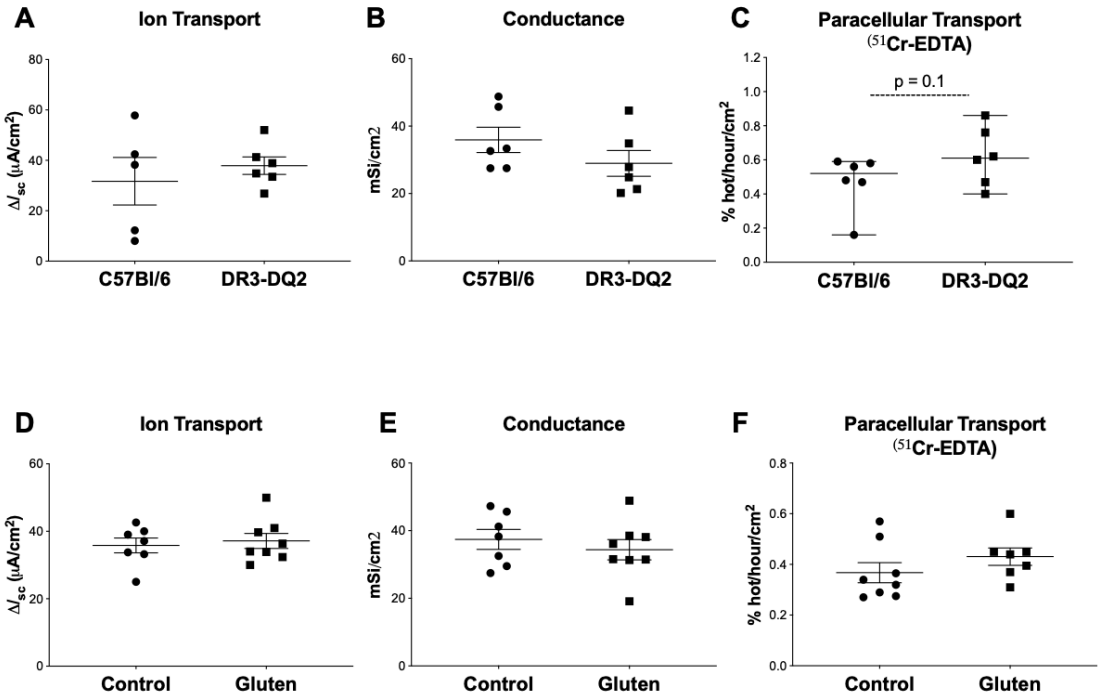


Figure 3.5 DR3-DQ2 mice have increased paracellular permeability

Proximal small intestinal permeability was assessed prior to gluten sensitization (**A**-**C**) and following gluten sensitization (**D**-**F**). Ion transport (**A**), conductance (**B**), and paracellular permeability to ^{51}Cr-EDTA (**C**) was assessed in DR3-DQ2 mice prior to sensitization, in comparison to naïve C57Bl/6 mice. Ion transport (**D**), conductance (**E**), and paracellular permeability to ^{51}Cr-EDTA (**F**) was assessed in gluten sensitized and control DR3-DQ2 mice. Each dot represents an individual mouse; horizontal line and whiskers depict mean ± SEM (**A, B, D, E, F**) or median ± range (**C**) ; p-value was determined using an unpaired t-test (**A, B, D, E, F**) or a Mann-Whitney test (**C**).

3.5. Discussion

The overall prevalence of CeD has increased significantly worldwide within the last few decades, suggesting that external environmental factors may play a role in CeD development (Murray *et al*., 2003; Rubio-Tapia *et al*., 2009; Tack *et al*., 2010). Despite this increase in prevalence, drug development and investigations into additional environmental factors have been hampered due to a lack of relevant preclinical animal models that can be used in a laboratory setting (Wungjiranirun *et al*., 2016). Previous studies have focused on the use of mice that transgenically express the HLA-DQ8 risk allele. No studies to date have investigated the characteristics of mice transgenically expressing the HLA-DQ2 risk allele at baseline and following gluten sensitization and challenge. Thus, the main objective of this study was to characterize physiological and immunological responses in DR3-DQ2 mice. The transgenic expression of the DR3-DQ2 haplotype ensures that T cells in these mice are selected in a DQ2-restricted manner. The DQ2 allele also allows for DCs to efficiently present immunogenic gluten fragments to $CD4^+$ T cells.

We show that gluten treatment, consisting of a sensitization phase using cholera toxin as a mucosal adjuvant, followed by short-term gluten challenge, increased the expression of proinflammatory genes in DR3-DQ2 mice. Some of the upregulated genes are involved in apoptotic pathways, such as *mapk3* (Cagnol & Chambard, 2010). Other upregulated genes are involved in immune activation in response to pathogens and the activation of $CD4^+$ T cells, including *il1a* and *tlr3* (Abadie & Jabri, 2014; Marafini et al., 2015; Novak et al., 2020; Zhou et al., 2007). We also observed a downregulation of *ccl2*, *irf3*, and *mmp9* in gluten treated DR3-DQ2 mice compared with controls. *Ccl2* encodes for

the CCL2 chemokine, which helps to recruit memory T cells, monocytes, and dendritic cells to areas of inflammation (Xu et al., 1996). *Irf3* encodes for the interferon regulatory factor 3, involved in immune responses to viruses (Collins et al., 2004). While both *ccl2* and *irf3* are important inflammatory genes, their downregulation in gluten treated DR3-DQ2 mice does not suggest that these mice have a decreased inflammatory response, as there are numerous other chemokines and interferon regulatory factors that may be compensating for the downregulation of these two genes. Lastly, *mmp9* encodes for matrix metalloproteinase (MMP) 9, a protein whose main function is to degrade extracellular matrix. There are a wide range of MMPs that may be playing a role in promoting immune responses in DR3-DQ2 mice. A study investigating the levels of MMPs in the duodenal mucosa of CeD showed no significant changes in the levels of *mmp9* expression between CeD patients and controls using RT-qPCR. However, the authors showed an increase in MMP-9 activity between CeD patients and controls using gel zymography, which suggests that MMP-9 is more tightly regulated at a post-transcriptional level (Ciccocioppo *et al*., 2005). Due to the small number of subjects in this study and since only RT-qPCR and gel zymography were used to measure MMP-9 activity, further experiments are needed to explore the role of MMP-9 in CeD.

An important gene induced by gluten treatment in DR3-DQ2 mice was *il15*, which encodes for a key cytokine that drives intestinal destruction in CeD by activating IEL cytotoxicity and suppressing regulatory T cells (Abadie & Jabri, 2014). The NOD-DQ8 mouse model inadequately expresses *il15* due to its NOD background, lowering the bioavailability of IL-15 within these mice (Suwanai *et al*., 2010). This makes the NOD-

DQ8 model less attractive for the study IL-15 driven mechanisms in CeD pathogenesis. The triple-transgenic DQ8-Dd-villin-IL15 mouse model, which overexpresses *il15* in the intestinal epithelium and lamina propria, allows for the study of IL-15 driven epithelial-NK cell interactions in mucosal atrophy but only in the context of HLA-DQ8 allele (Abadie *et al*., 2020). Our results suggest that DR3-DQ2 mice can be used to further study the role of this key cytokine and their triggers in CeD, without the need of transgenic overexpression.

To investigate adaptive immune responses in the model, we measured antibody development, namely anti-gliadin and anti-TG2 antibodies. Gluten treatment in DR3-DQ2 mice also led to an increase in anti-gliadin (IgA) in the intestinal lumen. An increase in anti-gliadin antibodies is seen in the majority of CeD patients, and the presence of these antibodies in the lumen suggests that B cell activation is occurring in the gut itself. However, the presence of these antibodies are not the most specific antibody test for CeD (Rubio-Tapia *et al*., 2013; Volta *et al*., 1985).

One of the most sensitive and specific tests for CeD is testing for anti-TG2 antibodies in the serum along with total IgA. This test is often the first diagnostic test conducted within the clinic and is an important first step to determining if a patient has an immune response to gluten (Rubio-Tapia *et al*., 2013). TG2, the autoantigen in CeD, plays a key role in CeD pathogenesis by deamidating immunogenic gluten peptides. These peptides are then taken up by dendritic cells within the lamina propria and presented to $CD4^+$ T cells. This crucial step allows immunogenic gluten peptides to strongly bind to the HLA groove in antigen presenting cells which in turn present these peptides to $CD4^+$ T cells. This activates the $CD4^+$ T cells, which leads to the induction of proinflammatory

cytokines such as IFN-γ. It is believed that these proinflammatory cytokines can then promote the activation of cytotoxic T cells. It has been proposed that B cells are able to produce anti-TG2 antibodies through the "hapten-carrier hypothesis". In this case, activated gluten specific $CD4^+$ T cells are able to activate B cells that then produce anti-TG2 antibodies (Fleur Du Pr & Sollid, 2015; Mesin et al., 2012; Schuppan et al., 1998). Anti-TG2 antibodies are a serological hallmark of the inflammatory and autoimmune process in CeD. Previous animal models have shown inconsistent results related to anti-TG2 antibodies, including the newly described triple-transgenic model (Abadie *et al*., 2020; Galipeau *et al*., 2011; Korneychuk *et al*., 2015; Verdu *et al*., 2007).

We show that there was an increase in anti-TG2 IgG antibodies in the serum of gluten treated DR3-DQ2 mice. This increase in anti-TG2 IgG antibodies is a unique to the DR3-DQ2 mouse model and these antibodies have not been seen in other models of gluten sensitivity. This finding suggests that B cell activation in systemic locations, such as the spleen, may be occurring in DR3-DQ2 mice following gluten treatment. These activated B cells are then producing anti-TG2 IgG that is then circulating in the serum (Iversen *et al*., 2017). However, anti-TG2 IgG antibodies likely originate from B cell activation at systemic locations. Measuring these antibodies in the serum are not as specific as testing for anti-TG2 IgA antibodies. Since anti-TG2 IgA antibodies are produced by plasma cells that originate from a B cell clone that was activated in the gut itself, these antibodies are the most specific method test for CeD. It would be beneficial to see if anti-TG2 IgA antibodies in the serum are increased in gluten treated DR3-DQ2 mice compared to controls to further characterize these mice (Iversen *et al*., 2017).

We also found a trend for increased anti-TG2 IgA antibodies within the intestinal content of gluten treated DR3-DQ2 mice compared to controls. The development of anti-TG2 IgA antibodies within the lumen often precedes celiac disease diagnosis, and anti-TG2 and IgA deposits typically develop in the mucosa in even potential CeD patients (Lionetti *et al*., 2019; Maglio *et al*., 2011). The development of anti-TG2 IgA antibodies within the intestinal content suggests that gluten treated DR3-DQ2 mice are activating B cells within the gut itself that are then secreting anti-TG2 IgA antibodies into the lumen (Iversen *et al*., 2017).

Intraepithelial lymphocytosis is one of the first, albeit non-specific, findings in CeD (Biagi *et al*., 2004; Pellegrino *et al*., 2011). The gold standard for diagnosing CeD in adult patients is a duodenal biopsy following a positive TG2 test to investigate the small intestinal architecture using the Marsh grading system (Rubio-Tapia *et al*., 2013). In line with previous mouse models transgenic for the HLA-DQ8 allele, gluten treatment in DR3-DQ2 mice led to moderate immunopathology, characterized by decreased villous-crypt ratios and increased $CD3^+$ IEL counts (Galipeau *et al*., 2011; Verdu *et al*., 2007). Gluten treated DR3-DQ2 mice developed decreases in villous to crypt ratios similar to a Marsh II lesion. The duration of gluten treatment in this study was short, and the mice may require longer exposure to develop more severe pathology similar to a Marsh IIIa lesion required for CeD diagnosis.

An important component of CeD pathogenesis is an increase in intestinal permeability that allows large, immunogenic gluten fragments to cross the epithelial barrier. It is currently unknown whether intestinal permeability precedes villous atrophy or

if it is a consequence of CeD inflammation that further exacerbates the severity of the disease. A study reported increased small intestinal permeability in irritable bowel syndrome patients without celiac disease, who were positive for the HLA-DQ2 allele, compared with IBS patients negative for the HLA-DQ2 allele (Vazquez-Roque *et al*., 2012). The study suggested a primary impaired barrier linked to the DQ2 allele. Other studies have reported that CeD patients treated with a GFD still have increased intestinal permeability compared with healthy controls. Alterations within the barrier structure also remain in patients on a GFD, further supporting the idea that intestinal permeability may be primary to inflammation (Ciccocioppo *et al*., 2006; Schulzke, Schulzke, Fromm, & Riecken, 1995; Schulzke, Bentzel, Schulzke, Riecken, & Fromm, 1998). We found that gluten treated DR3-DQ2 mice did not develop an increase in ion transport, conductance, or paracellular permeability, compared with controls. However, we saw that naïve DR3-DQ2 mice displayed increased paracellular permeability compared with wild type C57Bl/6 mice. This finding suggests that gluten treatment does not exacerbate barrier dysfunction in DR3-DQ2 mice, because these mice already have increased paracellular permeability in basal conditions. The exact mechanism driving this increase in paracellular permeability in naïve DR3-DQ2 mice remains to be elucidated and may be related to microbial factors.

In conclusion, we have shown that short-term gluten treatment leads to the development of moderate immunopathology and immune activation in DR3-DQ2 mice. DR3-DQ2 mice develop key features of CeD following a standard, and reproducible protocol. Thus, DR3-DQ2 mice constitute a useful tool to investigate DQ2 specific immune responses to gluten and as a relevant preclinical model for drug development and testing.

CHAPTER 4: INVESTIGATING GLUTEN-INDUCED ENTEROPATHY IN DR3-DQ2 MICE FOLLOWING A LONG-TERM GLUTEN EXPOSURE

Preface: The work I performed in this chapter was carried out from September 2019 until present. I planned and conducted the majority of the experiments and analyzed these data with my supervisor E. Verdu. I received technical support and guidance from H. Galipeau (immunohistochemistry, ELISAs), J. Jury (Ussing chamber experiments), L. Rondeau, and A. Caminero. DR3-DQ2 mice were provided by R. Anderson.

4.1. Summary & significance

CeD can develop at any point during one's life and diagnosis can occur from infancy to advanced age. It is hypothesized that the cause of disease onset between those that develop CeD later in life compared to in infancy is different, but the exact mechanisms that contribute to this are unknown. In **Chapter 3**, we characterized the immunological and physiological responses of DR3-DQ2 mice to gluten following sensitization using gliadin and cholera toxin. However, it is unknown if DR3-DQ2 mice will spontaneously develop key CeD features without sensitization, representing what may be occurring in patients diagnosed with CeD later in life or in potential CeD patients. These experiments investigate whether DR3-DQ2 mice spontaneously develop key CeD features when exposed to a wheat containing diet for twelve weeks. We show that DR3-DQ2 mice on a wheat containing diet, for 12 weeks, without sensitization spontaneously develop some key clinical CeD features including the development of anti-TG2 antibodies and decreased villous-crypt ratios. We also found that gluten sensitization and diet composition drive differential gene expression in the small intestine of DR3-DQ2 mice. These findings confirm the findings shown in **Chapter 3**, as well as indicate that DR3-DQ2 mice may spontaneously develop key CeD features without sensitization to gluten using an adjuvant.

4.2. Introduction

Celiac disease (CeD) is an immune-mediated enteropathy triggered by the ingestion of gluten proteins in individuals carrying either the HLA-DQ2 or -DQ8 alleles. Due to their high proline content, gluten peptides are resistant to degradation and large immunogenic fragments can translocate across the intestinal barrier (Matysiak-Budnik *et al*., 2008; Shan *et al*., 2002). CeD development relies on the interaction between these gluten peptides, the HLA-DQ2/DQ8 risk alleles, and tissue-transglutaminase 2 (TG2) within the lamina propria. Together, these factors lead to the development of an adaptive immune response to gluten that contributes to the intestinal enteropathy that is seen in CeD. In conjunction with this adaptive immune response, the innate immune system also needs to be activated in order for CeD to develop. The innate immune response is marked by increased intestinal permeability, the upregulation of epithelial stress markers, and intraepithelial lymphocyte (IEL) activation through increased interleukin (IL) 15 production (Tye-Din *et al*., 2018; Verdu *et al*., 2015). In contrast to the adaptive immune response, the initial trigger of the innate immune response remains unknown. Furthermore, approximately 30% of the general population carries either risk allele for CeD, while only 2-4% of those carrying either allele will develop CeD. This discrepancy suggests that other environmental factors may act as the initial trigger for the innate immune response and play a role in CeD development in those carrying either allele (Tack *et al*., 2010; Verdu *et al*., 2015).

CeD is a difficult disease to diagnose due to the heterogenous set of symptoms a patient could present with (Tack *et al*., 2010). Epidemiological studies have also shown that the age of onset of CeD varies widely. Elderly CeD patients, defined as patients that are

diagnosed with CeD when they are over the age of 60, make up a significant portion of CeD patients (Rashtak & Murray, 2009; Vivas, 2015). However, the factors that contribute to the loss of oral tolerance to gluten later in life remains unknown.

Potential CeD patients are defined as individuals that are genetically at-risk of developing CeD (carrying either the DQ8 or DQ2 allele) and present with one positive anti-TG2 blood test, but do not have villous atrophy (Rubio-Tapia *et al*., 2013). Only a small portion of potential CeD patients go on to develop CeD while eating a gluten-containing diet, while others only have transiently elevated anti-TG2 levels (Auricchio *et al*., 2014). Similar to elderly CeD patients, what causes potential CeD patients to go on to develop CeD, or what may protect them from developing CeD, remains to be elucidated.

In **Chapter 3**, we characterized a model of CeD using mice that transgenically express the DR3-DQ2 haplotype, which includes the HLA-DQ2 risk allele. These mice have increased paracellular permeability and develop moderate immunopathology after a short-term gluten treatment (**Chapter 3**). However, this protocol relies on initially sensitizing DR3-DQ2 mice to gluten using cholera toxin as an adjuvant. In this study, we used DR3-DQ2 mice to investigate a spontaneous phenotype following long-term exposure to a wheat containing diet. We show that, without a gluten sensitization phase, transgenic DR3-DQ2 spontaneously begin to develop enteropathy and anti-TG2 antibodies, when exposed to a wheat containing diet for at least 12 weeks. We also show that, without gluten sensitization, transgenic DR3-DQ2 mice have an upregulation of proinflammatory gene expression. These results suggest that transgenic DR3-DQ2 mice develop adaptive and innate immune responses when exposed to gluten in a long-term setting.

4.3. Materials & methods

Animals

All experiments were approved by the McMaster University Animal Care Committee. Female and male (12-16 weeks old) specific pathogen-free (SPF) DR3-DQ2 mice on a C57BL/6 background were obtained from Dr. R. Anderson (ImmusanT, Cambridge, MA) and bred at McMaster University's Central Animal Facility. All mice had unlimited access to food and water throughout the experiments.

Gliadin sensitization & diets

Mice were sensitized with pepsin-trypsin digested gliadin (PT-gliadin). PT-gliadin was prepared as previously described (Thomas *et al*., 2006). Briefly, gliadin (Sigma-Aldrich,) was dissolved in endotoxin-free 0.2 N HCl for 2 hours in a 37°C water bath with 1g pepsin (Sigma-Aldrich). After two hours of digestion, the pH was adjusted to 7.4 using endotoxin-free 2 M NaOH. Trypsin (Sigma-Aldrich) was added, the solution was boiled vigorously for 30 min, and the PT-gliadin was stored at −20°C. Mice then received an oral gavage containing 500μg of PT-gliadin with 25μg of cholera toxin (CT) once per week for three weeks to sensitize them to gluten (Galipeau *et al*., 2011). Non-sensitized mice received cholera toxin alone. Mice either remained on a gluten-free diet (GFD; Harlan Laboratories, Indianapolis, IN) or were placed on a standard lab diet, that contains wheat, until endpoint (WD; Figure 4.1). Mice that were sensitized and placed on a wheat containing diet were given the sen + WD treatment; mice that were non-sensitized and

placed on a wheat containing diet were given the NS + WD treatment. Mice that were non-sensitized and remained on a gluten free diet acted as controls (Table 4.1).

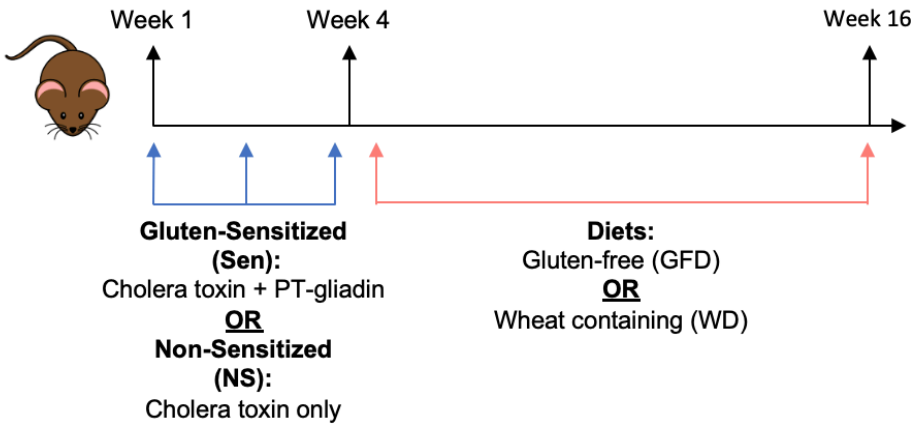


Figure 4.1 Experimental design

Adult SPF DR3-DQ2 mice were gavaged with cholera toxin and PT-gliadin or cholera toxin alone once a week for three weeks, this makes up the sensitization phase. The mice were then maintained on either a gluten-free diet or a wheat-containing diet for ~12 weeks. Mice that were non-sensitized and on a gluten-free diet served as controls. Mice were sacrificed at the beginning of week 17.

Table 4.1 Description of treatment groups

Treatment Name	Description
Sen + WD	Sensitization phase: Received cholera toxin and PT-gliadin Challenge phase: Changed to a wheat containing diet
NS + WD	Sensitization phase: Received only cholera toxin Challenge phase: Changed to a wheat containing diet
Control	Sensitization phase: Received only cholera toxin Challenge phase: Remained on a gluten-free diet

Nanostring gene expression analysis

Small intestinal sections were collected in RNAlater (Thermo Fisher, Waltham, MA), kept at 4˚C for 24 hours, and stored at -80˚C. Tissues were homogenized using a Tissue-Tearor homogenizer (Bispec, Bartlesville, OK). Total RNA was extracted from tissue sections using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA purity, quality, and concentration were verified using NanoDrop 2000 Spectrophotometer Bioanalyzer (Thermo Fisher). Gene expression was measured using a NanoString nCounter CodeSet (Mouse Inflammation Panel v2, 254 genes; NanoString Technologies, Seattle, WA) and analyzed with nSolver 4.0 (NanoString Technologies).

Anti-TG2 ELISA

Anti-TG2 antibodies were assessed in intestinal content and serum using a previously described protocol (Galipeau *et al*., 2011). TG2 was derived from guinea pig liver (Sigma-Aldrich). TG2 was diluted in PBS and 0.1μg was added to each well of a 96-well Nunc-Immuno plate (Nunc, Waltham, MA). Plates were incubated overnight at 4˚C. Plate were washed five times with PBS and blocked with 1% BSA in PBS for 1h at room temperature. Serum or intestinal washes were added to each plate (50μL per well) and incubated for 1h at room temperature. The plates were then washed five times, and secondary anti-mouse IgA or IgG (1:6000, Sigma-Aldrich) antibodies were added and incubated an additional 2h. Secondary antibodies were HRP conjugated, and tetramethylbenzidine was the substrate (SurModics, Eden Prairie, MN).

Anti-gliadin ELISA

Anti-gliadin antibodies were assessed in intestinal content using a previously described protocol (Galipeau *et al*., 2011). In brief, gliadin (Sigma-Aldrich) was dissolved in 70% ethanol and then diluted in PBS. A 96-well Nunc-Immuno plate (Nunc) was coated with gliadin by adding 5μg to each well. The plate was then incubated overnight at 4˚C. Plates were then incubated using 1% bovine-serum albumin (BSA) in PBS for 1h at room temperature. Intestinal content was diluted 1:10 into 1% BSA in PBS for detection of IgA before being added to plates for 2h at room temperature. Plates were then incubated with HRP conjugated secondary anti-mouse IgA (1:6000, Sigma-Aldrich). Antibodies were added to the plate for 1h at room temperature. Plates were washed with PBS-T, and tetramethylbenzidine (SurModics) was used as the substrate.

Villous-crypt analysis

Cross-sections of proximal jejunum were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Histological evaluation of the villous-crypt architecture was completed using light microscopy under 10× magnification (Olympus, Richmond Hill, ON, Canada). Two sections of jejunum per mouse were used for villous-crypt evaluation. Twenty villous-crypt pairs were measured for each mouse in a blinded fashion and villous-crypt ratios were determined from these pairs.

Intestinal permeability

Two sections of jejunum from each mouse were used for Ussing chamber experiments as previously described (Natividad *et al*., 2009; Verdu *et al*., 2007). A 4cm piece of jejunum was collected, cut into two sections, and placed in Krebs buffer aerated with 95% O_2 and 5% CO_2 (pH 7.3-7.4). Each segment of intestine was cut open along the mesenteric border to form a flat mucosal sheet and mounted into an Ussing chamber. The chamber exposed 0.25cm^2 of tissue surface area to 4mL of circulating oxygenated Krebs buffer containing 10mM glucose (serosal side) and 10mM mannitol (mucosal side), maintained at 37˚C. Net active transport across the epithelium was measured via short circuit current response (I_{sc}). Paracellular permeability was measured through mucosal-to-serosal flux of ^{51}Cr-EDTA. ^{51}Cr-EDTA was quantified in samples using a liquid scintillation counter and is expressed as % hot sample/h/cm^2. Tissue conductance (the passive permeability to ions) was calculated using Ohm's law and is expressed as ms/cm^2.

Statistics

Data were analyzed in GraphPad Prism 8.0. Parametric data (normal distribution as determined by D'Agustino-Pearson omnibus normality test; Shapiro-Wilk test; and Kolmogorov-Smirnov test) were analyzed using ANOVA or a Kruskal-Wallis test, where applicable, with the Bonferroni post-hoc test for multiple comparisons when comparing more than two groups. Outliers were determined using the ROUT method with a maximum false discovery rate of 1%. A p-value of < 0.05 was considered to be significant.

4.4. Results

Gluten sensitization and diet drive differential gene expression in DR3-DQ2 mice

We first measured transcript levels of 254 genes involved in inflammatory pathways in the proximal small intestinal sections from Sen + WD, NS + WD, or control treated DR3-DQ2 mice. Regardless of sensitization, DR3-DQ2 mice on a wheat containing diet had increased expression of a number of proinflammatory genes, including *cysltr2, cysltr1, ptgs2*, and *rela* (Figure 4.2, A) compared with DR3-DQ2 mice on a GFD. However, there was no difference in *il15* expression between DR3-DQ2 mice on a gluten free diet and on a wheat containing diet. We also found a decrease in expression in *ifit1, stat2, irf7, il18, mbl2,* and *mx2* in the DR3-DQ2 mice fed a wheat containing diet compared with DR3-DQ2 mice on a gluten free diet.

In addition, we found that DR3-DQ2 mice that were sensitized to gluten had increased proinflammatory gene expression in the small intestine compared with non-sensitized DR3-DQ2 mice, independent of diet (Figure 4.2, B). Some of these genes include *daxx, cystlr2,* and *ptgs2.* However, *il15* expression was not significantly different between sensitized and non-sensitized DR3-DQ2 mice. These findings suggest that previous sensitization and the presence of a wheat containing diet is driving different inflammatory gene expression patterns within the small intestine of DR3-DQ2 mice.

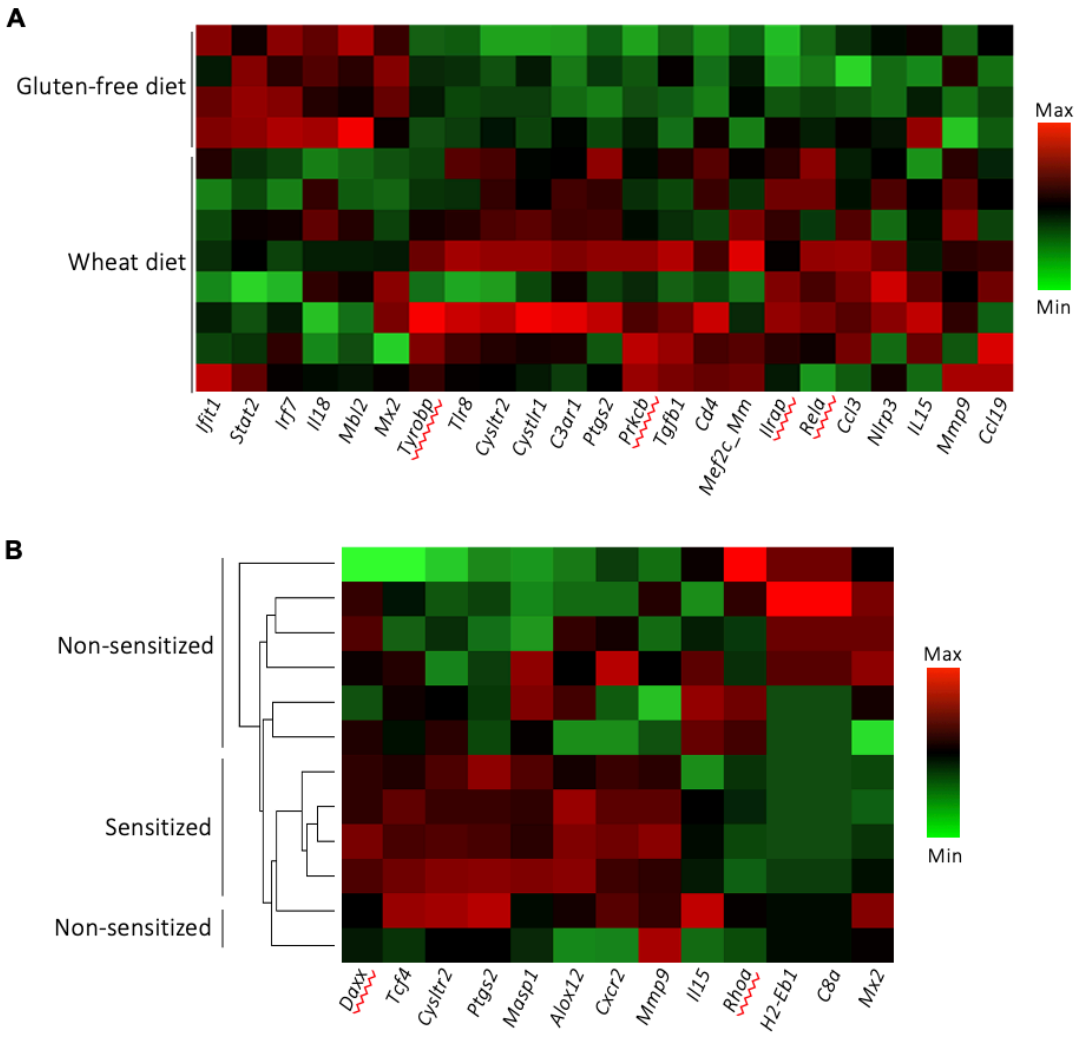


Figure 4.2 Gluten sensitization & diets drive different gene expression

Heat map of gene expression in the proximal small intestine of DR3-DQ2 on a gluten free diet and a wheat containing diet (**A**) and gluten sensitized or non-sensitized DR3-DQ2 mice (**B**). Only genes whose expression was significantly altered between the groups are shown, with the exception of *il15* in **A** and **B**. Each lane represents one mouse.

DR3-DQ2 mice on a wheat containing diet spontaneously develop anti-TG2 IgA antibodies

To further investigate whether DR3-DQ2 mice on a wheat containing diet can spontaneously develop immune responses to gluten, we evaluated markers of adaptive immune responses to gluten. We evaluated whether a wheat containing diet would lead to the development of anti-gliadin and TG2 antibodies in DR3-DQ2 mice, with and without gluten sensitization. In accordance to with experiments shown in **Chapter 3**, we found that Sen + WD treated DR3-DQ2 mice had increased anti-TG2 IgA and anti-TG2 IgG antibodies in the intestinal content and serum compared to control mice (Figure 4.3, A and B). We also found that Sen + WD treated DR3-DQ2 mice had a trend towards increased anti-gliadin IgA antibodies in the intestinal content compared to control mice (Figure 4.3, C). We found a trend towards increased anti-TG2 IgA antibodies in the intestinal content of NS + WD treated DR3-DQ2 mice (Figure 4.3, A). This finding suggests that NS + WD treated DR3-DQ2 mice are spontaneously producing anti-TG2 antibodies within the lumen of the intestine. However, we saw no increases in anti-TG2 IgG antibodies in the serum or anti-gliadin IgA antibodies in the intestinal content in NS + WD treated DR3-DQ2 mice compared to controls (Figure 4.3, B and C)

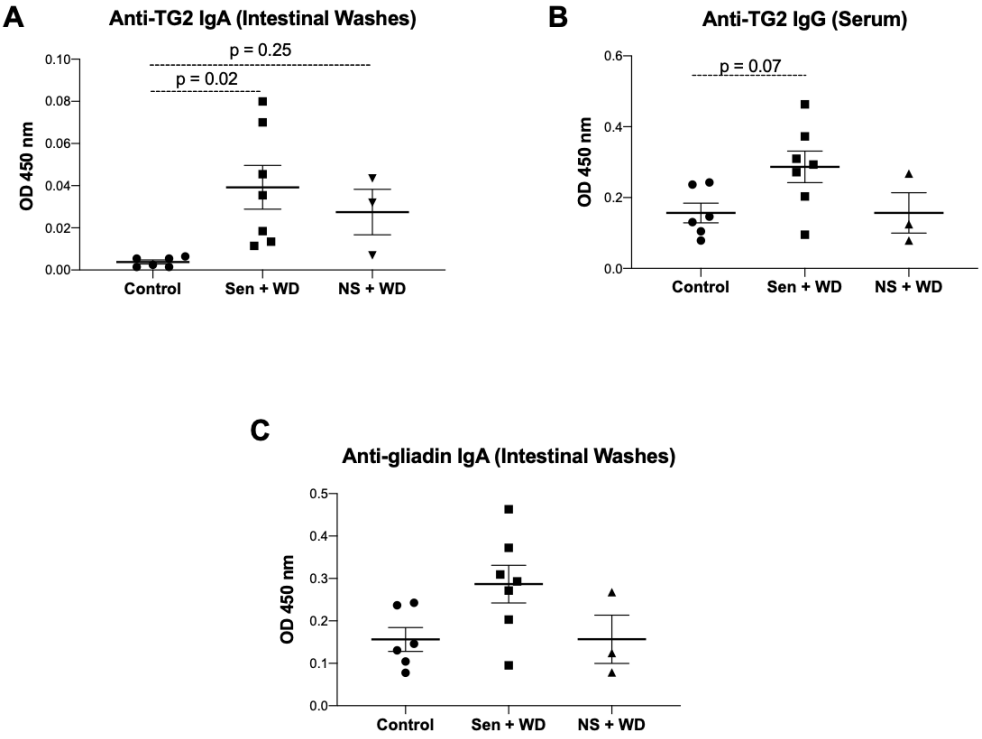


Figure 4.3 Development of anti-TG2 and anti-gliadin antibodies following a long-term gluten exposure

Serum and intestinal content were collected from Sen + WD, NS + WD, and control DR3-DQ2 mice. The presence of anti-TG2 (**A** and **B**) and anti-gliadin (**C**) antibodies were determined using ELISAs. Each dot represents an individual mouse; horizontal line and whiskers depict mean ± SEM; p-values were determined using ANOVA with post-hoc test for multiple comparisons.

DR3-DQ2 mice on a wheat containing diet develop villous-crypt abnormalities

To investigate whether DR3-DQ2 mice on a wheat containing diet develop enteropathy, we measured villous-crypt ratios in the proximal small intestine. In accordance to what we saw in **Chapter 3**, Sen + WD treated DR3-DQ2 mice had lower villous-crypt ratios compared to controls (Figure 4.4). On average, Sen + WD treated DR3-DQ2 mice

had a villous-crypt ratio of 3.4, compared to an average villous-crypt ratio of 4.5 in control mice. As well, we found that there was a trend for lowered villous-crypt ratios in the NS + WD treated DR3-DQ2 mice (Figure 4.4). We saw that NS + WD treated DR3-DQ2 had an average villous-crypt ratio of 3.8, compared to an average villous-crypt ratio of 4.5 in control mice. These findings suggest that Sen + WD treated mice are developing enteropathy in comparison to control mice, and that NS + WD treated mice are beginning to spontaneously develop villous-crypt abnormalities in comparison to control mice.

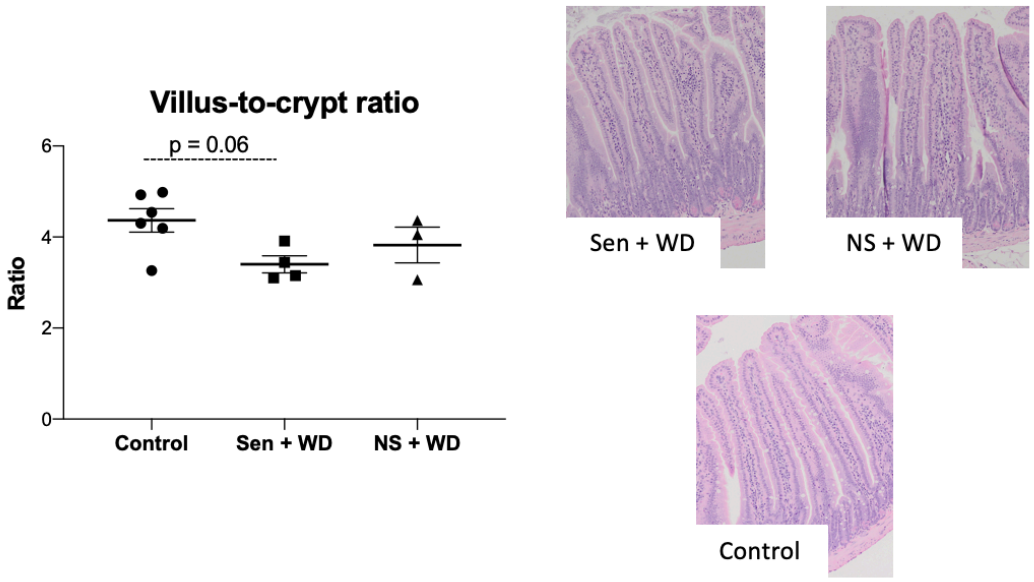
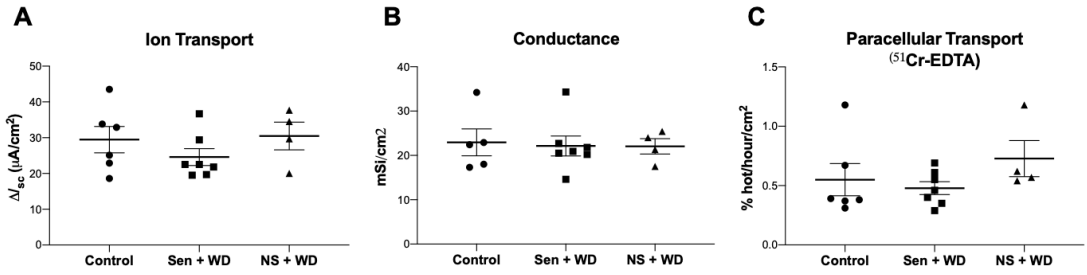


Figure 4.4 DR3-DQ2 mice on a wheat containing diet develop mild enteropathy

The villous-crypt ratios in the proximal small intestine were determined in Sen + WD, NS + WD, and control DR3-DQ2 mice. Villous-crypt ratios were measured from H&E stained slides. Representative photos are from Sen + WD (*top left*), NS + WD (*top right*), and control (*bottom*) DR3-DQ2 mice. Each dot represents an individual mouse; horizontal line and whiskers depict mean ± SEM; p-value was determined using an ANOVA test with post-hoc test for multiple comparisons.

DR3-DQ2 mice on a wheat containing diet did not have increased intestinal permeability

Lastly, we assessed if DR3-DQ2 mice on a gluten containing diet had increased intestinal permeability. In accordance to the experiments discussed in **Chapter 3**, we did not see a significant difference between Sen + WD, NS + WD, and control treated DR3-DQ2 mice when assessing ion transport and conductance (Figure 4.5, A and B). There was



no significant difference in paracellular permeability to ^{51}CR-EDTA between the Sen + WD, NS + WD, and control treated DR3-DQ2 mice (Figure 4.5, C)

Figure 4.5 DR3-DQ2 mice on a wheat diet do not develop changes in intestinal permeability

Ion transport (**A**), conductance (**B**), and paracellular permeability to ^{51}Cr-EDTA (**C**) was assessed in Sen + WD, NS + WD, and control DR3-DQ2 mice. No significant differences were seen. Each dot represents an individual mouse; horizontal line and whiskers depict mean ± SEM.

4.5. Discussion

CeD has long been thought of as a disease that only affects children, with it rarely impacting individuals over the age of 18. There is mounting evidence that CeD diagnosis rates are increasing rapidly amongst every age group, but particularly in adults over 60 (Hankey & Holmes, 1994; Rashtak & Murray, 2009). A study has also shown that a number of CeD cases in the elderly are the result of a loss of oral tolerance to gluten later in life, rather than a late diagnosis (Lohi *et al*., 2007). Similarly, potential CeD patients are defined as genetically at-risk individuals that present with one positive anti-TG2 blood test, but have yet to develop the villous atrophy needed for a full CeD diagnosis (Rubio-Tapia *et al*., 2013). Potential CeD patients represent a large number of cases that often go undetected but may provide interesting insight into how CeD pathogenesis evolves. The exact reason why some genetically susceptible individuals lose oral tolerance to gluten after being able to tolerate gluten for the majority of their life remains unknown. To date, no studies using DQ2 transgenic mice have been conducted to show the long-term impact of gluten exposure on the development of gluten-induced immunopathology. The objective of this study was to determine if DR3-DQ2 mice on a gluten containing diet could develop key CeD features without prior gluten sensitization, mimicking what may be occurring in older and potential CeD patients.

First, we show that gluten sensitization and diet composition drove different gene expression patterns within the small intestine of DR3-DQ2 mice. We found that exposure to a wheat containing diet, in both sensitized and non-sensitized DR3-DQ2 mice, led to an increase in expression of proinflammatory genes. These include *cysltr1* and *cysltr2*, both

genes whose expression have been linked to allergy, promotion of proinflammatory responses to certain antigens, and have been shown to increase permeability within the gastrointestinal tract of wildtype mice (Athari, 2019; Barajas-Espinosa *et al*., 2011; Kanaoka & Austen, 2019). The upregulation of *cysltr1* and *cysltr2* suggests that the continual ingestion of wheat through the diet of DR3-DQ2 mice, regardless of sensitization, may be promoting an increased immune response to gluten. We also observed an increase in *rela* and *nlrp3* in wheat-fed DR3-DQ2 mice, genes that help to activate NF-κB and promote apoptotic stress responses. This finding suggests that these genes could promote apoptosis within the small intestine and may contribute to enteropathy, however further experiments are needed to confirm this.

Sensitization to gluten using cholera toxin as an adjuvant increases the expression of proinflammatory genes within the small intestine of DR3-DQ2 mice. These genes are primarily involved in apoptotic stress responses and the induction of immune responses, including *cysltr2* and *daxx* (Brazina *et al*., 2015; Kanaoka & Austen, 2019). We also found that two out of four non-sensitized DR3-DQ2 mice that received a wheat diet had a gene expression pattern similar to the sensitized group on a wheat diet. This finding suggests that a spontaneous process similar to that of sensitization may be occurring in these mice due to their long-term exposure to gluten. However, further experimentation is needed to confirm these findings and to investigate how additional environmental triggers, perhaps from the microbiota, could affect the spontaneous development of inflammation in DR3-DQ2 mice. Notably, we did not see a significant difference in *il15* expression between gluten sensitized mice and non-sensitized mice on a wheat diet or controls. In **Chapter 3**

we determined that *il15* expression was significantly upregulated following gluten sensitization using the acute gluten challenge. The finding that *il15* was not different among groups in the long-term protocol is puzzling and suggests that *il15* may already be highly expressed across all DR3-DQ2 mice in these experiments, including controls. It would be beneficial to compare *il15* expression between the DR3-DQ2 mice in these experiments to wildtype C57Bl/6 mice to determine if *il15* expression is higher in all groups. Furthermore, there is currently no easy or reliable method to measure IL-15 protein levels directly and gene expression may not directly correlate with protein levels in DR3-DQ2 mice.

Patients with potential CeD are defined as having one elevated anti-TG2 antibody test, without the presence of villous atrophy (Rubio-Tapia *et al*., 2013). TG2 and IgA deposits are often found within the intestinal mucosa prior to the development of villous atrophy in potential CeD patients (Borrelli *et al*., 2018; Maglio *et al*., 2011). We wanted to test whether DR3-DQ2 mice treated with a wheat containing diet developed increased anti-TG2 antibodies in both the serum and intestinal content. We observed a trend for increased anti-TG2 (IgA) antibody levels in the intestinal content of non-sensitized DR3-DQ2 mice on a wheat containing diet, which suggests that B cell activation and plasma cell expansion is occurring within the lamina propria. Since the non-sensitized DR3-DQ2 mice on a wheat containing diet did not develop serum anti-TG2 (IgG), these mice are only spontaneously developing B cell activation within the gut rather than at systemic sites (Iversen *et al*., 2017). This may mimic the clinical situation observed in individuals at-risk of developing CeD that will progress to develop CeD, in whom TG2-IgA mucosal deposits in the absence of serum TG2 antibodies have been described (Borrelli *et al*., 2018; Gatti *et al*., 2014; Tosco

et al., 2011). However, it would be beneficial to determine if non-sensitized DR3-DQ2 on a wheat containing diet spontaneously develop anti-TG2 (IgA) antibodies in the serum to further confirm these findings.

Evaluating villous atrophy and crypt hyperplasia remains the gold standard in diagnosing CeD in adult patients (Korneychuk *et al*., 2015; Rubio-Tapia *et al*., 2013). We found that sensitized DR3-DQ2 mice on a wheat containing diet had significantly decreased villous-crypt ratios compared to control DR3-DQ2 mice, in accordance to previous experiments. We also saw a trend for decreased villous-crypt ratios in non-sensitized DR3-DQ2 mice on a wheat containing diet compared to control DR3-DQ2 mice. Interestingly, villous-crypt ratios were overall lower, when compared to those assessed in **Chapter 3**. It is important to note that mice in the long-term study were older than those in Chapter 3, as the protocol took 17 weeks to complete. This suggests that time may influence villous-crypt abnormalities in this model. Further experiments are needed to determine how aging changes the villous-crypt architecture within the small intestine, in the presence or absence of gluten exposure.

It is currently unknown whether intestinal permeability is a consequence or cause of CeD inflammation (Cardoso-Silva *et al*., 2019). We evaluated paracellular intestinal permeability in the sensitized and non-sensitized DR3-DQ2 mice on long term wheat containing diet. Similar to findings in **Chapter 3**, there were no significant differences in permeability between the groups studied. Since the permeability levels were similar to those seen in **Chapter 3**, it is most likely that intestinal permeability is elevated at baseline in the DR3-DQ2 mice and was not exacerbated by a wheat containing diet.

Overall, we have shown that sensitization to gluten and long-term exposure to a wheat containing diet leads to the development of enteropathy and immune activation in DR3-DQ2 mice. Exposing non-sensitized DR3-DQ2 mice to a wheat containing diet for an extended period of time led to the spontaneous development of some CeD features, including an increase in anti-TG2 (IgA) antibodies and a decrease in villous-crypt ratios indicating mild enteropathy. The number of DR3-DQ2 mice that were non-sensitized and on a wheat containing diet was small, and further experimentation is required to confirm these results. The findings are of particular interest since they may mimic potential CeD, and this may constitute a model to investigate additional triggers of breakdown of tolerance to gluten in older patients. However, further research is needed to determine whether aging alone or a combination of aging and the presence of the DR3-DQ2 transgene is contributing to a decrease in villous-crypt ratios even in the control DR3-DQ2 mice. These results show, for the first time, that even without prior sensitization, DR3-DQ2 mice spontaneously develop some key CeD features. This protocol does remain expensive and time-consuming to complete and may not be practical for the testing of pharmacological treatments. However, due to the unique findings seen within the non-sensitized DR3-DQ2 mice on a wheat containing diet, this protocol may be useful to study the factors that contribute to loss of oral tolerance to gluten. Lastly, using non-sensitized DR3-DQ2 mice on a wheat containing diet may provide greater insight into the mechanisms that may drive potential CeD patients into developing CeD and may allow deeper study into how to prevent CeD from developing in at-risk individuals.

CHAPTER 5: OVERALL DISCUSSION

5.1. Summary

An important component of survival is being able to maintain a stable internal environment, or homeostasis. Maintaining homeostasis is particularly important within the gastrointestinal (GI) tract, where the host is constantly exposed to microbes and their products, toxins, and dietary antigens. In the small intestine, the physical intestinal barrier, the immune system, host factors, and the microbiome interact to maintain homeostasis and allow for the proper function of the small intestine. The breakdown of any of these pathways leads dysregulation, loss of homeostasis, and improper functioning of the small intestine. This dysregulation may progress and lead to inflammatory responses towards typically innocuous antigens in the small intestinal lumen, particularly towards dietary antigens.

One of the most well-defined examples of how the dysregulation of all pathways needed to maintain homeostasis is can lead to disease is celiac disease (CeD). CeD is an immune-mediated enteropathy that develops in genetically susceptible individuals upon the ingestion of gluten proteins found in wheat, barley, and rye. While many aspects of CeD pathogenesis have been identified and described, the initial trigger and the development of this disease remains unknown. The only treatment for CeD is the lifelong adherence to a strict gluten-free diet, which is financially and socially burdensome for majority of patients to follow. Unfortunately, development of complementary pharmacological therapies to help CeD patients has been significantly hampered due to a lack of relevant, and easy-to-use, preclinical models. **Thus, the overall goal of this thesis was to characterize a novel mouse model of CeD that represents the majority of CeD patients that carry the HLA-**

DQ2 risk allele and could be used in future drug development studies. This central goal is addressed by the findings in **Chapter 3** and **Chapter 4**. In **Chapter 3**, I showed that transgenic DR3-DQ2 mice develop key features of the innate and adaptive immune response in CeD as well as moderate enteropathy after gluten sensitization and an acute gluten challenge. These results demonstrate that DR3-DQ2 mice are able to recapitulate many features of CeD, following an easily reproducible, standardized, and short-term gluten sensitization and challenge protocol. In **Chapter 4**, I showed that transgenic DR3-DQ2 mice are able to spontaneously develop key CeD features when exposed to gluten for a long period time, even without prior sensitization. These results suggest that DR3-DQ2 mice may be used to study mechanisms involved in the transition between potential CeD patients and active CeD later in life. Collectively, the work presented in **Chapter 3** and **Chapter 4** of this thesis characterizes the physiological and immunological responses of humanized DR3-DQ2 mice to gluten. These results show that DR3-DQ2 mice can be used as a novel animal model to study CeD pathogenesis with relevance to the majority of CeD patients expressing HLA-DQ2.

Results for **Chapter 3** and **Chapter 4** have been discussed in-depth within each chapter and will not be shown. In this chapter, connections between the experiments conducted in **Chapter 3** and **Chapter 4** and their significance is explored. Limitations and future directions are discussed at the end of this chapter.

5.2. Transgenic DR3-DQ2 mice can be used to study host-environment interactions

CeD is a challenging disease to model, as a combination of genetic factors, host immune responses, and environmental factors all contribute to disease development. In CeD, individuals must carry either the HLA-DQ2 or -DQ8 allele in order for CeD to develop. However, approximately 30% of the North American population carries these alleles, while only 2-4% of these individuals will go on to develop CeD (Tack *et al*., 2010; Tye-Din *et al*., 2018). This highlights the significance of environmental factors in contributing to CeD development. Many environmental factors have been proposed to trigger CeD pathogenesis, however, there has been no studies showing that one singular environmental factor can directly cause CeD (Abadie *et al*., 2011; Pinto-Sánchez *et al*., 2016; Verdu *et al*., 2015). Rather, it is likely that different combinations of environmental factors contribute to individual cases of CeD rather than one specific root cause.

In similar practice to clinical cases, I show that the presence of the DQ2 allele in C57Bl/6 background is not sufficient for the spontaneous development of moderate enteropathy and all CeD features without a change in environmental conditions. In **Chapter 3**, I show that sensitization to gluten using a mucosal adjuvant to help break tolerance is necessary for moderate enteropathy to occur. Similarly, in **Chapter 4**, I show that while non-sensitized DR3-DQ2 mice are able to spontaneously develop some mild features of CeD pathogenesis, these mice do not develop atrophy, or many of the features seen in sensitized DR3-DQ2 mice. This finding suggests that the use of a microbial adjuvant, in conjunction with the presence of PT-gliadin, helps to prime the immune system of these mice to gluten and enhances gluten-induced pathology. Using this adjuvant is similar to

current hypotheses surrounding CeD development, particularly of the "multiple hit hypothesis" where multiple bacterial or viral infections occur during critical times help to "prime" the patient's immune system to gluten fragments (Bouziat *et al*., 2017). Furthermore, in **Chapter 4**, I show that non-sensitized DR3-DQ2 mice develop some CeD features and mild enteropathy following long term exposure to a standard lab chow that contains gluten. Again, this finding suggests that environmental factors, in this case dietary factors such as ATIs, or perhaps microbiota or permeability changes associated with ageing may be influencing host immune responses. However, the specific dietary component driving these responses in DR3-DQ2 mice, and whether they act directly on the host or indirectly through modulating the composition or function of the gut microbiome, remains unknown.

The use of some sort of artificial method to inhibit oral tolerance to gluten is common in animal models used to study CeD research. For example, the NOD-DQ8 model also requires cholera toxin to be used to induce gluten immunopathology (Galipeau *et al*., 2011). As well, the recently reported triple transgenic model relies on the artificial overexpression of IL-15 within the lamina propria and epithelium of the small intestine and the DQ8 allele in order for enteropathy to occur (Abadie *et al*., 2020). This shows that environmental factors and immune response factors are necessary for gluten-induced enteropathy to occur in our current mouse models and supports the findings in **Chapter 3** and **4**. One of the advantages of using DR3-DQ2 mice and the protocols described in **Chapters 3** and **4** is that DR3-DQ2 mice develop many features of CeD pathogenesis while relying only on the DQ2 transgene and sensitization with a microbial adjuvant. This allows for a wider scope

of study in the future and shows that this model can be used to study components of both the innate and adaptive immune responses in CeD. This highlights their ability to be used to study a wide range of potential drugs, probiotics, or other therapies that may attenuate or exacerbate gluten-induced enteropathy. In contrast, animal models that focus on the overexpression of a specific cytokine can be used only to study a small number of interventions that focus specifically on attenuating that cytokine and its pathways. For example, as the triple transgenic model relies on an overexpression of IL-15 in the presence of the DQ8 allele, it can be used to study drugs or compounds that impact the IL-15 pathway in these mice (Abadie *et al*., 2020). However, it may not be a relevant model to use to study interventions that target intestinal permeability, for instance. Thus, using the DR3-DQ2 model may be beneficial in studying many aspects of CeD pathogenesis, rather than focusing on one specific feature.

In **Chapters 3** and **4**, I show that gluten sensitized DR3-DQ2 mice that are exposed to gluten, either in an acute challenge or long exposure, develop anti-TG2 IgG antibodies in serum. Presence of these antibodies has not been shown in other animal models used in CeD research and is a unique feature of the DR3-DQ2 mice. The presence of these antibodies indicate that systemic B cell activation can occur in systemic locations towards the autoantigen in CeD. These findings also suggest that DR3-DQ2 mice could be used in future studies investigating compounds that block the action of TG2. Thus, DR3-DQ2 mice can be used to study a wide range of different interventions to modulate gluten-induced immunopathology, particularly those that modulate TG2 activity, and will be a useful preclinical model to aid in CeD drug development.

5.3. Limitations & future directions

One of the largest obstacles in CeD research has been the lack of animal models that fully represent CeD pathogenesis. Similar to other models used in CeD research, the DR3-DQ2 mouse model outlined in this thesis has limitations. First, this model can be criticized for needing a microbial adjuvant to sensitize these mice to gluten to produce enteropathy in a short period of time. We show that DR3-DQ2 mice may develop key CeD features in the absence of using a microbial adjuvant and gluten sensitization, however, this process occurs slowly. While this long exposure model may be more pathophysiologically relevant to studying CeD development, the slow development of these features and expense to the user are major limitations of using this protocol. Utilizing a microbial adjuvant to sensitize DR3-DQ2 mice may not be fully representative of CeD development, but the short and reproducible protocol is an important benefit of this model.

Transgenic DR3-DQ2 mice develop moderate enteropathy following gluten sensitization and challenges rather than the complete atrophic enteropathy seen in CeD, in line with results from other HLA-DQ8 transgenic mouse models. With this limitation in mind, I wanted to characterize the transgenic DR3-DQ2 mouse model in terms of how these animals could model important aspects of the innate and adaptive immune response in CeD rather than just intestinal enteropathy. The findings outlined in **Chapter 3** show that the development of intestinal lymphocytosis and B cell activation occur in DR3-DQ2 mice independent of complete villous atrophy. These results are in line with CeD presentation in patients that is becoming more common, and in the movement away from using intestinal

biopsies to diagnosis CeD in children (Fasano & Catassi, 2001; S. K. Lee & Green, 2005; Werkstetter *et al*., 2017).

Many future studies have been opened due to the work presented in this thesis. While the list of future studies is essentially endless as there many unanswered questions left in the field of CeD research as a whole, a few immediate next steps are outlined below. To begin with, in **Chapter 3**, I demonstrated that there was an increase in $CD3^+$ IELs within the villous tips of gluten treated DR3-DQ2 mice. While this shows that there was a general increase in IELs in this group of mice, further studies should be conducted to determine which IEL subtypes are present in the villi after gluten treatment. I showed in **Chapter 3** that there was a significant increase in paracellular permeability to ^{51}Cr-EDTA in DR3-DQ2 mice at baseline. This is just one measure of intestinal permeability and it would be interesting to determine if DR3-DQ2 mice have increased permeability to other probes, such as specific bacteria, in the future. In conjunction with this, it would be beneficial to determine if there are gene expression differences between DR3-DQ2 mice and C57Bl/6 mice at baseline to see if there are any differences that may explain this increase in intestinal permeability, beyond just the presence of the DR3-DQ2 haplotype. Another important marker in this model would be the investigation of anti-deamidated gluten peptide (DGP) antibodies in the serum, as this test is often completed in the clinic in addition to anti-TG2 antibody tests. Measuring anti-DGP antibodies in the serum will confirm that DR3-DQ2 mice are developing an immune response specifically towards gluten peptides that have been deamidated by TG2. It would also be beneficial to measure anti-TG2 IgA antibodies in the serum to see if DR3-DQ2 mice do develop these antibodies following sensitization

and challenge. These antibodies are more specific to test for CeD than measuring anti-TG2 IgG antibodies, as these antibodies are produced by bone marrow plasma cells that originate from a B cell that was activated in the gut itself rather than in a systemic location, like the spleen (Iversen *et al*., 2017). Measuring anti-TG2 IgA antibodies would help to confirm the increase in anti-TG2 IgG that was seen in **Chapter 3**. Lastly, the gut microbiota composition of DR3-DQ2 mice before, during, and after gluten treatment may provide important insights into how this community may change and impact gluten-induced immunopathology in this model, particularly in the long-term exposure protocol. In the future, germ-free DR3-DQ2 mice can be used to further study the impact of the gut microbiota and its composition on gluten-induced immunopathology in the context of HLA-DQ2 positive patients.

Due to the 2020 COVID-19 pandemic, many of the experiments planned to be conducted and included in **Chapter 4** of this thesis were put on hold. Future studies should focus on completing these experiments. These include measuring $CD3^+$ IEL counts within the villi tips, determining if villous-crypt ratios decrease with aging, and measuring anti-deamidated gluten peptide antibodies following a long-term exposure to gluten. Furthermore, due to the small number of non-sensitized DR3-DQ2 mice on a wheat-containing diet, it would be beneficial to repeat this experiment with an increased number of mice in this group to confirm the findings outlined in **Chapter 4**. In the future, staining for TG2 and IgA deposits in the small intestinal mucosa of particularly the non-sensitized DR3-DQ2 on a wheat containing diet will provide further insight into whether these mice are spontaneously developing gluten-induced immunopathology. This staining will also

help to confirm whether TG2 and IgA deposits in the mucosa develop prior to intestinal damage, and may support looking for these deposits to track CeD development in potential CeD patients (Rubio-Tapia *et al*., 2013). Another area to explore is the role that IL-15 plays in the gluten immunopathology seen in DR3-DQ2 mice following gluten sensitization and challenge.

In **Chapter 3**, I show that *il15* was significantly upregulated in gluten sensitized DR3-DQ2 mice that were acutely challenged with gluten compared to control mice. In contrast, in **Chapter 4**, I show that *il15* was not significantly upregulated in gluten sensitized DR3-DQ2 mice compared to non-sensitized mice or in DR3-DQ2 mice on a wheat containing diet. This finding suggests that perhaps IL-15 plays a larger role in initiating gluten-immunopathology at the beginning of exposure to gluten but does not play a large role after a sustained, long-term gluten exposure. However, one of the major limitations of this finding is that only the relative gene expression of *il15* in small intestinal tissue was measured from the DR3-DQ2 mice in **Chapter 3** and **4**. This does not directly correlate with IL-15 protein levels within the tissue itself and is not a measurement of IL-15 activity. Unfortunately, measuring IL-15 through immunohistochemistry or ELISA is difficult as the current antibodies against IL-15 are not specific or sensitive. It would be beneficial to develop better IL-15 antibodies to directly measure IL-15 in the small intestinal tissue of DR3-DQ2 following acute and long-term challenge protocols to determine the exact role IL-15 is playing in gluten-immunopathology in this model.

Finally, a major limitation in the experiments performed in **Chapter 4** is the use of a standard lab chow rather than a matched diet. The use of the standard lab chow opens the

possibility that other dietary components, such as ATIs, that were not matched to the gluten-free diet may be contributing either directly or indirectly, through microbial metabolism, to the gut dysfunction seen in the non-sensitized DR3-DQ2 mice on a wheat containing diet. A matched diet has been designed where the only added component is gluten itself, however due to the COVID-19 pandemic these experiments could not be completed in time and will be conducted in the future. This will allow for more concrete findings on whether a long exposure to gluten itself is driving the spontaneous development of key CeD features in these mice.

5.4. Conclusions

The work presented in this thesis characterizes a novel transgenic animal model that can be used to study CeD and gluten-induced immunopathology. This work also highlights the importance of using different animal models to develop a comprehensive understanding of CeD, as each animal model is suited to recapitulate certain aspects of CeD within certain contexts. The animal model characterized provides a novel method for studying gluten-induced immunopathology in the context of HLA-DQ2. Furthermore, the model described in this thesis will help to support further research into pharmacological therapies that can be used by CeD patients to help alleviate their symptoms and increase their quality of life. While creating a picture-perfect animal model of CeD is an unattainable goal, the animal model described herein will help us continue to develop our understanding of CeD, other gluten related disorders, and their complexities.

REFERENCES

Abadie, V., Discepolo, V., & Jabri, B. (2012). Intraepithelial lymphocytes in celiac disease immunopathology. *Seminars in Immunopathology*, *34*(4), 551–556. https://doi.org/10.1007/s00281-012-0316-x

Abadie, V., & Jabri, B. (2014). IL-15: A central regulator of celiac disease immunopathology. In *Immunological Reviews* (Vol. 260, Issue 1, pp. 221–234). https://doi.org/10.1111/imr.12191

Abadie, V., Kim, S. M., Lejeune, T., Palanski, B. A., Ernest, J. D., Tastet, O., Voisine, J., Discepolo, V., Marietta, E. V., Hawash, M. B. F., Ciszewski, C., Bouziat, R., Panigrahi, K., Horwath, I., Zurenski, M. A., Lawrence, I., Dumaine, A., Yotova, V., Grenier, J. C., … Jabri, B. (2020). IL-15, gluten and HLA-DQ8 drive tissue destruction in coeliac disease. *Nature*, *578*(7796), 600–604. https://doi.org/10.1038/s41586-020-2003-8

Abadie, V., Sollid, L. M., Barreiro, L. B., & Jabri, B. (2011). Integration of Genetic and Immunological Insights into a Model of Celiac Disease Pathogenesis. *Annual Review of Immunology*, *29*(1), 493–525. https://doi.org/10.1146/annurev-immunol-040210-092915

Adelman, D. C., Murray, J., Wu, T. T., Mäki, M., Green, P. H., & Kelly, C. P. (2018). Measuring change in small intestinal histology in patients with celiac disease. *American Journal of Gastroenterology*, *113*(3), 339–347. https://doi.org/10.1038/ajg.2017.480

Ahadi, Z., Shafiee, G., Razmandeh, R., Keshtkar, A. A., Sani, M. N., Azemati, B., Sanaei, M., & Heshmat, R. (2016). Prevalence of celiac disease among the Iranian population: A systematic review and meta-analysis of observational studies. *Turkish Journal of Gastroenterology*, *27*(2), 122–128. https://doi.org/10.5152/tjg.2015.150191

Aidy, S. E., van den Bogert, B., & Kleerebezem, M. (2015). The small intestine microbiota, nutritional modulation and relevance for health. In *Current Opinion in Biotechnology* (Vol. 32, pp. 14–20). https://doi.org/10.1016/j.copbio.2014.09.005

Al-Toma, A., Goerres, M. S., Meijer, J. W. R., Peña, A. S., Bart, ¶ J, Crusius, A., & Mulder, C. J. J. (2006). *Human Leukocyte Antigen-DQ2 Homozygosity and the Development of Refractory Celiac Disease and Enteropathy-Associated T-Cell Lymphoma*. https://doi.org/10.1016/j.cgh.2005.12.011

Al Nabhani, Z., Dulauroy, S., Marques, R., Cousu, C., Al Bounny, S., Déjardin, F., Sparwasser, T., Bérard, M., Cerf-Bensussan, N., & Eberl, G. (2019). A Weaning Reaction to Microbiota Is Required for Resistance to Immunopathologies in the Adult. *Immunity*, *50*(5), 1276-1288.e5. https://doi.org/10.1016/j.immuni.2019.02.014

Araya, R. E., Gomez Castro, M. F., Carasi, P., McCarville, J. L., Jury, J., Mowat, A. M., Verdu, E. F., & Chirdo, F. G. (2016). Mechanisms of innate immune activation by gluten peptide p31-43 in mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *311*(1), G40–G49. https://doi.org/10.1152/ajpgi.00435.2015

Aronsson, C. A., Lee, H. S., Liu, E., Uusitalo, U., Hummel, S., Yang, J., Hummel, M., Rewers, M., She, J. X., Simell, O., Toppari, J., Ziegler, A. G., Krischer, J., Virtanen, S. M., Norris, J. M., & Agardh, D. (2015). Age at gluten introduction and risk of celiac disease. *Pediatrics*, *135*(2), 239–245. https://doi.org/10.1542/peds.2014-1787

Athari, S. S. (2019). Targeting cell signaling in allergic asthma. *Signal Transduction and Targeted Therapy*, *4*(1). https://doi.org/10.1038/s41392-019-0079-0

Auricchio, R., Tosco, A., Piccolo, E., Galatola, M., Izzo, V., Maglio, M., Paparo, F., Troncone, R., & Greco, L. (2014). Potential celiac children: 9-year follow-up on a gluten-containing diet. *American Journal of Gastroenterology*, *109*(6), 913–921. https://doi.org/10.1038/ajg.2014.77

Barajas-Espinosa, A., Ochoa-Cortes, F., Moos, M. P., Ramirez, F. D., Vanner, S. J., & Funk, C. D. (2011). Characterization of the cysteinyl leukotriene 2 receptor in novel expression sites of the gastrointestinal tract. *American Journal of Pathology*, *178*(6), 2682–2689. https://doi.org/10.1016/j.ajpath.2011.02.041

Biagi, F., Luinetti, O., Campanella, J., Klersy, C., Zambelli, C., Villanacci, V., Lanzini, A., & Corazza, G. R. (2004). Intraepithelial lymphocytes in the villous tip: Do they indicate potential coeliac disease? *Journal of Clinical Pathology*, *57*(8), 835–839. https://doi.org/10.1136/jcp.2003.013607

Black, K. E., Murray, J. A., & David, C. S. (2002). Mice Gluten Sensitivity in Transgenic Knockout Exogenous Wheat Proteins: A Model of HLA-DQ Determines the Response to. *J Immunol References*, *169*, 5595–5600. https://doi.org/10.4049/jimmunol.169.10.5595

Borrelli, M., Maglio, M., Korponay-Szabó, I. R., Vass, V., Mearin, M. L., Meijer, C., Niv-Drori, H., Ribes-Koninckx, C., Roca, M., Shamir, R., Troncone, R., & Auricchio, R. (2018). Intestinal anti-transglutaminase 2 immunoglobulin A deposits in children at risk for coeliac disease (CD): data from the PreventCD study. *Clinical and Experimental Immunology*, *191*(3), 311–317. https://doi.org/10.1111/cei.13078

Bouziat, R., Hinterleitner, R., Brown, J. J., Stencel-Baerenwald, J. E., Ikizler, M., Mayassi, T., Meisel, M., Kim, S. M., Discepolo, V., Pruijssers, A. J., Ernest, J. D., Iskarpatyoti, J. A., Costes, L. M. M., Lawrence, I., Palanski, B. A., Varma, M., Zurenski, M. A., Khomandiak, S., McAllister, N., … Jabri, B. (2017). Reovirus infection triggers inflammatory responses to dietary antigens and development of celiac disease. *Science*, *356*(6333), 44–50. https://doi.org/10.1126/science.aah5298

Božić, F., Forčić, D., Mažuran, R., Marinculić, A., Kozarić, Z., & Stojčević, D. (1998). γδTCR+ intestinal intraepithelial lymphocytes (i-IEL) in reaction against intestinal nematode Trichinella spiralis. *Comparative Immunology, Microbiology and Infectious Diseases*. https://doi.org/10.1016/S0147-9571(98)00014-9

Brandtzaeg, P., Kiyono, H., Pabst, R., & Russell, M. W. (2008). Terminology: Nomenclature of mucosa-associated lymphoid tissue. *Mucosal Immunology*, *1*(1), 31–37. https://doi.org/10.1038/mi.2007.9

Brazina, J., Svadlenka, J., Macurek, L., Andera, L., Hodny, Z., Bartek, J., & Hanzlikova, H. (2015). DNA damage-induced regulatory interplay between DAXX, p53, ATM kinase and Wip1 phosphatase. *Cell Cycle*, *14*(3), 375–387. https://doi.org/10.4161/15384101.2014.988019

Brown, E. M., Sadarangani, M., & Finlay, B. B. (2013). The role of the immune system in governing host-microbe interactions in the intestine. In *Nature Immunology* (Vol. 14, Issue 7, pp. 660–667). Nature Publishing Group. https://doi.org/10.1038/ni.2611

Cagnol, S., & Chambard, J.-C. (2010). ERK and cell death: Mechanisms of ERK-induced

cell death - apoptosis, autophagy and senescence. *FEBS Journal*, *277*(1), 2–21. https://doi.org/10.1111/j.1742-4658.2009.07366.x

Camarca, A., Anderson, R. P., Mamone, G., Fierro, O., Facchiano, A., Costantini, S., Zanzi, D., Sidney, J., Auricchio, S., Sette, A., Troncone, R., & Gianfrani, C. (2009). Intestinal T Cell Responses to Gluten Peptides Are Largely Heterogeneous: Implications for a Peptide-Based Therapy in Celiac Disease. *The Journal of Immunology*, *182*(7), 4158–4166. https://doi.org/10.4049/jimmunol.0803181

Caminero, A., Galipeau, H. J., McCarville, J. L., Johnston, C. W., Bernier, S. P., Russell, A. K., Jury, J., Herran, A. R., Casqueiro, J., Tye-Din, J. A., Surette, M. G., Magarvey, N. A., Schuppan, D., & Verdu, E. F. (2016). Duodenal Bacteria From Patients With Celiac Disease and Healthy Subjects Distinctly Affect Gluten Breakdown and Immunogenicity. *Gastroenterology*, *151*(4), 670–683. https://doi.org/10.1053/j.gastro.2016.06.041

Caminero, A., McCarville, J. L., Zevallos, V. F., Pigrau, M., Yu, X. B., Jury, J., Galipeau, H. J., Clarizio, A. V., Casqueiro, J., Murray, J. A., Collins, S. M., Alaedini, A., Bercik, P., Schuppan, D., & Verdu, E. F. (2019). Lactobacilli Degrade Wheat Amylase Trypsin Inhibitors to Reduce Intestinal Dysfunction Induced by Immunogenic Wheat Proteins. *Gastroenterology*, *156*(8), 2266–2280. https://doi.org/10.1053/j.gastro.2019.02.028

Cardoso-Silva, D., Delbue, D., Itzlinger, A., Moerkens, R., Withoff, S., Branchi, F., & Schumann, M. (2019). Intestinal barrier function in gluten-related disorders. In *Nutrients* (Vol. 11, Issue 10). MDPI AG. https://doi.org/10.3390/nu11102325

Chairatana, P., & Nolan, E. M. (2017). Defensins, lectins, mucins, and secretory immunoglobulin A: microbe-binding biomolecules that contribute to mucosal immunity in the human gut. In *Critical Reviews in Biochemistry and Molecular Biology* (Vol. 52, Issue 1, pp. 45–56). https://doi.org/10.1080/10409238.2016.1243654

Cheroutre, H., Lambolez, F., & Mucida, D. (2011). The light and dark sides of intestinal intraepithelial lymphocytes. In *Nature Reviews Immunology* (Vol. 11, Issue 7, pp. 445–456). https://doi.org/10.1038/nri3007

Ciacci, C., & Zingone, F. (2015). The Perceived Social Burden in Celiac Disease. *Diseases*. https://doi.org/10.3390/diseases3020102

Ciccocioppo, R., Di Sabatino, A., Bauer, M., Della Riccia, D. N., Bizzini, F., Biagi, F., Cifone, M. G., Corazza, G. R., & Schuppan, D. (2005). Matrix metalloproteinase pattern in celiac duodenal mucosa. *Laboratory Investigation*, *85*, 397–407. https://doi.org/10.1038/labinvest.3700225

Ciccocioppo, R., Finamore, A., Ara, C., Di Sabatino, A., Mengheri, E., & Corazza, G. R. (2006). Altered Expression, Localization, and Phosphorylation of Epithelial Junctional Proteins in Celiac Disease. *American Journal of Clinical Pathology*, *125*(4), 502–511. https://doi.org/10.1309/dtyr-a91g-8r0k-tm8m

Clemente, J. C., Ursell, L. K., Parfrey, L. W., & Knight, R. (2012). The impact of the gut microbiota on human health: An integrative view. In *Cell* (Vol. 148, Issue 6, pp. 1258–1270). https://doi.org/10.1016/j.cell.2012.01.035

Clevers, H. C., & Bevins, C. L. (2013). Paneth Cells: Maestros of the Small Intestinal

Crypts. *Annual Review of Physiology*, *75*(1), 289–311. https://doi.org/10.1146/annurev-physiol-030212-183744

Coates, M., Blanchard, S., & MacLeod, A. S. (2018). Innate antimicrobial immunity in the skin: A protective barrier against bacteria, viruses, and fungi. In *PLoS Pathogens* (Vol. 14, Issue 12). https://doi.org/10.1371/journal.ppat.1007353

Collado, M. C., Donat, E., Ribes-Koninckx, C., Calabuig, M., & Sanz, Y. (2009). Specific duodenal and faecal bacterial groups associated with paediatric coeliac disease. *Journal of Clinical Pathology*, *62*(3), 264–269. https://doi.org/10.1136/jcp.2008.061366

Collado, Maria Carmen, Donat, E., Ribes-Koninckx, C., Calabuig, M., & Sanz, Y. (2008). Imbalances in faecal and duodenal Bifidobacterium species composition in active and non-active coeliac disease. *BMC Microbiology*, *8*(1), 1–9. https://doi.org/10.1186/1471-2180-8-232

Collins, S. E., Noyce, R. S., & Mossman, K. L. (2004). Innate Cellular Response to Virus Particle Entry Requires IRF3 but Not Virus Replication. *Journal of Virology*, *78*(4), 1706–1717. https://doi.org/10.1128/jvi.78.4.1706-1717.2004

Dalton, J. E., Cruickshank, S. M., Egan, C. E., Mears, R., Newton, D. J., Andrew, E. M., Lawrence, B., Howell, G., Else, K. J., Gubbels, M., Striepen, B., Smith, J. E., White, S. J., & Carding, S. R. (2006). *Intraepithelial Lymphocytes Maintain the Integrity of Intestinal Epithelial Tight Junctions in Response to Infection Background & Aims: Intestinal epithelial integrity and*. https://doi.org/10.1053/j.gastro.2006.06.003

de Kauwe, A. L., Chen, Z., Anderson, R. P., Keech, C. L., Price, J. D., Wijburg, O., Jackson, D. C., Ladhams, J., Allison, J., & McCluskey, J. (2009). Resistance to Celiac Disease in Humanized HLA-DR3-DQ2-Transgenic Mice Expressing Specific Anti-Gliadin CD4 + T Cells . *The Journal of Immunology*, *182*(12), 7440–7450. https://doi.org/10.4049/jimmunol.0900233

De Palma, G., Nadal, I., Medina, M., Donat, E., Ribes-Koninckx, C., Calabuig, M., & Sanz, Y. (2010). Intestinal dysbiosis and reduced immunoglobulin-coated bacteria associated with coeliac disease in children. *BMC Microbiology*, *10*(1), 63. https://doi.org/10.1186/1471-2180-10-63

Di Cagno, R., De Angelis, M., De Pasquale, I., Ndagijimana, M., Vernocchi, P., Ricciuti, P., Gagliardi, F., Laghi, L., Crecchio, C., Guerzoni, M., Gobbetti, M., & Francavilla, R. (2011). Duodenal and faecal microbiota of celiac children: Molecular, phenotype and metabolome characterization. *BMC Microbiology*, *11*(1), 1–21. https://doi.org/10.1186/1471-2180-11-219

Duerksen, D. R., Wilhelm-Boyles, C., & Parry, D. M. (2005). Intestinal Permeability in Long-Term Follow-up of Patients with Celiac Disease on a Gluten-Free Diet. *Digestive Diseases and Sciences*, *50*(4), 785–790. https://doi.org/10.1007/s10620-005-2574-0

Fasano, A., & Catassi, C. (2001). Current approaches to diagnosis and treatment of celiac disease: An evolving spectrum. *Gastroenterology*, *120*(3), 636–651. https://doi.org/10.1053/gast.2001.22123

Fleur Du Pr, M., & Sollid, L. M. (2015). *T-cell and B-cell immunity in celiac disease*. https://doi.org/10.1016/j.bpg.2015.04.001

Galipeau, H. J., Mccarville, J. L., Huebener, S., Litwin, O., Meisel, M., Jabri, B., Sanz, Y., Murray, J. A., Jordana, M., Alaedini, A., Chirdo, F. G., & Verdu, E. F. (2015). Intestinal Microbiota Modulates Gluten-Induced Immunopathology in Humanized Mice. *The American Journal of Pathology*, *185*(11), 2969–2982. https://doi.org/10.1016/j.ajpath.2015.07.018

Galipeau, H. J., Rulli, N. E., Jury, J., Huang, X., Araya, R., Murray, J. A., David, C. S., Chirdo, F. G., McCoy, K. D., & Verdu, E. F. (2011). Sensitization to Gliadin Induces Moderate Enteropathy and Insulitis in Nonobese Diabetic-DQ8 Mice. *The Journal of Immunology*, *187*(8), 4338–4346. https://doi.org/10.4049/jimmunol.1100854

Gatti, S., Rossi, M., Alfonsi, S., Mandolesi, A., Cobellis, G., & Catassi, C. (2014). Beyond the intestinal celiac mucosa: Diagnostic role of anti-TG2 deposits, a systematic review. In *Frontiers in Medicine* (Vol. 1, Issue MAY). Frontiers Media S.A. https://doi.org/10.3389/fmed.2014.00009

Green, P. H. R. (2005). The many faces of celiac disease: Clinical presentation of celiac disease in the adult population. *Gastroenterology*, *128*(4 SUPPL. 1). https://doi.org/10.1053/j.gastro.2005.02.016

Green, P. H. R., & Cellier, C. (2007). Medical progress: Celiac disease. In *New England Journal of Medicine* (Vol. 357, Issue 17, pp. 1731–1743). Massachussetts Medical Society. https://doi.org/10.1056/NEJMra071600

Groschwitz, K. R., & Hogan, S. P. (2009). Intestinal barrier function: Molecular regulation and disease pathogenesis. In *Journal of Allergy and Clinical Immunology* (Vol. 124, Issue 1, pp. 3–20). https://doi.org/10.1016/j.jaci.2009.05.038

Hankey, G. L., & Holmes, G. K. T. (1994). Coeliac disease in the elderly. *Gut*, *35*(1), 65–67. https://doi.org/10.1136/gut.35.1.65

Ivarsson, A., Myléus, A., Norström, F., Der Pals, M. Van, Rosén, A., Högberg, L., Danielsson, L., Halvarsson, B., Hammarroth, S., Hernell, O., Karlsson, E., Stenhammar, L., Webb, C., Sandström, O., & Carlsson, A. (2013). Prevalence of childhood celiac disease and changes in infant feeding. *Pediatrics*, *131*(3), e687–e694. https://doi.org/10.1542/peds.2012-1015

Iversen, R., Snir, O., Stensland, M., Kroll, J. E., Steinsbø, Ø., Korponay-Szabó, I. R., Lundin, K. E. A., de Souza, G. A., & Sollid, L. M. (2017). Strong Clonal Relatedness between Serum and Gut IgA despite Different Plasma Cell Origins. *Cell Reports*, *20*(10), 2357–2367. https://doi.org/10.1016/j.celrep.2017.08.036

Järvinen, T. T., Kaukinen, K., Laurila, K., Kyrönpalo, S., Rasmussen, M., Mäki, M., Korhonen, H., Reunala, T., & Collin, P. (2003). Intraepithelial lymphocytes in celiac disease. *American Journal of Gastroenterology*, *98*(6), 1332–1337. https://doi.org/10.1111/j.1572-0241.2003.07456.x

Johansson, M. E. V., & Hansson, G. C. (2016). Immunological aspects of intestinal mucus and mucins. In *Nature Reviews Immunology* (Vol. 16, Issue 10, pp. 639–649). https://doi.org/10.1038/nri.2016.88

Kanaoka, Y., & Austen, K. F. (2019). Roles of cysteinyl leukotrienes and their receptors in immune cell-related functions. In *Advances in Immunology* (Vol. 142, pp. 65–84). Academic Press Inc. https://doi.org/10.1016/bs.ai.2019.04.002

Kim, C. Y., Quarsten, H., Bergseng, E., Khosla, C., & Sollid, L. M. (2004). Structural basis for HLA-DQ2-mediated presentation of gluten epitopes in celiac disease. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(12), 4175–4179. https://doi.org/10.1073/pnas.0306885101

Kim, Y. S., & Ho, S. B. (2010). Intestinal goblet cells and mucins in health and disease: Recent insights and progress. In *Current Gastroenterology Reports* (Vol. 12, Issue 5, pp. 319–330). https://doi.org/10.1007/s11894-010-0131-2

König, J., Wells, J., Cani, P. D., García-Ródenas, C. L., MacDonald, T., Mercenier, A., Whyte, J., Troost, F., & Brummer, R. J. (2016). Human intestinal barrier function in health and disease. *Clinical and Translational Gastroenterology*, *7*(10). https://doi.org/10.1038/ctg.2016.54

Korneychuk, N., Meresse, B., & Cerf-Bensussan, N. (2015). Lessons from rodent models in celiac disease. In *Mucosal Immunology* (Vol. 8, Issue 1, pp. 18–28). Nature Publishing Group. https://doi.org/10.1038/mi.2014.102

Kutlu, T., Brousse, N., Rambaud, C., Le Deist, F., Schmitz, J., & Cerf-Bensussan, N. (1993). Numbers of T cell receptor (TCR) αβ+ but not of TcR γδ+ intraepithelial lymphocytes correlate with the grade of villous atrophy in coeliac patients on a long term normal diet. *Gut*, *34*(2), 208–214. https://doi.org/10.1136/gut.34.2.208

Lebwohl, B., Ludvigsson, J. F., & Green, P. H. R. (2015). Celiac disease and non-celiac gluten sensitivity. In *BMJ (Online)* (Vol. 351). https://doi.org/10.1136/bmj.h4347

Lee, A. R., Ng, D. L., Zivin, J., & Green, P. H. R. (2007). Economic burden of a gluten-free diet. *Journal of Human Nutrition and Dietetics*, *20*(5), 423–430. https://doi.org/10.1111/j.1365-277X.2007.00763.x

Lee, S. K., & Green, P. H. R. (2005). Endoscopy in celiac disease. *Current Opinion in Gastroenterology*, *21*(5), 589–594. https://doi.org/10.1097/01.mog.0000174218.00333.19

Leonard, M. M., Sapone, A., Catassi, C., & Fasano, A. (2017). Celiac disease and nonceliac gluten sensitivity: A review. In *JAMA - Journal of the American Medical Association* (Vol. 318, Issue 7, pp. 647–656). American Medical Association. https://doi.org/10.1001/jama.2017.9730

Lionetti, E., Castellaneta, S., Francavilla, R., Pulvirenti, A., Naspi Catassi, G., & Catassi, C. (2019). Long-Term Outcome of Potential Celiac Disease in Genetically at-Risk Children: The Prospective CELIPREV Cohort Study. *Journal of Clinical Medicine*, *8*(2), 186. https://doi.org/10.3390/jcm8020186

Lionetti, E., Castellaneta, S., Francavilla, R., Pulvirenti, A., Tonutti, E., Arnarri, S., Barbato, M., Barbera, C., Barera, G., Bellantoni, A., Castellano, E., Guariso, G., Limongelli, M. G., Pellegrino, S., Polloni, C., Ughi, C., Zuin, G., Fasano, A., & Catassi, C. (2014). Introduction of gluten, HLA status, and the risk of celiac disease in children. *New England Journal of Medicine*, *371*(14), 1295–1303. https://doi.org/10.1056/NEJMoa1400697

Lionetti, E., Gatti, S., Pulvirenti, A., & Catassi, C. (2015). Celiac disease from a global perspective. *Best Practice and Research: Clinical Gastroenterology*, *29*(3), 365–379. https://doi.org/10.1016/j.bpg.2015.05.004

Lohi, S., Mustalahti, K., Kaukinen, K., Laurila, K., Collin, P., Rissanen, H., Lohi, O.,

Bravi, E., Gasparin, M., Reunanen, A., & Mäki, M. (2007). Increasing prevalence of coeliac disease over time. *Alimentary Pharmacology and Therapeutics*, *26*(9), 1217–1225. https://doi.org/10.1111/j.1365-2036.2007.03502.x

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. In *Nature* (Vol. 489, Issue 7415, pp. 220–230). https://doi.org/10.1038/nature11550

Mabbott, N. A., Donaldson, D. S., Ohno, H., Williams, I. R., & Mahajan, A. (2013). Microfold (M) cells: Important immunosurveillance posts in the intestinal epithelium. In *Mucosal Immunology* (Vol. 6, Issue 4, pp. 666–677). https://doi.org/10.1038/mi.2013.30

Macpherson, A. J., Gatto, D., Sainsbury, E., Harriman, G. R., Hengartner, H., & Zinkernagel, R. M. (2000). A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science*, *288*(5474), 2222–2226. https://doi.org/10.1126/science.288.5474.2222

Macpherson, A. J., & Uhr, T. (2004). Induction of Protective IgA by Intestinal Dendritic Cells Carrying Commensal Bacteria. *Science*, *303*(5664), 1662–1665. https://doi.org/10.1126/science.1091334

Maglio, M., Tosco, A., Auricchio, R., Paparo, F., Colicchio, B., Miele, E., Rapacciuolo, L., & Troncone, R. (2011). Intestinal deposits of anti-tissue transglutaminase iga in childhood celiac disease. *Digestive and Liver Disease*, *43*(8), 604–608. https://doi.org/10.1016/j.dld.2011.01.015

Mantis, N. J., Rol, N., & Corthésy, B. (2011). Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. In *Mucosal Immunology* (Vol. 4, Issue 6, pp. 603–611). Nature Publishing Group. https://doi.org/10.1038/mi.2011.41

Marafini, I., Monteleone, I., Di Fusco, D., Cupi, M. L., Paoluzi, O. A., Colantoni, A., Ortenzi, A., Izzo, R., Vita, S., De Luca, E., Sica, G., Pallone, F., & Monteleone, G. (2015). TNF-α producing innate lymphoid cells (ILCs) are increased in active celiac disease and contribute to promote intestinal atrophy in mice. *PLoS ONE*, *10*(5). https://doi.org/10.1371/journal.pone.0126291

Marietta, E., Black, K., Camilleri, M., Krause, P., Rogers, R. S., David, C., Pittelkow, M. R., & Murray, J. A. (2004). A new model for dermatitis herpetiformis that uses HLA-DQ8 transgenic NOD mice. *Journal of Clinical Investigation*, *114*(8), 1090–1097. https://doi.org/10.1172/jci21055

Martinez-Guryn, K., Hubert, N., Frazier, K., Urlass, S., Musch, M. W., Ojeda, P., Pierre, J. F., Miyoshi, J., Sontag, T. J., Cham, C. M., Reardon, C. A., Leone, V., & Chang, E. B. (2018). Small Intestine Microbiota Regulate Host Digestive and Absorptive Adaptive Responses to Dietary Lipids. *Cell Host and Microbe*, *23*(4), 458-469.e5. https://doi.org/10.1016/j.chom.2018.03.011

Matysiak-Budnik, T., Moura, I. C., Arcos-Fajardo, M., Lebreton, C., Ménard, S., Candalh, C., Ben-Khalifa, K., Dugave, C., Tamouza, H., Van Niel, G., Bouhnik, Y., Lamarque, D., Chaussade, S., Malamut, G., Cellier, C., Cerf-Bensussan, N., Monteiro, R. C., & Heyman, M. (2008). Secretory IgA mediates retrotranscytosis of intact gliadin peptides via the transferrin receptor in celiac disease. *Journal of Experimental Medicine*, *205*(1), 143–154. https://doi.org/10.1084/jem.20071204

Mauri, C., & Menon, M. (2017). Human regulatory B cells in health and disease: Therapeutic potential. In *Journal of Clinical Investigation* (Vol. 127, Issue 3, pp. 772–779). American Society for Clinical Investigation. https://doi.org/10.1172/JCI85113

Ménard, S., Cerf-Bensussan, N., & Heyman, M. (2010). Multiple facets of intestinal permeability and epithelial handling of dietary antigens. In *Mucosal Immunology* (Vol. 3, Issue 3, pp. 247–259). https://doi.org/10.1038/mi.2010.5

Menard, S., Lebreton, C., Schumann, M., Matysiak-Budnik, T., Dugave, C., Bouhnik, Y., Malamut, G., Cellier, C., Allez, M., Crenn, P., Schulzke, J. D., Cerf-Bensussan, N., & Heyman, M. (2012). Paracellular versus transcellular intestinal permeability to gliadin peptides in active celiac disease. *American Journal of Pathology*, *180*(2), 608–615. https://doi.org/10.1016/j.ajpath.2011.10.019

Mesin, L., Sollid, L. M., & Di Niro, R. (2012). The intestinal B-cell response in celiac disease. In *Frontiers in Immunology* (Vol. 3, Issue OCT). Frontiers Media SA. https://doi.org/10.3389/fimmu.2012.00313

Murray, J. A., Van Dyke, C., Plevak, M. F., Dierkhising, R. A., Zinsmeister, A. R., & Melton, L. J. (2003). Trends in the identification and clinical features of celiac disease in a North American community, 1950-2001. *Clinical Gastroenterology and Hepatology*, *1*(1), 19–27. https://doi.org/10.1053/jcgh.2003.50004

Natividad, J. M., Huang, X., Slack, E., Jury, J., Sanz, Y., David, C., Denou, E., Yang, P., Murray, J., McCoy, K. D., & Verdú, E. F. (2009). Host responses to intestinal microbial antigens in gluten-sensitive mice. *PLoS ONE*, *4*(7). https://doi.org/10.1371/journal.pone.0006472

Nilsen, E. M., Lundin, K. E. A., Krajči, P., Scott, H., Sollid, L. M., & Brandtzaeg, P. (1995). Gluten specific, HLA-DQ restricted T cells from coeliac mucosa produce cytokines with Th1 or Th0 profile dominated by interferon γ. *Gut*, *37*(6), 766–776. https://doi.org/10.1136/gut.37.6.766

Novak, J., Zamostna, B., Vopalensky, V., Buryskova, M., Burysek, L., Doleckova, D., & Pospisek, M. (2020). Interleukin-1α associates with the tumor suppressor p53 following DNA damage. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-63779-x

Olivares-Villagómez, D., & Van Kaer, L. (2018). Intestinal Intraepithelial Lymphocytes: Sentinels of the Mucosal Barrier. In *Trends in Immunology* (Vol. 39, Issue 4, pp. 264–275). https://doi.org/10.1016/j.it.2017.11.003

Olsson, C., Hrnell, A., Ivarsson, A., & Sydner, Y. M. (2008). The everyday life of adolescent coeliacs: issues of importance for compliance with the gluten-free diet. *Journal of Human Nutrition and Dietetics*, *21*(4), 359–367. https://doi.org/10.1111/j.1365-277X.2008.00867.x

Olsson, Cecilia, Hernell, O., Hornell, A., Lönnberg, G., & Ivarsson, A. (2008). Difference in celiac disease risk between swedish birth cohorts suggests an opportunity for primary prevention. *Pediatrics*, *122*(3), 528–534. https://doi.org/10.1542/peds.2007-2989

Pabst, O., & Mowat, A. M. (2012). Oral tolerance to food protein. In *Mucosal Immunology* (Vol. 5, Issue 3, pp. 232–239). Nature Publishing Group.

https://doi.org/10.1038/mi.2012.4
Pagliari, D., Cianci, R., Frosali, S., Landolfi, R., Cammarota, G., Newton, E. E., & Pandolfi, F. (2013). The role of IL-15 in gastrointestinal diseases: A bridge between innate and adaptive immune response. In *Cytokine and Growth Factor Reviews* (Vol. 24, Issue 5, pp. 455–466). https://doi.org/10.1016/j.cytogfr.2013.05.004
Parish, C. R., Glidden, M. H., Quah, B. J. C., & Warren, H. S. (2009). Use of the intracellular fluorescent dye CFSE to monitor lymphocyte migration and proliferation. *Current Protocols in Immunology*, *SUPPL. 84*. https://doi.org/10.1002/0471142735.im0409s84
Parra-Medina, R., Molano-Gonzalez, N., Rojas-Villarraga, A., Agmon-Levin, N., Arango, M. T., Shoenfeld, Y., & Anaya, J. M. (2015). Prevalence of celiac disease in Latin America: A systematic review and meta-regression. In *PLoS ONE* (Vol. 10, Issue 5). Public Library of Science. https://doi.org/10.1371/journal.pone.0124040
Pellegrino, S., Villanacci, V., Sansotta, N., Scarfì, R., Bassotti, G., Vieni, G., Princiotta, A., Sferlazzas, C., Magazzù, G., & Tuccari, G. (2011). Redefining the intraepithelial lymphocytes threshold to diagnose gluten sensitivity in patients with architecturally normal duodenal histology. *Alimentary Pharmacology & Therapeutics*, *33*(6), 697–706. https://doi.org/10.1111/j.1365-2036.2011.04578.x
Perrier, C., & Corthésy, B. (2011). Gut permeability and food allergies. *Clinical & Experimental Allergy*, *41*(1), 20–28. https://doi.org/10.1111/j.1365-2222.2010.03639.x
Pinto-Sánchez, M. I., Verdu, E. F., Liu, E., Bercik, P., Green, P. H., Murray, J. A., Guandalini, S., & Moayyedi, P. (2016). Gluten Introduction to Infant Feeding and Risk of Celiac Disease: Systematic Review and Meta-Analysis. *Journal of Pediatrics*, *168*, 132-143.e3. https://doi.org/10.1016/j.jpeds.2015.09.032
Ploski, R., Ek, J., Thorsby, E., & Sollid, L. M. (1993). On the HLA-DQ(α1*0501, β1*0201)-associated susceptibility in celiac disease: A possible gene dosage effect of *DQB1*0201*. *Tissue Antigens*, *41*(4), 173–177. https://doi.org/10.1111/j.1399-0039.1993.tb01998.x
Rashtak, S., & Murray, J. A. (2009). Celiac Disease in the Elderly. In *Gastroenterology Clinics of North America* (Vol. 38, Issue 3, pp. 433–446). NIH Public Access. https://doi.org/10.1016/j.gtc.2009.06.005
Reilly, N. R., & Green, P. H. R. (2012). Epidemiology and clinical presentations of celiac disease. *Seminars in Immunopathology*, *34*(4), 473–478. https://doi.org/10.1007/s00281-012-0311-2
Rescigno, M. (2011). The intestinal epithelial barrier in the control of homeostasis and immunity. In *Trends in Immunology* (Vol. 32, Issue 6, pp. 256–264). https://doi.org/10.1016/j.it.2011.04.003
Rooks, M. G., & Garrett, W. S. (2016). Gut microbiota, metabolites and host immunity. In *Nature Reviews Immunology* (Vol. 16, Issue 6, pp. 341–352). https://doi.org/10.1038/nri.2016.42
Rostom, A., Murray, J. A., & Kagnoff, M. F. (2006). American Gastroenterological Association (AGA) Institute Technical Review on the Diagnosis and Management of Celiac Disease. *Gastroenterology*, *131*(6), 1981–2002.

https://doi.org/10.1053/j.gastro.2006.10.004
Rubio-Tapia, A., Hill, I. D., Kelly, C. P., Calderwood, A. H., & Murray, J. A. (2013). ACG clinical guidelines: Diagnosis and management of celiac disease. *American Journal of Gastroenterology*, *108*(5), 656–676. https://doi.org/10.1038/ajg.2013.79
Rubio-Tapia, A., Kyle, R. A., Kaplan, E. L., Johnson, D. R., Page, W., Erdtmann, F., Brantner, T. L., Kim, W. R., Phelps, T. K., Lahr, B. D., Zinsmeister, A. R., Melton, L. J., & Murray, J. A. (2009). Increased Prevalence and Mortality in Undiagnosed Celiac Disease. *Gastroenterology*, *137*(1), 88–93. https://doi.org/10.1053/j.gastro.2009.03.059
Sarra, M., Cupi, M. L., Monteleone, I., Franzè, E., Ronchetti, G., Di Sabatino, A., Gentileschi, P., Franceschilli, L., Sileri, P., Sica, G., Del Vecchio Blanco, G., Cretella, M., Paoluzi, O. A., Corazza, G. R., Pallone, F., & Monteleone, G. (2013). IL-15 positively regulates IL-21 production in celiac disease mucosa. *Mucosal Immunology*, *6*(2), 244–255. https://doi.org/10.1038/mi.2012.65
Schulzke, J. D., Schulzke, I., Fromm, M., & Riecken, E. O. (1995). Epithelial barrier and ion transport in coeliac sprue: Electrical measurements on intestinal aspiration biopsy specimens. *Gut*, *37*(6), 777–782. https://doi.org/10.1136/gut.37.6.777
Schulzke, Jörg Dieter, Bentzel, C. J., Schulzke, I., Riecken, E. O., & Fromm, M. (1998). Epithelial tight junction structure in the jejunum of children with acute and treated celiac sprue. *Pediatric Research*, *43*(4 I), 435–441. https://doi.org/10.1203/00006450-199804000-00001
Schuppan, D., Dieterich, W., Ehnis, T., Bauer, M., Donner, P., Volta, U., & Riecken, E. O. (1998). Identification of the autoantigen of celiac disease. *Annals of the New York Academy of Sciences*, *859*, 121–126. https://doi.org/10.1111/j.1749-6632.1998.tb11116.x
Schuppan, D., & Zevallos, V. (2015). Wheat amylase trypsin inhibitors as nutritional activators of innate immunity. *Digestive Diseases*, *33*(2), 260–263. https://doi.org/10.1159/000371476
Senger, S., Maurano, F., Mazzeo, M. F., Gaita, M., Fierro, O., David, C. S., Troncone, R., Auricchio, S., Siciliano, R. A., & Rossi, M. (2005). Identification of Immunodominant Epitopes of α-Gliadin in HLA-DQ8 Transgenic Mice following Oral Immunization. *The Journal of Immunology*, *175*(12), 8087–8095. https://doi.org/10.4049/jimmunol.175.12.8087
Shah, S., Akbari, M., Vanga, R., Kelly, C. P., Hansen, J., Theethira, T., Tariq, S., Dennis, M., & Leffler, D. A. (2014). Patient perception of treatment burden is high in celiac disease compared with other common conditions. *The American Journal of Gastroenterology*, *109*(9), 1304–1311. https://doi.org/10.1038/ajg.2014.29
Shan, L., Molberg, Ø., Parrot, I., Hausch, F., Filiz, F., Gray, G. M., Sollid, L. M., & Khosla, C. (2002). Structural basis for gluten intolerance in Celiac Sprue. *Science*, *297*(5590), 2275–2279. https://doi.org/10.1126/science.1074129
Shewry, P. R., Halford, N. G., Belton, P. S., & Tatham, A. S. (2002). The structure and properties of gluten: An elastic protein from wheat grain. In *Philosophical Transactions of the Royal Society B: Biological Sciences* (Vol. 357, Issue 1418, pp. 133–142). The Royal Society. https://doi.org/10.1098/rstb.2001.1024

Singh, P., Arora, A., Strand, T. A., Leffler, D. A., Catassi, C., Green, P. H., Kelly, C. P., Ahuja, V., & Makharia, G. K. (2018). Global Prevalence of Celiac Disease: Systematic Review and Meta-analysis. *Clinical Gastroenterology and Hepatology*, *16*(6), 823-836.e2. https://doi.org/10.1016/j.cgh.2017.06.037

Sollid, L. M., Molberg, Mcadam, S., & Lundin, K. E. A. (1997). Autoantibodies in coeliac disease: Tissue transglutaminase guilt by association? In *Gut* (Vol. 41, Issue 6, pp. 851–852). https://doi.org/10.1136/gut.41.6.851

Sollid, Ludvig M, & Thorsby, E. (1993). HLA Susceptibility Genes in Celiac Disease: Genetic Mapping and Role in Pathogenesis. In *GASTROENTEROLOGY* (Vol. 105).

Suwanai, H., Wilcox, M. A., Mathis, D., & Benoist, C. (2010). A defective Il15 allele underlies the deficiency in natural killer cell activity in nonobese diabetic mice. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(20), 9305–9310. https://doi.org/10.1073/pnas.1004492107

Szajewska, H., Shamir, R., Mearin, L., Ribes-Koninckx, C., Catassi, C., Domellof, M., Fewtrell, M. S., Husby, S., Papadopoulou, A., Vandenplas, Y., Castillejo, G., Kolacek, S., Koletzko, S., Korponay-Szabo, I. R., Lionetti, E., Polanco, I., & Troncone, R. (2016). Gluten introduction and the risk of coeliac disease: A position paper by the european society for pediatric gastroenterology, hepatology, and nutrition. *Journal of Pediatric Gastroenterology and Nutrition*, *62*(3), 507–513. https://doi.org/10.1097/MPG.0000000000001105

Tack, G. J., Verbeek, W. H. M., Schreurs, M. W. J., & Mulder, C. J. J. (2010). The spectrum of celiac disease: Epidemiology, clinical aspects and treatment. In *Nature Reviews Gastroenterology and Hepatology* (Vol. 7, Issue 4, pp. 204–213). https://doi.org/10.1038/nrgastro.2010.23

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A. N., Mackay, C. R., & Macia, L. (2014). The Role of Short-Chain Fatty Acids in Health and Disease. *Advances in Immunology*, *121*, 91–119. https://doi.org/10.1016/B978-0-12-800100-4.00003-9

Thomas, K. E., Sapone, A., Fasano, A., & Vogel, S. N. (2006). Gliadin Stimulation of Murine Macrophage Inflammatory Gene Expression and Intestinal Permeability Are MyD88-Dependent: Role of the Innate Immune Response in Celiac Disease. *The Journal of Immunology*, *176*(4), 2512–2521. https://doi.org/10.4049/jimmunol.176.4.2512

Todd, J. A., & Bell, J. I. (1988). HLA antigens and insulin-dependent diabetes. *Nature*, *333*, 710.

Tordesillas, L., & Berin, M. C. (2018). Mechanisms of Oral Tolerance. In *Clinical Reviews in Allergy and Immunology* (Vol. 55, Issue 2, pp. 107–117). Humana Press Inc. https://doi.org/10.1007/s12016-018-8680-5

Tortora, G. J., & Derrickson, B. H. (2017). *Principles of Anatomy and Physiology* (15th ed.). Wiley-Blackwell.

Tosco, A., Salvati, V. M., Auricchio, R., Maglio, M., Borrelli, M., Coruzzo, A., Paparo, F., Boffardi, M., Esposito, A., D'Adamo, G., Malamisura, B., Greco, L., & Troncone, R. (2011). Natural History of Potential Celiac Disease in Children. *Clinical Gastroenterology and Hepatology*, *9*(4), 320–325. https://doi.org/10.1016/j.cgh.2010.09.006

Trynka, G., Hunt, K. A., Bockett, N. A., Romanos, J., Mistry, V., Szperl, A., Bakker, S. F., Bardella, M. T., Bhaw-Rosun, L., Castillejo, G., De La Concha, E. G., De Almeida, R. C., Dias, K. R. M., Van Diemen, C. C., Dubois, P. C. A., Duerr, R. H., Edkins, S., Franke, L., Fransen, K., … Van Heel, D. A. (2011). Dense genotyping identifies and localizes multiple common and rare variant association signals in celiac disease. *Nature Genetics*, *43*(12), 1193–1201. https://doi.org/10.1038/ng.998

Turner, J. R. (2009). Intestinal mucosal barrier function in health and disease. In *Nature Reviews Immunology* (Vol. 9, Issue 11, pp. 799–809). Nature Publishing Group. https://doi.org/10.1038/nri2653

Tye-Din, J. A., Galipeau, H. J., & Agardh, D. (2018). Celiac disease: A review of current concepts in pathogenesis, prevention, and novel therapies. *Frontiers in Pediatrics*, *6*. https://doi.org/10.3389/fped.2018.00350

Vazquez-Roque, M. I., Camilleri, M., Smyrk, T., Murray, J. A., O'Neill, J., Carlson, P., Lamsam, J., Eckert, D., Janzow, D., Burton, D., Ryks, M., Rhoten, D., & Zinsmeister, A. R. (2012). Association of HLA-DQ gene with bowel transit, barrier function, and inflammation in irritable bowel syndrome with diarrhea. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *303*(11). https://doi.org/10.1152/ajpgi.00294.2012

Verdu, E. F., Huang, X., Natividad, J., Lu, J., Blennerhassett, P. A., David, C. S., McKay, D. M., & Murray, J. A. (2007). Gliadin-dependent neuromuscular and epithelial secretory responses in gluten-sensitive HLA-DQ8 transgenic mice. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *294*(1). https://doi.org/10.1152/ajpgi.00225.2007

Verdu, Elena F, Galipeau, H. J., & Jabri, B. (2015). Novel players in coeliac disease pathogenesis: Role of the gut microbiota. In *Nature Reviews Gastroenterology and Hepatology* (Vol. 12, Issue 9, pp. 497–506). https://doi.org/10.1038/nrgastro.2015.90

Vivas, S. (2015). Age-related differences in celiac disease: Specific characteristics of adult presentation. *World Journal of Gastrointestinal Pharmacology and Therapeutics*, *6*(4), 207. https://doi.org/10.4292/wjgpt.v6.i4.207

Volta, U., Lenzi, M., Lazzari, R., Cassani, F., Collina, A., Bianchi, F. B., & Pisi, E. (1985). Antibodies to gliadin detected by immunofluorescence and a micro-ELISA method: Markers of active childhood and adult coeliac disease. *Gut*, *26*(7), 667–671. https://doi.org/10.1136/gut.26.7.667

Wambre, E., & Jeong, D. (2018). Oral Tolerance Development and Maintenance. In *Immunology and Allergy Clinics of North America* (Vol. 38, Issue 1, pp. 27–37). W.B. Saunders. https://doi.org/10.1016/j.iac.2017.09.003

Werkstetter, K. J., Korponay-Szabó, I. R., Popp, A., Villanacci, V., Salemme, M., Heilig, G., Lillevang, S. T., Mearin, M. L., Ribes-Koninckx, C., Thomas, A., Troncone, R., Filipiak, B., Mäki, M., Gyimesi, J., Najafi, M., Dolinšek, J., Dydensborg Sander, S., Auricchio, R., Papadopoulou, A., … Eftekhar Sadat, A. T. (2017). Accuracy in Diagnosis of Celiac Disease Without Biopsies in Clinical Practice. *Gastroenterology*, *153*(4), 924–935. https://doi.org/10.1053/j.gastro.2017.06.002

White, L. E., Bannerman, E., & Gillett, P. M. (2016). Coeliac disease and the gluten-free diet: a review of the burdens; factors associated with adherence and impact on

health-related quality of life, with specific focus on adolescence. *Journal of Human Nutrition and Dietetics*, *29*(5), 593–606. https://doi.org/10.1111/jhn.12375

Wungjiranirun, M., Kelly, C. P., & Leffler, D. A. (2016). Current Status of Celiac Disease Drug Development. *American Journal of Gastroenterology*, *111*(6), 779–786. https://doi.org/10.1038/ajg.2016.105

Xu, L. L., Warren, M. K., Rose, W. L., Gong, W., & Wang, J. M. (1996). Human recombinant monocyte chemotactic protein and other c-c chemokines bind and induce directional migration of dendritic Cells in vitro. *Journal of Leukocyte Biology*, *60*(3), 365–371. https://doi.org/10.1002/jlb.60.3.365

Zhou, R., Wei, H., Sun, R., Zhang, J., & Tian, Z. (2007). NKG2D recognition mediates Toll-like receptor 3 signaling-induced breakdown of epithelial homeostasis in the small intestines of mice. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(18), 7512–7515. https://doi.org/10.1073/pnas.0700822104

Zoetendal, E. G., Raes, J., Van Den Bogert, B., Arumugam, M., Booijink, C. C., Troost, F. J., Bork, P., Wels, M., De Vos, W. M., & Kleerebezem, M. (2012). The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. *ISME Journal*, *6*(7), 1415–1426. https://doi.org/10.1038/ismej.2011.212